FEED ADDITIVES AND ANIMAL WASTE PHOSPHOROUS REACTIONS

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ABSTRACT

Ph.D. G.M. Barnett Renewable Resources

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Organic phosphorus (P_o) in farm animal wastes must be mineralized to inorganic P for subsequent plant use. This study was conducted to determine if feed additives affect P_o mineralization, manure decomposition, and plant growth. Feed additives in aqueous systems affected the P mineralization of inositol hexaphosphate by phytase and of adenosine monophosphate by alkaline phosphatase. Pronounced effects were produced by bacitracin and both enzymes and by neomycin on phytase. Feed additives in dairy cattle (*Bos taurus* L.) manure produced effects on microbial activity as measured by gas production that differed from those produced on fecal phosphatase activity. Additives applied directly or with manure to Ste. Rosalie clay, Greensboro loam, or silica sand had no effect on barley (*Hordeum vulgare* L.) yield but did produce additive, rate, growth medium, and manure dependent effects on plant P concentration and soil phosphatase activity. Therefore, each feed additive must be independently evaluated to determine its effect on biological systems.

Key words: Fecal phosphorus, phytase, alkaline phosphatase, feed additives

RÉSUMÉ

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LES ADDITIFS DE MOULÉE ET LES RÉACTIONS PHOSPHORIQUES DANS LE FUMIER

Pour être assimilé par les plantes, il faut que le phosphore organique (P_o) dans le fumier soit minéralisé sous forme inorganique. Cette étude a examiné l'effet des additifs de moulée sur la minéralisation du P_o, la décomposition du fumier (*Bos taurus* L.), et la croissance des plantes. En systèmes aqueux, les additifs ont affecté la minéralisation de l'inositol hexaphosphate par le phytase et de l'adénosine monophosphate par la phosphatase alkaline. L'effet de la bacitracine sur les deux enzymes et celui de la néomycine sur le phytase étaient importants. Les activités phosphatasiques, fécales et microbiennes, telles que mesurées par la production de gaz, n'ont pas été affectées de la même façon par les additifs. Appliqués seuls ou dans le fumier, les additifs n'ont pas eu d'effet sur le rendement de l'orge (*Hordeum vulgare* L.) cultivé dans de l'argile Ste-Rosalie, de la terre franche Greensboro, et de la silice. Par contre, les effets dépendant de l'additif, du taux d'application, du milieu de croissance, et du fumier, ont été produits sur la concentration en P dans la plante et l'activité phosphatasique du sol. Alors, pour déterminer les effets sur les systèmes biologiques, il faut évaluer chaque additif.

Mots clés: Phosphore fécal, phytase, phosphatase alkaline, additifs de moulée.

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PREFACE

This document examines the proportion of the various phosphorous forms in animal manures and the effects of antibiotic feed additives on manure decomposition, phosphatase activity, and crop growth. The thesis is presented as a series of manuscripts preceded by an introduction and an overall literature review. The manuscripts are followed by chapters with an overall conclusion and contributions to knowledge. Chapters three, four, five, six, and seven are preceded by a connecting paragraph. The thesis is composed of the following chapters:

Chapter One: Introduction. This chapter establishes the P status of animal wastes and the use of feed additives by the farm industry.

Chapter Two: Literature Review: Manure P and Feed Additives. This chapter covers manure phosphorus, enzyme activity, agrochemicals, and feed additives, in relation to subsequent research objectives.

Chapter Three: Manure P Fractionation. This chapter examines extraction methods for forms of manure P. These modified methods were used in the subsequent research for this thesis.

Chapter Four: Phosphorous Forms in Animal Manures. This section reports results of P forms in dairy, beef, hog, and poultry manures. It discusses the results of an experiment that evaluated the effects of harvest date and hay species on calf manure P forms.

Chapter Five: Feed Additives and Phosphatase Activity. Experiments conducted to evaluate the effects of some feed additives on phytase and phosphatase activity in pure solutions of inositol hexaphosphate and adenosine monophosphate are reported.

Chapter Six: Feed Additives and Manure Transformations. This section reports the results of an experiment concerning the effects of pH on phosphatase activity in manure and of feed additives on short-term manure digestion under anaerobic conditions.

Chapter Seven: Feed Additives, Crop Growth, and Soil Phosphatase. The results of an experiment conducted to determine the influence of feed additives in manure on barley growth and soil phosphatase activity are reported in this chapter.

Chapter Eight: General Discussion.

Chapter Nine: Contributions to Knowledge.

The research results reported in chapters three to seven are based on hypotheses and experiments designed solely by the author. The laboratory and greenhouse work was conducted with the assistance of Richard Magny, Marc Benoit, and Gaston Dionne. The manuscript was written entirely by the author.

MANUSCRIPTS AND AUTHORSHIP[1]:

Candidates have the option, **subject to the approval of their Department,** *of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.*

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[1] Guidelines concerning thesis preparation. Faculty of Graduate Studies and Research, McGill University.

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CHAPTER 1

INTRODUCTION

1.1 Manure P

Since soils generally do not furnish sufficient P to meet crop requirements, supplemental P is applied as fertilizer and/or when available and practical, as animal wastes. Because the effectiveness of manure as a fertilizer replacement depends on P concentration, form, and availability, any factor influencing these characteristics will affect the value of animal wastes.

Although the actual amount depends on many factors such as age and state, quality of feed, and Ca level, domestic farm animals excrete some 40 to 90% of ingested P. Consequently, levels of less than 1 to 10 or more kg of P per tonne (wet basis) are found in animal wastes. Because of the large volumes generated, roughly 21 million tonnes in Quebec (7) and 129 million cubic metres in Canada (5), animal manures are an important P source. In Quebec this source amounts to about 31,000 and in Canada, perhaps 200,000 tonnes of P. At a fertilizer value of some $1.50 per kg of P, manure phosphorus is worth $46.5 million in Quebec and $300 million in Canada.

1.2 Form in manure

In animal manures, P is found in both organic and inorganic forms, the organic form varying from 20 to 80% of the total (3,6). Organic P is composed mainly of inositol hexaphosphate and nucleic acid type material plus small amounts of phospholipid P and traces of sugar phosphates and other forms. The relative proportion of these varies among species. For instance, grain is high in inositol hexaphosphate which is essentially unavailable to monogastric animals, since they do not possess the phytase enzyme necessary to decompose this form. By contrast, ruminants can digest this form of organic P. Therefore, feces from nonruminants contain more inositol hexaphosphate than ruminant fecal material.

1.3 Disposition of animal manure as a P fertilizer

Since animal waste is recycled to soils and plants and because plants use only inorganic P (8), it is important that organic P be converted to the mineral form. This is accomplished by an array of specific and nonspecific enzymes such as phytase, nucleases, and phosphatases.

The activity of enzymes has been shown to be affected both positively and negatively by agrochemicals (1).

1.4 Feed additives

The meat industry, especially the feeder cattle, hog, and poultry sectors, and to some extent the dairy sector, all use considerable amounts of feed additives (4). These include many types varying from clays to conditioners to antibiotics. Antibiotics are used at low levels as feed additives to improve rate of gain and feed conversion (4). However, significant amounts (2), if not almost all, are excreted in feces or urine.

1.5 Objective of this document

The question that therefore arises is, what are the effects of additives on manure decomposition? Biological oxygen demand measurements have shown that manure from chlortetracycline-fed animals decomposes more slowly and that the microbes are less active (2) compared with manure from non-treated animals. However, the effects of additives on fecal, organic P mineralization, in particular, does not appear to have been examined.

It was therefore the objective of this research to examine forms of P in animal wastes and to determine the effects of feed additives on the mineralization of some fecal organic P forms, on enzyme activity in manure and soil, and on crop growth.

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CHAPTER 2

LITERATURE REVIEW: MANURE P AND FEED ADDITIVES

2.1 Phosphorous sink

Animal wastes vary widely in P concentration both within and among species. Reported values of manure P from farms in the St. Francis River Valley (12) on a wet wt. basis were: dairy cattle from 0.59 to 1.96 kg t^{-1} with a mean of 1.21 and a coefficient of variation (CV) of 30%, beef cattle from 0.40 to 1.58 kg t^{-1} with a mean of 0.80 and a CV of 46%, pig from 0.08 to 3.64 kg t^{-1} with a mean of 1.16 and a CV of 98%, sheep from 1.39 to 3.04 kg t^{-1} with a mean of 1.97 and a CV of 33%, goat from 1.17 to 1.95 kg t^{-1} with a mean of 1.56 and a CV of 35%, and poultry from 3.13 to 11.78 kg t^{-1} with a mean of 8.63 and a CV of 25%. Clearly, animal wastes contain significant amounts of P and in Quebec totals about 31,000 t.

Almost all of this P is recycled to the soil-plant system. Since plants require P and because many Quebec soils are deficient in this element, its availability to plants is of considerable significance. Of particular importance is short-term availability of manure P for short-season crops.

2.2 Excretion of phosphorus by farm animals

Of the total P ingested by actively producing domestic farm animals, some 70% is excreted in wastes, although this will vary from about 40% to 100% (31). For instance, cattle excreted 70% of the ingested P, pigs 77%, layers 91%, and broilers 61%. The respective dietary levels were, with the recommended P levels in parentheses: cattle 0.60% ± 0.10 (0.50), hogs 0.75% ± 0.08 (0.55 - 0.60), laying hens 0.83 ± 0.10 (0.65), and broilers 0.80% ± 0.07 (0.65 - 0.80) (31). The proportion excreted depends on many factors some of which are: dietary levels, source of P, Ca, vitamin D, state of the animal, management, and other nutritional and physiological factors (9). However, it is clear that a significant proportion of ingested P is found in the animal waste.

2.3 Form of phosphorus in animal waste

Phosphorus in animal wastes occurs in both inorganic (P_i) and organic (P_o) forms. The organic form constitutes from 10 to 80% of the total P

in animal wastes (27), the proportion generally being higher in fresh manure and decreasing with age of manure. Results from long-term storage indicate that the ratio of P_i:P_o tends to be in the order of 4:1 (14,27). Therefore with time, mineralization of P_o occurs, the rate being influenced by storage conditions (15).

Manure P_o is found mainly as phospholipids (2%), inositol phosphates (up to 60%) and nucleic acid or residual phosphorus (up to 80%) (27). Much of the residual form has not been identified but is believed to be nucleic acid type compounds. The relative proportions of these forms vary according to source.

Most P_i is only slightly soluble and, based on solubility data, occurs as mono-calcium phosphate, apatites, and struvite (17). Although variscite and strengite may occur, the major part of Al and Fe would appear to be in organic complexes (17).

2.4 Plant availability

Total animal waste P has sometimes been found to be less available to plants on a short-term basis than equivalent amounts of fertilizer P (19,29). This is because the P_o must be mineralized to the inorganic forms of $H_2PO_4^-$ and $HPO_4^{=}$ for plant uptake (36). A wide C:P ratio may also reduce P availability in a fashion similar to the C:N ratio where an increase in C decreases N availability in soils or manure (36). Since the C:P ratio is increased by the presence of bedding, this may explain literature reports of manure P being less available than fertilizer P. Because of the lag in P_o mineralization, the requirement for fast growing plants cannot always be met from animal wastes at rates equivalent to those of mineral fertilizers, where P is largely water-soluble.

2.5 Enzymes

The mineralization of P_o is effected by enzymes known as phosphatases (5,14,33). The term "phosphatase" groups many different enzymes which derive their specific name from the substrate that is mineralized (32). Phosphatases belong to the hydrolase group.

These enzymes are long-chain proteins produced by living organisms. In any given system such as the soil or animal wastes, the total phosphatase activity is dependent on accumulated enzymes, either free in their environment or adsorbed on clays or soil organic matter (termed

abiontic, spelled with an "n" by Skujins (32)) and those associated with living organisms, mainly microorganisms, plant roots, and fungi. In addition, some enzymes occur in dead organisms in various stages of decomposition. Therefore there are essentially two sources of phosphatases: abiontic and those within living organisms, which would be mainly microorganisms.

2.6 Phosphatase in manure and soil

If Michaelis-Menten kinetics apply, the decomposition rate of organic phosphorus by phosphatase is described by the Lineweaver-Burk linear transform (42):

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[S]}$$

where v = observed velocity

V = maximum velocity

$[S]$ = molar substrate concentration

K_m = reaction constant

This model would apply strictly only to a single pure enzyme and not to systems involving numerous isoenzymes or groups of enzymes. Such systems occur in most complex media such as soils or manure. Although constants can be calculated for such systems, they represent a composite of those enzymes present which are particular to the given situation.

The reaction constant for the dephosphorylation of p-nitrophenyl phosphate in pig slurry has been measured at 680 to 1000 μmoles L^{-1} while in soils it was 140 to 175 (14). The maximum rate of decomposition was 700 to 1100 μmoles g^{-1} h^{-1} in pig slurry (dry weight basis) while in soil it was 80 in the 0 to 20 cm, 25 in the 20 to 50 cm, and 4 μmoles g^{-1} h^{-1} in the 50 to 100 cm layers. Thus phosphatase activity, although of the same order, was considerably higher in manure than in soil. The reduced activity in soil may be apparent rather than real because sorption of enzymes may increase the turnover time of substrate decomposition.

The enzyme concentration, which is proportional to V_{max} has been found to increase in pig slurry-soil mixtures after 30 weeks but not in inositol hexaphosphate-soil or soil itself (18). The K_m was significantly greater in pig slurry-soil than in the other two cases indicating an inhibitory effect of pig slurry. A decrease in water soluble organic P coincided

with an increase in phosphatase activity. Phytate P did not decrease as fast nor did the phosphatase activity increase as much over that of soil alone. Clearly, there are animal waste-soil interactions concerning phosphatase.

It is of interest to note that the centrifugation of pig slurry at 2000 *g* resulted in significant phosphatase loss in solution with complete loss at 4000 *g* (16). Therefore phosphatase is sorbed to the manure solids. Freeze-drying pig slurry caused no significant effect on phosphatase activity but air-drying soils reduced its activity. There was no difference in phosphatase concentration V_{max} at pH 5 or pH 8 in soils and manure thereby indicating one enzyme. The V_{max} was found to be strongly correlated with organic matter, organic and total P in soils, and to P_o and organic matter in slurry. Thus phosphatase activity in soils and manure is significant, is related to organic matter and P_o, and may be related to only one enzyme.

2.7 Agrochemicals and enzyme inhibition

The effect of an agrochemical on an enzyme – in soil, at least – will depend on the rate of decay of the chemical, the ability to react with the enzyme itself, whether the chemical is adsorbed or not, how tightly it is adsorbed, soil pH, water content, and other factors (5). It will depend not only on the global soil properties but also on those of the microsites within the soil. Adsorbed enzymes do not react in the same manner as free solution enzymes. Hydrolysis of a substrate will occur when the enzyme effect overcomes the matrix effect.

An inhibitory effect will occur if the inhibitor is an analogue of the substrate and reacts at the active site of the enzyme by binding covalently or noncovalently (10). Inhibitors that bind noncovalently are close analogues of the substrate and compete for the active site or fit an allosteric position on the enzyme and are in reversible equilibrium.

Steric hindrance may occur if the space around the enzyme is too small or wrongly orientated. The rate of substrate diffusion will also affect the interaction. If the substrate is opposite charged to the matrix it will be concentrated in the microenvironment around the enzyme if it is sorbed on the matrix thereby requiring less substrate to react with the enzyme. Similarly, like-charged substrate and matrix

necessitates higher substrate concentration for interaction. The clay mineral type will affect the buffer capacity of the soil which in turn affects the relative growth of different microorganisms (34) and hence enzyme production. Clays that have a higher buffer capacity will resist pH change and favor bacteria and actinomycete growth at the expense of fungae. The clay type can therefore influence the microbial population.

Agrochemicals likely affect enzymes in several ways (5). The microorganism may be physically altered reducing enzyme accumulation. The metabolism may be changed and if the chemical serves as a substrate, it will cause an increase in the extracellular enzyme. Numerous endocellular effects may be produced which influence the amount of endo and extracellular enzymes. These are protein synthesis, membrane structure (enzymes are bound to phospholipids in the membrane), lipid synthesis, membrane permeability, and growth regulator effects. If the microorganisms are killed, their lysis will lead to a temporary enzyme increase. Furthermore, if the adsorption of agrochemicals occurs on the same surfaces occupied by microorganisms, the ecological sequence will be affected (34) and thereby influence quantity and type of enzymes produced.

In normal farm practice, manure is stored (incubated) and therefore storage is an important factor in transformations. However, animal wastes are ultimately applied to the soil-plant system.

The application of agrochemicals to soils have no permanent effects on total soil populations (5). Their application may temporarily favor one class over another and therefore influence the amount, type, and rate of enzyme production. Agrochemicals may cause the death of sensitive microorganisms with the release of the cell contents upon lysis which may serve as substrate leading to the development of secondary populations. In fact all these effects would likely occur simultaneously. Thus, the agrochemical may be decomposed immediately or a lag may occur if the appropriate enzyme is absent and must be accumulated.

Therefore the application of an agrochemical to soil may influence the growth of microbial populations and change ecological sequences.

2.8 Effect of agrochemicals on phosphatase activity

It was found that 23 of 27 acid phosphatases isolated from germinating barley seeds treated with antibiotics, were sensitive to cyclohexamide and 16 to chloramphenicol while some were sensitive to both (26). The mechanisms are unknown but the antibiotics may affect the overall metabolism and in particular that concerning protein.

The miticide Kelthane (Rohm and Haas, Philadelphia, PA) was found to inhibit cell-wall phosphatase activity in cotton, which decreased resistance of susceptible varieties to verticillium (11).

The rate of inositol hexaphosphate dephosphorylation in solution was increasingly depressed by increasing rates of the herbicides atrazine, linuron, diuron, and the insecticides crotoxyphos, dichlorvos, and phosphamidon (6). The inhibitory effect decreased with increasing substrate concentration for all levels of inhibitors. The same authors have shown that many organic phosphates are hydrolysed by alkaline phosphatase with mineralization being affected by some vinyl phosphate insecticides (6); dichlorvos, tetrachlorvinos, crotoxyphos, and phosphamidon being competitive inhibitors.

The level of alkaline phosphatase in the duodenal loop of broiler chicks was reduced by 20 and 100 ppm dietary zinc bacitracin, the effect increasing with age (40). By contrast, dietary virginiamycin did not affect the activity of alkaline phosphatase in isolated enterocytes from the jejunum of broiler chicks (21). However, tetracycline administered by stomach tube to piglets at 50 mg kg^{-1} day^{-1} for 21 days reduced the alkaline and acid phosphatase activity in the jejunum, blood plasma, and liver.

Similarly, several examples of the effects of agrochemicals on phosphatase activity in insects can be found. For instance the insecticides Cyolane, Dursban, Phosvel, and Nuvacron caused inhibition of acid phosphatase in susceptible and Endrin-resistant strains of the cotton leafworm but activated it in Sumithion-resistant and field strains (30).

Acid phosphatase activity in the larval cells of *F. hepatica* was increased several times with Lebaycid, Chwastox, and Foschlor pesticides (23). Vapam had no effect while Lebaycid and Chwastox had intermediate effects with Foschlor being most effective. The effect was greater on

phosphatases than on other enzymes such as ß-glucuronidase and acetylcholinesterase.

Parathion increased acid phosphatase activity in the liver of rats but decreased hepatic phosphatase activity (7), these changes being more severe in protein deficient diets. Protein level and quality have been shown to affect pesticide toxicity in laboratory animals. Zinc deficiency in rats reduced not only the activity but also the quantity of alkaline phosphatase in serum and several cell tissues (1). However, sufficient Zn also reduced activity, probably through Mg displacement. Zinc deficiency inhibited activity in some tissues more than others and this is thought to be due to the existence of several isoenzymes, these occurring in different ratios in different tissues which are more or less sensitive. Magnesium deficiency affected the amount more than the activity of serum alkaline phosphatase and its isoenzymes (25). Inorganic pyrophosphatase was similarly affected.

Thus phosphatase is affected by factors in addition to agrochemicals and these may increase or decrease enzyme effects.

Penicillin and streptomycin inhibit soil phosphatase markedly (2). Copper and Zn are also strong inhibitors, Cu being more effective than Zn (38). Copper compounds are used to improve feed efficiency in hogs and 95% is excreted (4). Zinc carbonate or sulfate compounds are fed to hogs to control parakeratosis, which is a skin disease. Some of the Zn would be excreted in the manure.

Each soil type has its own enzymatic pattern (32). Often enzymatic activity is not correlated to other biochemical and microbial properties. Soil enzymes appear to be much less sensitive to factors that affect plant, microbial, or animal tissue enzymes (5). For instance malathion esterase is only affected by very high levels of heavy metals.

2.9 Feed additives

Feed additives are extensively used in the domestic animal industry (8,20). These include antibiotics, antibacterial agents, hormones, stabilizers, emulsifiers, anticaking agents, and many others (24). They are used to improve growth rate and/or feed efficiency, disease control, handling, preservation, flavour, and for other special purposes. In addition there are other compounds such as herbicides, fungicides, and

insecticides often contaminating feedstuffs by default (28). Naturally occurring substances that are ingested and affect animal performance are plant estrogens (37).

Most antimicrobial, antibacterial, antifungal, and other additives are used in the meat industry for the production of beef, hogs, poultry, and sheep (22). They may be used alone or in mixtures of 2 or 3 at levels of a fraction of a gram to 50 g t^{-1} (24). Some popular broad spectrum combinations used in the hog industry are chlortetracycline-penicillin-sulfonamide, tylosin-sulfonamide, and penicillin-streptomycin for the control of atrophic rhinitis, respiratory diseases, dysentery, and bacterial enteritis (24). These are fed at relatively high levels for short periods of time for the control of infectious diseases in the gastro-intestinal tract or body. As growth promotors, they may be used at low levels either continuously or intermittently.

At low levels over extended periods, typical improvement in gains are in the order of 2.5 to 5% with up to 20% in runt pigs (28). Beneficial effects tend to be more marked in young animals. For example growth rate and feed efficiency in calves was improved by 10 to 30% using tetracyclines.

The principal mode of action may be in controlling the indigeneous intestinal populations of *C. perfringens*, *S. fecalis*, and *E. coli* which produce toxins causing an increase in weight and thickness of the intestinal wall. This reduces nutrient absorption even on adequate diets (28). Antibiotics are believed to inhibit specific bacterial enzymes which do not exist in the cells of higher animals (23).

Production improvers include any compound which improves animal performance. A growth permittent is an antimicrobial agent which permits animals with depressed growth rates and feed efficiency caused by naturally occurring competitive intestinal bacteria, to realize their potential. A growth effector does the same by controlling clinical and sub-clinical diseases (24). These compounds differ widely in composition, bactericidal and bacteriostatic effects, absorption, metabolism, excretion, and in the microbial spectrum affected (20). Some of these compounds and their mode of action are listed in Table 1.

2.10 Fate of feed additives

It might be expected that once the additive had played its role in the animal, there would be no residual effect in the waste. There is some evidence to demonstrate that this is not the case.

Significant levels are often found in the feces. For instance, 12.5 $\mu g\ g^{-1}$ chlortetracycline (CTC) was found in the litter of chickens continuously fed this additive mixed in the feed (3). In cattle, 75% of the daily 70 mg dose of CTC was excreted as such to constitute 14 $\mu g\ g^{-1}$ in fresh manure and 0.34 $\mu g\ g^{-1}$ in aged material (13). The half-life was over 20 days at 4 or 28°C and 1 week at 37°C. Similarly, many other feed additives including other antibiotics, hormones, plant estrogens, and antibacterial agents are excreted in whole, in part, or as metabolites in animal waste, mainly in the feces (3).

Additives such as bacitracin, bambermycin, and virginiamycin are not absorbed from the gastro-intestinal tract (24). Their mode of action is to alter the bacterial population. Others such as the tetracyclines are absorbed and act against diseases in the tissues as well. Although unabsorbed additives are excreted in the feces, as more and more of the additive is absorbed, a greater proportion is found in the urine. However those absorbed and excreted in the bile, mainly as metabolites, are also found in the feces. Little is known about the metabolism of most additives in animals and even less about their activities in animal wastes (3).

2.11 Effects on animal waste decomposition

Some 38% more organic matter was oxidized in nonCTC-manure than CTC-contaminated manure (13). Microbes isolated from CTC-manure removed 40% more organic matter in nonCTC manure than in CTC-manure. Non CTC-manure microbes removed more organic matter in CTC-manure than CTC-manure organisms. This indicated that the microbial population in CTC-manure was less efficient either through selection or physiological modification. It was suggested that in addition, digestive processes in the CTC-treated animal may be altered rendering the manure less biodegradable. Although the mechanisms of action remain undefined, it is clear that degradation processes in animal waste can be affected by feed additives.

The residual effects of additives can be quite dramatic. For example, the treatment of a disease outbreak produced contaminated manure which resulted in digester kill (39). Recovery generally takes several months.

