December 11, 1957

To the Graduate Council:

I am submitting herewith a thesis written by Harold Baxter entitled "The Effect of Ortho, Meta, Pyro, and Phytin Phosphates upon Cellulose Digestion by Rumen Microorganisms in Vitro." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

O Glen Hall
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We have read this thesis
and recommend its acceptance:

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Accepted for the Council:

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THE EFFECT OF ORTHO, META, PYRO, AND PHYTIN PHOSPHATES UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS *IN VITRO*

A THESIS

Submitted to
The Graduate Council
of
The University of Tennessee
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for the degree of
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by

Harold Baxter

December 1957

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Harold Baxter

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CHAPTER I

INTRODUCTION

Microorganisms have long been known to play an intergral part in the nutrition of ruminant animals. The requirement of rumen microbes for specific nutrients has become an important area of investigation. In recent years considerable research has been conducted concerning minerals that would increase microbial activity in the rumen. Through increased rumen microbial activity, cattle and sheep can be expected to better utilize roughages and thus make more economical gains.

Extensive studies have shown that phosphorus is particularly important from the standpoint of efficient feed utilization by ruminants. It is believed that phosphorus stimulates rumen microbial activity which in turn enhances feed utilization. Insufficient data are available, however, to give a clear understanding of the influence of the phosphorus-microorganism relationship upon feed utilization.

The introduction of suitable artificial rumen techniques has enabled a deeper understanding of the mineral requirements of rumen microorganisms. In addition, it has presented an excellent method for the study of the requirements of phosphorus by rumen microorganisms. Although one can not extrapolate laboratory data to apply to the live animal, the artificial rumen technique is valuable as a preliminary method to explore rumen nutritional problems.

Several reports compare the effect of phosphorus from various phosphorus sources to ruminants. In many cases these sources were various

compounds of the same chemical form of phosphorus. No data are available comparing the ortho, meta, pyro, and phytate forms of phosphorus, which are the common chemical forms of this element. More complete knowledge of the availability of phosphorus from these chemical forms to rumen microorganisms would be of basic interest and would, in all probability, be of practical importance.

There were three objectives of the present investigation: (1) to determine if rumen microorganisms were critical in their requirement of phosphorus as to amount and source; (2) to determine if the ortho, meta, pyro, and phytate forms were utilized by rumen microorganisms; and (3) to determine the relative availability of the above forms.

CHAPTER II

REVIEW OF LITERATURE

Phosphorus has been shown to influence the activity of rumen microorganisms materially both *in vitro* and in the live animal. The addition of phosphorus supplements to rations of ruminants has been a common practice. Extensive research has been conducted with poultry and laboratory animals comparing the availability of phosphorus from various sources. Some studies have been made comparing the availability of different phosphorus sources for ruminant animals.

Early biochemists and nutritionists recognized the importance of phosphorus as a constituent of plant and animal life. Forbes and Keith (1914) reviewed the literature on phosphorus in animal metabolism and published a literature review and bibliography of some seven hundred pages. This gives some insight into the volume of early research done on this mineral.

A review of the broader aspects of phosphorus in ruminant nutrition will not be undertaken in this thesis. The present literature review is primarily concerned with the procedures and results of experiments that deal with the availability of phosphorus sources to ruminants and to rumen microorganisms.

Many of the earlier research workers, who studied phosphorus deficient animals, observed that they sometimes chewed old bones. Logically the first phosphorus supplement fed to livestock was bone meal. However, as ranchers, stockmen, and feed manufacturers realized the value of

phosphorus in feeding ruminant animals, the quantity of available bone meal could not meet the demand. The quest for other phosphorus sources brought on the market a number of phosphorus bearing materials. Several experiments have been conducted to compare or evaluate these phosphorus sources.

Knox and Neale (1937) compared bone meal, monocalcium phosphate and dicalcium phosphate on the New Mexico range and found them to be equal in supplying the cattle needs and equal in palatability when mixed with salt.

Ammerman et al. (1955) fed a semipurified, low phosphorus basal ration to lambs. After a four week depletion period, dicalcium phosphate, Curacao Island phosphate (imported rock phosphate), soft phosphate in colloidal clay, and deflourinated rock phosphate were fed as supplements to the respective lots. The criterion used to evaluate the phosphorus sources was the amount of increase in blood serum phosphorus. Blood serum phosphorus levels, expressed in milligrams per cent increase above the control lot (no added phosphorus), for the supplements were as follows: dicalcium phosphate, 2.4 mg.; Curacao Island phosphate, 2.6 mg.; colloidal clay phosphate, 1.3 mg.; deflourinated rock phosphate 1.3 mg. The differences among the values were not statistically significant.

Using a balance technique with 550 pound steers, Ammerman et al. (1954) observed no significant difference in phosphorus retention between steers fed various inorganic phosphates as sources of phosphorus. Supplements tested were dicalcium phosphate, bone meal, deflourinated rock phosphate, imported rock phosphate, and colloidal clay phosphate.

Dicalcium phosphate and colloidal clay were evaluated in a feeding experiment conducted by Burroughs et al. (1956). Both phosphorus sources were added to fattening rations at a level of 0.33 per cent. Average daily gains were used as a response criterion. Steers receiving dicalcium phosphate in their ration gained 2.80 lbs. per head daily as compared to 2.44 lbs. per head daily for steers fed colloidal clay.

Three groups of eight heifers each were used by Long et al. (1956), to determine the relative value of soft phosphate with colloidal clay as an inorganic phosphorus supplement for growing beef heifers. The heifers were fed a basal ration which was sub-optimum in phosphorus content. Heifers in lot one received the basal ration whereas those in lot two and three were fed the basal ration supplemented with 0.05 per cent phosphorus as colloidal clay and as dicalcium phosphate, respectively. Principal criteria of response were weight gains, plasma inorganic phosphorus, and ash content of cannon bone and mandible. The differences in response at the end of a 98-day feeding period were statistically significant in favor of dicalcium phosphate. Heifers fed colloidal clay exhibited pica, coprophagy, and walked with difficulty. The heifers fed dicalcium phosphate were apparently normal in every way.

Steam bone meal, Curacao Island phosphate, and dicalcium phosphate were compared as sources of phosphorus with heifers in feeding trials by Long et al. (1957). One group of heifers was fed a basal ration containing 0.07 per cent phosphorus. Additional groups received the same ration to which phosphorus from the above sources was added to raise the total ration phosphorus to a level of 0.15 per cent. Heifers on the basal ration ate less feed, made smaller gains, and had lowered plasma phosphorus

levels than supplemented groups of heifers. Differences in performance among the groups of heifers receiving the various phosphorus supplements were small and not statistically significant. The results indicated equal availability of phosphorus in the three supplements.

Phosphorus has been shown to influence feed utilization by Eckles et al. (1926, 1927, 1932). Digestion trials by these workers showed that it took 116 to 150 per cent more feed to maintain body weight of phosphorus deficient animals considering Morrisons Feeding Standards as 100 per cent than for animals on an adequate level of phosphorus intake. Riddell et al. (1934) substantiated the findings of Eckles and his co-workers. Increased feed utilization has been accepted as a function of adequate phosphorus nutrition. It is interesting to note that some of the response to phosphorus observed by these workers could have been due to the phosphorus influence on rumen microorganisms. Burroughs et al. (1951), while studying the stimulatory influences of alfalfa ash, observed that the addition of phosphorus stimulated cellulose digestion by rumen microorganisms in vitro. Hall (1955), using an artificial rumen technique, showed that rumen microorganisms required between 50 and 200 p.p.m. of phosphorus for maximum cellulose digestion.