2.12 Effects on soils and plants

The application of poultry manure containing either 20 $\mu g\ g^{-1}$ amprolium or 22.5 $\mu g\ g^{-1}$ aureomycin at 250 or 750 mg dry manure per 50 g soil had no effect on N mineralization (41). Hog manure from animals treated with various antibiotics and applied to soils had no effect on oat yields at low N rates (manure) but as they increased, zinc bacitracin, oleandomycin-1, ocytetracycline and flavophospholipol caused yield reductions while oleandomycin-2, virginiamycin, and particularly carbodox caused yield increases (35). At the same time, N content in grain and straw were greatly increased by oxytetracycline and flavophospholipol while zinc bacitracin and virginiamycin depressed N contents. Thus manure contaminated by feed additives can have significant effects on crop growth and probably soil processes.

2.13 Conclusions

Animal wastes can contain a high proportion of the P in the organic state which must be mineralized to the inorganic form to become available to plants. This mineralization is accomplished by the phosphatase activity of microorganisms and abiontic enzymes and is affected by many factors: substrate concentration, time, moisture, oxygen, temperature, and inhibitors. Phosphatase activity in plants, animals, insects, yeasts, and soils has been shown to be influenced by inhibitors, mainly antibiotics, antibacterial agents, insecticides, and herbicides.

Domestic animals are fed low and high levels of growth promotors which include antibiotics, antibacterial agents, and growth hormones. Natural estrogens and toxins may also be ingested by default. It has been shown that all or part of these compounds are excreted unchanged, partially changed, or completely metabolized in animal wastes. It has also been demonstrated that manure contaminated with some of these additives can affect plant growth but the nature of these effects has not been established.

Therefore a subject of interest, and for which no published information has been found, is the influence of feed additives in animal waste on mineralization of fecal P_o, availability of manure P to plants, and reactions in the soil.

2.14 Hypothesis

Main hypothesis

It was therefore hypothesized that some unabsorbed (by the animal) feed additives will reduce phosphatase activity, delay the conversion of fecal P_o to the inorganic form, and reduce the short-term P availability from this source to plants.

Sub-hypothesis

1. Some unabsorbed feed additives will reduce the activity of phosphatase in the mineralization of organic compounds.

2. Some feed additives such as neomycins, bacitracins and streptomycins added directly to animal feces will reduce the short-term phosphatase activity, delay the increase in P_i, and consequently the short-term P availability to plants.

2.15 Objectives

The objectives therefore were:

1. To conduct an evaluation of the effects of several groups of non-absorbed feed additives on the phosphatase activity and mineralization of P_o, specifically phytin and a nucleic acid.

2. To evaluate the effects of selected unabsorbed feed additives applied to dairy cattle feces on phosphatase activity in the feces and in feces-soils mixtures, the increase in fecal P_i, and fecal P availability to plants.

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Table 1. Description of non-nutritive feed additives for swine, poultry, cattle and sheep.

Additive	Species	Mode of Production Improvement
Antibiotics		
Bacitracin[a]	Swine, Poultry, Cattle	Gram-positive, not absorbed, growth permittant, enteric diseases
Bambermycin	Swine, Poultry	Gram-positive, not absorbed, growth permittant
Chlortetracycline	Swine, Poultry, Cattle, Sheep	Broad-spectrum and mycobacteria, absorbed, growth permittant, systemic and enteric diseases
Erythromycin	Swine, Poultry, Cattle	Gram-positive, absorbed, growth permittant, systemic diseases
Lincomycin	Poultry	Gram-positive, absorbed, growth permittant
Monensin	Cattle	Gram-positive, absorbed, rumen additive
Neomycin	Swine, Poultry, Cattle, Sheep	Broad-spectrum, limited absorption, enteric diseases
Novobiocin	Poultry	Broad-spectrum, absorbed, systemic diseases
Oleandomycin	Swine, Poultry	Gram-positive, absorbed, growth permittant
Oxytetracycline	Swine, Poultry, Cattle, Sheep	Broad-spectrum and mycobacteria, absorbed, growth permittant, systemic and enteric diseases
Penicillin	Swine, Poultry, Cattle	Gram-positive, limited absorption, growth permittant, enteric diseases
Streptomycin	Swine, Poultry	Broad-spectrum, limited absorption, growth permittant, enteric diseases
Tylosin	Swine, Poultry, Cattle	Gram-positive and mycobacteria, absorbed, systemic and enteric diseases
Virginiamycin	Swine	Gram-positive, limited absorption, growth permittant, enteric diseases
Antibacterial Agents		
Arsanilic Acid[b]	Swine, Poultry	Weak antimicrobial activity, absorbed, enteric diseases
Carbadox	Swine	Broad-spectrum, absorbed, growth permittant, enteric diseases
Ethylenediamine Dihydriodide	Cattle	Weak antimicrobial activity, absorbed, systemic diseases
Furazolidone	Swine, Poultry	Broad-spectrum, absorbed, systemic and enteric diseases
Nihydrazone	Poultry	Broad-spectrum, absorbed, systemic and enteric diseases
Nitrofurazone	Swine	Broad-spectrum, absorbed, enteric diseases
Roxarsone	Swine, Poultry	Weak antimicrobial activity, absorbed, enteric diseases
Sulfamethazine	Swine	Broad-spectrum, absorbed, systemic and enteric diseases
Sulfathiazole	Swine	Broad-spectrum, absorbed, systemic and enteric diseases
Sulfaquinoxaline	Poultry	Broad-spectrum, absorbed, systemic and enteric diseases
Antifungals		
Gentian Violet	Poultry	Fungi, enteric diseases
Nystatin	Poultry	Fungi, enteric diseases

[a] Includes bacitracin, bacitracin methylene disalicylate and zinc bacitracin.
[b] Includes sodium arsanilate.

Muir et al (1979)

CONNECTING PARAGRAPH

According to the literature, animal wastes contain variable amounts of P_o and P_i. To evaluate the proportion of P_i, residual (nucleic acid), acid-soluble P_o (inositol hexaphosphate), and phospholipid P in animal waste, a published method using sequential extractants was adapted. Effort was devoted to improving phospholipid extraction. Chapter 3 describes the efforts to improve this method of fecal P fractionation, especially phospholipid P extraction.

CHAPTER 3

MANURE P FRACTIONATION

3.1 Summary

The proportion of organic (P_o) and inorganic P (P_i) in animal wastes affects their value as crop fertilizers. This study was conducted to develop a manure P fractionation method. Samples of beef and dairy cattle manure were lyophilized and ground to pass a 2 mm screen. The fecal P in these samples was then partitioned into phospholipid (P_l), inorganic (P_i, acid-soluble organic (P_{aso}) and residual (P_r) forms using the McAuliffe and Peech (MP) method. Methods (MP and an Association of Official Analytical Chemists (AOAC) procedure developed for flour), sequential extractions, and grinding size (2 vs. 0.25 mm) were compared for fecal P_l extraction. The AOAC method removed more P_l with less variability, less chemicals, and required less time than the MP procedure and there was no effect on other P forms. All lipid dry matter was removed in the first ether or ethanol extraction but not P. More lipid dry matter and P were removed in the first extraction of coarser (2 mm) dairy cattle fecal material than from feces ground finer (0.25 mm). Two beef and one dairy cattle fecal samples contained 6.4, 7.2 and 8.8 mg g^{-1} of total P (P_t) with 50.0, 51.5 and 70.0% as P_i, 27.0, 25.3 and 15.9% as P_r, 23.0, 22.2 and 12.4% P_{aso}, and 0.0, 0.8 and 1.7% as P_l, respectively. The AOAC flour method can be used to extract fecal P_l and the MP procedure for P_i, P_{aso}, and P_r forms.

Key words: Manure, feces, phosphorus, organic, inorganic, fractionation

Résumé

La proportion du P organique (P_o) et inorganique (P_i) dans le fumier affecte sa valeur comme fertilisant. Cette étude a été conduite pour développer une méthode de partition du P fécal. Des échantillons de fumier de bovins laitiers et de boucherie ont été lyophilisés et moulus pour passer dans un tamis de 2 mm. Le P fécal a été divisé sous forme phospholipide (P_l), inorganique (P_i), organique soluble dans l'acide (P_{aso}), et résiduelle (P_r) par la méthode McAuliffe et Peech (MP). Deux méthodes (MP et une méthode de l'Association of Official Analytical (AOAC) développée pour la farine), les extractions séquentielles, et la finesse de mouture (2 vs. 0.25 mm) ont été comparées pour l'extraction des

phospholipides fécaux. La méthode AOAC a extrait plus de P_i avec moins de variabilité, moins de chimique, et moins de temps que la procédure MP et il n'y a pas eu d'effet sur les autres formes de P. Toute matière sèche lipide était extraite dans la première extraction par l'ether ou l'éthanol, mais pas le P. Il y a eu plus de matière sèche lipide et de P extraits dans la première extraction des fèces moulues à 2 mm qu'à 0,25 mm. Deux échantillons de fèces de bovins de boucherie et un de bovins laitiers avaient 6,4, 7,2 et 8,8 mg g^{-1} de P total (P_t) sur base de matière sèche avec 50,0, 51,5 et 70,0% sous forme P_i 27,0, 25,3 et 15,9% P_r, 23,0, 12,4 et 22,2% P_{aso}, et 0, 0,8 et 1,7% P_l, respectivement. La méthode AOAC développée pour la farine est applicable pour l'extraction des phospholipides fécaux et la méthode MP pour les formes P_i, P_{aso}, et P_r.
Mots clés: Fumier, fèces, phosphore, organique, inorganique, fractionnement

3.2 Introduction

Animal wastes contain significant amounts of P. For instance, a survey of numerous farms reported the following average P values for several species of animal manure, which included litter (in mg P g^{-1} of dry matter): dairy 6.37, beef 3.92, hog 22.66, poultry 22.64, and sheep 6.57 (4). Others have found total P in fresh manure unmixed with litter to vary in the following manner (mg P g^{-1} dry basis): horse 4.03 to 7.25, cow 4.32 to 7.27, steer 8.35 to 11.52, sheep 11.92, poultry 7.25 to 29.85 (12). Values for manure mixed with litter were lower (mg P g^{-1} dry basis): cow 3.86 to 6.06, hog 2.66 to 7.75, poultry 4.88 to 5.74 (12). The significant point is that most literature reports total P (19), and it is rare that the proportions of the various P forms are reported.

Total P is important for monitoring loading rates and long-term applications. However, it is the amount of plant-available P that is significant, because it has been demonstrated that crop response is directly related to the P_i forms in manure (2). Although, the lower esters of inositol, such as the mono, di, tri, or tetraphosphates, are somewhat available (20), myo-inositol hexaphosphate (IHP) was equivalent to KH_2PO_4 only at high concentration (200 ppm) on low P retention soils (10). At low P rates (20 ppm) or on high P-retention soils, P from myo-IHP was unavailable. Since IHP's constitute up to 60% of soil organic

P (P_o) they appear to be more stable and less available than phospholipid and nucleic acid P which form only 1 and 5% respectively (7).

It is clear that P forms vary in their availability to plants and therefore it is important to evaluate their relative abundance when animal manure is used to fertilize crops.

Schemes to fractionate manure P into P_o and P_i components by means of appropriate extractants have been developed from chemical principles (11). Most procedures have used ether-alcohol mixtures to extract the phospholipids, and dilute acids for the P_i and P_{aso} forms which are mainly IHP's (11,12). Acid-soluble P can be separated into P_i and P_o forms by difference between the total and colorimetrically determined P_i (11,13). Chromatographic separation has also been used to determine IHP independently in acid extracts (3) and gel filtration and HPLC to measure IHP, adenosine triphosphate, and adenosine monophosphate in liquid manure (6).

Often, total P is simply separated into P_o and P_i forms as is the case for the trichloroacetic acid (TCA)–EDTA (13), HF–HCl–$TiCl_4$ (17) and 2.5 N HCl (13) methods. This is done by determining total and P_i in the extracts, the difference between the two being organic P_o.

It was the object of this work to develop a method capable of fractionating manure P into P_l, P_i, P_{aso}, and P_r or nucleic acid forms. It was also an objective to decrease variability and to eliminate heating ether because of its explosiveness (5). Other objectives were to examine the appropriateness of automated methods for phospholipid extraction, to compare plasma spectrometer (ICP) and colorimetric analysis for soluble P, and to evaluate the role of charcoal as an adsorption agent for soluble organic compounds.

3.3 Materials and Methods

Samples of uncontaminated beef and dairy cattle feces were obtained, freeze-dried, and homogenized by grinding with a Wiley mill to 2 mm or less.

The McAuliffe and Peech (11) method was modified for P_l extraction from manure. To a 5 g sample of dried beef or dairy fecal material placed in a teflon screw-cap 250 mL erlenmyer flask, 100 mL of an ether-alcohol mixture (1:3 diethyl ether:ethanol) was added, capped, and heated to

boiling (70°C) in a water bath. This mixture was then shaken at room temperature for 1 h, vacuum-filtered through a No. 54 paper, washed with 50 mL of ether, and then evaporated to dryness in a digestion tube (boiling chips, 50°C for 1 h, 150°C for 4 h). This procedure was repeated, the extract evaporated in the same tube, digested with 6 mL H_2SO_4: H_2SeO_3 (40:1) plus 4 mL H_2O_2 at 400°C for 45 min (8) and diluted to 30 mL with distilled water. Phospholipid P was then determined by the phosphomolybdate-blue method (16).

An Association of Official Analytical Chemists (AOAC) method (1) for flour phospholipid was adapted for manure-P fractionation. A 5 g sample was placed in a screw-cap erlenmyer flask, 45 mL of 70% ethanol added, capped, shaken for 15 min at 75°C in a water-bath, cooled, 45 mL diethyl ether added, shaken at room temperature for another 15 min, filtered (No. 54), decanted, washed with a total of 50 mL of ether in several steps, shaking, decanting and filtering each time, with all residue transferred to the filter paper. The rest of the procedure regarding drying temperatures, digestion, dilution to volume, and digestion was as described above.

The following procedure was then applied (independently) to the resulting residues from each of the lipid methods. The residue was washed from the filter paper with 5% TCA, shaken with 100 mL of the acid for 1 h at room temperature, centrifuged at 5000 *g*, vacuum-filtered through a No. 54 paper and washed with small portions of TCA. The extraction procedure was repeated, extracts combined, and then made to 250 mL with 5% TCA. A 50 mL aliquot was evaporated to dryness in the presence of boiling chips, and then digested and analysed as above for total acid-soluble P.

Inorganic P was determined by shaking 50 mL of the TCA extract with 3 g of Darco-60 activated charcoal for 15 min in a 125 mL erlenmyer flask, filtered through a No. 42 paper, diluted 1:9 with 5% TCA solution, and measured colorimetrically as above.

The difference between acid soluble P_t and acid soluble P_i was P_{aso} (11).

The residual or nucleic acid P (11) was analysed as follows. The ethanol-ether-TCA residue was washed with water, the residue transferred

to a 250 mL erlenmyer flask, dried at 70°C, weighed, and homogenized with a blender. A 500 mg sample was then digested with H_2SO_4–H_2SeO_3–H_2O_2, made to 250 mL with distilled water, P determined by colorimetry, and corrected to original sample weight.

A Tecator Soxtec fat extractor was used as described (9,15). Extraction thimbles, aluminum cups, boiling chips, and cotton plugs were conditioned with the extractant to remove any traces of fat. A 3 g sample was weighed into the thimbles and plugged. The thimbles were placed in position and immersed in 50 mL of the extractant in the previously tared extraction cups and boiling chips. The solvent was brought to boil and held in that position for 15 min and then rinsed for the desired length of time. After evaporation and recovery of the extractant, samples were dried for 30 min at 100°C in an oven (9).

To verify the completeness of the two-step extraction procedures for phospholipids, P was determined in each of the following combinations of sequential extractions: flour and feces by four 15-min AOAC ether-ethanol; feces by one 1-h and four 15-min ethanol-ether; feces by three 1.5-h ethanol by Soxtec; feces by two 1.5-h and one 1-h by Soxtec; feces by four 45-min ethanol and four 45-min ether by Soxtec on separate samples for weight and P; feces by four-45 min ethanol by Soxtec on 0.25 and 2 mm material with both weight and P being determined on each extract.

The completeness of three sequential extractions by TCA was also determined by measuring P in the combined extracts of the first two and separately in a third extract.

The effect of decoloring an aliquot of the TCA extract of each of 15 dairy fecal samples on P determined by colorimetry or by ICP spectrometry was measured as well. The procedure is described above for P_i.

In all appropriate places, blanks were included to correct for background and standard National Bureau of Standards (NBS) citrus samples were used to verify accuracy (18). To test differences *t* tests were used where appropriate (14).

3.4 Results and Discussion

Initial trials produced results with relatively high coefficients of variability (CV). Partition of beef cattle fecal P resulted in CV's of 15% for P_i, 21% for P_r, 21% for P_{aso} (TCA, IHP), and 21% for acid-soluble

P_t. This sample had a P_t concentration of 6.4 mg g^{-1} distributed as follows: 50% as P_i, 27% as P_r, 23% as P_{aso}, and no P_l. After numerous trials and changes, variability was reduced to more acceptable levels of 5% for P_i, 8% for P_r, and 8% for acid soluble P_t, although P_{aso} was still high at 22%. However, this is a value calculated by differences which may explain the variation. Coefficients of variation for the two forms were quite acceptable at 8 and 5% for acid soluble P_t and P_i. This sample had 7.2 mg g^{-1} of P_t with 52% P_i, 25% P_r, 22% P_{aso}, and 1% in the P_l form.

Reduction in CV values was accomplished by meticulous attention to laboratory details which were especially important for sequential extractions of the same sample.

Of the two methods used to extract beef fecal P_l, the AOAC (1) procedure produced significantly ($P<0.05$) higher P_l levels of 0.06 mg g^{-1} dry matter compared to 0.03 mg g^{-1} with the McAuliffe and Peech (11) method. Furthermore, the AOAC method had a CV of 0% compared to 30% for the McAuliffe and Peech method. The AOAC procedure required half the time or less and used less extractant. A comparison of the two methods on a dairy fecal sample revealed that although there were significant differences for P_l, there were none for P_i, P_r, P_{aso}, and P_t forms (Fig. 1). Even though the AOAC method produced significantly higher P_l results, nevertheless this form remained a small constituent of P_t and therefore the procedure had little consequence on other forms which were comparable in quantity to those removed by the McAuliffe and Peech procedure.

It is difficult to determine why the variation is less with the AOAC method but may be related to a lower initial extraction volume and hence less surface contact with the extraction vessel walls. The reduced volume would be of greater significance at the evaporation and digestion stage because less wall contact would occur in the digestion tubes. Material that remained attached to the upper walls of the vessel after evaporation would not necessarily be attained during digestion since refluxing occurs mainly in the lower parts.

It was found that "phospholipid" P continued to be removed even in the last of four successive extractions, both for dairy feces and for flour (Fig. 2). Since the AOAC method was developed for flour, the similar reaction of flour and feces to successive extractions demonstrated that the method was not material specific.

Modifying extraction procedures with a longer initial period did not change the fact that "phospholipid" P continued to be found in subsequent extracts (Fig. 3) which would imply that lipid extraction was not complete or that other forms of P were being removed.

Even with a Soxtec, which is an apparatus specifically designed for lipid extraction, P continued to be removed in the third extraction, both in ether (Fig. 4) and ethanol (Fig. 5). Coefficient of variation values were less than 10%. There was a sharp reduction between first and second extractions for weight, both with ethanol (Fig. 6A) and ether (Fig. 7A) but not for phosphorus (Fig. 6B and 7B respectively). Therefore in all subsequent work, P_l was considered to be that obtained from a single extraction.

Ethanol (Fig. 5) produced considerably higher P_l levels than ether (Fig. 4). It has been demonstrated elsewhere that ethanol extracted more fat-oil from feed than ether (9) and this was stated to be due to carbohydrate and mineral dissolution by the former. Nonpolar extractants are recommended for lipids (5). Therefore, ethanol may be extracting compounds other than lipids and produce measured levels higher than actual levels. Nevertheless, ethanol is an accepted lipid extractant (1, 11).

The above work has revealed that the P_l component was small for the samples analysed and whether one or two times as much is extracted by one procedure or another had little effect on other P components. However, the decrease in concentration from forage to sheep feces was greatest for P_l, less for P_{aso}, and unchanged for P_r (11) which may indicate less resistance to decomposition and therefore relatively greater availability of P_l.

Both acid soluble P_t and P_i were completely removed in the first two extractions, there being only traces in the third (Fig. 8). Three extractions were therefore not necessary if adequate washing occurs.

Four sequential ethanol extractions of dairy cattle feces revealed that more weight and P were removed in the first extraction of material ground to pass a 2 mm sieve compared to 0.25 mm stock (Fig. 9). However there was generally more of both weight and P_l extracted from 0.25 mm material in the three subsequent steps. In the final analysis, the same amount of total P_l and somewhat more total weight were obtained from 2 mm stock.

Lerique et al. (9) in studying lipid extraction of feeds, stated that finer milling produced higher lipid yields. This was not the case for the dairy cattle fecal sample used (Fig. 9A) and is believed to be due to the fact that coarser manure tended to be better dispersed in the extractant than finer stock.

There was no effect of activated carbon (Darco) on the amount of P found in the TCA extract when analysed by ICP (Fig. 10A). However, when analysed colorimetrically, the Darco-treated TCA extract was slightly, albeit significantly, higher in P (Fig. 10B) when analysed on different days but lower when analysed on the same day (Fig. 10C). It has been stated that the activated carbon treatment removes all but very slight amounts of P_o compounds (11). If this is true and since ICP analyzes total P in solution, the amount of P_o removed would have had to equal the amount of P_i added by supposedly contaminated C since there was no difference between treated and untreated TCA extract (Fig. 10A). In the case of colorimetry where only P_i is analysed (16), more P_i would have been added than P_o removed from the TCA extract (Fig. 10B) but less in (Fig. 10C). Darco contained 0.3 $\mu g\ g^{-1}$ inorganic P, but this was corrected by means of a blank. Since the No Darco (N) and Darco (D) groups in Fig. 10B were analysed on two different days, this would contribute to the difference and indicate that in spite of strict precautions, slight but significant differences can occur. However in Fig. 10C when analyses for N and D were conducted on the same day and with the same gains and calibration curves, it would appear that the Darco may be removing some P_o but the quantities are small. Nevertheless this indicates the necessity of taking precautions regarding how, in what sequence, and when samples are analysed. Activated carbon did not remove P_o, or certainly not all of it, and therefore using ICP spectrometry cannot be used to analyse P_i in TCA extracts of fecal material.

Since ICP-P in the series of TCA extracts treated with Darco (Fig. 10E) was similar in magnitude to acid-soluble P_t determined by ICP or colorimetry on digested TCA extract (Fig. 10F), clearly acid-soluble P_t was being measured which means that the Darco removed very little if any P_o. Colorimetric methods, which measure P_i only, produced much lower results than ICP (Fig. 10D, E), due to the inclusion of organic P_o in ICP analyses.

Although the same matrix-matched standards were used and analyses of NBS standards were found to be within accepted values (data not shown), nevertheless ICP results gave slightly, but significantly higher results for acid soluble P_t than colorimetry (Fig. 10F). Since P_i must be determined colorimetrically, even though ICP analysis for acid-soluble P_t requires much less time, results were not comparable, although the differences were small.

Therefore fecal P_i was effectively removed in a single extraction using an AOAC method developed for flour. This involved shaking five g of material, for 15 min in 45 mL of 70% ethanol in a screw-cap erlenmyer flask placed in a water bath, cooling, adding 45 mL ether and shaking at room temperature for 15 min, filtering, washing, acid-digesting an aliquot, and analysing for P. Variability was lower and the amount extracted higher than for the McAuliffe and Peech method. Higher initial lipid yield was effected with 2 mm than 0.25 mm material. Although significant, P_i in these dairy cattle manure samples only constituted about 2% of P_t. Most P_o was in the P_r and P_{aso} forms.

3.5 Acknowledgements

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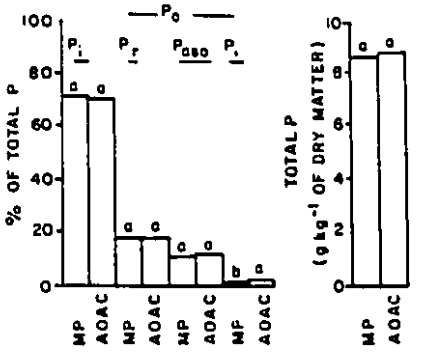

Fig. 1. Phosphorous forms in lyophilized dairy cattle feces. McAuliffe and Peech (MP) for inorganic (P_i), residual (P_r), acid-soluble organic (P_{aso}), lipid (P_l) and Association of Official Analytical Chemists (AOAC) P_i. P_o = organic P methods. (Columns capped by the same letter are not significantly ($P \leq 0.05$) different by a paired t test. Coefficients of variation (%) were P_i: 5, P_r: 8, P_{aso}: 15, P_l: 10, total: 10).

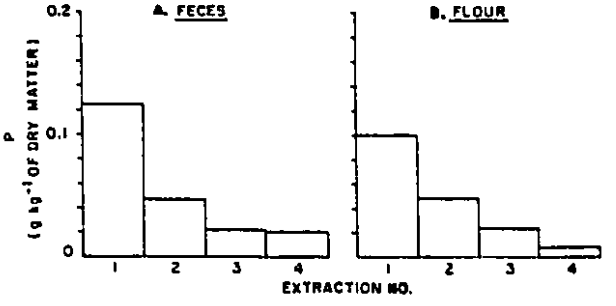

Fig. 2. Phosphorus in each of four sequential 15-min ethanol-ether extractions of flour and lyophilized dairy cattle feces. Association of Official Analytical Chemists method. (Coefficient of variation (%) were A.1: 10, A.2: 11. A.3: 7, A.4:8, B.1: 7, B.2: 6, B.3: 6, B.4: 7).

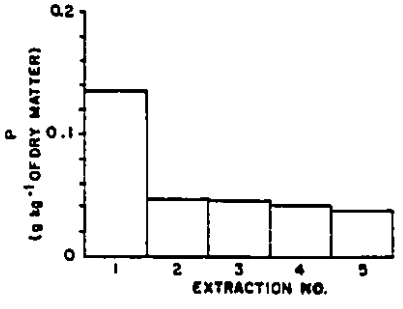

Fig. 3. Phosphorus in each of five sequential ethanol-ether extractions of lyophilized dairy cattle feces. First extraction heated 1 h, next 4 for 15 min. Association of Official Analytical Chemists method. (Coefficients of variation (%) were - 1: 7, 2: 12, 3: 7, 4: 7, 5: 9).

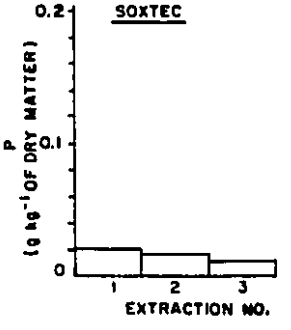

Fig. 4. Phosphorus in each of two 1.5-h and one 1-h ether extractions of lyophilized dairy cattle feces by Soxtec. Coefficients of variation (%) were: 1 = 7, 2 = 5, 3 = 23.

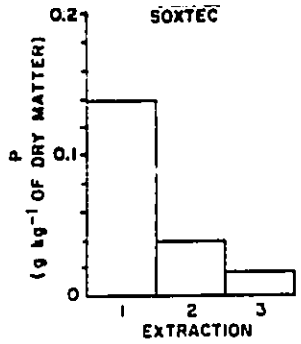

Fig. 5. Phosphorus in each of three sequential 1.5-h ethanol extractions of lyophilized dairy cattle feces by Soxtec. Coefficients of variation (%) were: 1 = 8, 2 = 8, 3 = 4).

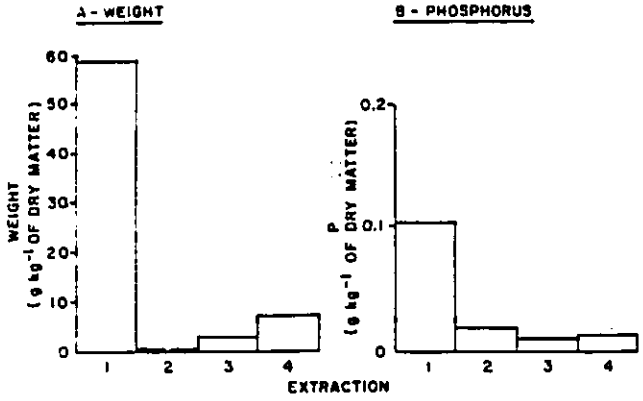

Fig. 6. Weight (A) and P (B) removed in each of four sequential 45-min Soxtec ethanol extractions of lyophilized dairy feces. (Coefficients of variation (%) were - A.1: 1, A.2: 173, A.3: 9, A.4: 26, B.1: 14, B.2: 17, B.3: 34. B.4: 0).

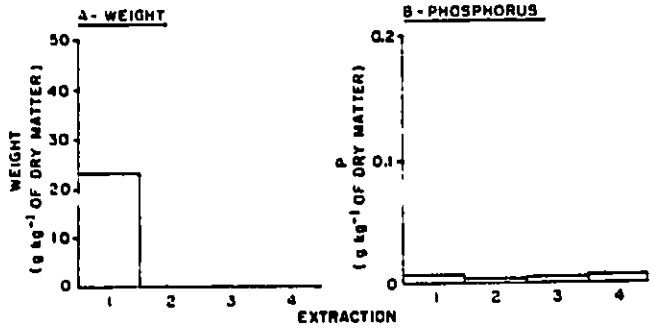

Fig. 7. Weight (A) and P (B) removed in each of four sequential 45-min Soxtec ether extractions of lyophilized dairy feces. (Coefficients of variation (%) were - A.1: 12, A.2 to A.4: 0, B.1: 10, B.2: 12, B.3: 14, B.4: 13).

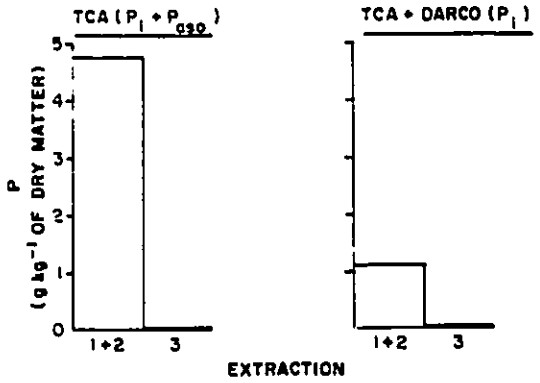

Fig. 8. Phosphorus removed in three sequential trichloroacetic (TCA) extractions. (P_i = inorganic P, P_{aso} = acid-soluble organic P, Darco = activated carbon. Coefficients of variation (%) were: A.1+2: 7, A.3: 5, B.1+2: 7, 3: 6).

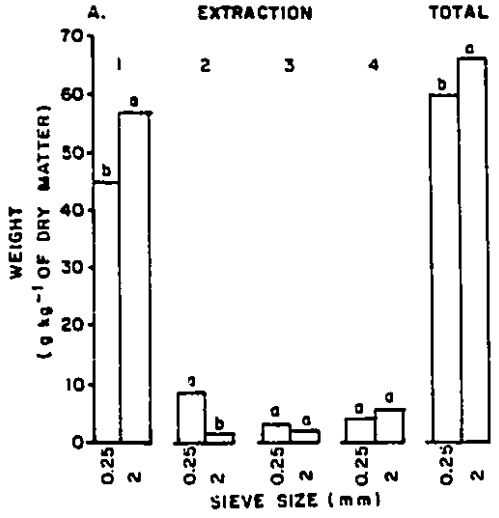

Fig. 9A. Effect of sieve size on weight removed in each of four sequential 45-min Soxtec ethanol extractions of lyophilized dairy feces. (Columns capped with the same letter are not significantly ($P \leq 0.05$) different by a paired t test. Coefficients of variation (%) were - A.1: 6, A.2: 7, A.3: 7, A.4: 6, A. total: 6).

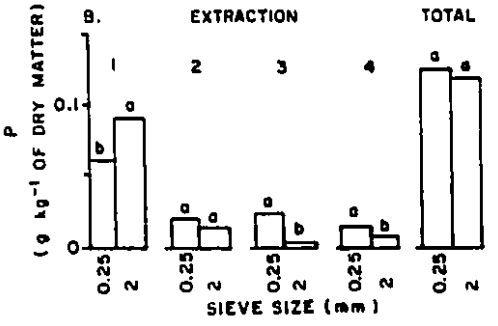

Fig. 9B. Effect of sieve size on P removed in each of four sequential 45-min Soxtec ethanol extractions of lyophilized dairy feces. (Columns capped by the same letter are not significantly ($P \leq 0.05$) different by a paired t test. Coefficients of variation (%) were B.1: 5, B.2: 6, B.3: 8, B.4: 7, B. Total = 6.).

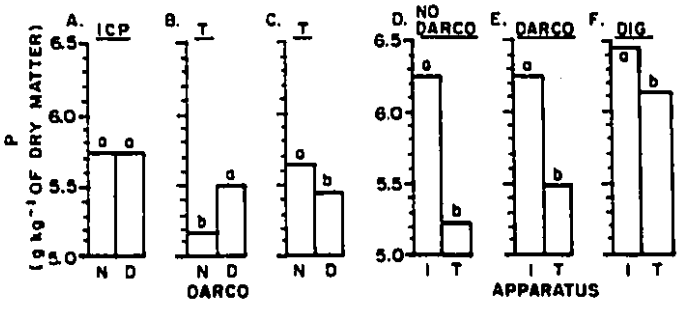

Fig. 10. Effect of activated carbon decoloring agent and apparatus used to determine P content in the trichloroacetic acid extract of lyophilized dairy cattle manure. (Columns capped by the same letter are not significantly ($P \leq 0.05$) different by a paired t test. N = no Darco, D = Darco, I = ICP, T = Technicon, Digest = digested. B. and C. were conducted at two different times. Coefficients of variation were (%) - A:5, B:3, C:4, D:8, E:2, F:2).

CONNECTING PARAGRAPH

In chapter 3, a method to partition fecal phosphorus into P_i, P_r, P_{aso}, and P_l forms was adapted and improved. There is very little literature on the proportion of the various forms of P in animal wastes and what little that exists is thirty to forty years old. Animal rearing systems, type and quality of feedstocks, and attention to nutrition have all changed greatly and therefore it was thought to be important to evaluate the P status in the fecal material of common farm animals. When animal wastes are used to fertilize crops, the proportion of P_i in particular has an impact on crop nutrient requirement. Samples of animal wastes from commercial farms and a controlled calf nutrition experiment were obtained and the P partitioned into its various forms. Chapter 4 reports the results.

CHAPTER 4

PHOSPHOROUS FORMS IN ANIMAL MANURES

4.1 Summary

The proportion of inorganic (P_i) and organic (P_o) P in animal feces is affected by rearing conditions. This study was conducted to evaluate the P status of farm animal wastes and the effects of some management factors. Total (P_t), P_i, residual (P_r), acid-soluble organic (P_{aso}), and lipid (P_l) phosphorus were determined in freeze-dried, ground (2 mm screen) samples of fresh uncontaminated dairy (*Bos taurus* L.), beef (*Bos taurus* L.), hog (*Sus scrofa domestica* L.), and poultry (*Gallus gallus domesticus* L.) feces from commercial farms. As well, feces from calves (*Bos taurus* L.) fed cut-1 and cut-2 of three reed canarygrass (*Phalaris arundinacea* L.) cultivars and one timothy (*Phleum pratense* L.) cultivar were analysed. Total P varied from 6.7 for feeder cattle feces to 29.1 g kg^{-1} for hog feces on a dry matter basis. Of the P_t, P_i ranged from 34.8 (broilers) to 63.2% (dairy), P_r from 11.0 (broiler) to 40.8% (finisher beef), P_{aso} from 7.8 (dairy) to 53.4% (broilers), and P_l from 0.4 (hog) to 2.1% (feeders). Dry matter ranged from 14.3 (dairy) to 67.5% (broilers). Ruminant feces varied more in P_t, P_{aso}, P_l but less in P_i and P_r than non-ruminant fecal material. Total P and P_i were closely related. Fecal P_i and P_l were higher for cut-2 hay than in cut-1. Calves fed timothy forage produced feces with less P_i than those fed reed canarygrass. Some calves on cut-2 forage produced feces with lower P_i and less P_r on cut-1 material than other animals. Published values were found to be unreliable indicators of fecal P status.

Key words: Phosphorus, feces, P form.

Résumé

Les conditions d'élevage affectent la proportion du P inorganique et organique dans les fèces des animaux. Cette étude a été conduite pour déterminer les formes de P dans les fèces des animaux de ferme commerciale et les effets de quelques facteurs de gestion. Le phosphore total (P_t), inorganique (P_i), résiduel (P_r), organique soluble dans l'acide (P_{aso}) et lipide (P_l) ont été dosés dans les échantillons lyophilisés et moulus (tamis 2 mm) de fèces fraiches non contaminées de bovins laitiers et de

boucherie (*Bos taurus* L.), de porcs (*Sus scrofa domestica* L.) et de volailles (*Gallus gallus domesticus* L.) des fermes commerciales. Aussi, les fèces de veaux (*Bos taurus* L.) recevant la coupe 1 ou la coupe 2 de un de quatre foins - trois d'alpiste roseau (*Phalaris arundinacea* L.) et un mil (*Phleum protense* L.) ont été analysés. Le P_t a varié de 6.7 g kg^{-1} de fèces de bovins de boucherie en croissance sur base sèche à 29.1 pour les fèces de porcs. Du P_t, il y avait 34.8% sous forme P_i dans les fèces des poulets à chair à 63.2% dans les fèces de bovins laitiers. Le P_r a varié de 11.0 (poulets à chair) à 40.8% (bovins de boucherie en finition), P_{aso} de 7.8 (bovins laitiers) à 53.4% (poulets à chair), P_l de 0.4 (porc) à 2.1% (bovins en croissance), et la matière sèche a varié de 14.3% dans les fèces de bovins laitiers à 67.5% dans les fèces de poulets à chair. Les fèces des ruminants étaient plus variables en P_t, P_{aso}, et P_l mais plus stables en P_i et P_r que les fèces des non-ruminants. Le P_t et P_i étaient fortement corrélés. Il y a eu plus de P_i et P_l dans les fèces de veaux alimentés au foin de coupe-1 que de coupe-2. Aussi ceux recevant du mil ont produit des fèces plus faibles en P_i. Certains veaux alimentés au foin de coupe-2 ont produit des fèces plus faibles en P_i et P_r sur coupe-1 que d'autres. Les valeurs publiées ne reflètent pas le status du P fécal.

4.2 Introduction

Of the P_t ingested by domestic farm animals, about 70% is excreted (6). The actual amount depends on the state of the animal, dietary levels, source, Ca, vitamin D, management and other nutritional and physiological factors (6). A young growing animal will excrete less, perhaps 40%, while typical values for more mature animals are: cattle 70%, hogs 77%, layers 91%, and broilers 61% (22).

In animal nutrition, the preoccupation is to ensure that at least the minimum nutritional requirements are met, less attention being given to excessive levels. Consequently, dietary P often exceeds animal needs. Furthermore it has been shown that export is directly dependent on dietary content (22). For instance, as dietary content increases from 0.4 to 1% of dry matter, excretion by the pig increases from 0.8 to 2.1 g per day per animal.

Total P content in manure varies considerably and one study (9) reported that dairy cattle manure had a mean of 1.22 kg t^{-1} on a wet basis and an inter-farm coefficient of variation (CV) of 30% while beef cattle manure had a mean of 0.82 kg t^{-1} and a CV of 46%. The manure of other farm animals had the following P_t values: hogs – a mean of 1.16 kg t^{-1} and a CV of 98%, sheep – 1.97 kg t^{-1} and a CV of 32%, goats – 1.56 kg t^{-1} and a CV of 35%, and poultry manure had a mean of 8.63 kg t^{-1} and a CV of 41%. Feces contain virtually all P excreted by ruminants and 90% of total P excreted by sheep and hogs (13). Only under exceptional circumstances does urinary P become important (2,3,11). Poultry produce no urine.

It has been shown that plant yield and P uptake responses are directly related to manure P_i content (5). Furthermore, not all P forms in manure are of equal plant availability. For instance, on high P retention soils and at low rates on low P retention soils the P in myo-inositol hexaphosphate was relatively unavailable to ryegrass (*Lolium perenne* L.). At high rates on a low P retention soil myo-inositol P was, however, as available as that from an inorganic source (14). It has also been found that as total P in manure increases, so does the proportion of P_i (5). Therefore it is important to evaluate the composition of manure P, particularly the inorganic portion.

Manure P is composed of inorganic and organic parts, the latter varying from 10 to 80% of total and decreasing with age of the manure (11,20). However not all organic constituents decreased at the same rate (20).

In various samples of fresh manure from several animal species, phospholipid P (P_l) constituted 0.02 to 0.14 mg g^{-1} dry basis or 0.06 to 1.1% of P_t, inositol hexaphosphate (IHP) 0.07 to 3.51 mg g^{-1} or 0.5 to 12.3% of the total, P_r from 0.14 to 6.34 mg g^{-1} or 1.9 to 27.4%, other P_{aso} forms from 0.08 to 2.21 mg g^{-1} or 1.1 to 18.5% and P_i from 2.62 to 24.15 mg g^{-1} or 60.6 to 80.9% (20). For cow and sheep feces, both aged and fresh, P_t was composed of the following constituents: P_l 0.27 to 0.74%, P_{aso} (IHP and other forms) 2.69 to 9.40%, P_r (nucleic acid) 12.1 to 32.5%, and P_i 50.3 to 83.0% (16). Some 70 to 90% of P_t in liquid hog manure was in the P_i form (11).

Most of the above results were from work conducted some years ago and was based on a very limited number of samples, often only one or two for any single species of animal. Furthermore rearing conditions, age, and nutrition varied.

There does not appear to be any recent information on the forms of P in animal wastes other than in hog manure (10,11). Since the days when most of the above data was gathered, animal production has changed drastically. Today much more attention is given to the quality of feedstuffs, nutritional balance, and supplementation to meet growth and maintenance requirements. Therefore older data may not reflect the composition of todays' manure.

Because animal wastes are returned to the soil and serve to fertilize crops, it is important to know the proportion of the P that will be plant available, short-term crop response being dependent mainly on the inorganic form. Therefore, it was the objective of this work to evaluate the fecal P status of animal wastes from commercial forms and from calves fed different types of hay.

4.3 Materials and Methods

Fresh undisturbed fecal samples were collected from dairy and beef cattle, hogs, and poultry from the floors of the rearing facilities of several farms. The samples were taken during the winter, transported to the laboratory, frozen, freeze-dried, and ground to 2 mm.

Dietary data were also obtained but are not presented here because of the many ingredients and combinations employed. Nevertheless nearly all dairy cows were fed dry hay, almost always timothy (*P. pratense* L.) but sometimes in association with alfalfa (*M. sativa* L.) or red clover (*T. repens* L.). Silages of these same species and of corn (*Z. mays* L.) were also common. Grain was always fed to milkers with all diets containing dry, rolled, or high moisture corn and quite often barley (*H. vulgare* L.) as well. In addition a premix was also added since all producers made their own rations and often were on a total mixed ration. The most frequent constituents were limestone, dicalcium phosphate, soda, magnesium oxide, and salt. Some producers used commercial premixes while others confected their own from basic ingredients.

In addition fecal samples were obtained twice daily over a 6-day period directly from calves in the third week of a nutrition experiment, mixed together for each animal-treatment-period combination, frozen, freeze-dried, and ground to 2 mm. The eight nutrition treatments were composed of two cuts (June, August) of four hay types: one timothy (cv. Climax) and three reed canarygrasses of different alkaloid contents (low American cv. Vantage, low Canadian cv. Beaverlodge NG, high cv. Frontier). The basic design was a four by four latin square (four animals for four periods) for each cut, replicated three times. The hay was grown, harvested, prepared, and fed similar to described procedures (15) except that it was fed to Holstein calves of 200 kg initial weight.

The Association of Official Analytical Chemists (1) procedure was used to extract P_i and the McAuliffe and Peech (16) method was used to fractionate manure P into P_i, P_r, and P_{aso} forms. A 5 g sample was extracted by agitation for 15 min with 45 mL of 70% ethanol at 70 to 80°C, cooled, 45 mL of diethyl ether added, reextracted for a further 15 min without heating. The liquid and solids were separated by vacuum filtering through a Whatman No. 54 paper. The residue was carefully washed and all filtrates transferred quantitatively to a 250 mL BD-20 digester tube, evaporated at 50°C to near dryness, and digested with a H_2SO_4–$H_2Se_2O_3$–H_2O_2 system (12). The digested material was diluted to volume with distilled water and the P_i determined colorimetrically (24).

The residue was extracted with 100 mL of 5% trichloroacetic acid (TCA) at room temperature for one h with agitation, centrifuged at 5000 *g*, and filtered through a No. 54 paper. The procedure was repeated once and the residue transferred to the filter paper and washed. The filtrate was diluted to volume with 5% TCA solution, 50 mL was shaken for 15 min with three g of Darco G-60 charcoal, filtered with Whatman No. 42, and then analysed colorimetrically for P_i. Another aliquot was evaporated at 150°C to dryness, digested as for P_i, diluted to volume with distilled water, and analysed for P_i. This was the P_{aso} fraction considered to contain inositol hexa- and pentaphosphates and other unknown organic compounds (16).

The residue was dried and weighed in order to correct to original weight. A subsample was then digested in BD-20 tubes as above, diluted

to volume with distilled water, and P_i determined colorimetrically. This was the P_r or nucleic acid P.

Total P was calculated by summation. Dry matter (DM) was determined on all samples. Standard National Bureau of Standards plant samples were also digested and analysed to verify accuracy where appropriate (25).

Means, standard errors, and coefficients of variation were calculated for the survey data and analysis of variance performed on the calf experiment (23). Concentrations and proportions of each P form were compared over species. Pearson and partial correlations between P forms, P_t and DM were also calculated.

4.4 Results and Discussion

Total P for feces free of litter and urine for dairy cattle fecal samples gathered in this study (Table 1) were nearly double most reported values of some 30 years ago (20).

The largest component in dairy feces was P_i, which constituted an average of 63.2% of the total, and is comparable to literature values of 50.3% (16) and 60.6 to 87.3% (20) for cattle under various conditions.

Literature values for dairy feces were 32.5% (16) and 3.3 to 16.7% (20) of total P in the P_r form. Thus values obtained in this work (Table 1) are in the same order as those of the first authors (16) but considerably higher than those found in the second reference (20), which may be due to different methods. Residual P was found to be much less available to plants in the short-term than superphosphate or whole manure (17) and therefore high proportions of this form would be undesirable. Residual P is considered to be nucleic acid type material (16) and indeed, soluble high molecular weight P_o compounds in hog manure have been identified as polydeoxyribonucleotides (10).

Acid-soluble organic P (IHP plus other organic P compounds) in dairy feces as a percentage of P_t (Table 1) compared to literature values of 9.5% (16) and 9.0 to 27.1% (20). Differences may be due to diets.

Phospholipid P in dairy feces formed only a small part of P_t (Table 1) and this compared favorably with literature values of 0.74% (16) and 0.4 to 1.5% (20).

It is therefore clear that P_i is the most important component. This is significant since plant response is directly dependent on it. The fact

that P_r also constitutes an important part indicates significant residual value. Furthermore, because the feces used in this study are much higher in P_t than literature values, the actual concentrations of all forms are also higher.

Total P values for only two samples of beef cattle feces were found in the literature and these were 8.4 g kg^{-1} for 10 month-old steers and 11.5 g kg^{-1} for 18 month-old steers (20). Therefore values from this study (Table 1) were generally lower for feeders and finisher steers than published figures.

Literature values were 59.8% for beef cattle fecal P_i of the total for steers (20). Thus, values from this study Table 1 were lower than the published data. Lower supplementation of inorganic sources in the diets of cattle involved in this study, and feedstuff would likely explain the differences.

Published values for beef cattle fecal P_r were 19.4% and 13.2% of P_t for 10 and 18 month-old steers (20). Therefore, P_r in beef cattle feces from this study (Table 1) was much higher than the published value.

Published values for P_{aso} were 9.4% (16) and 19.7% and 22.1% of P_t for 10 and 18 month-old steers (20). Therefore values from this study (Table 1) differ with the data of Peperzak et al. (20) but are relatively close to the single value of McAuliffe and Peech (16). Differences would be due to amount of grain in the diet since IHP is found only in grain (22) and in the enzymatic activity of the rumen since ruminants possess phytase which can decompose IHP (6).

Published data for beef cattle fecal P_i were 1.1% and 0.8% of P_t for 10 and 18 month-old steers (20). Thus values from the current study (Table 1) were higher than published data.

Total P in finisher hog feces (Table 1) compared to a literature value of 11.0 g kg^{-1} (20) which is not really applicable since it is for sow feces. Little comparable data were found because published values were for hogs on litter, a rearing system no longer in use. One comparable source reported values of 14.8 to 18.0 g kg^{-1} (11). Thus values in this study (Table 1) are much higher than those reported in the literature. This probably indicates the tendency among nutritionists to discount feed P and supplement with inorganic sources (7).

Inorganic P in hog feces (Table 1) was lower than literature values of 82.9% (20) and 80 to 90% (11) of P_t. Values for Table 1 are for fresh feces while some literature data (11) was based on aged material and this may explain the difference.

Hog fecal P_r values (Table 1) are much higher than the single reported value of 3.1% (20).

Acid-soluble organic P values in hog feces from the current study (Table 1) were generally twice as large as reported values of 12.5% (20) of P_t and likely reflects diet. It may be undesirable to have large amounts of this form in manure because it has been shown to be essentially unavailable to plants (14).

Hog fecal P_i values (Table 1) were comparable to a published value of 0.5% (20) of P_t.

Therefore, literature values for some P forms were not always reliable indicators for current hog feces composition.