Phosphorus from dicalcium phosphate, acidulated phosphate, bone meal, Curacao Island phosphate, colloidal clay phosphate and a standard sodium-potassium phosphate mixture were compared as to their availability to rumen microorganisms by Anderson et al. (1956). Dicalcium phosphate appeared to be as available as the sodium-potassium phosphate mixture. The acidulated phosphorus product and steam bone meal were intermediate

in phosphorus availability while Curacao rock phosphate and the colloidal clay phosphate were less available. Not only did the rumen microorganisms in these experiments show response to inorganic phosphorus but they showed a greater response to certain sources which were more available to them. These workers proposed their artificial rumen technique as a method for determining the phosphorus availability of cattle and sheep phosphorous supplements.

Studies carried out on rats, swine, poultry, and dogs have indicated that phytic acid, an organic bound phosphorous compound found in plants, is not completely utilized by fowls and simple stomach animals. Common (1939) observed that pullets failed to hydrolyze a considerable proportion of the phytic acid present in their rations. Growing-finishing swine did not utilize plant phosphorous as efficiently as inorganic phosphorous as shown by Chapman et al. (1955). Mellanby (1949) reported that phytin phosphorous had a ricket-producing and an anti-calcifying action when fed to dogs. Using adult birds, Gillis et al. (1953) reported that phytin phosphorous was approximately one-half as effective as inorganic phosphates in supplying phosphorous. Earlier work by the same workers (Gillis et al. 1949) gave evidence that phytin phosphorous was not efficiently utilized by the young chick. Rats were shown to utilize phytin phosphorous poorly in the absence of vitamine D in work by Boultwell et al. (1946).

Cattle and sheep, unlike the simple stomach animals, have been shown to utilize phytin phosphorus very efficiently. Hart et al. (1927) observed that feeding phytin phosphorus in wheat bran brought recovery from phosphorus deficiency symptoms in dairy cows. Using a radio active isotope

of phosphorus as a tracer, Lofgreen and Kleiber (1953) differentiated body orgin fecal phosphorus from fecal phosphorus of feed orgin in lambs fed on alfalfa hay. They showed that phosphorus in alfalfa hay was 88 per cent utilized by the lambs.

Australian work by Reid et al. (1947) indicated that phytates ingested under various conditions of calcium and phosphorus intake were completely hydrolyzed in the alimentary tract of sheep. These workers slaughtered sheep which had been fed on three different high cereal rations for several days. The contents of the rumen, reticulum, abomasum, small intestine, and rectum were removed and subjected to chemical tests. They found no phytin phosphorus further along in the digestive tract than the rumen. These workers concluded that phytin phosphorus had been completely hydrolyzed by enzymes produced by the rumen microorganisms.

Washed suspensions of rumen microorganisms were used by Raun et al. (1956) to study the availability of phytate phosphorus to rumen microorganisms. Cellulose digestion was the criterion of response. Phytin phosphorus appeared to be close to 100 per cent available to the bacteria. It thus appeared that the rumen microorganisms produced the enzyme, phytase, which was capable of hydrolyzing the phytate molecule.

CHAPTER III

EXPERIMENTAL

An artificial rumen technique employing washed suspensions of rumen microorganisms was used in these studies. The technique was a slight modification of that described by Cheng et al. (1955) and Anderson et al. (1956). Details of the technique are described below.

Fistulating the Steer

A representative sample of rumen microorganisms is the basis of the artificial rumen technique. A port of entry into the rumen was needed to facilitate the collection of such a sample. This need was met by one of the college staff veterinarians who prepared a fistula in the side of a Hereford steer. The surgical procedure was carried out in two steps: (1) the entry through the abdominal wall; (2) the entry through the rumen wall.

In preparation for the first operation the animal was held off feed for twenty-four hours to reduce the amount of fill. The steer was held firmly for the operation in a metal stanchion. Prior to making the incision the area to be fistulated was shaved and locally anesthetized with procaine-hychrochloride. A circular hole, approximately five inches in diameter, was made in the left side, below the loin and posterior to the last rib. The epidermis about the rim of the hole was sutured to the protruding exterior of the rumen. Medication was applied to the traumatized area which was then covered by a cotton compress. A sixteen day waiting period permitted healing of the epidermis to the rumen.

The first step in the second operation was to anesthetize locally. The sutures of the first operation were removed and the area was examined to insure that the healing was continuous around the fistula rim. The fistula was then extended through the rumen wall by the removal of the portion of the rumen wall within the ring of sutures of the first operation. A plastic cannula, described by Stoddard et al. (1951) with inside and outside retaining flanges was assembled in the fistula. The opening was then closed by a screw-type plastic lid as is shown in Figure 1.

Artificial Rumen Technique

Rumen contents were obtained from a fistulated Hereford steer grazing on permanent pasture consisting mainly of blue grass, orchardgrass, and Ladino clover. A mineral mixture containing equal parts of dicalcium phosphate and common salt and located in a conveniently placed mineral box was available ad libitum to the fistulated steer. On sampling mornings, the steer was driven quitely to the barn along with the other cattle in the pasture and was released to the pasture as soon as the sample was taken.

The liquid portion of the rumen contents was strained through four layers of cheese cloth and collected in thermal-neutral containers. In a typical experiment 1200 ml. of rumen fluid were collected from the fistulated steer. The strained rumen fluid was centrifuged in a Servall angle centrifuge at about 1000 r.p.m. for one minute to separate the feed debris and protozoa from the rumen liquid. The feed debris and protozoa were discarded. The supernatant was next centrifuged at a speed of 5000 r.p.m.



Figure 1. The collection of rumen contents from a fistulated steer with plastic cannula and screw-type plastic lid.

for fifteen minutes and the centrifuge was then allowed to glide to a stop. The resulting supernatant was poured off and the remaining sediment consisting principally of rumen bacteria was suspended in 360 ml. of distilled water saturated with carbon dioxide gas. A waring Blendor was used to suspend the cells in the washing solution so that clumps of cells would be dispersed. This bacterial suspension was centrifuged again for fifteen minutes at 5000 r.p.m. and the sediment reconstituted in 360 ml. of carbon dioxide saturated distilled water. The washing process was repeated. The final sediment obtained from the third high speed centrifugation was suspended in 600 ml. of a nutrient solution prepared according to a formula described by Anderson et al. (1956) and shown in Table I.

Three grams of purified cellulose, a commercial product known as Solka-Floc, was added to the suspension making the concentration of this insoluble cellulose 0.5 per cent. The solution was bubbled with a stream of carbon dioxide for ten minutes. The pH was adjusted to 7.0 with a saturated solution of sodium carbonate. Nine milliliters of specially hydrolyzed feather meal by a process reported by Hall et al. (1954) was added to the suspension. The suspension was then incubated 24 hours in a one-liter Erlenmeyer flask fitted with a rubber stopper and inlet glass tube and outlet condenser tube. A constant flowing stream of carbon dioxide gas was passed through the flask to establish and maintain anaerobiosis as well as to agitate the fermenting suspension. The flask was incubated in a constant temperature water bath thermostatically controlled at 39° Centigrade. The purpose of this 24-hour preliminary digestion period was to deplete any phosphorus that was not removed in the washing process or any

TABLE I

COMPOSITION OF NUTRIENT SOLUTION

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

phosphorus impurities in the basal medium.

At the end of the 24-hour preliminary fermentation period, one-half of the contents of the flask was discarded and the flask made up to 600 ml. by adding phosphorus deficient basal medium and three grams of cellulose. The suspension was again bubbled with carbon dioxide gas and the pH was adjusted to 7.0 with sodium carbonate. Aliquotes of 20 ml. each of the suspension were pipetted into 75-milliliter centrifuge tubes containing graded amounts of phosphorus from various sources. Each tube also contained 0.3 ml. of feather meal hydrolysate. The tubes were incubated at 39° Centigrade for twenty hours. Each tube was fitted with a rubber stopper and inlet and outlet glass tubing for bubbling the solution with a constant flowing stream of carbon dioxide gas. The apparatus for these studies is shown in Figure 2.