Literature values for P_t in chick feces were 13.2 to 23.2 g kg^{-1} and 7.2 to 29.8 g kg^{-1} for hens (20), which are of the same order of those realized in this study (Table 1).

Reported values of P_i in chick feces were 31.0% to 55.5% of P_t and for layers, 53.7% to 79.3% (20). Hence, values realized in this (Table 1) and other studies were in the same range.

Literature residual P_r values were 2.0 to 27.4% of P_t for chicks and 0.1 to 12.4% for hens (20). Therefore values were similar for broilers but considerably higher for layer feces gathered in this study (Table 1) compared to literature values.

Reported P_{aso} values for chick feces were 16.5 to 44.3% and 8.9% to 33.2% of P_t for hen feces (20). Therefore values from this study (Table 1) were much higher than literature data for broilers but similar for layers. The much lower levels in layer feces relative to broiler feces may be related to diet. The layers had a high proportion of wheat in their diet compared to more corn in broiler rations and it is known that wheat possesses strong phytases active down to pH 3 while corn has essentially none (18).

Fecal P_i values for broiler and layer feces (Table 1) were comparable to literature (20) values of 0.6% to 1.0% of P_t in chick feces and 0 to 0.6% for layer feces.

It was generally not possible to make truly valid comparisons with published values. Literature data were extremely few in number, incomplete, and often based on a single analysis or a single animal. The sampling details were generally not given nor were diets reported. Often the class of animal and rearing conditions for published values were not at all comparable to the current situation. Current diets are generally based on better-quality and different feedsteefs, varieties and hybrids are not the same, greater attention is paid to nutritional balance, and diets are universally adjusted to growth stage. All these factors and others such as breed differences contribute to changes in fecal composition over the last few decades. The results of this study demonstrate that concentrations and proportions of P forms generally differ significantly from published values and therefore indicates the necessity of reevaluating the proportion of P_t in various forms because of its importance to crop fertilization.

There was generally a sharp difference in coefficient of variation (CV) values for ruminant and nonruminant fecal variables. The average CV value for ruminant and non-ruminant feces were: P_t – 31.6 vs. 17.4%, P_i – 14.7 vs. 24.3%, P_r – 19.7 vs. 40.9%, P_{aso} – 42.6 vs. 28.7%, P_l – 33.1 vs. 22.9%, and DM – 13.2 vs. 16.2% (Table 1).

The greater instability in P_t, P_{aso}, and P_l and greater stability in P_i and P_r in ruminant compared to nonruminant feces is likely a reflection of diet and physiology. Forages, which are eaten only by ruminants, vary greatly in composition depending on growing conditions (8). The level of grain in ruminant diets will vary according to production levels for a dairy cow and age for a beef animal, increasing in the finishing stages. Although ruminants possess phytase, the amount of this enzyme will depend on age being much less in a young animal (6). Both grain proportion and phytase amount would affect P_{aso}. Residual P for nonruminant feces would likely be less stable because diets are formulated according to availability and costs of different grains and therefore vary in composition.

Variation in fecal P concentration can be affected by many factors some of which are feedstock composition (2,6,7,8) energy level (21), protein intake (2), P intake (4), Ca:P ratio (2), parturition (2), fetal

development and lactation (2), age of the animal (2), hormones (2), and plasma P (2). These and numerous other physiological factors would all contribute simultaneously to influence fecal P composition.

It is of interest to note the greater variability in some P forms and the dependence on species. This would affect the number of samples necessary to detect differences at a given probability level. The observed variation also reflects the necessity of taking precautions in sampling, and the uniqueness of each farm.

Hog feces had by far the highest P_i concentration at 15.8 g kg^{-1} (Fig. 1A). Layer feces were also high at 12.0 g kg^{-1} and there was no difference between dairy and broiler feces in P_i nor between the feeder and finisher cattle which had only 3.2 g kg^{-1}. There was more P_i in dairy than in beef cattle feces. There are no strictly comparable literature values but hen feces did have up to 22.63 g kg^{-1} of P_i while a sow sample had only 9.08 (20). Recommendations are 5.0 g kg^{-1} of feed dry matter for cattle, 5.5 to 6.0 g kg^{-1} for hogs, 6.5 for layers, and 6.5 to 8.0 g kg^{-1} for broilers and since 70% of ingested P was excreted by cattle and 77% by pigs (22), such large differences between ruminants and non-ruminants would not be expected. Experience has shown that feed levels of P are often considerably in excess, especially for nonruminant animals.

The highest percentage of the P_t in the P_i form was found in dairy and the lowest in broiler feces (Fig. 1G). There was no difference between the other species and categories. It is clear that the order of concentration and proportion can be quite different.

Hog and layer feces had the highest amount of P_r while beef cattle, dairy, and broiler feces had the lowest concentration (Fig. 1B). These differences would be a function of diet in the case of the poultry, and probably of physiology as well for the ruminants. By contrast, beef cattle feces had the highest proportional amount, dairy was intermediate, while the nonruminants had the least (Fig. 1H).

There were no differences among the feces of nonruminants in the concentration of P_{aso} which was much higher than in the feces of the ruminants (Fig. 1C). There were no differences between the dairy and beef cattle feces. Ruminants possess phytase which permit them to digest this P form while for nonruminants, P_{aso} which is mainly inositol

hexaphosphate, is indigestible (6,7,22) and is partly or completely discounted (7). Furthermore, this form is found in cereals, there being little or none in vegetative parts (22) and this would also contribute to the difference since monogastric diets are based mainly on grains while ruminants are fed both. The same relative distribution also occurred for the proportion of P_t composed of this form except that broiler feces had significantly more than hog or layer feces (Fig. 1I).

Lipid P concentration was highest in broiler and lowest in hog feces (Fig. 1D), the values for the other species being intermediate (Fig. 1D). These intermediate values and that for hogs were similar statistically but different from poultry feces.

On a proportional basis, the ruminants had the highest percentage of total P in P_i form, beef cattle feces being significantly higher than dairy (Fig. 1J). Hogs had the lowest proportion in this form. Small amounts of total P were found in lipid form for all fecal material.

Total fecal P was significantly higher in all monogastric species than in ruminants (Fig. 1E). Total fecal P was different for all species and classes except beef cattle, which were statistically similar. Higher total nonruminant fecal P was mainly related to P_{aso} (Fig. 1C), and perhaps to P_i (Fig. 1A) and P_r forms (Fig. 1B) for layers and hogs being higher in the feces of these species.

Ruminants had the wettest feces, hogs and layers were intermediate, and broilers had the driest. Moisture content reflects mainly diet and rearing conditions. Broilers are raised on litter in well-ventilated heated buildings thereby drying fecal material rapidly.

When all species were included, the principal correlations were between P_i and P_t (Fig. 2A) and P_{aso} and P_t (Fig. 2C) since the magnitudes of the coefficients were of the order of 0.9 and 0.8 respectively. The relations for P_i and P_r, P_i and P_{aso} (Fig. 2A), P_r and P_{aso}, P_r and P_t (Fig. 2B), P_{aso} and DM (Fig. 2C), P_t and DM (Fig. 2E), albeit significant, were generally 0.6 or less. Thus, with the species factor included, P_i and P_t tended to vary together in the same direction and this probably reflects the fact that P supplementation is in the inorganic form and any excess, which is excreted in the feces, is found as mainly P_i. Generally, P_i was in the highest concentration. It has been demonstrated elsewhere that P_t and P_i are related (5).

When adjusted for the species effect, all correlations were lower or disappeared (Fig. 2F, J). Nevertheless, a strong relationship remained between P_i and P_t (Fig. 2F). Although P_t and P_r, P_t and P_{aso}, P_t and P_l, and P_{aso} and DM were significantly correlated, the coefficients were generally below $r = 0.5$.

Both P_i and P_l were significantly higher in the feces of calves fed second cut hay compared to first cut, there being no difference for P_r and P_{aso} (Fig. 3). The higher fecal concentrations in the second-cut material reflects the fact that forages are often more concentrated in P_t than is first cut material. For example second-cut hay averaged 2.2 g kg^{-1} of P_t while first-cut contained 1.9 g kg^{-1}.

There were no cultivar effects on the P_r, P_{aso}, or P_l fractions but P_i in the feces of calves fed timothy was considerably lower than in the feces produced from reed canarygrass cultivars (Fig. 4). This may reflect slightly lower P_i levels of 2.0 g kg^{-1} (average of two cuts) compared to 2.1 g kg^{-1} in the reed canarygrasses. Cut-1 timothy at 1.8 g kg^{-1} of P_i was lower than the average of the reed canarygrasses at 2.0 g kg^{-1}, while there was little difference in cut-2 concentrations at 2.2 in timothy compared to 2.1 in the reed canarygrasses. Timothy P may be more available to calves than reed canarygrass P.

The amount of P_i and P_r in the feces also depended on the animal (Fig. 5, 6). For cut-2, animals 6 and 7 had significantly less P_i in their feces than did 8 and 5. For cut-1, although animal 1 had substantially more P_i in the feces, the difference was not significant (Fig. 5). Animal 3 also had less P_r in its feces than did animals 2, 3, and 4 for cut 1, there being no difference for cut 2 (Fig. 6).

From the literature (4), inter-animal variation for various physiological parameters were calculated: rate of P loss in feces had a coefficient of variation (CV) of 14%; CV for rate of endogenous fecal P loss was 29%, CV for rate of dietary P absorption was 15%; CV for P absorbed was 17%; P accretion into non-exchangeable pools had a CV of 31%; P resorption CV was 34%. Furthermore, because inter-animal variation of the same order occurred for the various Ca mechanisms (3), and since there is a relatively close relationship between Ca and P (3), Ca metabolism will also influence P physiology. It is also known that vitamin D

influences P and more directly, Ca metabolism (19) and it is likely that inter-animal variation also occurs for this nutrient. The inter-animal variation in these parameters and others such as the enzyme and buffer systems are mainly reflections of genetic differences. These inter-animal variations would also contribute to the variability observed in Table 1.

Therefore fecal P concentrations and proportion of P_t often varied significantly from literature values of which there were few in any event. Total P, P_i, and P_{aso} varied more and P_i and P_r varied less in ruminant than nonruminant feces. Generally, P_i constituted half or more of P_t in fresh feces. Fecal P concentrations were affected by date of forage cut, variety, and animal.

4.5 Acknowledgements

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Table 1. Total P, proportion of four P forms, and dry matter in fresh, uncontaminated feces of various classes of livestock raised on commercial farms.

Livestock class Variable	Statistical parameters			
	Mean	Range	Standard deviation	Coefficient of variation (%)
Dairy (15 herds)				
Total P (g kg^{-1})†	9.3	6.0 - 16.0	3.0	32.4
Inorganic P (%)‡	63.2	55.3 - 71.2	5.1	8.1
Residual P (%)‡	27.7	18.1 - 35.8	5.1	18.4
Acid-soluble organic P (%)‡	7.8	2.4 - 13.9	3.6	46.7
Lipid P (%)‡	1.4	1.1 - 1.8	0.3	19.6
Dry matter (g kg^{-1})	143.0	135.0 - 162.0	9.0	6.4
Feeder cattle (8 herds)				
Total P (g kg^{-1})	6.7	4.5 - 10.7	2.0	30.3
Inorganic P (%)	47.1	37.8 - 53.3	5.2	11.0
Residual P (%)	37.0	30.8 - 44.4	4.8	12.9
Acid-soluble organic P (%)	13.9	7.1 - 15.6	5.0	36.3
Lipid (%)	2.1	1.0 - 4.0	0.9	45.3
Dry matter (g kg^{-1})	156.0	125.0 - 180.0	18.0	11.6
Finisher cattle (9 herds)				
Total P (g kg^{-1})	6.7	3.7 - 10.6	2.1	32.0
Inorganic P (%)	48.3	28.2 - 63.2	12.1	25.1
Residual P (%)	40.8	20.7 - 56.4	11.3	27.8
Acid-soluble organic P (%)	8.9	3.0 - 14.9	4.0	44.7
Lipid P (%)	1.9	1.5 - 3.3	0.7	34.3
Dry matter (g kg^{-1})	172.0	136.0 - 241.0	37.0	21.8
Hogs (16 herds)				
Total P (g kg^{-1})	29.1	19.7 - 40.0	5.3	18.3
Inorganic P (%)	54.7	42.2 - 76.7	11.1	20.3
Residual P (%)	15.2	9.2 - 26.9	5.5	36.0
Acid-soluble organic P (%)	29.7	13.7 - 45.3	12.3	41.6
Lipid P (%)	0.4	0.3 - 0.5	0.1	15.8
Dry matter (g kg^{-1})	272.0	210.0 - 365.0	37.0	13.5
Broiler (13 flocks)				
Total P (g kg^{-1})	18.0	13.1 - 23.3	3.0	16.7
Inorganic P (%)	34.8	21.4 - 58.4	10.8	31.1
Residual P (%)	11.0	1.5 - 16.6	5.0	45.7
Acid-soluble organic P (%)	53.4	26.2 - 75.5	13.0	24.4
Lipid P (%)	0.9	0.4 - 1.3	0.3	33.4
Dry matter (g kg^{-1})	675.2	371.1 - 855.2	144.0	21.3
Layer (11 flocks)				
Total P (g kg^{-1})	24.2	16.2 - 30.3	4.2	17.4
Inorganic P (%)	49.3	39.8 - 70.0	10.6	21.4
Residual P (%)	17.3	6.6 - 31.8	7.1	41.2
Acid-soluble organic P (%)	33.2	24.7 - 44.4	6.7	20.2
Lipid P (%)	0.6	0.4 - 0.8	0.1	19.5
Dry matter (g kg^{-1})	280.3	236.1 - 382.5	39.0	14.0

† of dry matter

‡ % of total P

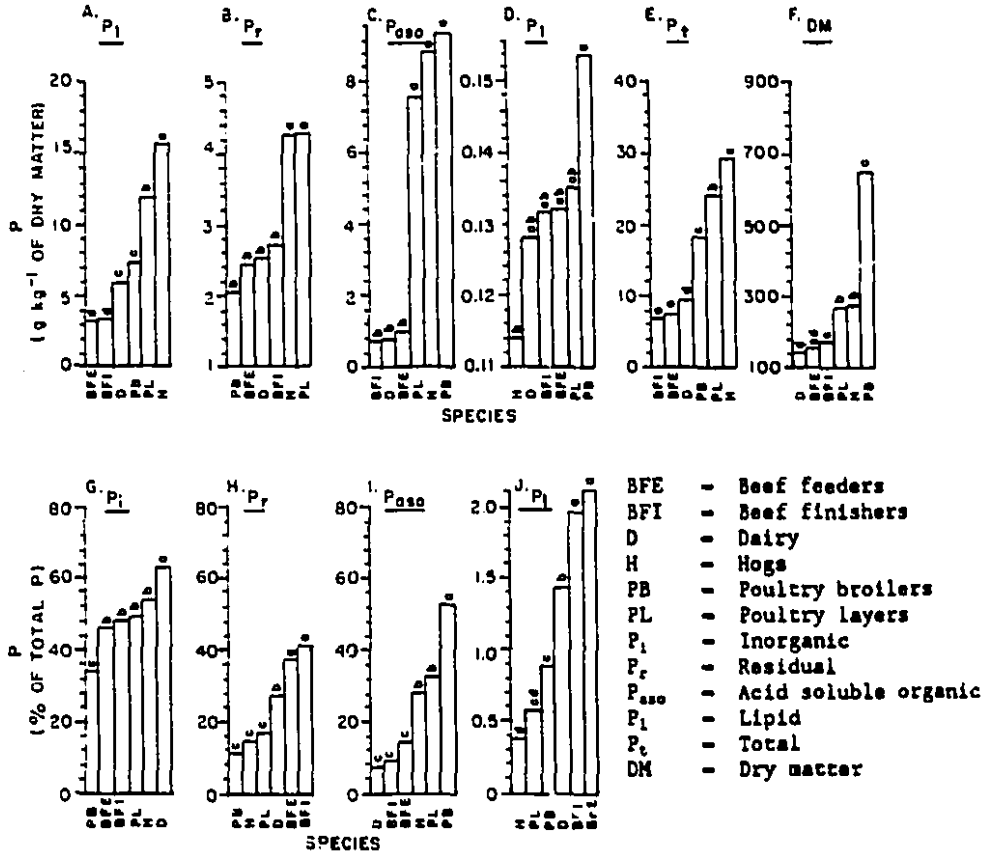

Fig. 1. Concentration and proportion of P forms in the feces of beef and dairy cattle, hogs, and poultry. (Letters are Duncan's Multiple Range groupings P = 0.05.).

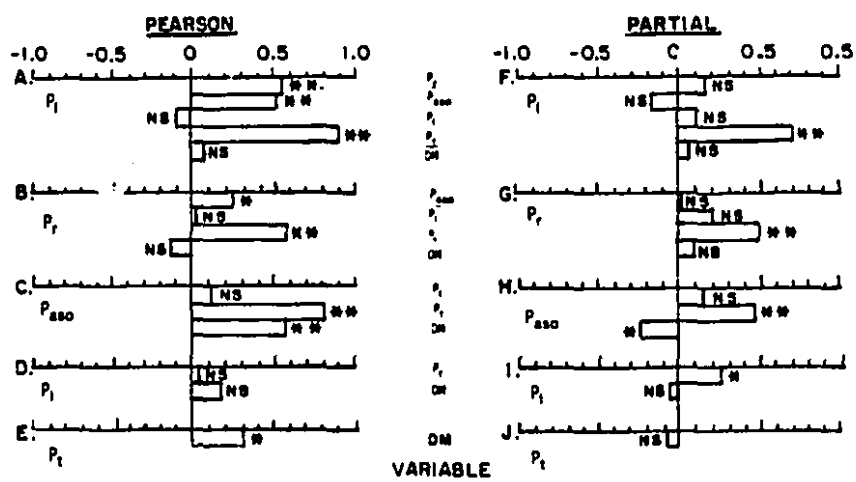

Fig. 2. Correlations between inorganic (P_i), residual (P_r), acid-soluble organic (P_{aso}), lipid (P_l), total P_t, and dry matter (DM) in the feces of dairy and beef cattle, hogs, and poultry. (*, ** = significant at P = 0.05 and P = 0.01 respectively. NS = non-significant at P = 0.05).

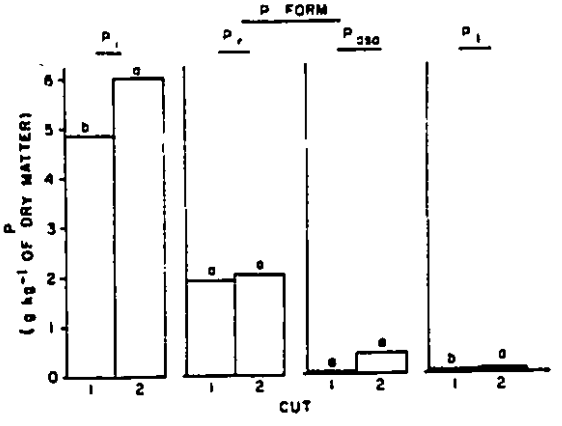

Fig. 3. Effect of cut on inorganic (P_i), residual (P_r), acid-soluble organic (P_{aso}), and lipid (P_l) phosphorus in calf feces. (Columns capped by same letter are not significantly ($\underline{P}$ = 0.05) different by F test).

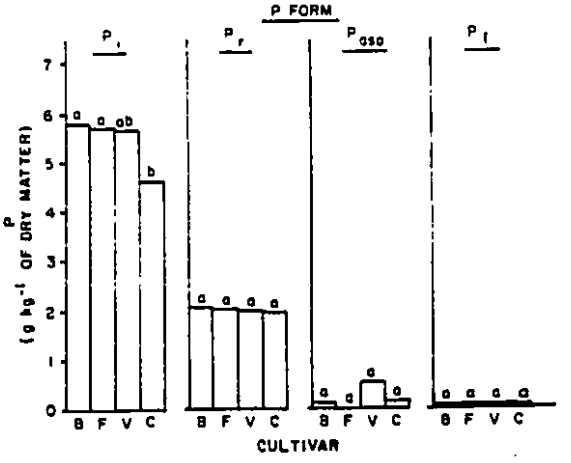

Fig. 4. Forage cultivar and species effects on P forms in calf feces. (Reed canarygrass: B = Beaver, F = Frontier, V = Vantage. Timothy: C = Climax. Columns capped by the same letter are not ($\underline{P}$ = 0.05) significantly different according to Duncan's Multiple Range Test).

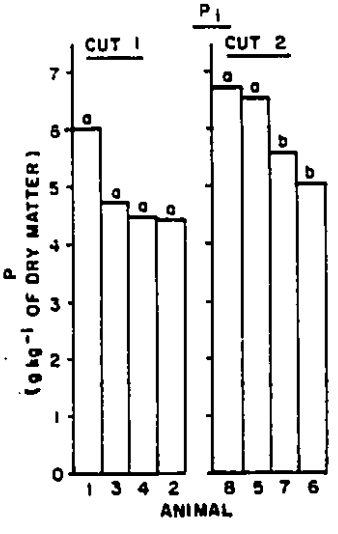

Fig. 5. Mean animal effect across all reed canarygrass and timothy cultivars of cut-1 and cut-2 on calf fecal inorganic P concentration. (Columns capped by the same letter are not significantly ($\underline{P}$ = 0.05) different according to Duncan's Multiple Range Test).

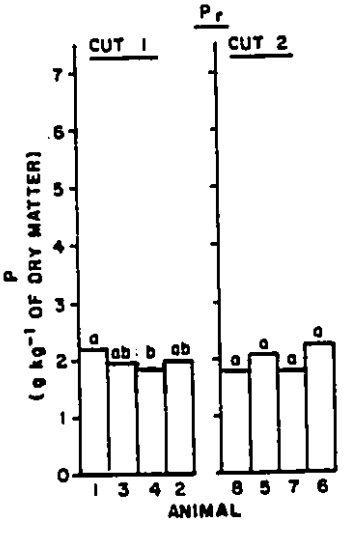

Fig. 6. Mean animal effect across all reed canarygrass and timothy cultivars of cut-1 and cut-2 on calf fecal residual P concentration. (Columns capped by the same letters are not significantly ($\underline{P}$ = 0.05) different according to Duncan's Multiple Range Test).

CONNECTING PARAGRAPH

In chapter 4, it was reported that 36.8 to 52.9% of animal fecal P was in the organic form. Feed additives are commonly used in the farm industry, especially the meat sector. It is known that agrochemicals such as herbicides and insecticides can affect phytase activity, generally in a negative fashion, and that intestinal phosphatase activity has been reduced by antibiotics. It is therefore asked whether feed additives, especially those not absorbed into the cells, could negatively affect the enzymes involved in the breakdown of the two major P_o groups found in animal wastes. Therefore chapter 5 reports the results of experiments conducted to evaluate the effects of several common feed additives on the dephosphorylation activities of phytase on inositol hexaphosphate and of alkaline phosphatase on 5'-adenosine monophosphate.

CHAPTER 5

FEED ADDITIVES AND PHOSPHATASE ACTIVITY

5.1 Summary

The mineralization of organic phosphates (P_o) in animal wastes, which is necessary to render the P available to plants, can be inhibited by agrochemicals. The effects of some feed additives on the enzymatic mineralization of inositol hexaphosphate and adenosine 5'-monophosphate were studied. The mineralization of 2, 5, 10, 20 and 50 x 10^{-5} *M* aqueous solutions of sodium inositol hexaphosphate in 0.1 *M* acetate by 6-phytase was measured in the presence of increasing rates of bacitracin, lincomycin hydrochloride, neomycin sulfate, sodium and procain benzlpenicillin, streptomycin sulfate and virginiamycin. Similar experiments were conducted for alkaline phosphatase dephosphorylation of 50, 100, 200, 400, and 800 x 10^{-5} *M* aqueous solutions of adenosine 5'-monophosphate in 1 *M* diethanolamine. Phytase activity was stimulated by bacitracin at 110 $\mu g\ mL^{-1}$, by 60 $\mu g\ mL^{-1}$ of neomycin, 10 and 50 $\mu g\ mL^{-1}$ of Na-penicillin, by 10 and 90 $\mu g\ mL^{-1}$ of streptomycin but inhibited by 4 and 6 $\mu g\ mL^{-1}$ of lincomycin, 120 and 180 $\mu g\ mL^{-1}$ of neomycin, and 50 $\mu g\ mL^{-1}$ of procain penicillin when compared to the zero rate. Virginiamycin had no effect. Neomycin at 120 $\mu g\ mL^{-1}$, streptomycin at 50 $\mu g\ mL^{-1}$, virginiamycin at 3 and 5 $\mu g\ mL^{-1}$, bacitracin at 300 $\mu g\ mL^{-1}$, lincomycin at 18 $\mu g\ mL^{-1}$, and Na and procain penicillin at 50, 100, and 150 $\mu g\ mL^{-1}$ stimulated alkaline phosphatase activity while bacitracin at 10 and 100 $\mu g\ mL^{-1}$ and streptomycin at 300 $\mu g\ mL^{-1}$ inhibited this enzyme. Effects of hydrolysis therefore depended on additive and concentration but were generally small except for neomycin on phytase and bacitracin on both enzymes. Effects could be positive or negative.

Key words: Phytase, alkaline phosphatase, feed additives, organic phosphorus

Résumé

La minéralisation des phosphates organiques (P_o) des fumiers et lisiers, qui est nécessaire pour rendre le phosphore disponible aux plantes, peut être empêchée par les chimiques agricoles. Les effets de quelques additifs de moulée sur la minéralisation enzymatique de

l'inositol hexaphosphate et l'adenosine monophosphate ont été étudiés. La minéralisation des solutions aqueuses de 2, 5, 10, 20, et 50 x 10^{-5} *M* de l'inositol hexaphosphate de sodium dans 0.1 *M* d'acétate par le 6-phytase a été mesurée dans la présence des taux croissants de bacitracine, de l'hydrochlorure, de lincomycine, du sulfate de streptomycine et de la virginiamycine. Des expériences semblables ont été conduites sur la déphosphorylation par la phosphatase alkaline des solutions aqueuses de 50, 100, 200, 400, et 800 x 10^{-5} *M* du 5'-monophosphate d'adenosine dans 1 *M* diethanolamine. L'activité phytasique a été stimulée par la bacitracine à 110 μg mL^{-1}, par la néomycine à 60 μg mL^{-1}, par la pénicilline de sodium à 10 et 50 μg mL^{-1}, par la streptomycine à 10 et 90 μg mL^{-1} mais a été réduite par la lincomycine à 4 et 6 μg mL^{-1}, la néomycine à 120 et 180 μg mL^{-1}, et la pénicilline de procain à 50 μg mL^{-1}, toujours en comparaison avec le niveau zéro. La virginiamycine n'a pas eu d'effet. La phosphatase alkaline a été stimulée par la néomycine à 120 μg mL^{-1}, la streptomycine à 50 μg mL^{-1}, la virginiamycine à 3 et 5 μg mL^{-1}, la bacitracine à 300 μg mL^{-1}, la lincomycine à 18 μg mL^{-1}, la pénicilline de sodium et de procain à 50, 100 et 150 μg mL^{-1} tandis que la bacitracine à 10 et 100 μg mL^{-1} et la streptomycine à 300 μg mL^{-1} en comparaison avec les tratements sans additifs, ont réduit l'activité de cet enzyme. Alors, les effets dépendaient de l'additif et sa concentration et pouvaient être positifs ou négatifs. Généralement les effets étaient relativement petits excepté pour l'action de la néomycine sur la phytase et la bacitracine sur les deux enzymes.

Mots clés: Phytase, phosphatase alkaline, additifs de moulée, phosphore organique.

5.2 Introduction

In the United States, recent statistics indicate that about one million kilograms of antibiotics or 40% of the total are used annually as feed supplements (9). Of the hundreds in existence (300 up to 1952), 13 are approved for the livestock and poultry industry as therapeutic agents and feed additives (9). Antibiotics are used as feed additives because they improve feed conversion efficiency and rate of gain. Studies have reported increases ranging from 1.8 to 11.1% for feed conversion efficiency, being most frequent around 5%; and for rate of gain from 3.8

to 22.0% with 5.6% being the most common (9). The variation in rate of response is dependent on other factors such as rate of gain. Nevertheless the point is that considerable amounts of these products are used, especially in the meat sector of the farm industry.

The modes of action of additives in animals are metabolic, nutrient sparing, and disease control (9), only the first being pertinent here. Tetracyclines have been found to have inhibitory effects on cell membranes by disrupting the physical structure and/or transport and hence influencing enzyme cell-wall complexes and phosphatase activity (15), the mechanisms of secretion and release of enzymes from cells as occurred for alkaline phosphatase (11), energy, and other mechanisms involved in microbial activity (1).

The above effects are indirect and involve the formation of phosphatases. However, agrochemicals act at the molecular level by decomposing enzymes or inhibiting them either competitively or non competitively (1).

For instance, Zn-bacitracin at 20 and 100 ppm reduced the intestinal alkaline phosphatase activity in the duodenum of chicks (21). In pure solution, the herbicides atrazine, diuron, and linuron and the insecticides crotoxyphos, dichlorvos, and phosphamidon all inhibited phytase activity (3). The same insecticides also reduced alkaline phosphatase activity (2). Thus, it is clear that agrochemicals can affect the dephosphorylation of P_o compounds but that the knowledge of the mechanisms and modes of action is very scanty.

It has been shown that feed additives can be excreted directly in the feces of farm animals (20). Their presence may affect the mineralization of fecal P_o.

Thus, it is of interest to determine if the fundamental process of mineralization is affected as opposed to indirect effects through reduced bacterial enzyme production. The objective of this work was therefore to evaluate the effects of some commonly used nonabsorbed antibiotics on the mineralization by phytase of inositol hexaphosphate, a common P_o compound, especially in the feces of monogastrics, and on the dephosphorylation by alkaline phosphatase of a nucleotide. Nucleic acid type materials are found in all feces.

5.3 Materials and methods

The enzyme myo-inositolhexakiphosphate 6-phosphohydrolase (6-phytase, phytate 6-phosphatase, 6-phosphohydrolase, EC.3-1-3-26, 0.04 units per mg, extracted from wheat) was reacted with various concentrations of the sodium salt of inositol hexaphosphoric acid (IHP, 98% purity, 11% moisture, 12 Na mole^{-1}) in the presence of different concentrations of various feed additives.

The reaction mixture (3) consisted of 10 mL of a salt solution of 2.5 g L^{-1} each of $MgSO_4$, K_2SO_4, and KCl plus 500 mg L^{-1} of $CaCl_2$; 30 mL of 0.1 *M* acetate buffer of pH 5.1 (8) with or without additive; 5 mL of 2, 5, 10, 20 or 50 x 10^{-5} *M* solutions of IHP in 0.1 *M* acetate buffer at pH 5.1; and 5 mL of phytase solution of 0.5 enzyme units in 50 mL of 0.1 *M* acetate buffer at pH 5.1 in a 100 mL erlenmyer flask.

Various concentrations of feed additives were prepared in 0.1 *M* acetate solution, 30 mL of which was used in the above reaction mixture. They were (in $\mu g\ mL^{-1}$): bacitracin (73,000 units g^{-1}) at 0, 10, 60, and 110; lincomycin hydrochloride (850 units mg^{-1}): 0, 2, 4, and 6; neomycin sulfate (657 $\mu g\ mg^{-1}$, 90-95% B, rest C): 0, 60, 120, and 180; benzlpenicillin sodium salt (1662 units mg^{-1}): 0, 10, 30, and 50; benzylpenicillin procain salt (1000 units mg^{-1}): 0, 10, 30, and 50, streptomycin sulfate (750 units mg^{-1}): 0, 10, 50, and 90, and virginiamycin (95%): 0, 1, 3, and 5. The middle concentration of these additives is common in feed rations, levels occurring in manure being unknown.

Each additive was studied in a separate experiment of split-plot design. The four additive rates constituted main plots and the five IHP concentrations were sub-plots. Each experiment was replicated three times.

All reaction mixtures, without the enzyme were preheated to 53°C in the 100 mL flask. The enzyme was added, the flask stoppered, and reaction mixture incubated at 53°C for 60 min in a reciprocating waterbath (50 cycles min^{-1}). The enzyme was added at one-minute intervals to each consecutive reaction mixture in the order determined by the experimental design. At precisely 60 min, a 5 mL aliquot was removed from each mixture and added to the ascorbic-trichlorocetic acids, solution A of Dick and Tabatabai (5). After adding the reaction mixture to A, solutions B and

then C were immediately added (5). Inorganic P was then determined by colorimetry (5) and corrected for the enzyme and the appropriate IHP plus additive combination (3).

Alkaline phosphatase from bovine intestinal mucosa Type VII-T 2010 units mg^{-1} protein (diethanolamine pH 9.8) in a solution of 3 *M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, and 30 m*M* triethanolamine pH 7.6 was used to dephosphorylate the sodium salt (1 mole/mole) of adenosine 5'-monophosphate (AMP) (18).

The reaction mixture (18) consisted of 30 mL of 1.0 *M* diethanolamine, 10 mL of 0.5 m*M* $MgCl_2$, 5 mL of adenosine 5'-monophosphate at 50, 100, 200, 400, and 800 x 10^{-5} *M*, and 22 μL of alkaline phosphatase (9400 units in 50 mL of 1.0 *M* diethanolamine), reaction occurring at pH 9.8.

Two experiments were conducted, the first with the same additives at the same concentrations as in the phytase experiment except they were dissolved in 1.0 *M* diethanolamine, and the second with much higher additive rates ($\mu g\ mL^{-1}$) bacitracin: 0, 100, 200, and 300; lincomycin: 0, 6, 12, and 18; neomycin: 0, 200, 400, and 600; Na-penicillin: 0, 50, 100, and 150; procain penicillin: 0, 50, 100, and 150; and streptomycin 0, 100, 200, and 300. Virginiamycin was not used in the second trial. The 30 mL of 1.0 *M* diethanolamine in the reaction mixture was replaced with 30 mL of the same buffer containing the additive at the appropriate level. The reaction mixture was incubated at 37°C for one h in a reciprocating waterbath. The reaction was stopped with 1 mL of 30% trichloroacetic acid and inorganic P analysed colorimetrically (19).

The experiment was conducted in a split-plot design with additive rate in main plot and AMP concentration in subplot. Treatments were replicated three times.

A Lineweaver-Burk transform (6,10,14) was used to convert the original substrate and product concentrations to the inverse form. Regression (7) was performed on the transformed data permitting the determination of K_m and V. Confidence limits (7) were calculated for the intercepts and slopes of the "0" rate for each additive. The values of these parameters for each of the other rates within an additive were then compared to the "0" rate confidence interval to determine if the regression lines were different or not.

5.4 Results and discussion

Initial trials demonstrated that the amount of orthophosphate mineralized from a 50 x 10^{-5} *M* IHP solution by phytase decreased for samples analysed towards the end of a run (Fig. 1). Several changes were evaluated as variability control measures, such as IHP preheat time (Fig. 2), none of which had any effect. All other results are not presented. However, what was found to be of importance was delaying the addition of solution C (5) for about 10 s since a lag occurred before the reaction was complete (Fig. 3). This was confirmed in subsequent trials as well. Nevertheless the major factor producing the variability was probably the heat sensitivity of the enzyme, its activity decreasing with time (Fig. 4). Consequently, the enzyme solution was not preheated.

Bacitracin, lincomycin, neomycin, sodium and procain penicillin, and streptomycin had significant effects on phytase activity which were dependent on additive level (Table 1). Virginiamycin had no effect on phytase activity at any level.

Bacitracin enhanced phytase activity at the 110 $\mu g\ mL^{-1}$ level (Table 1). This is reflected in the lower K_m, the substrate concentration required to reach half the maximum velocity (Table 1) but with no significant ($P \leq 0.05$) difference in V values. Similarly the neomycin 60, $\mu g\ mL^{-1}$, Na-penicillin 10 and 50 $\mu g\ mL^{-1}$, and streptomycin 10 and 90 $\mu g\ mL^{-1}$ (Table 1) stimulated phytase activity significantly.

Inhibited phytase activity was indicated by a simultaneous increase or decrease in both V and K_m, a decrease in V and increase in K_m, or no change in V and an increase in K_m, when compared to the 0 rate V and K_m coefficients. Phytase activity was reduced by lincomycin at the 4 and 6 $\mu g\ mL^{-1}$ rate, by neomycin at 120 and 180 $\mu g\ mL^{-1}$, and by procain penicillin at 50 $\mu g\ mL^{-1}$ (Table 1). For the other additive-rate combinations, there were no significant effects based on confidence limit criteria.

Therefore, phytase mineralization of IHP was affected by the presence of feed additives, but not always in a negative fashion as reported for herbicides and insecticides (3). Even when there was a strong negative effect by an additive such as neomycin, the same antibiotic also stimulated mineralization at a lower rate.

For those regressions in Table 1 which intercept the vertical axis close to the same point (no significant difference in V) as occurred for bacitracin, competitive inhibitory effects were produced (22). This means that the additive competed with the substrate for the enzyme but the complex formed was temporary and could be overcome by increasing the substrate concentration. The additive may not react with the enzyme at all but simply block access to it physically.

Some of the inhibitions may be noncompetitive (22) in nature, since the intercepts, V in Table 1 (actually 1/V), for the additive curves are significantly different from nonadditive curves. Examples are lincomycin 4 $\mu g\ mL^{-1}$, neomycin 120 and 180 $\mu g\ mL^{-1}$, and procain penicillin 50 $\mu g\ mL^{-1}$ (Table 1).

Some enzymes are activated by other molecules and ions (22) and in the case of some of the additives at some rates – bacitracin 110 $\mu g\ mL^{-1}$, Na-penicillin 10 and 50 $\mu g\ mL^{-1}$, streptomycin 10 and 90 $\mu g\ mL^{-1}$ – this was the case.

Additive effects on alkaline phosphatase mineralization of AMP depended on the substrate level for bacitracin, neomycin, Na-penicillin, streptomycin, and virginiamycin (Table 2). Procaine penicillin and lincomycin had no effect.

Bacitracin at 10 $\mu g\ mL^{-1}$ (Table 2) was the only combination to have an inhibitory effect while neomycin at 120 $\mu g\ mL^{-1}$ (Table 2), streptomycin at 50 $\mu g\ mL^{-1}$ (Table 2), Na-penicillin at 10 and 50 $\mu g\ mL^{-1}$ (Table 2), and virginiamycin at 3 and 5 $\mu g\ mL^{-1}$ (Table 2) stimulated phosphatase activity. There were no other effects. Constants were not calculated because the intercepts oscillated around the origin (Table 2). However, it is clear that V was high.

The effects of low additive rates on alkaline phosphatase activity were therefore not marked.

In comparing the high (Table 3) and low (Table 2) rate experiments the following effects were found – lincomycin: slightly stimulatory at 18 $\mu g\ mL^{-1}$ versus no effect, neomycin: none vs. slightly positive at 120 $\mu g\ mL^{-1}$, bacitracin: stimulatory at 300 $\mu g\ mL^{-1}$ but inhibitory at 100 $\mu g\ mL^{-1}$ vs. inhibitory at 10 $\mu g\ mL^{-1}$, Na-penicillin: stimulatory at all rates vs. positive at 50 $\mu g\ mL^{-1}$, procain penicillin: stimulatory at all rates vs.

none, and streptomycin: slightly inhibitory at 300 $\mu g\ mL^{-1}$ vs slight enhancement at 50 $\mu g\ mL^{-1}$, respectively. Therefore, the effects were often different and dependent on the rate of additive.

The greatest difference between the low and high rate experiments occurred for bacitracin (Table 2 vs. 3) where concentrations of 10 $\mu g\ mL^{-1}$ caused inhibition but concentrations of 300 $\mu g\ mL^{-1}$ caused enhancement of enzyme activity. It was also found that alkaline phosphatase in lepidoptera larvae could be inhibited or stimulated depending on insect strain, insecticide, and sampling delay (17).

The high coefficients of determination, which were generally greater than 0.9 (Tables 1 to 3), indicated that the linear regression model fitted the Lineweaver-Burk transformed data very well (Tables 1,2,3), which means that the velocity was directly dependent on substrate concentration and therefore was a normal enzyme reaction (22).

For the dephosphorylation of adenosine 5'-monophosphate, alkaline phosphatase was used. This is a nonspecific enzyme and it is unknown if it would play a major role in the mineralization of nucleic acids. The possibility therefore exists that a more specific enzyme, adenosine 5'-monophosphatase (5'-nucleotidase), a nucleinase, may be more important and be more affected by additives. For instance, it has been found that Mg(Na+K)-ATPase and 5'-nucleotidase in enterocytes from the jejunum of broiler chicks were affected by virginiamycin while alkaline phosphatase was not (12). Also it has been found that the enzyme form was important. Oligomycin-sensitive Mg^{+2}-ATPase was strongly inhibited by DDT while Na^{+}-K^{+}-ATPase was not (16). Furthermore there are many other nucleic acids (22) perhaps of much greater importance in animal waste decomposition.

The same comments also apply to IHP mineralization. It is not known if the myo form predominates in animal wastes. However, it would be expected that this form would be prevalent since only the myo isomer is found in plants (4). As well, in soils the mono, di, tri, tetra, penta, and hexa esters of myo-inositol are found, the most common being the latter two.

Although the additives used in this study are essentially non absorbed by animals, the degree of breakdown is generally unknown. For instance, part of penicillin may be metabolized as penicillin acid (20)

and it was found that in the chicken, this antibiotic was rapidly deactivated to penicilloic acid, penicillamine, and penicillic acid and that little reached the caecum (13). The effects of such metabolites on phosphatase are unknown. Another factor to consider is the excretory pathway, penicillin for example being largely eliminated through the kidneys although 10-20% is excreted through the intestine (20). Nevertheless, the excretory pathway is probably immaterial since feces and urine are stored together in any event. It would be expected that there would be increased additive concentration in fecal material derived from medicated feed since the animal uses about half the carbon but will try to eliminate all of any foreign substances. However, dilution will occur from other feedstuffs and water. Furthermore, in the real situation of manure decomposition, the presence of a complex of other compounds -- proteins, amino and carboxylic acids, esters, fats, carbohydrates and many others may play a role. Thus, the degree to which dephosphorylation of IHP and AMP can be affected in manure by feed additives remains to be determined.

Therefore, the presence of feed additives, especially bacitracin and neomycin did affect phytase and alkaline phosphatase activity. Effects depended on the additive and its' concentration and could not be generalized. Most additives produced only small effects on phytase and alkaline phosphatase activity in pure solution.

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5.6 References

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Table 1. Constants calculated from Lineweaver-Burk form† of Michaelis-Menten equation for phytase mineralization of inositol hexaphosphate in the presence and absence of feed additives.

Additive Parameter	Additive rate ($\mu g\ mL^{-1}$) Enzyme constants				95% confidence limits‡ Lower	Upper
Bacitracin	0	10	60	110		
V ($\mu g\ mL^{-1}\ h^{-1}$)	13.77	13.86^{NS}	14.81^{NS}	13.48^{NS}	0.0558	0.0893
K_m (10^{-5} M)	15.36	14.06^{NS}	14.93^{NS}	11.22*	1.0467	1.1829
R^2	0.996	0.998	0.996	0.996		
Lincomycin	0	2	4	6		
V ($\mu g\ mL^{-1}\ h^{-1}$)	10.11	9.51^{NS}	8.13*	9.55^{NS}	0.0782	0.1196
K_m (10^{-5}M)	9.45	8.67^{NS}	8.31*	10.45*	0.8503	1.0195
R^2	0.991	0.990	0.998	0.970		
Neomycin	0	60	120	180		
V ($\mu g\ mL^{-1}\ h^{-1}$)	13.61	10.60^{NS}	9.51*	8.53*	0.0465	0.1004
K_m (10^{-5} M)	17.65	12.40*	13.33*	18.22*	1.1875	1.4065
R^2	0.808	0.996	0.996	0.989		
Na-penicillin	0	10	30	50		
V ($\mu g\ mL^{-1}\ h^{-1}$)	13.66	14.53^{NS}	14.70^{NS}	14.86^{NS}	0.0538	0.0926
K_m (10^{-5} M)	15.10	14.77*	15.69^{NS}	14.98*	1.0277	1.1849
R^2	0.998	0.995	0.998	0.999		
Procain penicillin	0	10	30	50		
V ($\mu g\ mL^{-1}\ h^{-1}$)	12.27	13.40^{NS}	12.30^{NS}	15.41*	0.0678	0.0952
K_m (10^{-5} M)	13.76	15.08^{NS}	13.15^{NS}	19.93*	1.0664	1.1765
R^2	0.997	0.997	0.998	0.998		
Streptomycin	0	10	50	90		
V ($\mu g\ mL^{-1}\ h^{-1}$)	15.17	13.00^{NS}	15.62^{NS}	13.93^{NS}	0.0351	0.0965
K_m (10^{-5} M)	15.61	11.11*	15.44^{NS}	12.29*	0.9039	1.1533
R^2	0.999	0.997	0.999	0.997		
Virginiamycin	0	1	3	5		
V ($\mu g\ mL^{-1}\ h^{-1}$)	13.70	14.00^{NS}	14.30^{NS}	17.33^{NS}	0.0450	0.1010
K_m (10^{-5} M)	14.16	13.74^{NS}	14.01^{NS}	18.90^{NS}	0.9192	1.1482
R^2	0.999	0.999	0.999	0.997		

† $\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[S]}$

v = observed velocity

V = maximum velocity

[S] = molar substrate concentration

K_m = molar substrate concentration at half maximum velocity

‡ on regression coefficients (1/V and K_m/V)

*, NS outside and inside 95% confidence limits for regression coefficients for 0 additive rate

R^2 = coefficient of determination

Table 2. Intercepts and slopes† for alkaline phosphatase mineralization of adenosine 5'-monophosphate in the presence and absence of feed additives (low additive rates).

Additive / Parameter	Additive rate ($\mu g\ mL^{-1}$) — Regression coefficients				95% confidence limits‡	
					Lower	Upper
Bacitracin	0	10	60	110		
1/V	0.0107	-0.0124^{NS}	0.0082^{NS}	0.0088^{NS}	-0.0275	0.0488
K_m/V (x 10^{-5})	53.6899	60.2067*	52.9675^{NS}	52.5056^{NS}	49.9746	57.4052
R^2	0.999	0.997	0.999	0.999		
Lincomycin	0	2	4	6		
1/V	0.0049	-0.0018^{NS}	-0.0055^{NS}	0.0074^{NS}	-0.0519	0.0617
K_m/V (x 10^{-5})	54.6871	56.5782^{NS}	56.7927^{NS}	53.0870^{NS}	49.1740	60.2002
R^2	0.999	0.999	0.997	0.999		
Neomycin	0	60	120	180		
1/V	-0.0026	-0.0059^{NS}	0.0055^{NS}	-0.0040^{NS}	-0.0026	0.0275
K_m/V (x 10^{-5})	61.2084	59.1957^{NS}	55.3674*	58.6447^{NS}	58.2827	64.1333
R^2	0.997	0.998	0.999	0.998		
Na-penicillin	0	10	30	50		
1/V	0.0124	0.0091^{NS}	0.0002^{NS}	-0.0118^{NS}	-0.0446	0.0692
K_m/V (x 10^{-5})	57.9907	57.2463^{NS}	58.7280^{NS}	56.5583^{NS}	52.4439	63.5376
R^2	0.999	0.999	0.999	0.999		
Procain penicillin	0	10	30	50		
1/V	0.0031	-0.0023^{NS}	-0.0011^{NS}	0.0053^{NS}	-0.0248	0.0311
K_m/V (x 10^{-5})	66.7225	59.0751^{NS}	58.7251^{NS}	58.4904^{NS}	58.0096	64.4354
R^2	0.998	0.999	0.999	0.999		
Streptomycin	0	10	50	90		
1/V	0.0077	0.0004^{NS}	0.038^{NS}	0.0132^{NS}	-0.0337	0.0491
K_m/V (x 10^{-5})	57.0042	57.4256^{NS}	51.2482*	56.2646^{NS}	52.9797	61.0282
R^2	0.999	0.999	0.997	0.999		
Virginiamycin	0	1	3	5		
1/V	-0.0002	-0.0039^{NS}	-0.00007^{NS}	0.0024^{NS}	-0.0236	0.0232
K_m/V (x 10^{-5})	45.5089	43.8845^{NS}	43.1257*	42.5642*	43.2347	47.7831
R^2	0.998	0.998	0.999	0.998		

*, ^{NS} Outside and inside 95% confidence limits for 0 additive rate

† $\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[S]}$

v = observed velocity

V = maximum velocity

K_m = molar substrate concentration at half maximum velocity

$[S]$ = molar substrate concentration

‡ on regression coefficients (1/V and K_m/V)

R^2 = coefficient of determination

Table 3. Alkaline phosphatase constants calculated from Lineweaver-Burk† form of Michaelis-Menten equation for mineralization of adenosine 5'-monosphosphate, in the absence and presence of feed additives (high rates).