Zero control tubes were prepared at the beginning of the last digestion period. Twenty milliliters of the rumen bacterial suspension were aliquoted to the zero control tubes in the same manner as aliquoted to the digestion tubes. Microbial activity in the zero control tubes was stopped immediately by addition of 12.5 ml. of glacial acetic acid. The tubes were then set aside for chemical cellulose determination later. At the end of the 20-hour fermentation period, chemical determination for cellulose was made on the tubes by the method described by Crampton and Maynard (1938) with slight modifications. An average of the cellulose content of the zero control tubes represented the initial cellulose in the digestion tubes. Digestion coefficients were calculated for each tube.

Raun and coworkers (1956), using basically the same technique as

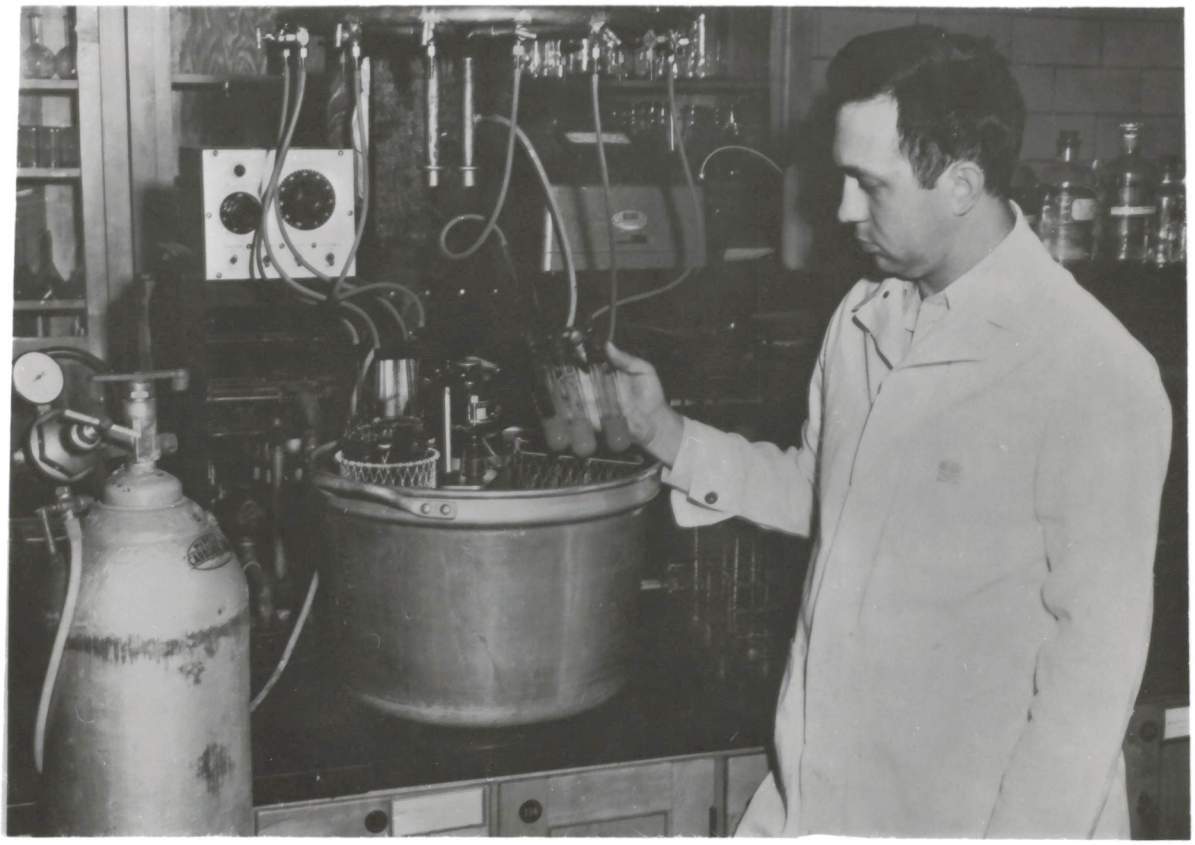

Figure 2. Apparatus for studying rumen microbial cellulose digestion in vitro.

described above, found that the length of time of the second digestion period was an important factor in the utilization of phosphorus by rumen microorganisms. This observation had important implications to the study of the availability of phosphorus by the artificial rumen technique. It was believed that a less available phosphorus source would be utilized as completely as an available source of phosphorus if the incubation was permitted long enough. Hall (1955) found that the hourly cellulose digestion rate by rumen microorganisms was more rapid between sixteen and twenty hours. In light of the above discussion, twenty hours were selected as the incubation time in these experiments. This was a slight modification of the digestion time used by Anderson et al. (1956).

Except for the phosphorus added in the treatments, the incubating tubes must be as devoid of phosphorus as possible to be critical of the element. All possible steps were taken to provide this condition. The tubes were washed in mild hydrochloric acid solution and rinsed twice in distilled water to dislodge any phosphate ions that might have adhered to the glass surface. This process was carried out prior to the beginning of each experiment.

Phosphorus Sources

The ortho, meta, pyro, and phytin forms of phosphorus were evaluated in these experiments. The sources used were chemical reagent grade materials, which were made to known concentrations in distilled water. These solutions were pipetted in desired levels into the tubes. A standard phosphorus source, which was a mixture of two orthophosphate compounds, was the fifth source used. These compounds were used in the

same proportion as they were used in the nutrient solution of Cheng et al. (1955) The standard source was the same as the standard source used in earlier work by Anderson et al. (1956).

Since phosphorus salts are named for the parent acid from which they are derived, one phosphate salt was used to represent each of the above forms. The orthophosphoric acid form was represented by its monosodium salt. The pyro-phosphoric acid form was represented by its sodium salt. The metaphosphoric acid form was represented by a hexa-multiple complex of its sodium salt. The phytin acid form was represented by its calcium salt.

The ortho, meta, and pyrophosphates have in common that they were all derived from the same phosphoric acid anhydride (P_2O_5). The phytin form is a hexaphosphoric ester of an organic compound known as inositol. The elemental phosphorus in each of the four forms has a plus five electron valence.

Text by Mack et al. (1949) reported that sodium hexametaphosphate, the meta source that was used in these studies, ionized only partly. A diagram of this ionization is as follows: $Na_6P_6O_{18} \rightarrow 2\ Na^+ + Na_4P_6O_{18}^{--}$. Sodium hexametaphosphate has the property of reacting with calcium or magnesium ions in such a way as to include them in the above complex ion, thus removing them from the solution. Since the nutrient solution contained magnesium, this reaction must have taken place. It was of interest to this study, to see if the rumen microorganisms were capable of breaking down this complex and obtaining the phosphorus and magnesium they needed.

The organic bound phytate phosphorus did not completely go into solution in distilled water. However, when agitated the phytate particles

went into a colloidal suspension which enabled pipetting. Very little variation was observed in the first experiment between triplicate tubes within treatment. This technique of aliquoting phytate was contained throughout the experiments.

Each phosphorus source was tested singularly in a series of experiments at 0, 20, 40, 60, 70, 80, 90, and 100 mcg. per ml. of basal medium. Triplicate tubes were used at each level. Two experiments each were run for the ortho, meta, and pyro phosphates. Three experiments each were run for the standard and phytate phosphates.