Additive Parameter	Additive rate ($\mu g\ mL^{-1}$) Enzyme constants				95% confidence limits‡ Lower	Upper
Bacitracin	0	100	200	300		
V ($\mu g\ mL^{-1}\ h^{-1}$)	41.86	64.11^{NS}	48.22^{NS}	31.15^{NS}	0.0008	0.0396
K_m (10^{-2} M)	2.29	4.14*	2.59^{NS}	1.16*	50.5127	58.7332
R^2	0.999	0.998	0.999	0.999		
Lincomycin	0	6	12	18		
V ($\mu g\ mL^{-1}\ h^{-1}$)	33.78	35.46^{NS}	42.02^{NS}	30.03^{NS}	0.0079	0.0513
K_m (10^{-2} M)	1.80	1.92^{NS}	2.19^{NS}	1.46*	51.1164	55.3249
R^2	0.999	0.999	0.999	0.999		
Neomycin	0	200	400	600		
V ($\mu g\ mL^{-1}\ h^{-1}$)	26.04	35.97^{NS}	26.95^{NS}	31.95^{NS}	0.0147	0.0621
K_m (10^{-2} M)	1.32	1.89^{NS}	1.35^{NS}	1.64^{NS}	48.3282	52.9294
R^2	0.999	0.999	0.999	0.999		
Na-penicillin	0	50	100	150		
V ($\mu g\ mL^{-1}\ h^{-1}$)	34.13	24.75^{NS}	32.78^{NS}	27.78^{NS}	0.0038	0.0572
K_m (10^{-2} M)	1.76	1.17*	1.64*	1.34*	50.5644	52.7339
R^2	0.999	0.999	0.999	0.999		
Procain penicillin	0	50	100	150		
V ($\mu g\ mL^{-1}\ h^{-1}$)	45.04	37.04^{NS}	39.84^{NS}	32.47^{NS}	0.0037	0.0407
K_m (10^{-2} M)	2.42	1.90*	2.05*	1.63*	51.9423	55.5449
R^2	0.999	0.999	0.999	0.999		
Streptomycin	0	100	200	300		
V ($\mu g\ mL^{-1}\ h^{-1}$)	24.33	25.25^{NS}	24.75^{NS}	25.51^{NS}	0.0083	0.0739
K_m (10^{-2} M)	1.14	1.20^{NS}	1.18^{NS}	1.31*	43.8748	50.2680
R^2	0.999	0.999	0.999	0.999		

† $\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[S]}$

v = observed velocity

V = maximum velocity

K_m = molar substrate concentration at half maximum velocity

[S] = molar substrate concentration

*, ^{NS} Outside and inside 95% confidence limits for 0 additive rate

R^2 = coefficient of determination

‡ on regression coefficients (1/V and K_m/V)

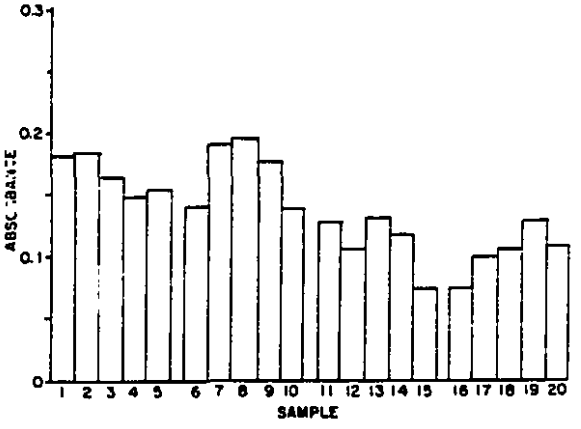

Fig. 1. Inorganic P determinations from mineralization of 50×10^{-5} M inositol hexaphosphate by phytase at 53°C. The enzyme was added at 1 minute intervals in the order 1 to 20. Samples and enzyme preheated together until used. CV = 26%.

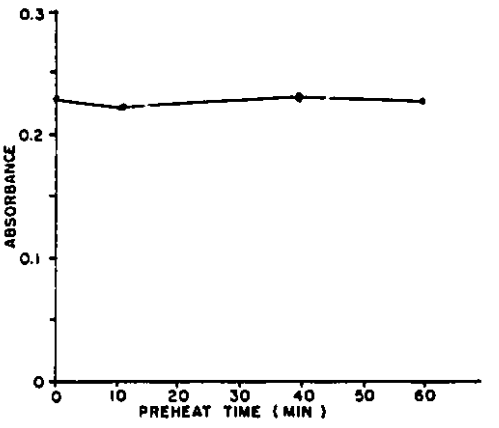

Fig. 2. Inorganic P concentrations from the mineralization of inositol hexaphosphate (IHP) by phytase as affected by time of IHP preheating. (Time^{NS}, coefficient of variation = 3.2%. NS, = nonsignificant at $P = 0.05$ respectively).

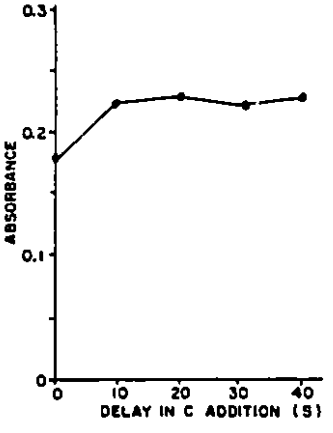

Fig. 3. Inorganic P concentration from inositol hexaphosphate mineralization by phytase at 53°C as affected by delay in the addition of C (sodium citrate). (Delay ** (quadratic**), Coefficient of variation = 4.3%. ** = significant at $P = 0.01$).

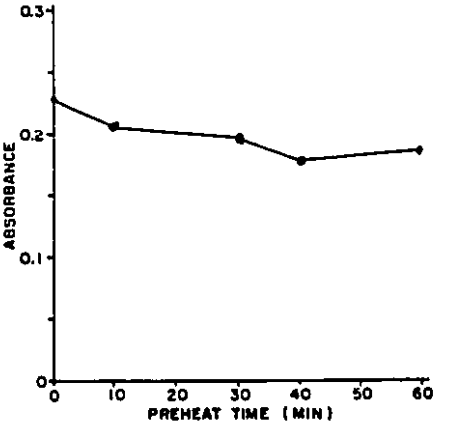

Fig. 4. Inorganic P concentration resulting from inositol hexaphosphate mineralization by phytase at 53°C as affected by time of phytase preheating. (Time** (quadratic**), Coefficient of variation = 2.2%. ** = significant at $P = 0.01$).

CONNECTING PARAGRAPH

In chapter 5, it was demonstrated that the dephosphorylating activities of phytase and alkaline phosphatase in pure systems could be both negatively or positively affected by the presence of feed additives. The question was therefore posed whether the presence of feed additives in decomposing animal wastes would affect bacterial and fecal phosphatase activities and if the proportion of the various P forms would be affected. Therefore chapter 6 reports the results of a digester experiment which measured the effects of four additives at four rates on gas production, fecal phosphatase activity, and P_o and P_i forms in anaerobically decomposing dairy cattle wastes.

CHAPTER 6

FEED ADDITIVES AND MANURE TRANSFORMATIONS

6.1 Summary

Animal waste properties affect decomposition during storage. This study was conducted to evaluate the effect of feed additives on manure decomposition and fecal organic P (P_o) mineralization. Four feed additives, each at four rates (bacitracin: 0, 10, 60, 110; lincomycin: 0, 0.75, 4.50, 8.25; neomycin: 0, 20, 120, 220; penicillin: 0, 5, 30, 55 in $\mu g\ g^{-1}$ wet feces), were mixed with fecal material from dairy cattle (*Bos taurus* L.) fed high or low quality feedstuffs and phosphatase activity (PA) measured on nonincubated material. After 20 h incubation in flasks, total (P_t), inorganic (P_i), acid-soluble (P_{aso}), and residual (P_r) forms were measured on incubated fecal material and gas volume determined. Phosphatase activity was initially depressed by all additives in a linear fashion when added before incubation. After incubation, the depressive effects disappeared with lincomycin and neomycin, increased to 25 $\mu g\ g^{-1}$ for penicillin and then decreased, and was depressed to a greater extent by bacitracin. Gas production, which occurred only on the high-quality diet fecal material, was depressed by bacitracin, increased linearly by penicillin, not affected by neomycin, and increased up to 4.5 $\mu g\ g^{-1}$ and then was depressed thereafter by lincomycin. Gas production and PA were therefore affected by additives but not necessarily in the same fashion. Total P, P_i, and P_r were not affected by additives but acid-soluble P_t and P_{aso} were increased quadratically by increasing additive rate. By comparison to bacitracin and penicillin, lincomycin and neomycin tended to depress soluble P_t. Therefore additives affected short-term P_o mineralization.

Key words: Feces, antibiotics, phosphatase, phosphorus

Résumé

Les propriétés du fumier affectent sa décomposition pendant l'entreposage. Cette étude a été faite pour évaluer l'effet des additifs de moulée sur la décomposition des fèces et la minéralisation du P organique fécal. Quatre additifs chacun à quatre taux (bacitracine 0, 10, 60, 110; lincomycine: 0, 0.75, 4.50, 8.25; néomycine: 0, 20, 120, 220;

pénicilline: 0, 5, 30, 55 en $\mu g\ g^{-1}$ humide) ont été mélangés avec de la matière fécale provenant des bovins laitiers (*Bos taurus* L.) sur diètes de haute qualité ou de basse qualité et l'activité phosphatasique (AP) a été mesurée avant l'incubation. Après 20 h d'incubation en flacons, le P total (P_t), inorganique (P_i), organique soluble dans l'acide (P_{aso}), et résiduel (P_r) a été mesuré dans les fèces et le volume de gaz a été déterminé. L'activité phosphatasique a été diminuée de façon linéaire avant l'incubation par tous les additifs. Après l'incubation, les effets dépressifs sur l'AP ont disparu pour la lincomycine et la néomycine, l'AP a augmenté jusqu'à 25 $\mu g\ g^{-1}$ de pénicilline pour diminuer par la suite et a diminué encore plus avec la bacitracine. Il y a eu de la production de gaz seulement sur les fèces de diète haute qualité et a diminué avec la bacitracine, augmenté de façon linéaire avec la pénicilline, n'a pas été affectée avec la néomycine et a augmenté jusqu'à 4,5 $\mu g\ g^{-1}$ de la lincomycine pour diminuer par la suite. Alors, la production de gaz et l'AP ont été affectées par les additifs mais pas nécessairement de la même façon. Le P total, P_i, et P_r n'ont pas été affectés par les additifs mais le P_t soluble et P_{aso} ont augmenté de façon quadratique avec les doses croissantes d'additifs. En comparaison avec la bacitracine et la pénicilline, la lincomycine et la néomycine ont diminué le P_t soluble. Alors, la présence d'additifs ont affecté la minéralisation du P_o fécal.

Mots clés: Fèces, antibiotiques, phosphatase, phosphore

6.2 Introduction

Feed additives include many compounds added to farm animal diets for various reasons. The antibiotic group is commonly used in the hog, poultry, and beef industries. Although well over 300 of these substances have been discovered, only a small percentage find widespread use (11). Nevertheless, over one million kg are sold annually in the United States, some 40% of which is destined for nonmedical use, mainly as feed additives (11). Antibiotics are used in this fashion at low rates because of increased rate of gain and improved feed conversion efficiency (11,17). Of course, these substances are also used at much higher rates over short periods for therapeutic purposes. Since feed additives can be found in the manure (7,18,25), manure decomposition and therefore nutrient availability are likely affected.

The fecal material from feeder cattle receiving 70 mg $head^{-1}$ d^{-1} of chlortetracycline (CTC) or oxytetracycline (OTC) contained 5.3 and 11.3 $\mu g\ g^{-1}$ of the respective antibiotics (18), which caused increased manure K. There was also a tendency towards reduced ammonia and higher nitrate in the feces. Similarly, fresh fecal material from feeder steers receiving the same level of CTC, contained 14 $\mu g\ g^{-1}$ of the additive while aged material contained 0.34 $\mu g\ g^{-1}$, some 75% of the CTC having been excreted (7). What was noteworthy was that the fed antibiotic selected for microbes which were relatively inefficient in decomposing manure and that the fecal material itself was less biodegradable, according to biological oxygen demand tests.

Mixing manure with the soil seems to have a mitigating effect on antibiotic contamination because it was found that Zn-bacitracin, spiramycin, tetracycline, and flavophospholipol were found to persist much longer in feces alone whether under aerobic or anaerobic conditions, than in soil-feces mixtures (14). It was also reported that manure containing CTC or OTC also decomposed more rapidly in soil than uncontaminated material and that N transformation was unaffected (18). Amprolium fed to White Leghorn hens (*Gallus gallus domesticus* L.) at 125 g t^{-1} resulted in fecal concentrations of 204 $\mu g\ g^{-1}$ and when manure was mixed with soil at 56.1 t ha^{-1}, remained detectable for 80 days, but had no effect on soil respiration (25). Rapid initial disappearance was believed to be due to adsorption. The effect on manure decomposition was not evaluated. However, gas production from methane digesters has been shown to be inhibited when manure containing antibiotics was used as stock (10,24) which indicates that manure decomposition was retarded.

Feed additives are fed at relatively low rates over a long period of time. However, the same antibiotics are also used at much higher, therapeutic rates, but for a shorter period of time. Effects may be more dramatic under such conditions (20).

Thus, feed additives can affect manure decomposition, the effects being dependant on the particular compound and the rate applied. Animal wastes contain P_o, sometimes up to 80% (19), and this form must be mineralized by phosphatases to be of use to plants. However, the possible mechanisms involved in manure decomposition which might be affected by the

additives were not identified in the literature and it is not known if P_o mineralization is affected.

It was the objective of this study to evaluate whether a soil phosphatase method (22) could be used to measure phosphatase activity in fecal material, whether the pH in soil work was appropriate, and to apply this method in the measurement of any additive effects on fecal phosphatase activity during manure decomposition. The effects of antibiotic feed additives on gas production and on fecal P forms were also evaluated.

6.3 Materials and methods

Uncontaminated feces were collected directly from gestating Holsteins (*Bos taurus* L.) fed a diet of straw (*H. vulgare* L.) and mature hay (*P. pratense* L.) (low-quality) and from producing cows of the same breed fed mainly high moisture corn grain (*Z. mays* L.), haylage (*P. pratense* L.), supplements, and minerals (high-quality) (Table 1). Manure samples were analysed as described (6) and results are found in Table 1. Carbon was determined by resistance furnace (16). A weighed amount of feces was added to water to make a suspension of about 50 g L^{-1}, NH_3 was direct-distilled and measured with a micro-kjeldahl apparatus on a measured aliquot. Another aliquot was digested in a block digester at 400°C with H_2SO_4-$H_2Se_2O_3$-H_2O_2 (13) and after appropriate dilutions, total N was determined by colorimetry and P, K, Ca and Mg by plasma spectrometer. Total P and P_i were determined on a trichloroacetic acid extract of freeze-dried feces (15). Density was determined by weighing a known volume of manure. Dry matter was determined at 70°C. Fecal samples were frozen until needed at which time they were thawed and mixed in a food processor. Considerable care was taken in homogenizing and preparing the samples since this was found to influence variation in the subsequent operations.

To each of two 50-g fecal samples placed in 250 mL erlenmyer flasks, the appropriate volume of additive in aqueous solutions was added and then diluted to 100 g with distilled water. The mixture was shaken, stoppered with a one-hole outlet connected by Tygon tubing to a closed-end glass column filled with water and inverted in a beaker of the same liquid, which permitted the capture of any gas generated. The erlenmyer flasks were placed in a reciprocating water bath (20 cycles min^{-1}) at 37°C and

incubated for 20 h after which the mixture was freeze-dried. A duplicate of each of the treatments at preparation time was immediately frozen and freeze-dried. The purpose of this sample was to evaluate initial effects of additive presence on enzyme activity.

The appropriate additive quantities from aqueous solutions were added to separate flasks of manure to produce the following concentrations chosen to encompass those which might occur in the feed and the highest possible levels in the feces (on a wet fecal basis, $\mu g\ g^{-1}$) bacitracin: 0, 10, 60, and 110, Na-penicillin: 0, 5, 30, and 55, lincomycin: 0, 0.75, 4.50, and 8.25; and neomycin: 0, 20, 120, and 220.

The incubation experiment consisted of the factorial combination of four additives each at four rates applied to the two fecal types, all combinations being replicated three times. Treatments were arranged in split-plot, additives forming the main plots, with rate by fecal type in subplots.

The volume of gas generated in 20 h was measured and air pressure and room temperatures were noted permitting correction to standard temperature and pressure conditions. The rest of the variables were measured on freeze-dried material.

Total P was determined by digesting a 200 mg subsample with H_2SO_4–$H_2Se_2O_3$–H_2O_2 (13), diluting to volume, and analysing the resulting solution colorimetrically (23). Soluble (in trichloroacetic acid (TCA)) P_t (P_i plus P_{aso}, mainly inositol hexaphosphate) and P_i were extracted from a one g sample with 5% TCA at room temperature for one h with agitation, centrifuged at 5000 *g* and filtered through a No. 54 paper (15). The extraction was repeated, the residue transferred to the filter paper, washed with TCA, and the filtrate was made to volume with TCA. Soluble P_t was determined as above (13,22). To measure P_i, a 50 mL aliquot of the TCA extract was shaken for 15 min with three g of Darco G-60 charcoal, filtered through a No. 42 paper, washed, and diluted to volume, both with TCA, and then analysed colorimetrically (23). Acid-soluble organic P (inositol hexaphosphate) was calculated as the difference of soluble P_t less P_i. Similarly, P_r (nucleic acid) was calculated as the difference between P_t and soluble P_t.

The p-nitrophenyl phosphate method (22) was modified to evaluate phosphatase activity in the fecal samples. A 50 mg (150 for soils) sample was placed in a 100 mL erlenmyer flask, four mL of modified universal buffer at pH 6.5 and one mL of p-nitrophenyl phosphate (0.115 *M*) was added, swirled, stoppered and incubated at 37°C in a waterbath for one h. The stopper was removed, one mL of 0.5 *M* $CaCl_2$ and four mL of 0.5 *M* NaOH added, the suspension was swirled and filtered through a No. 54 paper. The p-nitrophenol yellow was then measured with a colorimeter at 400 mμ calibrated to p-nitrophenol standards of 0 to 10 $\mu g\ mL^{-1}$. Background correction was accomplished by following the above procedure except that p-nitrophenyl phosphate was added after the $CaCl_2$ and NaOH solutions. Correction for adsorption was not made since none was found to occur as noted by Tabatabai and Bremner (21).

The pH effect on the phosphatase activity curve was determined to ascertain that the recommended pH was appropriate. To do this, the p-nitrophenyl phosphate method described above was assayed at all unitary pH levels from 4 to 12 in freeze-dried dairy cattle manure, sandblast silica 40 sand, Greensboro loam (orthic humo-ferric podzol) and Ste-Rosalie clay (orthic humic gleysol) soils (2) (Table 1) by adjusting the modified universal buffer pH with 0.1 N HCl. The experiment which consisted of the factorial combination of nine pH levels and four media replicated three times was conducted in randomized complete block. Blank corrections were made as described (21) for each pH. The soils and silica were analysed for the following characteristics: extractable P, K, Ca, and Mg by Mehlich-3 (5), lime requirement by SMP and water pH (1:1) (5), cation exchange capacity by the $BaCl_2$ method (12), C by resistance furnace (16), and texture by hydrometer (3). Results are found in Table 1.

The data were statistically analysed according to design and source of variation (9) and the results appear in Table 2. Significant ($P \leq 0.05$) interactions were partitioned to verify the significance of simple effects (Table 3). When the simple effect was significant and was quantitative in nature, the highest significant orthogonal polynomial was fitted while Duncan's multiple range test (P=0.05) was used to group qualitative treatments. Unless otherwise indicated only significant ($P \leq 0.05$) effects involving additives are presented in this paper.

6.4 Results and discussion

As pH of the two soils increased beyond 6.5, phosphatase activity dropped thereby indicating that the acid form predominated (Fig. 1). It has been reported that most soils produce maximum phosphatase activity at a pH near neutral (6.2 to 7.0) generally peaking at about 6.5 (21). Unlike other soils (21) there was no peak for the two soils employed in this work over the pH range of 4 to 12 (Fig. 1A, B). This difference may be due to the greater natural acidity of the soils used in this study (Table 1) compared to those used by others (21). For the silica sand, activity was low over the whole pH range (Fig. 1C). This activity would be expected to be chemical in origin since sandblast silica would not have supported plants and little if any microbial life.

The dairy cattle manure reacted quite differently and demonstrated a large increase in activity above pH 6 (Fig. 1D). Although the pH rises to near neutrality as digested food passes through the duodenum to the caecum (4) which is further confirmed by the basic nature of most manures (6), it appears that fecal phosphatase activity is greatest above natural pH levels. Optimum pH for amylolytic and proteolytic enzymes active in the digested food has also been found to be around 7 (4). As no optimum pH was found and activities varied less in the pH 4 to 7 range, the recommended 6.5 was used in this work.

Fecal phosphatase activity was immediately affected by the addition of additives, even before incubation. As additive rate increased phosphatase activity decreased linearly independent of the additive (no interaction) (Fig. 2). This indicated that the enzyme was inhibited by the presence of all antibiotics before any transformations of the additives had occurred. After 20 h of incubation bacitracin had a sharp negative effect on phosphatase activity of incubated material (Fig. 3A), the initial negative effects of lincomycin (Fig. 3B) and neomycin (Fig. 3C) disappeared, and penicillin increased activity up to 30 $\mu g\ g^{-1}$ but had an inhibitory effect above this level (Fig. 3D). These effects were independent of fecal type. The fecal material from the high-quality diet had much higher activity ($P < 0.01$) (545 $\mu g\ 0.05\ g^{-1}h^{-1}$) than from the low quality diet (212 $\mu g\ 0.05\ g^{-1}h^{-1}$).

The increased activity produced by increasing penicillin rates up to 30 $\mu g\ g^{-1}$ may mean that the additive preserved the enzyme from bacterial attack while enzymes in the absence of additives were more subject to degradation. Therefore, the additive probably did not enhance enzyme activity itself. By contrast, increasing rates of bacitracin (Fig. 3A) and penicillin above 30 $\mu g\ g^{-1}$ (Fig. 3D) promoted a decrease in phosphatase activity over the 20 h period which are probably inhibitory in nature. These effects were independent of fecal type. In fact, penicillin has been shown to have a bacteriostatic effect (21).

Gas production depended on the kind of additive, additive rate, and type of feces (Tables 2,3). Except for a small amount with neomycin (Fig. 4C), no gas was produced under any conditions from fecal material produced by animals on a low-quality diet. In contrast feces from animals on a high-quality diet produced significant gas amounts (Fig. 4). This is indicative of easily fermentable materials such as carbohydrates in the feces from rich diets. Feces from beef cattle fed diets with increasing corn silage and decreasing proportions of grain were found to produce less methane (10). Furthermore the proportion of lignin in straw or mature hay in the low-quality diet would be higher. Lignin has been found to inhibit methanogenesis (8).

However, gas production from the rich material was influenced by additive and rate in the following fashion: bacitracin had a negative effect (Fig. 4A), lincomycin up to 4.5 $\mu g\ g^{-1}$ stimulated gas formation but had an inhibitory effect thereafter (Fig. 4B), neomycin had no impact (Fig. 4C) while penicillin increased gas production (Fig. 4D) in a linear fashion.

In contrast to gas production, no statistically significant effect was produced on manure C concentration (Table 2). This may indicate that C is a less sensitive indicator of manure decomposition than gas. A small amount of C-compound decomposition would lead to a large volume of gas, in effect a magnification of the effect. The 20 h incubation was undoubtedly too short to produce measurable effects on manure C.

Thus, bacitracin negatively affected both gas production and phosphatase activity. This could indicate several things: gas-producing microorganisms did not multiply and/or may have been killed, or their

activity may have been restricted by inhibition of enzymes active in the decomposition of carbohydrates, proteins, and other compounds thereby reducing gas production, regardless of whether phosphatase activity was affected. The reduction in phosphatase activity (Fig. 3A) could reflect less active or dead bacteria, and/or direct deleterious effects on free enzyme activities. If additives had no effect on phosphatase activity but killed the microbes, it would be expected that there would be a temporary decrease in the activity of any enzyme since there would be no further active exchange with the media until such time as the cells lysed and released their contents. Furthermore, if the additive acted at the bacterial as opposed to the enzyme level, any effect would also depend on the ratio of intra to extracellular enzyme. Whether or not enzyme production is affected by additives, it is clear (Fig. 2) that additives did negatively affect phosphatase activity since these data are from the same experimental units but sampled immediately after additive addition and before any significant effect on microbial growth could be produced. Therefore, additives had a direct negative effect on initial phosphatase activity dependent on rate but independent of additive (no interaction) (Fig. 2).

The initial negative effect may rapidly disappear for some additives since no significant effect on phosphatase activity was measured for lincomycin (Fig. 3B) or neomycin (Fig. 3C) after 20 h incubation. There is some indication that gas production and enzyme activity may be independent in some cases since penicillin continuously increased gas production at all rates (Fig. 4D) but had a deleterious effect on phosphatase activity at high rates (Fig. 3D). Therefore additives do not necessarily affect all mechanisms (eg. phosphatase activity, gas production) in the same fashion.

Both gas production (Fig. 4) and phosphatase activity were lower for feces from the low-quality diet animals. This would indicate much lower microbial activity on this diet, hence less gas and less phosphatase activity.

Additives had no effects on P_t, P_i, or P_r. All of these forms were significantly ($P < 0.05$) lower in the feces from animals on the low-quality diet than in the fecal material from animals on the high-quality

diet (Table 2). The low-quality compared to high-quality diet feces had the following concentrations in g kg^{-1} of dry matter: P_t 7.6 vs. 10.4, P_i 6.3 vs. 6.9, and P_r 1.2 vs. 1.8. Total P and P_i concentration in sheep feces also were directly related to feed quality (1).

Although there were no additive effects on P_i, the form most important in crop fertilization, nevertheless phosphatase activity was affected even after 20 h, and hence might influence the mineralization of organic P during a longer period of time.

As in soils, measurements of P_i are likely less sensitive to changes than phosphatase activity and therefore do not reflect treatment effects as well. Furthermore the lower phosphatase activity in the low-quality diet feces would mean that a longer period is needed to reflect differences. The proportion of fecal P_o may not have been high enough in the fecal material to produce measurable P_i effects, especially during a short term incubation.

The increase in P_{aso} produced by all additives (Fig. 5) would not be due to increased inositol hexaphosphate since this form occurs in plants. This may simply indicate a greater decomposition of this form at low additive rates because of less inhibition effect on enzyme activity as seen in Fig. 2.

There was less ($P < 0.01$) P_{aso} in the feces of cattle on the low-quality diet (0.08 g kg^{-1} dry matter) compared to those on the high-quality diet (1.76 g kg^{-1} dry matter). The difference was especially marked for P_{aso}, which reflects the influence of the presence of grain in the high-quality diet. Grain contains inositol hexaphosphates while very little is found in forages (4).

After incubation, both P_{aso} (Fig. 5) and soluble P_t (Fig. 6A) increased with increasing additive rate, but decreased at the higher rates. Since soluble P_t includes P_{aso}, the rate effect on the latter would also be reflected on the former. Furthermore, soluble P_t was also diet and additive dependent, for both incubated and nonincubated material (Fig. 6B, C). Except for penicillin in the incubated fecal material from low-quality diet animals (Fig. 6B), lincomycin and neomycin produced less soluble P_t than bacitracin or penicillin.

Thus, the p-nitrophenyl phosphatase method developed for soils was found to be useful to detect feed additive treatment effects in animal waste. All assayed antibiotics linearly depressed fecal phosphatase activity initially but this effect rapidly disappeared for some, became more negative or changed direction. These effects were independent of diet. No measurable effect was produced on P_i but there was some indication that P_{aso} mineralization may be depressed. Gas production was sensitive to antibiotics but was produced only on feces from cattle on a rich diet. Animal waste decomposition was therefore affected by the presence of feed additive antibiotics but there was no effect on P_i produced during short-term incubation. Longer term incubation and a higher proportion of P_o in feces could however influence the amount of P_i produced.

Therefore additives affected fecal decomposition, but the nature of the effect depended on additive, its rate, sometimes diet, and the variable measured.

6.5 Acknowledgements

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Table 1. Soil and fecal characteristics.

Dairy cattle fecal sample	Characteristic								
	N	NH_3	P†	K	Ca	Mg	C‡	Dry matter	Density
	(kg t^{-1} wet)						g kg^{-1}		(kg L^{-1})
Low-quality diet	2.63	0.14	1.21	2.48	2.50	0.69	461	221.8	0.886
High-quality diet	4.37	0.21	1.54	1.99	2.11	0.81	458	154.4	0.925

Soil or media	pH	Mehlich III pH	Mehlich III P (buffer)	Mehlich III K	Mehlich III Ca	Mehlich III Mg	C	Sand	Silt	Clay	Cation exchange capacity
			kg ha^{-1}				g kg^{-1}				cmol(+)kg^{-1}
Sand 40	7.1	7.64	7	38	1659	42	1	991	7	2	1.3
Greensboro	4.8	6.48	45	281	654	156	20	817	53	130	6.7
Ste Rosalie	5.3	6.83	36	263	9119	1460	25	276	145	579	25.3

† Low and high-quality diets: 706 and 944 g kg^{-1} P_i respectively (dry matter basis)
‡ Dry basis

Table 2. F values and their significance for sources of variation.

Source	DF	Variable PA(N)	PA(I)	Sol P_t (N)	GAS	P_t	P_i	P_r	Sol P_t	P_{aso}	C
Additive (A)	3	1.05	1.25	1.87	32.33**	0.85	1.80	0.60	0.50	0.43	2.02
EMS (A)	6	5467.83	110.68	0.02	14.76	0.0179	0.0216	0.0275	0.0229	0.0549	2.05
Rate ratio (R)	3	3.52*	1.42	0.36	2.68	0.82	1.06	1.20	4.50**	3.22*	1.07
AxR	9	0.61	3.14**	0.54	6.26**	0.65	1.26	0.55	1.08	1.42	1.55
Diet (D)	1	1069.16**	966.66**	205.59**	565.02**	176.57**	11.61**	4.67*	415.78**	49.20**	0.11
AxD	3	0.27	1.89	3.29*	12.53**	1.22	0.91	1.22	3.37*	1.92	0.50
DxR	3	0.61	1.38	1.11	3.99*	1.11	0.30	0.91	0.35	0.17	0.34
AxRxD	9	0.94	0.61	0.83	5.82**	0.68	0.47	0.78	1.31	0.65	1.07
EMS (B)	56	2023.09	2799.02	0.0030	36.32	0.0103	0.0082	0.0146	0.0028	0.0122	1.47

Legend: PA(N) = phosphatase activity (non-incubated), PA(I) = phosphatase activity (incubated), Sol P_t (N) = total soluble P (nonincubated), P_t = P total incubated, P_i = inorganic P, P_r = residual P, Sol P_t = total soluble P (incubated), P_{aso} = acid soluble organic P, C = carbon, EMS(A) = error mean square main plots, EMS(B) = error mean square subplots, DF = degrees of freedom. R = additive rate ratio. *, ** = significant at $P = 0.5$, $P = 0.01$, respectively. F values with no * or ** are not significant at $P = 0.05$.

Table 3. F values for partitioned sources of variation.

Figure	Simple effects - F value
1.	A. pH (G) 99.19** B. pH (SR) 146.32** C. pH (SS) 2.48^{NS} D. pH (DCM) 6563.08**
3.	A. R (B) 6.62** B. R(Li) 0.65^{NS} C. R(N) 0.77^{NS} D. R(Pe) 2.79*
4.	A. R (B H) 30.36** B. R (Li H) 9.33** C. R (N H) 0.30^{NS} D. R (Pe H) 2.58^{NS} A. R (B L) - B. R (Li L) - C. R (N L) 0.32^{NS} D. R (Pe L) -
6.	A. R 4.50** B. A (L) 3.52* A(H) 3.89* C. A(L) 4.86** A(H) 10.72**

S = Source, DCM = Dairy cattle manure, G= Greensboro, SR = Ste Rosalie SS = Sandblast Sand, A = Additive, R = Rate, T = Type, B = Bacitracin, Li = Lincomycin, N = Neomycin, P_c = penicillin, H = High, L = Low, EMS = error mean square. Brackets mean within: ex. pH (G) = pH within Greensboro.

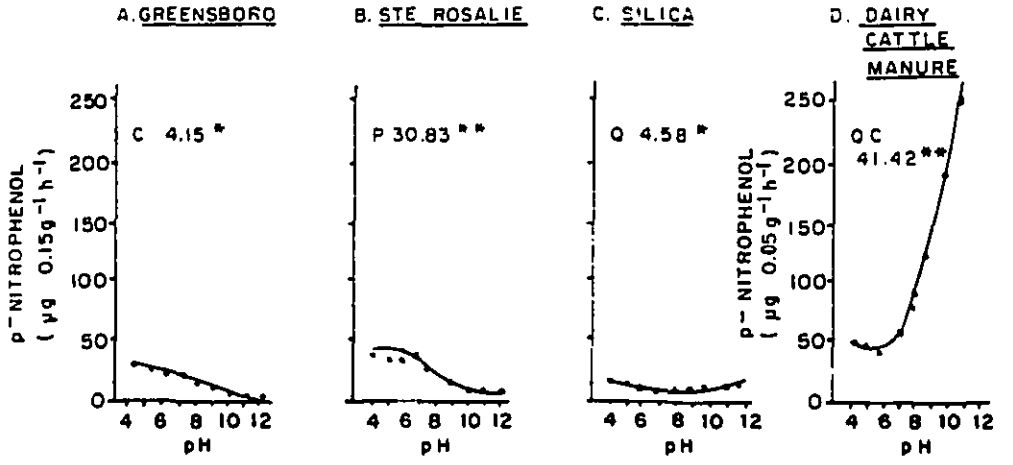

Fig. 1. Effect of buffer pH on the release of p-nitrophenol from p-nitrophenyl phosphate by dairy cattle manure, 2 soils, and sandblast sand. (Q = quadratic, C = cubic, QC = quartic, P = pentic, numbers are F values for highest significant orthogonal polynomial; *, ** = significant at $\underline{P}$ = 0.05 and $\underline{P}$ = 0.01 respectively. • = observed. F ratios for main sources: media 10888.53**, pH 586.95**, media x pH 1224.92**, SD(A-C) = 3.59 µg 0.15 g^{-1} h^{-1}, SD(D) = 3.59 µg 0.05 g^{-1} h^{-1})

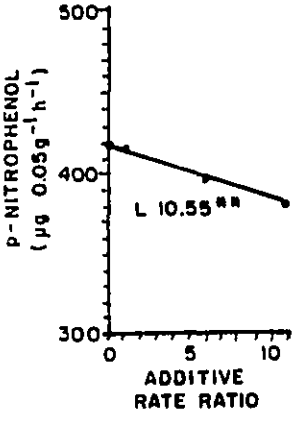

Fig. 2. Additive rate ratio effect on phosphatase activity in non-incubated dairy cattle feces. (L = linear. Number is F value for highest significant orthogonal polynomial. ** = significant at $\underline{P}$ = 0.01. Rate ratios were 0, 0, 0, 0; 1 = 10, 0.75, 20, 5; 6 = 60, 4,50, 120, 30; 11 = 110, 8.25, 220, 55 µg g^{-1} of wet feces for bacitracin, lincomycin, neomycin, and Na-penicillin, respectively. • = observed. SD = 44.97 0.05 g^{-1} h^{-1})

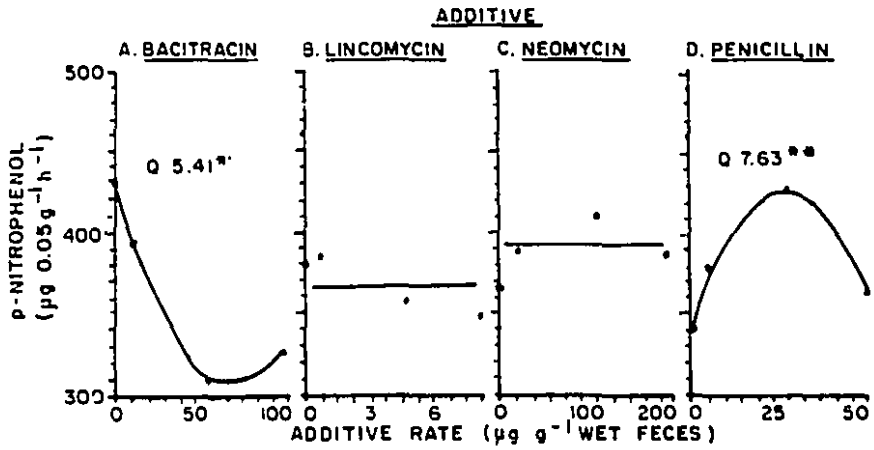

Fig. 3. Effects of additive rate on phosphatase activity in incubated dairy cattle feces. (Q = quadratic. Numbers are F value for highest significant orthogonal polynomial. *, ** = significant at $P = 0.05$ and $P = 0.01$. • = observed. SD = 52.91 μg 0.05 g^{-1} h^{-1})

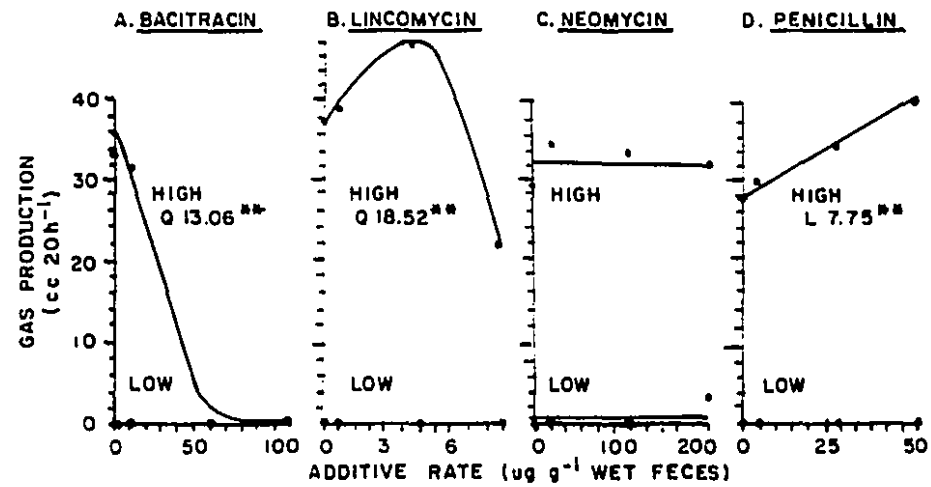

Fig. 4. Gas production during 20 h anaerobic incubation of dairy cattle feces from animals on a high or low quality diet. Q = quadratic, L = linear. Numbers are F values for highest significant orthogonal polynomial. • = observed. ** = $P = 0.01$. SD = 6.02 cc 20 h^{-1})

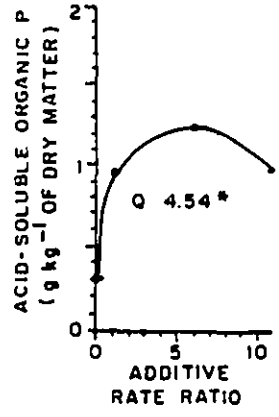

Fig. 5. Additive rate effect on acid-soluble organic P in dairy cattle feces. (Q = Quadratic, number is F ratio for quadratic effect. * = significant at $P = 0.05$. Rate ratios are in the order bacitracin, lincomycin, neomycin, and penicillin respectively, in $\mu g\ g^{-1}$ wet feces: 0 = 0, 0, 0, 0; 1 = 10, 0.75, 20, 5; 6 = 60, 4.50, 120, 30; 11 = 110, 8.25, 220, 55. • = observed. SD = 0.11 g kg^{-1})

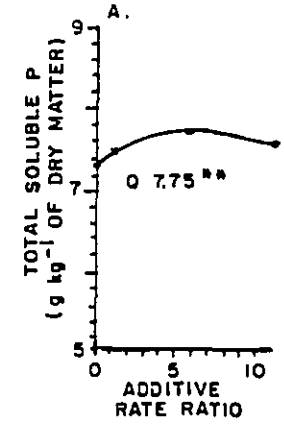

Fig. 6A. Total soluble P in incubated dairy cattle feces. (Q = quadratic, ** = significant at $P = 0.01$. Figure is F value for highest significant orthogonal polynomial. Rate ratios are, in order bacitracin, lincomycin, neomycin, and Na-penicillin ($\mu g\ g^{-1}$ wet feces): 0 = 0, 0, 0, 0; 1 = 10, 0.75, 20.5; 6 = 60, 4.50, 120, 30; 11 = 110, 8.25, 220, 55. . = observed. SD = 0.05 g kg^{-1})

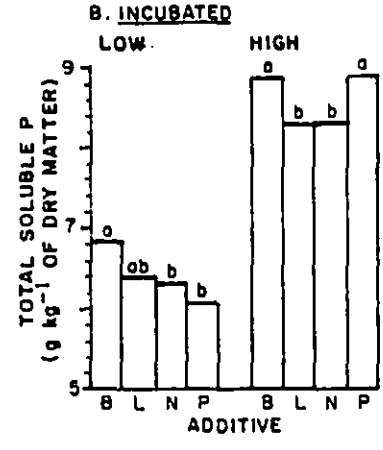

Fig. 6B. Total soluble P in incubated dairy cattle feces. (B = bacitracin, L = lincomycin, N = neomycin, P = penicillin. LOW, HIGH = feces from cattle fed low and high qaulity diets. Columns capped by the same letter are not significantly different by Duncan's Multiple Range Test at $P = 0.05$. SD = 0.05 g kg^{-1})

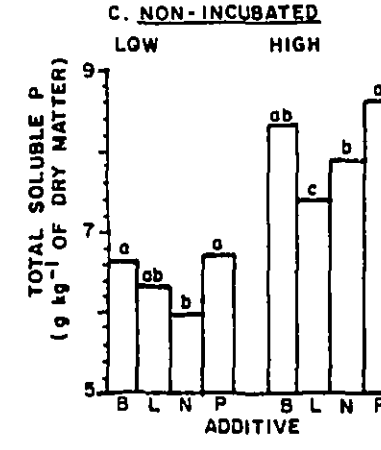

Fig. 6C. Total soluble P in nonincubated dairy cattle feces. (B = bacitracin, L = lincomycin, N - Neomycin, P = penicillin. LOW, HIGH = feces from cattle fed low and high quality diets. Columns capped by the same letter and not significantly different by Duncan's Multiple Range Test at $P = 0.05$. SD = 0.05 g kg^{-1})

CONNECTING PARAGRAPH

In chapter 5, phytase and alkaline phosphatase activities in pure solution were reported to be affected by the presence of feed additives. Similarly, feed additives also affected fecal phosphatase and bacterial activities the nature of which depended on the additive and its rate in decomposing dairy cattle manure. Since animal wastes are applied to crops and soils, would those containing feed additives have any effect on soil phosphatase activity and or crop growth? Chapter 7 examines this question.

CHAPTER 7

FEED ADDITIVES, SOIL PHOSPHATASE, AND CROP GROWTH

7.1 Summary

Soil activity and crop growth are affected by animal waste application. This greenhouse study was conducted to evaluate the effects of feed additives on soil phosphatase activity (SPA) and plant growth and composition. Barley (*Hordeum vulgare* L.) was grown on Ste. Rosalie clay (orthic humic gleysol), Greensboro loam (orthic humo-ferric podzol), and sandblast 40 silica to which these factorial treatments were applied: four feed additives each at three levels (in $\mu g\ g^{-1}$ of wet feces: bacitracin 10, 60, 110; lincomycin 2, 4, 6; neomycin 60, 120, 180; Na-penicillin 10, 30, 50) and two types of P fertilization (45 kg P ha^{-1} applied fertilizer (N) (10-6-16) N, P_2O_5, K_2O (%) and dairy cattle (*Bos taurus* L.) feces (M), 37.3 t ha^{-1}). Additives produced no effects on barley dry matter yields. Cut-1 plant P concentration was reduced by penicillin on manured silica relative to the other additives while cut-2 plant P was reduced on silica by bacitracin (N) and penicillin (M) while all additives produced a positive quadratic effect on silica (M). Root P concentration was reduced by lincomycin on all M media. Penicillin increased P uptake on all N treatments and on all Ste. Rosalie clay treatments. Bacitracin reduced SPA at 30 d on N clay and penicillin depressed SPA on M clay and loam soils with positive quadratic effects by penicillin on N Ste. Rosalie and Greensboro and by lincomycin on M loam. Additive effects decreased at 60 d. Effects therefore were dependent on the additive and its environment.

Résumé

L'activité du sol et la croissance des cultures sont affectées par l'apport du fumier. Cette étude en serre a évalué les effets des additifs de moulée sur l'activité phosphatasique du sol (APS), la croissance des plantes, et leur composition. L'orge (*Hordeum vulgare* L.) a été semé dans de l'argile Ste-Rosalie (gleysol humique orthique), de la terre franche Greensboro (podzol humo-ferrique orthique) et de la silice jet de sable 40. Les traitements en combinaison factorielle étaient: quatre additifs chacun à trois niveaux (en $\mu g\ g^{-1}$ de fèces humides: bacitracine 10, 60, 110; Na-pénicilline 10, 30, 50; lincomycine 2, 4, 6; néomycine 60, 120,

180) et deux types de fertilisation au P (45 kg P ha^{-1}: l'engrais 10-6-16 (N) (N, P_2O_5, K_2O (%)) et les fèces (M) de bovins laitiers (*Bos taurus* L.) à 37.3 t ha^{-1}). Il n'y a pas eu d'effet sur le rendement de l'orge. La teneur en P de la coupe une dans la plante a été réduite par la pénicilline sur la silice en présence de fumier (F) en comparison avec les autres additifs. La teneur en P de la coupe deux a été réduite sur la silice par la bacitracine (SF) et la pénicilline (F) et il y a eu un effet quadratique positif par tous les additifs sur la silice (F). Le P dans les racines a été réduit par la lincomycine sur les traitements F. La pénicilline a augmenté le prélèvement de P sur les traitements SF et sur l'argile. La bacitracine a produit une réduction dans APS à 30 j par bacitracine sur l'argile (SF), par la pénicilline sur l'argile (F) et le Greensboro (F). Sur l'APS, il y a eu un effet quadratique positif produit par la pénicilline sur Ste. Rosalie (SF) et Greensboro (SF) et par la lincomycine sur Greensboro (F). Les effets des additifs sur APS ont été réduits à 60 j. Alors, les effets dépendaient de l'additif et son environnement.

7.2 Introduction

Numerous feed additives are used in all sectors of the animal industry including dairy, poultry, swine, beef, and sheep (5,15). These production improvers as they are known in the feed industry, include antimicrobial substances, growth promotors, antibacterial agents, rumen additives, hormones, surfactants, and specialty additives.

Antimicrobials are used at relatively low levels over long periods to improve production and at medicinal or high levels for short periods. These agents are used as feed additives because feed conversion efficiency and rates of gain are improved from 5 to 10% with a 3 to 4 times return on investment (10).

An animal will seek to eliminate foreign substances from the body as rapidly as possible. Therefore these compounds are of interest from an animal waste management point of view because they or their metabolites are found in fecal material and affect its properties. For instance, 75% of the oral chlortetracycline fed to yearling steers (70 mg $head^{-1}$ day^{-1}) was excreted in the feces which consequently were less degradable (8). The half-life of the antibiotic was found to be greater than 20 days at

4°C or 28°C and aged manure still contained 0.34 $\mu g\ g^{-1}$. Anaerobic decomposition in digesters has also been shown to be severely affected by some additives (20). Therefore, at least a portion of fed-antibiotics and/or their metabolites can be excreted in animal wastes.

Organic phosphates through the activity of enzymes known as phosphatases are hydrolysed to release inorganic P. However, different soils have their own unique enzyme patterns which are not correlated to other biochemical and microbial properties and are often less sensitive than tissue enzymes (17). Of 27 acid phosphatases isolated from germinating barley seeds, 23 were negatively affected by cyclohexamide and 16 by chloramphenicol, some being sensitive to both (16). The activity of alkaline phosphatase from the duodenal loop of broiler chicks was reduced by 20 and 100 ppm of dietary zinc bacitracin (22) and although virginiamycin did not affect the alkaline phosphatase activity in isolated enterocytes from the jejunum of broiler chicks, tetracyclines did reduce the acid and alkaline phosphatase in the jejunum, blood plasma, and liver (13). It has been found that penicillin and streptomycin applied to soils reduced phosphatase activity (1). Therefore, the presence of antibiotics can reduce phosphatase activity in many systems.

Manure from animals and birds fed additive and nonadditive diets when applied to oats was found to have effects that were product-dependent (19). For instance manure from hogs fed carbadox at 50 mg kg^{-1} of feed increased oat dry matter remarkably over nonadditive manure but flavophospholipol at 15 mg kg^{-1}, bacitracin and oxytetracycline each at 80 mg kg^{-1}, and oleandomycin at 10 mg kg^{-1} depressed yields at higher fertilizer N rates. Manure from chickens fed quindoxin at 20 mg kg^{-1} produced higher yields compared to manure from nonquindoxin-fed birds. Payzone at 12 mg kg^{-1} had little effect. Flavophospholipol, chlortetracycline, and zinc bacitracin applied alone at 0.8 and 14.2 mg kg^{-1} soil resulted in no effect on dry matter or N content but when mixed with poultry manure and incorporated into soil, reduced dry matter and increased N content dramatically as did streptomycin applied directly to the soil at the same rates. The effects of antibiotics in manure were found to persist much longer in the feces alone, whether stored under aerobic or anaerobic conditions than when mixed with soil (14). Soil

therefore had a mitigating effect. Some of the mechanisms in plants thought to be affected by additives are growth, flowering, necrosis, chlorosis, interactions with Mg, Ca, Mn, and Fe, respiration, and probably other unknown processes (2).

No work was found which reported the effects of feed additives in manure on soil phosphatase activity, P availability from manured soil, or plant P concentration. The objective of this work was therefore to determine the effects of some common nonabsorbed feed additives, alone and in manure, on P availability, crop growth, and soil phosphatase activity.

7.3 Materials and methods

Barley (*Hordeum vulgare* L., cv Laurier) was grown in a greenhouse experiment on three media (two soils and sandblast silica No. 40) chosen for low available P and to give a range of cation exchange capacities and textures (Table 1). Treatments consisted of the factorial combination of four feed additives each applied separately at three rates in the presence and absence of dairy cattle (*Bos taurus* L.) feces. Thus, there were 72 treatments arranged in a randomized complete block design. These were replicated twice.