All of the above phosphorus sources were also compared simultaneously at levels of 0, 20, 40, and 60 mcg. per ml. of basal medium in three different experiments. Duplicate tubes were used at each level in these three experiments. The data were subjected to an analysis of variance according to Snedecor (1957) and the least significant difference (L.S.D.) was determined for each experiment. Cellulose digestion coefficients for the individual tubes were used for the statistical analysis.

CHAPTER IV

RESULTS AND DISCUSSION

The effects of various levels of standard phosphorus upon cellulose digestion by rumen microorganisms are shown in Table II. The addition of 20 mcg. of phosphorus per ml. of basal medium resulted in a highly significant ($P < 0.01$) increase in cellulose digestion. When compared with the 20 mcg. level, the addition of 40 mcg. of phosphorus per ml. of basal medium in one experiment gave a significant ($P < 0.05$) increase in cellulose digestion but this increase was not observed in subsequent experiments. Except for this one increase, which may have been due to chance variation, no appreciable response was observed from additional levels of the standard phosphorus above the 20 mcg. level.

Cellulose digestion in the control tubes (no added phosphorus) was assigned a value of 100 per cent. The response to the addition of phosphorus was calculated as a per cent increase over the control and is shown graphically in Figure 3.

The response of rumen microorganisms to graded levels of monosodium orthophosphate is summarized in Table III. A marked increase in cellulose digestion by rumen microorganisms was observed upon addition of 20 mcg. of phosphorus per ml. of basal medium. This response was highly significant ($P < 0.01$). Slight increase in cellulose digestion were observed at levels above 20 mcg. but these increases were not statistically significant. Figure 4 shows the average cellulolytic response to graded levels of orthophosphate. The response curve of orthophosphate compared very closely to

TABLE II

EFFECT OF PHOSPHORUS FROM A STANDARD PHOSPHORUS SOURCE[a] UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO

Level of added Phosphorus	Cellulose Digestion Coefficients[c]			
	Experiment I	Experiment II	Experiment III	Average
Mcg./ml.[b]	%	%	%	%
0	50.5	56.5	65.9	57.6
20	69.8	68.6	76.1	71.5
40	76.1	69.7	74.3	73.2
60	69.0	71.9	72.7	71.2
70	74.0	71.2	75.1	73.4
80	77.9	73.4	75.5	75.6
90	75.4	71.3	76.7	74.5
100	64.7	70.1	75.6	70.1
L. S. D. (5% level)	7.7	6.8	2.8	12.0
L. S. D. (1% level)	10.7	9.4	3.8	16.7

[a] A mixture of KH_2PO_4 and Na_2HPO_4 routinely used in basal media for rumen microorganisms.

[b] Micrograms of phosphorus per milliliter of phosphorus deficient basal medium.

[c] Each value is an average of three replicates.

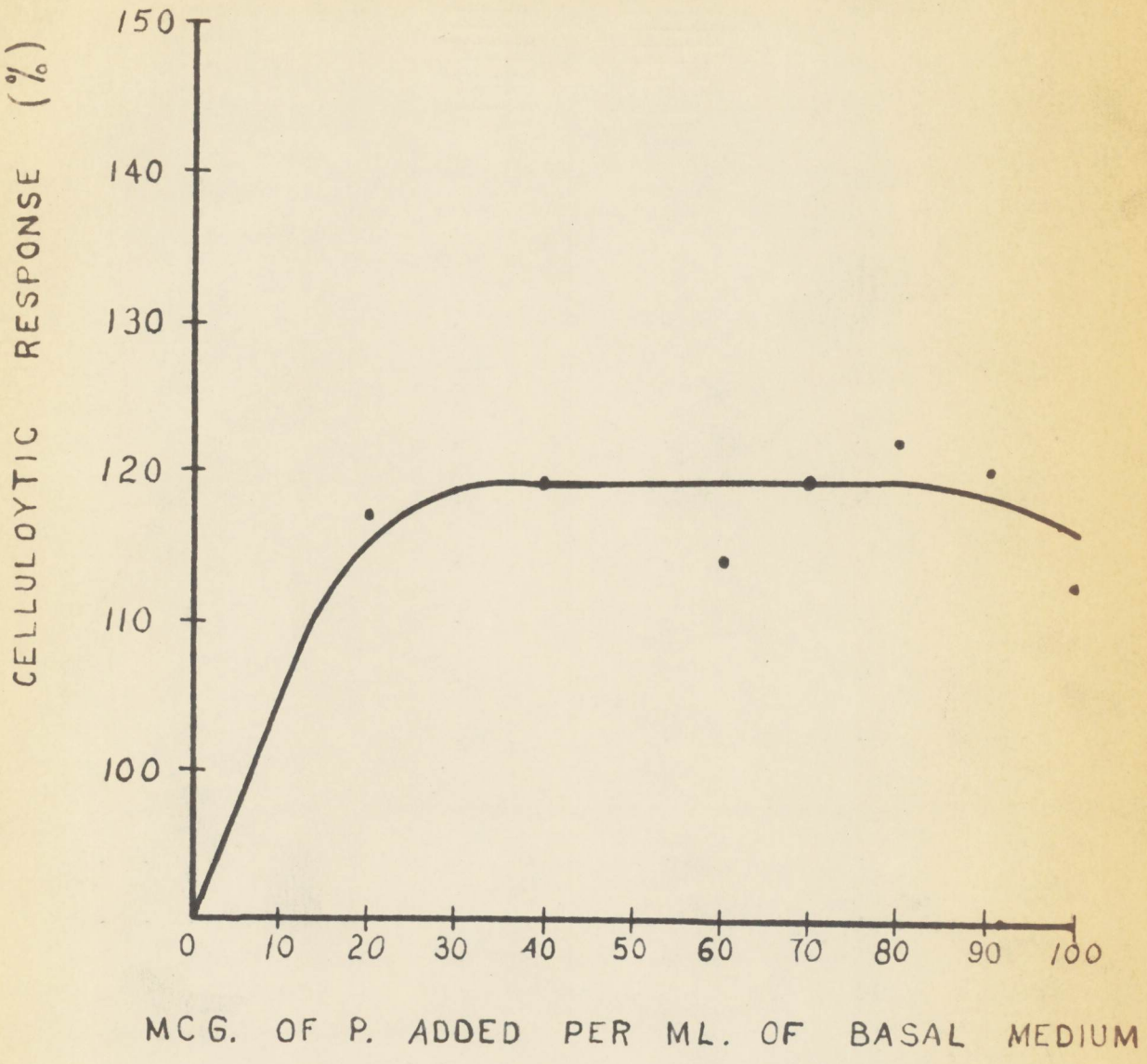

Figure 3. Cellulolytic response of rumen microorganism to graded levels of standard phosphorus

TABLE III

EFFECT OF PHOSPHORUS FROM MONOSODIUM ORTHOPHOSPHATE UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO

Level of Added Phosphorus	Cellulose Digestion Coefficients[b]		
	Experiment I	Experiment II	Average
Mcg./ml.[a]	%	%	%
0	22.6	16.9	19.7
20	61.9	34.4	48.1
40	60.1	36.8	48.4
60	62.8	31.2	47.0
70	66.5	40.8	53.6
80	66.3	40.4	53.3
90	66.9	41.1	54.0
100	71.4	31.5	51.4
L. S. D. (5% level)	4.7	5.9	15.8
L. S. D. (1% level)	6.4	8.1	----

[a]Micrograms of phosphorus per milliliter of phosphorus basal medium.

[b]Each value is an average of three replications.

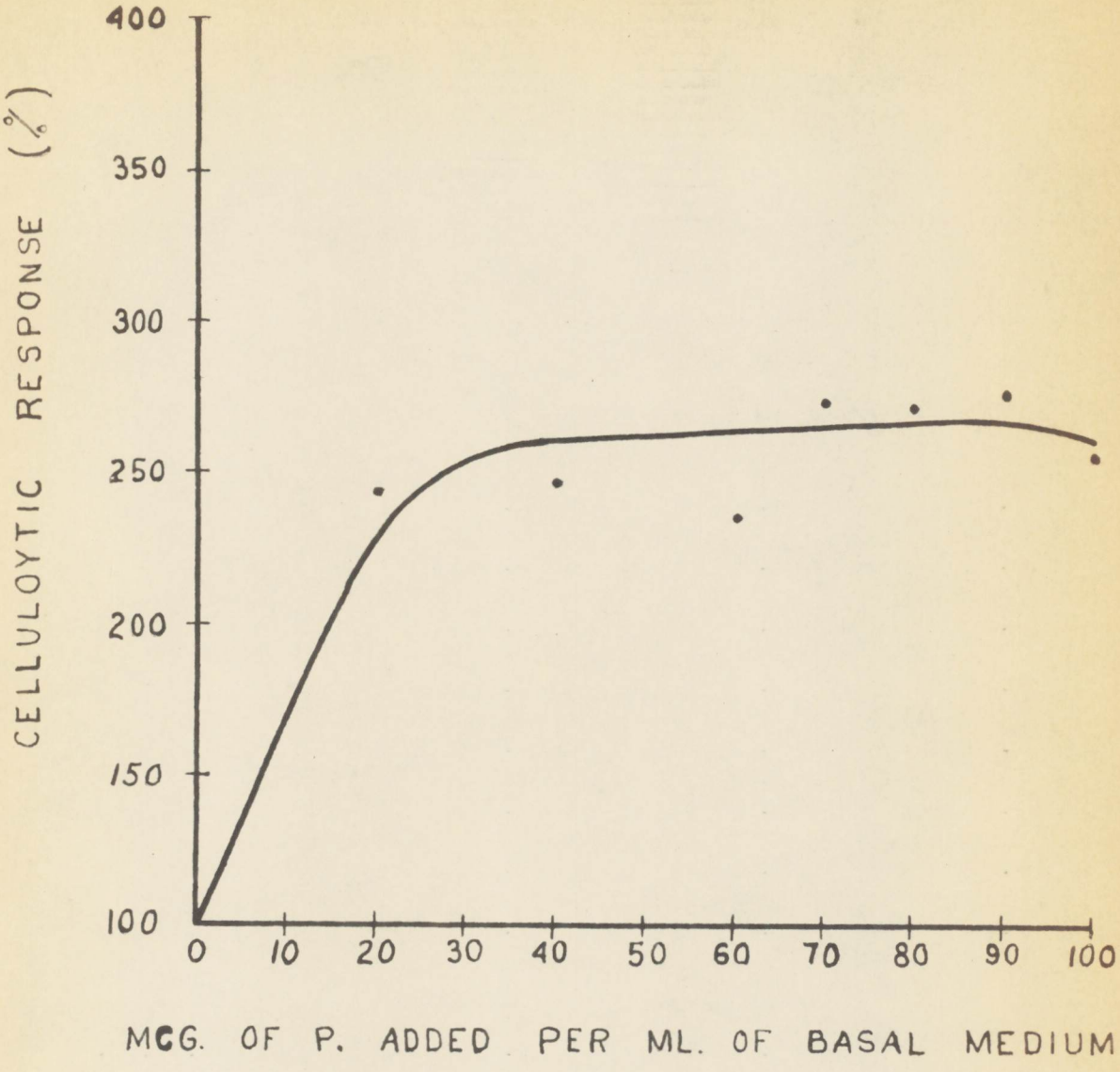

Figure 4. Cellulolytic response of rumen microorganism to graded levels of monosodium ortho phosphate.

the response curve of the standard phosphate. This had been expected since the standard phosphate was a mixture of orthophosphates.

As shown in Table IV and Figure 5 the addition of 20 mcg. of phosphorus from sodium acid pyrophosphate per ml. of basal medium resulted in a highly significant (P 0.01) increase in cellulose digestion. In Experiment I there was a highly significant (P 0.01) increase in cellulose digestion at the 40 mcg. level when compared to cellulose digestion at the 20 mcg. level. This same trend was apparent in Experiment II but the difference in cellulose digestion at the two levels of phosphorus addition was not statistically significant. A progressive upward trend in cellulose digestion was observed as the amount of pyrophosphate added was increased above 40 mcg. per ml. of basal medium.

The response of rumen microorganisms to graded levels of sodium hexametaphosphate is summarized in Table V. The response to the addition of 20 mcg. of phosphorus per ml. from this source resulted in a highly significant (P 0.01) increase in cellulose digestion. The increased response at the 40 mcg. level when compared with the 20 mcg. level was also significant (P 0.05). Further additions of phosphorus from this source above the 40 mcg. level did not appreciably increase cellulose digestion. Figure 6 shows the average cellulolytic response to graded levels of sodium hexametaphosphate. The increased microbial activity which occurred in the presence of this source of phosphorus is evidence that rumen microorganisms can utilize phosphorus from the sodium hexametaphosphate complex. The high cellulose digestion coefficients at the 100 mcg. level seems proof that the sodium hexametaphosphate complex did not

TABLE IV

EFFECT OF PHOSPHORUS FROM SODIUM ACID PYROPHOSPHATE UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISM IN VITRO

Level of added Phosphorus	Cellulose Digestion Coefficients[b]		
	Experiment I	Experiment II	Average
Mcg./ml.[a]	%	%	%
0	27.8	11.9	19.8
20	61.9	30.3	46.1
40	81.8	37.3	60.0
60	87.0	36.3	61.6
70	88.7	30.2	59.4
80	85.4	40.3	62.8
90	90.5	40.7	65.6
100	90.3	49.4	69.8
L. S. D. (5% level)	3.3	7.6	20.8
L. S. D. (1% level)	4.5	10.5	----

[a]Micrograms of phosphorus per milliliter of phosphorus deficient medium.

[b]Each value is an average of three replicates.

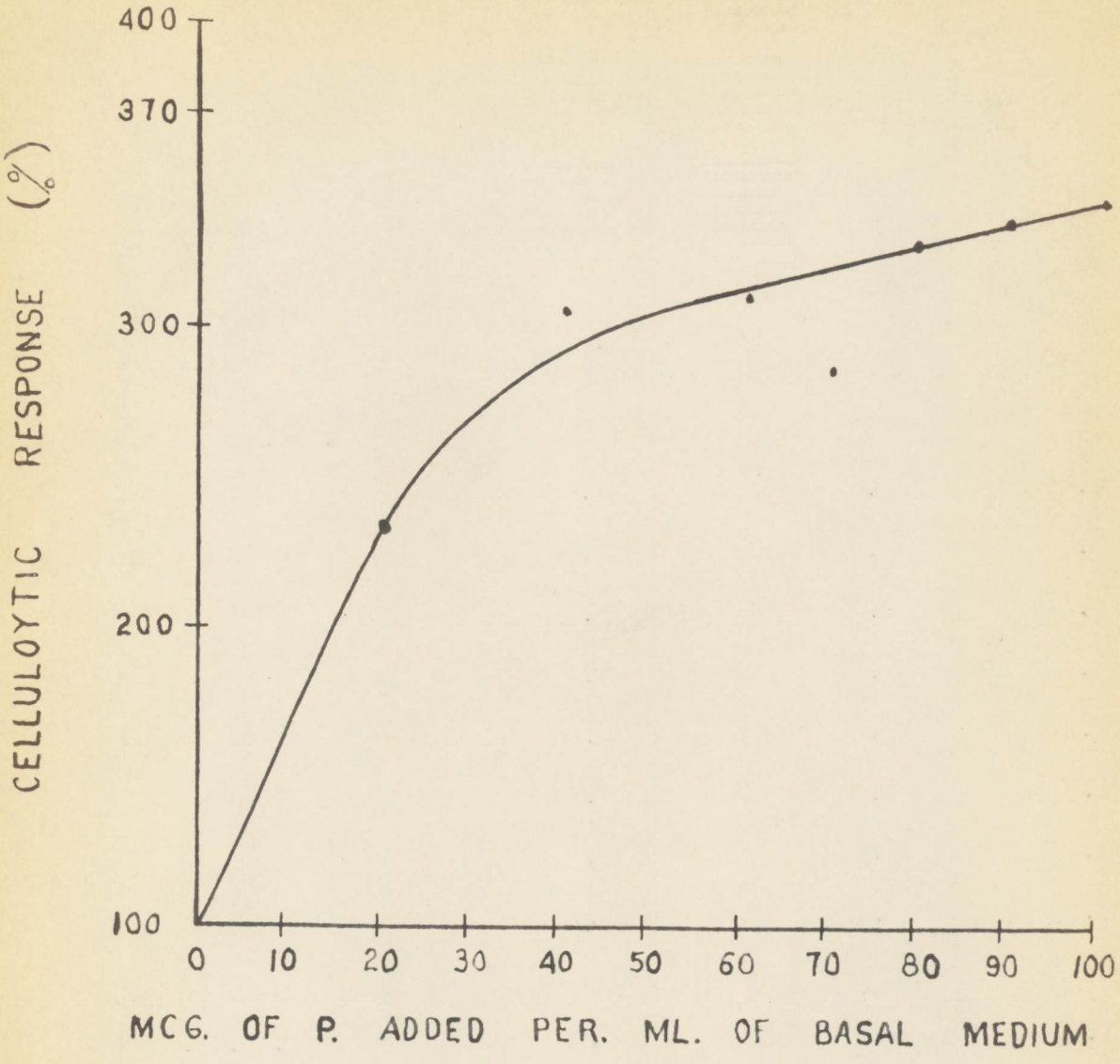

Figure 5. Cellulolytic response of rumen microorganism to graded levels of sodium acid pyrophosphate.

TABLE V

EFFECT OF PHOSPHORUS FROM SODIUM HEXAMETAPHOSPHATE UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO

Level of Added Phosphorus	Cellulose Digestion Coefficients[b]		
	Experiment I	Experiment II	Average
Mcg./ml.[a]	%	%	%
0	51.9	33.8	42.8
20	77.3	76.8	77.0
40	90.5	87.8	89.1
60	91.1	85.8	88.4
70	91.8	87.6	89.7
80	92.6	87.8	90.2
90	91.5	88.1	89.8
100	92.6	88.8	90.7
L. S. D. (5% level)	2.1	1.7	9.6
L. S. D. (1% level)	2.9	2.4	14.2

[a]Micrograms of phosphorus per milliliter of phosphorus deficient basal medium.

[b]Each value is an average of three replicates.

effect microbial activity by making the magnesium unavailable in the basal medium.

A summarized statement of the response of rumen microorganisms to graded levels of the organic calcium phytate is shown in Table VI and Figure 7. A highly significant ($P < 0.01$) increase in cellulose digestion was obtained by the addition of 20 mcg. per ml. of phosphorus from this source. Two of three experiments showed a highly significant ($P < 0.01$) increase in cellulose digestion at the 40 mcg. level as compared to the 20 mcg. level. The increase in digestion at the 40 mcg. level exhibited in the third experiment was not significant. The addition of phosphorus from calcium phytate at levels above 40 mcg. per ml. of basal medium did not produce significant increases in cellulose digestion. These data substanuate the findings of Raun et al. (1955), who reported that rumen bacteria utilize the phytin form of phosphorus.

Cellulolytic response by rumen microorganisms to the five chemical sources of phosphorus previously discussed was compared simultaneously. The sources were compared at three levels: 20 mcg., 40 mcg., and 60 mcg. per ml. of basal medium. These three levels were selected because they bracketed the breaking points of the response curves of the respective phosphorus sources. The establishment of the response curves was one of the objectives of the trials for each individual phosphorus source discussed in the preceding paragraphs. The results of these experiments are summarized in Table VII and are presented graphically in Figure 8.

An average of three experiments in which all sources of phosphorus were tested simultaneously showed that the addition of 20 mcg. of phosphorus from calcium phytate resulted in 44.5 per cent of the cellulose

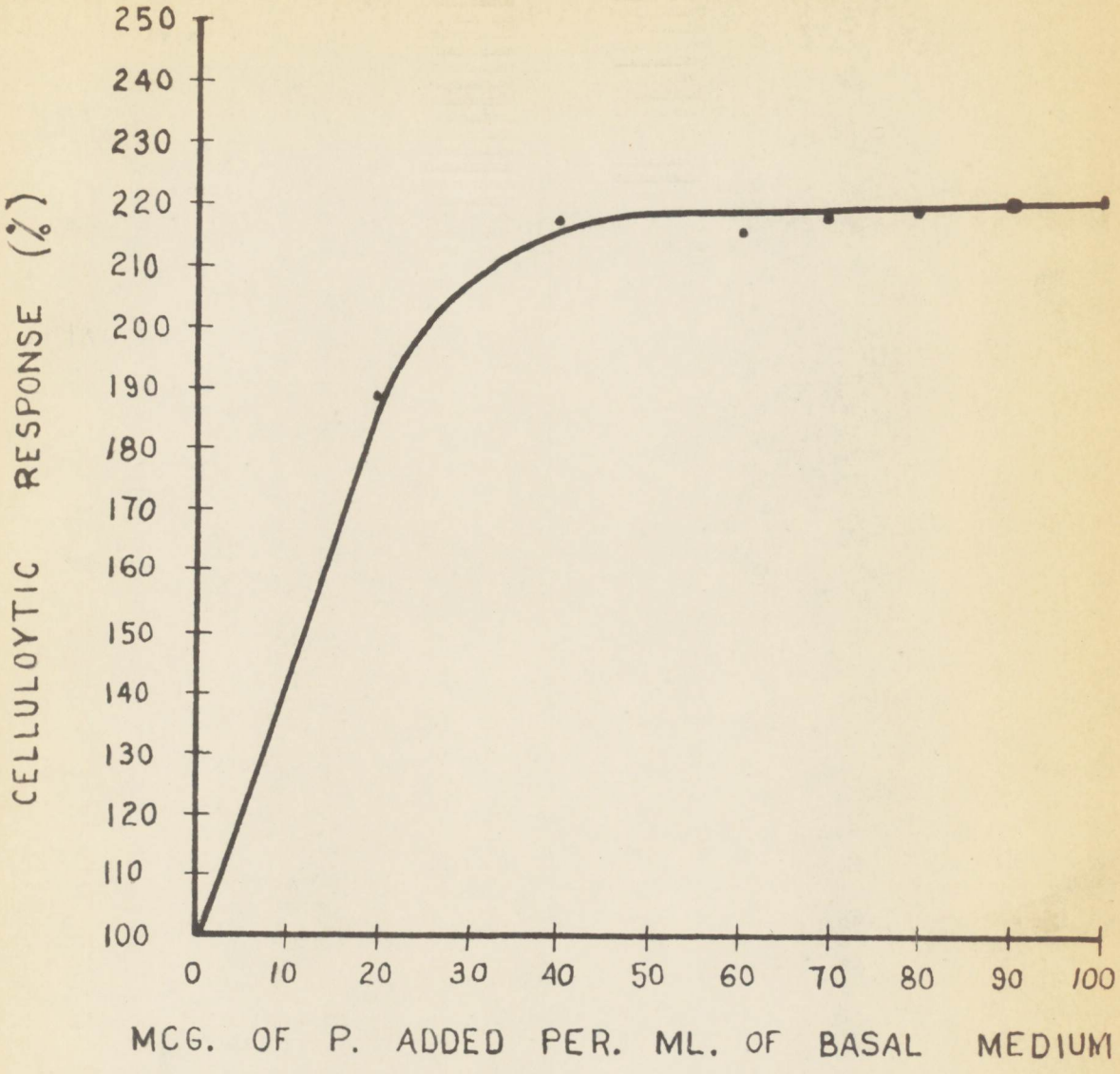

Figure 6. Cellulolytic response of rumen microorganism to graded levels of sodium hexameta phosphate.

TABLE VI

EFFECT OF PHOSPHORUS FROM CALCIUM PHYTATE UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO

Level of Added Phosphorus	Cellulose Digestion Coefficients[b]			
	Experiment I	Experiment II	Experiment III	Average
Mcg./ml.[a]	%	%	%	%
0	14.3	14.9	37.0	22.1
20	34.0	20.3	64.4	46.8
40	41.8	24.0	82.3	49.6
60	40.8	25.5	25.1	50.5
70	43.0	31.2	89.5	54.6
80	43.4	33.8	88.9	55.4
90	43.8	30.6	89.1	54.5
100	53.5	23.5	86.3	54.4
L. S. D. (5% level)	4.0	6.8	2.4	12.2
L.S.D. (1% level)	5.5	9.4	3.3	18.0

[a]Micrograms of phosphorus per milliliter of basal medium.

[b]Each value is an average of three replicates.

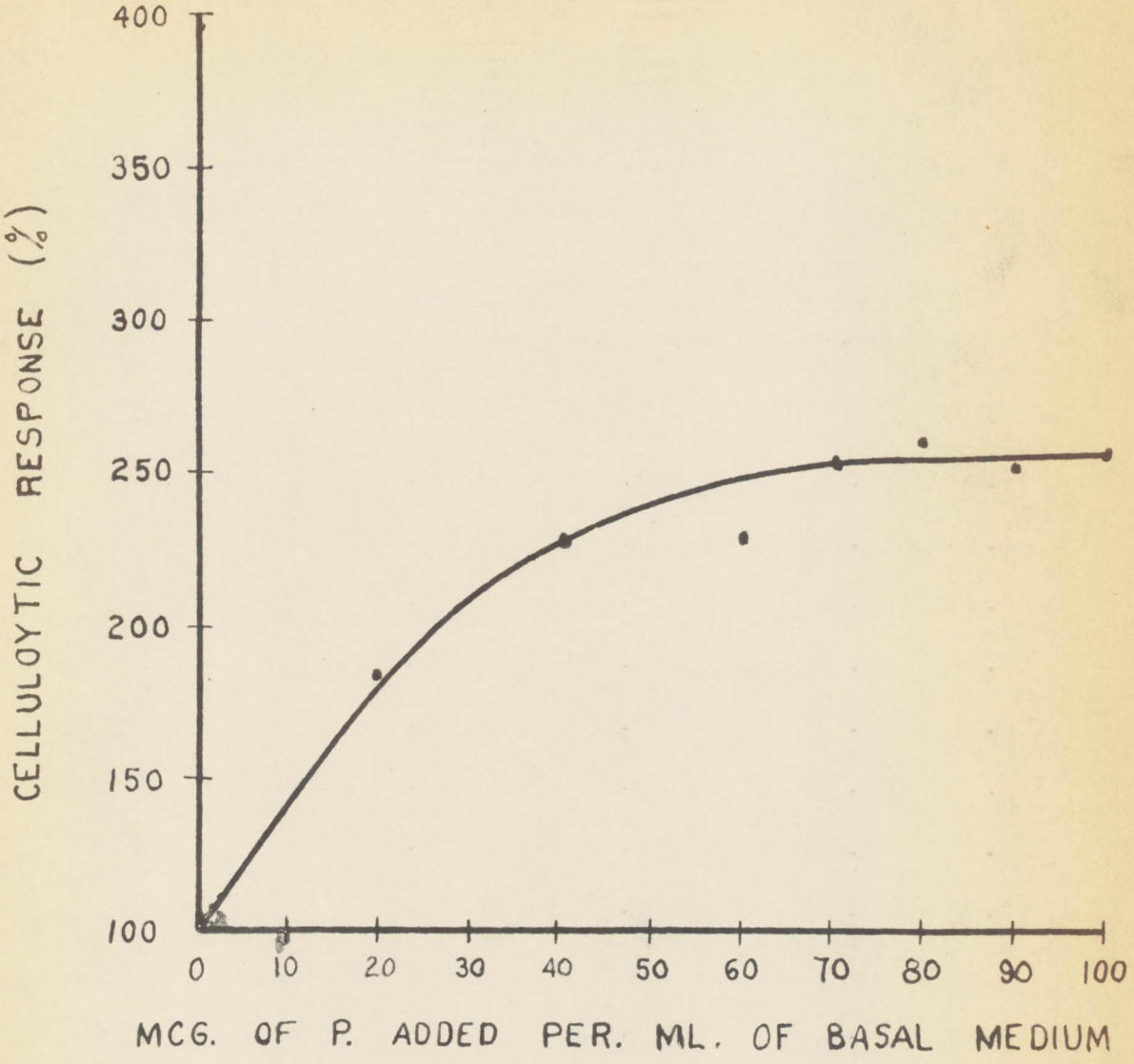

Figure 7. Cellulolytic response of rumen microorganism to graded levels of calcium phytate.

TABLE VII

EFFECT OF PHOSPHORUS IN DIFFERENT CHEMICAL FORMS AT VARIOUS LEVELS UPON CELLULOSE DIGESTION BY RUMEN MICROORGANISMS IN VITRO

Phosphorus Source	Level Added	Cellulose Digestion Coefficients[b]			
		Experiment I	Experiment II	Experiment III	Average
	Mcg./ml.[a]	%	%	%	%
--	0	37.9	17.5	32.3	29.3
Standard	20	66.8	46.0	48.9	53.8
	40	75.3	43.1	52.0	56.5
	60	76.3	47.3	53.1	59.0
Sodium Hexameta Phosphate	20	61.4	40.9	46.6	49.5
	40	73.7	48.2	48.1	56.7
	60	72.5	45.4	50.1	55.7
Calcium Phytate	20	54.4	29.5	44.6	44.5
	40	71.4	41.8	45.5	52.8
	60	73.0	45.6	53.1	57.2
Monosodium Ortho-phosphate	20	66.9	39.3	48.7	51.5
	40	75.5	46.1	53.3	58.2
	60	75.9	42.3	54.9	57.6
Sodium acid pyro-phosphate	20	69.2	46.0	50.3	55.0
	40	75.4	52.9	56.2	61.3
	60	73.8	50.0	55.0	59.5
L. S. D. (5% level)		5.0	6.0	4.9	5.4
L. S. D. (1% level)		6.9	8.2	6.7	7.3

[a]Micrograms of phosphorus per milliliter of phosphorus deficient basal medium.

[b]Each value is an average of duplicates.

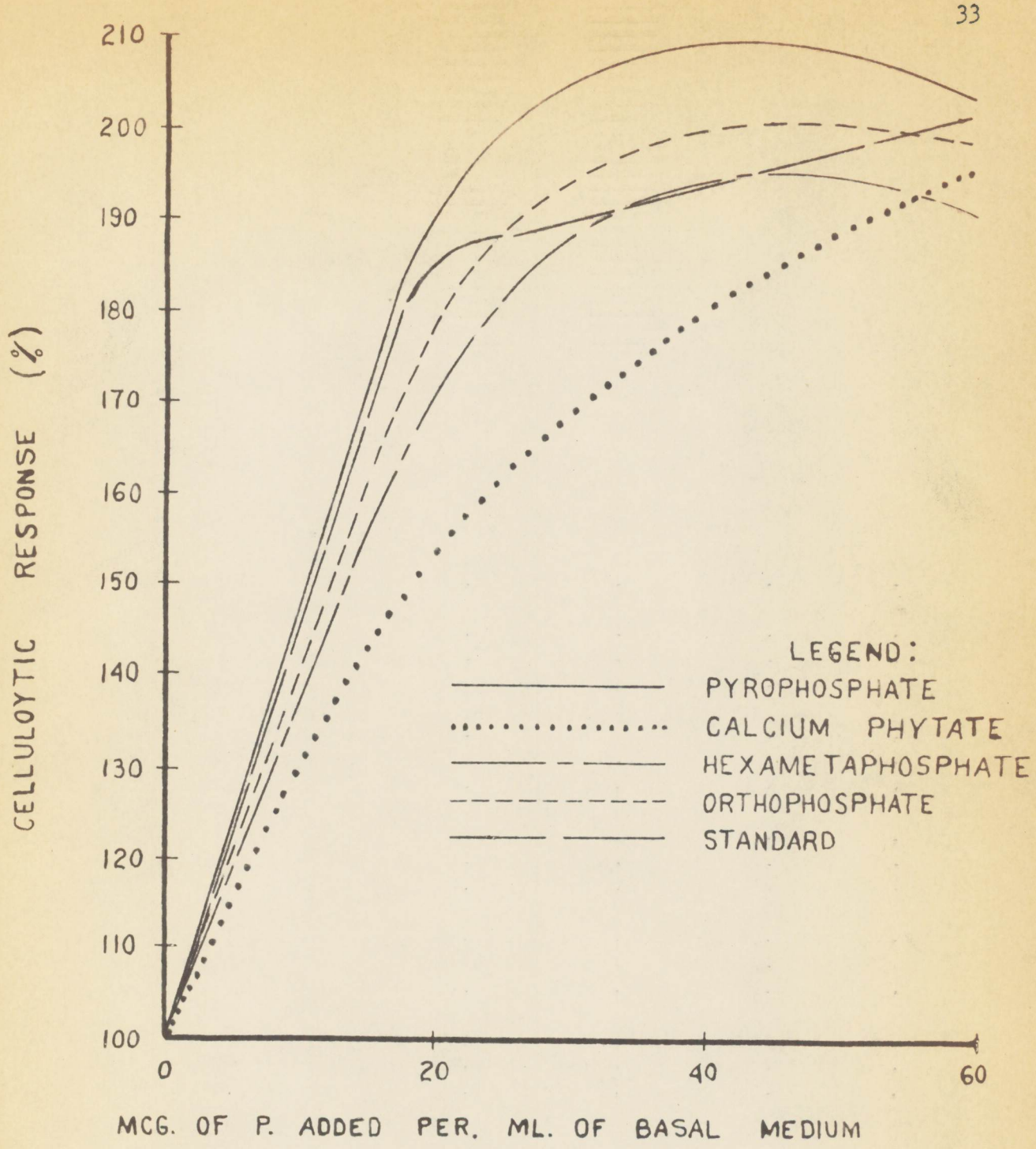

Figure 8. Cellulolytic response by rumen microorganisms to graded levels of phosphorus from various chemical forms *in vitro*.

being digested as compared to 50 to 55 per cent for the other forms of phosphorus. This difference in cellulose digestion by rumen microorganisms was statistically significant ($P < 0.01$). Differences in cellulose digestion coefficients resulting from the addition of meta, ortho, and standard phosphates at a level of 20 mcg. per ml. of basal medium were not statistically significant. However, the difference between pyro and meta phosphates was significant at this level.

Cellulose digestion coefficients resulting from additions of the various forms of phosphorus at a level of 40 mcg. per ml. level ranged from about 53 per cent for the phytate to 61 per cent for pyrophosphate. The difference in cellulose digestion resulting from phytate at this level and that of pyro and orthophosphate were also statistically significant ($P < 0.05$). No significant difference resulted when all phosphorus sources were added to fermentation tubes at a level of 60 mcg. per ml. of basal medium. All of these data have been interpreted to mean that phytin phosphorus can be utilized by rumen bacteria but that this source of phosphorus is slightly less available than is phosphorus from the other sources studied.

The results of all the data reported herein emphasizes the importance of phosphorus for maximum cellulose digestion by rumen microorganism. In many of the experiments two and three-fold increases in cellulose digestion resulted from addition of various chemical forms of phosphorus. Ortho, meta, pyro, and phytate forms of phosphates were all shown to be highly effective in stimulating cellulose breakdown by rumen microbes although the phytate and meta phosphates were slightly less effective than

the other forms.

The data also showed that the artificial rumen technique employed lends itself very effectively to phosphorus availability studies. Since it was shown that relatively little difference existed in the availability of phosphorus from some of the common chemical forms, it would be of interest in future experiments to study the effect that various processing methods have upon the availability of phosphorus from common phosphorus supplements for ruminants.

While the artificial rumen technique is of value and has definite advantages in rumen nutritional studies, it should be pointed out that results obtained in vitro are not directly applicable to the live animal and that such results should be corroborated with the live animal. However, it is felt that in vitro studies can help expand our knowledge of some of the fundamental as well as practical aspects of rumen nutrition.

CHAPTER V

SUMMARY

Washed suspensions of rumen microorganisms were used in a series of studies to determine the effects of adding various levels of phosphorus in different chemical forms upon cellulose digestion *in vitro*.

Samples of rumen liquid were collected from a fistulated Hereford steer which was permitted to graze a mixed blue grass-Ladino clover-orchard grass pasture. The rumen liquid was collected in thermal-neutral containers and taken immediately to the laboratory for processing. The rumen microorganisms were separated from the feed debris and rumen liquid by a process of differential centrifugation. They were washed twice with carbon dioxide-buffered distilled water before being suspended in a phosphorus deficient basal medium. After a 24-hour preliminary fermentation period in a liter Erlenmeyer flask, 20 ml. aliquotes were further incubated for 20 hours in 75-milliliter digestion tubes containing graded amounts of phosphorus from the various phosphorus sources.

Sodium orthophosphate, sodium pyrophosphate, sodium hexametaphosphate, and calcium phytate were the chemical compounds studied. The response to additions of phosphorus from 0 to 100 mcg. per ml. of basal medium was studied. The amount of cellulose digested in a 20-hour fermentation period was used as a criterion to evaluate microbial activity. Two and three-fold increases in cellulose digestion resulted from additions of each of the above phosphorus forms. Phosphorus from the ortho, meta, pyro, and phytate forms was utilized by the rumen microorganisms.

Results of experiments in which all of the above chemical forms

of phosphorus were tested simultaneously at 0, 20, 40, and 60 mcg. per ml. of basal medium showed that the phytate was less available ($P < 0.05$) than phosphorus from the other sources at the 20 and 40 mcg. levels. However, at the 60 mcg. level the phytate appeared to be equal to the ortho, meta, and pyro forms. The pyro form was significantly more ($P < 0.05$) available as shown by cellulose digestion coefficients than the meta form at the 20 mcg. level. The addition of 40 mcg. of phosphorus from pyrophosphate resulted in slightly higher cellulose digestion coefficient than the addition of a similar level of metaphosphate. No significant differences were observed between the pyro and the ortho, meta, and phytate forms at the 60 mcg. level.

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