Surface samples of Ste. Rosalie clay obtained near Marieville, QC and Greensboro loam from Hatley, QC were shredded and sieved to pass a 2 mm screen. Ste. Rosalie clay is a dark gray orthic humic gleysol common to the St.Lawrence Plain while the Greensboro loam is an orthic humo-ferric podzol common to the Appalachian Highlands of the Eastern Townships, QC (4). The third medium was No. 40 sandblasting silica. Lime ($CaCO_3$) was mixed with soils at the rates of 5.5 g kg^{-1} of Greensboro, and 2.1 g kg^{-1} of Ste Rosalie soils (oven-dried basis), according to lime requirement (6). The two soils and silica were analysed for P, K, Ca and Mg as described (6). Cation exchange capacity was determined by the $BaCl_2$ method (11). The hydrometer method was used to determine the texture (3).

The additives and their respective rates (in μg g^{-1} of wet feces) were: bacitracin 10, 60, and 110; Na-penicillin, 10, 30, and 50; lincomycin 2, 4, and 6, and neomycin 60, 120, and 180. These rates were chosen to encompass those used in the feed industry, the rationale being

that higher concentrations would not be found in fecal material. Appropriate quantities of these common nonabsorbed soluble materials were added in solution to 65.9 g of feces. A compensating amount of water was added such that the total volume was constant. The slurry made from the feces, additive, and water was mixed with two kg of soil and then put in a 15 cm by 15 cm undrained, round plastic pot. The same quantities of additives and water were also mixed with another series of soils, but in the absence of feces.

Fecal material was obtained directly from dairy cattle in production (Table 1) and applied at the rate of 37.3 t ha^{-1} (65.9 g pot^{-1}) to furnish 45 kg ha^{-1} of inorganic P based on surface area. For the pots not receiving manure, 10-6-16 (% N, P_2O_5, K_2O) fertilizer was applied at a rate to supply the same amount of P contained in the feces (1.32 g pot^{-1}). Feces were analysed as described (6).

Three treated (carbathiin) seeds were planted in each of ten equally spaced holes and covered to one cm. Seedlings were subsequently chemically (paraquat) thinned to ten plants per pot at 30 d. Plants were harvested twice at the late tillering stage. The material was dried to constant weight at 70°C and then ground in a Wiley mill to one mm. During the experiment, soil water content was maintained approximately at field capacity (3) and checked every two days by weighing. Temperature was maintained between 15 and 20°C and natural daylight was the only light source. The experiment was seeded in mid-April and harvested in June and again in July.

Yield and plant number were determined at each cut. Roots were harvested after the last cut and weighed. The ground aerial material for each of the two harvests and the root dry matter were digested and analysed for total P concentration (6,12). Standard National Bureau of Standards samples were used to verify accuracy where appropriate (21). Phosphorus uptake was also calculated. A small tube was used to obtain a soil core from each pot at 30 and 60 d. On these samples, phosphatase activity corrected for background color (blanks) was determined (18).

Analyses of variance was then performed on the data (9). Significant ($P \leq 0.05$) interactions were partitioned to determine the nature of the simple effects. Duncan's Multiple Range Test (P=0.05) was used to group qualitative treatments while the highest significant orthogonal polynomial

was fitted to factors involving levels. Unless otherwise stated, only significant ($P \leq 0.05$) effects involving additives are presented.

7.4 Results and Discussion

Antibiotics affected soil phosphatase activity (SPA1) at the first sampling but the effect was dependent on the antibiotic, rate of application, the presence of manure, and on soil type (Tables 2 and 3, Fig. 1). The simple effects were quite complex (Table 3).

Bacitracin alone had a negative linear effect on SPA1 (Fig. 1A) in the Ste. Rosalie clay but no effect in the presence of manure and no effect on Greensboro or silica sand. Penicillin by contrast had a negative linear effect on SPA1 in the presence of manure (Fig. 1D, 1H) but without manure produced a positive quadratic effect on both Ste. Rosalie and Greensboro loam (Fig. 1D, H). This antibiotic was found to produce a negative effect on SPA elsewhere (1). The only other effect was a positive quadratic reaction to lincomycin in the presence of manure (Fig. 1F). There were no effects produced in the silica (Fig. 1, I-L).

At the second sampling, the treatment effects on soil phosphatase activity (SPA2) were simpler than at the first (Table 2), the second order interaction being explained by a negative quadratic reaction of antibiotics in the presence of manure and positive in its absence on the Ste. Rosalie soil, there being no effect on the Greensboro and silica (Fig. 2A). The additive by rate interaction (Fig. 2B) was due to a positive quadratic response to lincomycin and a negative quadratic reaction to neomycin, there being no effects produced by bacitracin or penicillin (Fig. 2B). The magnitude of the responses to additives were less at the second sampling (Fig. 2) compared to the first (Fig. 1).

Although all antibiotics produced an effect on SPA in this experiment, the effects were not universal and were dependent on specific conditions. Antibiotic effects occurred only on the two soils, there being none on the silica. In contrast to soils, silica would not be expected to contain enzymes produced by biological activity since presumably there has been none. Activity in manured media would reflect both soil and manure sources. However, the medium would be the major source since the weight of manure would only be a fraction of that of the soil. Even the enzymes contained in the manure treatment were not

measurably affected, perhaps due to this dilution effect. The small amount of SPA in silica was probably chemical in nature.

The nature of the effects was dependent on the antibiotic and was often negative. The reduction in activity would likely be due to the blockage of active enzymatic sites (7).

The effects were less pronounced and less complex as time passed which would concur with the statement that agrochemicals such as pesticides, herbicides, and fertilizers have no permanent effect on total soil microbial populations and that recovery will occur over time from single and even repeated applications (7). It has been observed (1) that penicillin markedly inhibited SPA and indeed this antibiotic was found to be more consistent in its effects. Therefore, soil phosphatase activity in both manured and nonmanured soils did prove to be sensitive to some additives.

The magnitude of blank determinations was found to be additive and soil dependent (Table 2, Fig. 3) for both samplings. Therefore it was necessary to conduct a correction for each treatment combination because different amounts of background color were produced by different soil and antibiotic combinations.

Barley yield (leaves plus stems) proved to be insensitive to antibiotics, the only effects being manure by soil interactions for both cuts and for additive and nonadditive treatments (Table 2). No additive effect was produced on the root yield (Table 2). The lack of effect contrasts with other work (19) and may be due to different crops, soils, and additives.

Additives did influence plant P content, the effects being dependent on particular treatment combinations (Tables 2, 3, Fig. 4, 5). First-cut P content was significantly decreased by penicillin on the silica in the presence of manure when compared to other additives (Fig. 4). Bacitracin also reduced P concentration slightly on the silica-manure combination for cut-1. For cut-2 (Fig. 5A), bacitracin and penicillin produced significant reductions again on the silica no-manure and manure treatments respectively. An additive independent positive quadratic effect was produced in the manured silica (Fig. 5B) which was the only significant effect for the rate by manure by soil interaction (Table 3). Thus, the

penicillin depressive effect on plant P concentration in the manured silica (Fig. 4) persisted from cut-1 to cut-2. Most effects occurred on the manured silica and indicates an interaction of antibiotics, especially penicillin, with manure on that medium. Lincomycin and neomycin depressed root P concentration in the presence of manure but independent of additive and rate compared to the other additives (Fig. 6).

There was a large increase in plant P concentration on the manure-silica treatment compared to the nonmanured treatment (Fig. 4). This was in inverse relation to the yield and is a reflection of the fact that plants grew poorly in manured silica. On the second cut (Fig. 5A) the P concentrations were more similar reflecting the relatively lesser yield difference. Phosphorus concentrations on silica-grown material were relatively higher than on the other two soils probably reflecting greater availability due to less fixation and relatively smaller yield, especially for the first cut.

The additive effects on P concentrations do not appear related to SPA. For instance, bacitracin had no effect on barley P concentration grown on the Ste. Rosalie soil in the absence of manure (Fig. 4). However, under the same conditions SPA was depressed by bacitracin (Fig. 1) which should depress P availability to the plant on these low available-P soils. Although penicillin in manured Ste. Rosalie and Greensboro soils depressed SPA in a linear fashion, there was no effect on cut-1 P concentration on the same treatments (Fig. 4). This lack of relation may be due to soil contributions, even though available P levels were low, and relatively high manure inorganic P which may have swamped any additive effect on organic P mineralization. It is also possible that the use of p-nitrophenyl phosphate, which is an artificial substrate, may not reflect effects on soil-manure phosphatase activity. Fixation of mineralized P by soils and temporary immobilization in the microbial biomass may also have reduced the availability of newly mineralized P to plants. Plant P concentration was therefore affected quite markedly by some additives on the silica mainly, and the effect could be negative or positive. No literature was found that related to P-drug interactions.

Cut-1 P uptake in the leaves and stems was higher for penicillin than for the other additives in the absence of manure but not in its presence

(Tables 2, 3, Fig. 7A) the effect being independent of additive rate and soil. Similarly, penicillin increased P uptake on the clay soil but not on the coarser Greensboro and silica, the effect being independent of additive rate and manure (Fig. 7B).

There were no significant treatment effects for cut-2 P uptake (Table 2) but additive rate in the absence of manure did produce a negative quadratic effect on P uptake by roots harvested at the time of cut-2 on the silica only (Tables 2, 3, Fig. 8).

The increased P uptake in cut-1 material by the penicillin treatment on the clay soil (Fig. 7B) and in the absence of manure (Fig. 7A) reflected the increased P concentration produced in the leaves and stems by this additive (Fig. 4) since there were no yield effects. Similarly, there were no treatment effects on root yield and root P uptake effects would reflect P concentration differences (Fig. 6).

At the first cut, additives caused a plant number reduction as rate increased, but only in the absence of manure (Tables 2, 3, Fig. 9A). The effect was independent of growth medium. Additives had no effect on Ste. Rosalie or Greensboro but bacitracin and penicillin, when compared to the other additives, provoked a reduction on the silica medium (Fig. 9B) and this effect persisted to the second cut (Fig. 10). Neomycin also provoked a reduction in plant number at second cut (Fig. 10). (Since plant number exceeded 10, especially on the Ste. Rosalie clay, it would appear that the chemical thinning was not always effective). Bacitracin and penicillin caused both plant number (Fig. 9B) and P concentration depressions on the manured silica (Fig. 4) indicating that these two variables were sensitive. However, there were no statistical differences for yield or P uptake on this media indicating that increased plant size compensated for reduced plant numbers. The nature of additive toxicity to plants is complex (2).

Therefore additives, especially penicillin and bacitracin did produce effects on SPA but only in the two soils, and on plant P concentration but only on the silica. The additive effects on SPA declined over a two month period. No yield effects were produced. Since the additives were added directly to the soil or manure at prevalent rates used in the feed industry up to those used in a disease situation, it would therefore not

be expected to find additives at higher rates in manure. Furthermore additive effects would probably be reduced during manure storage.

7.5 Acknowledgements

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Table 1. Soil and fecal characteristics.

Dairy cattle fecal sample	Characteristic								
	N	NH_3	P†	K	Ca	Mg	C‡	DM	Density
	(kg t^{-1} wet)						g kg^{-1}		kg L^{-1}
Feces	2.63	0.14	1.21	2.48	2.50	0.69	461	221.8	0.886

Soil or media	pH§	pH buffer	Mehlich III P	Mehlich III K	Mehlich III Ca	Mehlich III Mg	C	Sand	Silt	Clay	Cation exchange capacity
			kg ha^{-1}				g kg^{-1}				cmole(+)kg^{-1}
Sand 40	7.1	7.64	7	38	1659	42	1	991	7	2	1.3
Greensboro	4.8	6.48	45	281	654	156	20	817	53	130	6.7
Ste Rosalie	5.3	6.83	36	263	9119	1460	25	276	145	579	25.3

† 706 g kg^{-1} inorganic per kg of total P
‡ dry matter basis
§ before liming

Table 2. F values and their significance for sources of variation in Fig. 1 to 10

Source	DF	Variables† DM1	DM2	DMR	SPA1	SPA2	SPAB1	SPAB2
Additive (A)	3	2.62	0.39	2.64	3.76*	5.43**	13.47**	3.72*
Rates (R)	2	0.99	0.22	2.55	4.53*	0.77	0.45	0.16
A x R	6	0.71	1.02	1.48	1.33	2.44*	1.23	1.08
Manure (M)	1	123.10**	95.87**	74.83**	1.76	17.62**	0.24	0.33
A x M	3	0.59	0.89	2.11	4.06**	1.10	0.54	0.93
R x M	2	3.05	0.85	1.38	1.72	2.27	0.57	0.18
A x R x M	6	0.20	0.86	0.69	4.94**	1.38	0.73	0.77
Soil (S)	2	33.30**	167.65**	9.74**	821.58**	964.18**	4.66*	13.08**
A x S	6	0.81	0.62	0.67	1.85	1.68	0.32	0.92
R x S	4	0.56	1.35	0.84	2.71*	0.08	0.28	0.74
A x R x S	12	0.79	0.57	0.67	1.12	1.09	0.56	0.48
M x S	2	145.36**	125.95**	14.40**	12.14**	12.87**	0.06	0.08
A x M x S	6	0.37	1.00	0.45	1.61	0.58	0.15	0.32
R x M x S	4	1.43	0.57	1.68	3.41*	2.52*	0.18	0.64
A x R x M x S	12	0.50	0.64	0.75	2.11*	0.84	0.24	0.54
EMS		0.20	0.29	0.147	877.80	462.23	123.97	64.95
EMS DF		71	71	71	71	71	71	71

Source	DF	C1	C2	CR	U1	U2	UR	N1	N2
Additive (A)	3	1.40	0.17	1.21	0.72	0.44	2.50	0.99	6.02**
Rate (R)	2	0.74	0.05	0.06	0.00	0.12	3.38*	0.26	0.06
A x R	6	0.72	1.31	0.72	0.28	0.10	1.48	1.02	1.64
Manure (M)	1	93.14**	19.47**	18.47**	0.36	25.55**	47.00**	20.49**	21.82**
A x M	3	5.09**	3.07*	3.57*	3.52*	2.66	0.28	0.96	0.53
R x M	2	0.30	1.73	1.17	0.89	0.12	0.13	3.43*	0.44
A x R x M	6	0.48	1.99	0.15	0.96	0.89	1.19	0.62	0.41
Soil (S)	2	165.26**	53.24**	33.58**	49.05**	60.22**	31.96**	49.23**	56.38**
A x S	6	4.40**	1.87	1.12	3.56**	0.83	0.72	3.56**	7.87**
R x S	4	0.43	1.94	0.47	0.66	0.74	0.84	1.13	0.73
A x R x S	12	0.34	1.36	0.30	0.67	0.75	0.70	1.78	0.61
M x S	2	57.02**	16.62**	0.23	6.46**	25.31**	25.00**	11.92**	1.78
A x M x S	6	2.64*	2.76*	1.62	1.19	0.67	1.41	0.73	0.70
R x M x S	4	1.28	2.90*	0.40	1.13	0.13	2.81*	0.29	0.78
A x R x M x S	12	0.58	1.50	1.13	0.33	0.71	1.06	1.26	1.59
EMS		0.015	0.0079	0.0019	0.131	0.032	0.0038	9.63	8.75
EMS DF		70	70	71	70	70	71	70	69

† Codes: DM = dry matter; 1,2 = cuts 1 and 2; SPA = soil phosphatase activity; SPAB = blanks as described in the soil p-nitrophenyl phosphatase method; C = plant P concentration; R = roots; U = plant P uptake; N = number of plants pot^{-1}; EMS = error mean square; *, ** = significant at $P=0.05$, 0.01 levels respectively. No symbol on F values = nonsignificant at the 0.05 level.

Table 3. Partition of the significant interactions, simple effects.

Figure	Simple effects	F	Figure	Simple effect	F
1A-D	AxRxM (SR)†	4.08**	2B	R (B)	0.41^{NS}
	AxR (M SR)	3.25**		R (L)	4.57* Q 5.74**
	R (B M SR)	0.30^{NS}		R (N)	2.97^{NS} Q 4.52*
	R (L M SR)	0.86^{NS}		R (P)	0.17^{NS}
	R (N M SR)	0.09^{NS}			
	R (P M SR)	10.68** Li 19.53**			
	AxR (NM SR)	3.54**			
	R (B NM SR)	7.47** Li 14.55**			
	R (L NM SR)	0.77^{NS}	4	AxM (SR)	0.80^{NS}
	R (N NM SR)	0.40^{NS}		A (SR)	1.86^{NS}
	R (P NM SR)	10.60** Q 18.75**		M (SR)	0.56^{NS}
1E-H	AxRxM (G)	4.85**		AxM (G)	0.02^{NS}
	AxR (M G)	2.25*		A (G)	0.04^{NS}
	R (B M G)	2.21^{NS}		M (G)	2.73^{NS}
	R (L M G)	2.48^{NS} Q 4.97*		AxM (S)	10.62**
	R (N M G)	1.03^{NS}		A (NM S)	0.19^{NS}
	R (P M G)	4.86** Li 8.37**		A (M S)	18.45**
	AxR (NM G)	3.14*	5A	AxM (SR)	0.56^{NS}
	R (B NM G)	1.35^{NS}		A (SR)	1.43^{NS}
	R (L NM G)	0.01^{NS}		M (SR)	53.39**
	R (N NM G)	0.50^{NS}		AxM (G)	0.86^{NS}
	R (P NM G)	11.16** Q 12.25**		A (G)	0.00^{NS}
1I-L	AxRxM (S)	0.24^{NS}		M (G)	0.87^{NS}
	AxR (S)	0.01^{NS}		AxM (S)	6.62*
	AxM (S)	0.29^{NS}		A (NM S)	3.30*
	RxM (S)	0.08^{NS}		A (M S)	4.49**
	A(S)	0.38^{NS}	5B	RxM (SR)	0.74^{NS}
	R(S)	0.04^{NS}		R (SR)	1.87^{NS}
	M(S)	0.11^{NS}		M (SR)	53.00**
2A	RxM (SR)	6.11**		RxM (G)	0.25^{NS}
	R (M SR)	4.18* Q 7.19**		R (G)	0.93^{NS}
	R (NM SR)	2.52^{NS} Q 4.90*		M (G)	0.00^{NS}
	RxM (G)	1.16^{NS}		RxM (S)	8.44**
	R(G)	0.17^{NS}		R (M S)	9.67** Q 17.89**
	M(G)	1.77^{NS}		R (NM S)	0.94^{NS}
	RxM (S)	0.04^{NS}	6	A (NM)	1.67^{NS}
	R (S)	1.10^{NS}		A (M)	12.77**
	M (S)	1.30^{NS}			

Table 3 (continued)

Figure	Simple effects	F	Figure	Simple effect	F
7A	A (NM)	2.57*		RxM (S)	2.13^{NS}
	A (M)	1.08^{NS}		R (NM S)	2.75^{NS} Q 4.96*
7B	A (SR)	6.04**		R (M S)	2.21^{NS}
	A (G)	0.03^{NS}	9A	R (M)	1.35^{NS}
	A (S)	1.35^{NS}		R (NM)	2.75* Q 2.85*
8	RxM (SR)	0.40^{NS}	9B	A (SR)	1.48^{NS}
	R (NM SR)	2.03^{NS}		A (G)	1.55^{NS}
	R (M SR)	0.31^{NS}		A (S)	22.08**
	RxM (G)	3.03^{NS}	10	A (SR)	2.94*
	R (NM G)	2.42^{NS}		A (G)	0.83^{NS}
	R (M G)	1.26^{NS}		A (S)	18.41**

† A = additive, R = rate, M = manure, NM = no manure, B = bacitracin, L = lincomycin, N = neomycin, P = penicillin, S = soil, SR = Ste. Rosalie, G = Greensboro, S = silica 40, Li = linear, Q = quadratic, *, ** = significant at $\underline{P}$ = 0.05 and 0.01, NS = non-significant $\underline{P}$ = 0.05.

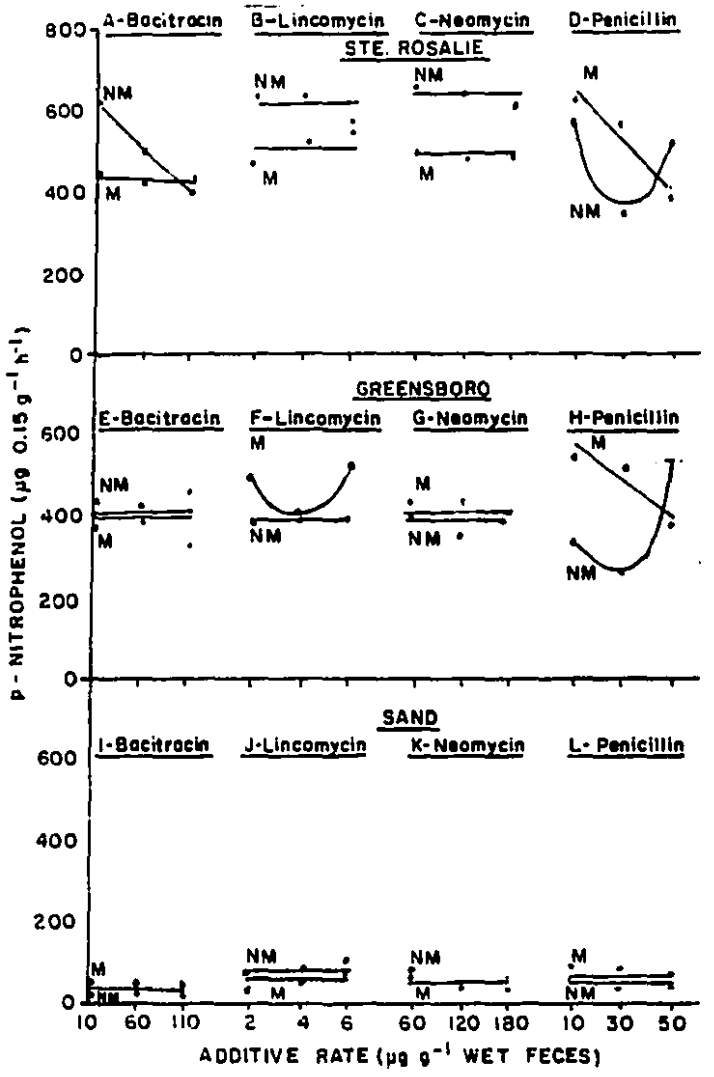

Fig. 1. Feed additive and manure effects on soil phosphatase activity in three soils after 30 d. (NM = no manure, M = manure. SD = 29 $\mu g\ g^{-1}$).

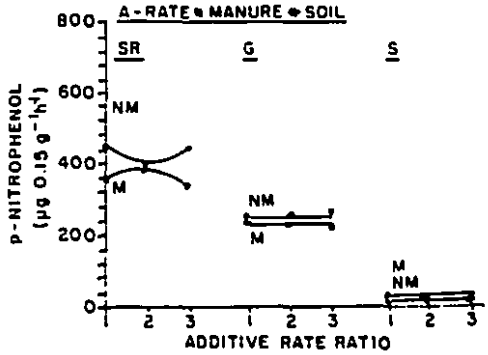

Fig. 2A. Additive and manure effects on soil phosphatase activity at 60 d. (SR = Ste. Rosalie, G = Greensboro, S = silica, NM = no manure, M = manure. Additive rate ratios are, in order bacitracin, lincomycin, neomycin, and Na-penicillin (in $\mu g\ g^{-1}$ wet feces): 1 = 10, 2, 60, 10; 2 = 60, 4, 120, 30; 3 = 110, 6, 180, 50. SD = 21 $\mu g\ g^{-1}$).

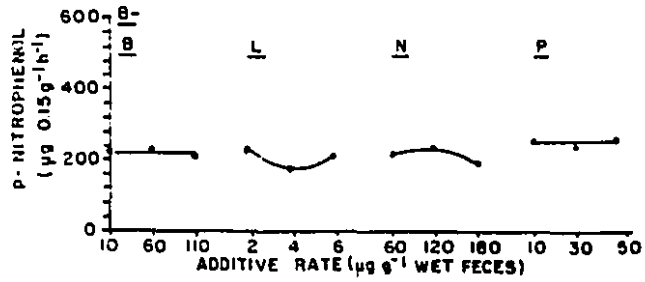

Fig. 2B. Additive and rate effects on soil phosphatase activity at 60 d. B = bacitracin, L = lincomycin, N = neomycin, P = Na-penicillin. SD = 21 $\mu g\ g^{-1}$.

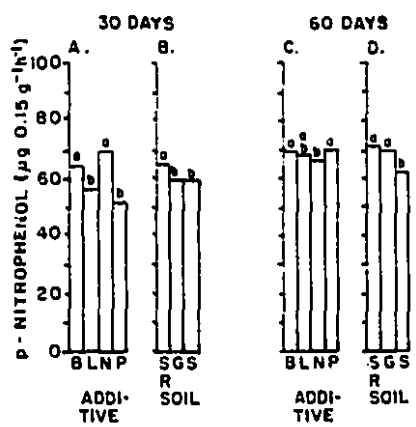

Fig. 3. Apparent soil phosphatase activity at two sampling dates. Treatments within additives or media at each date having the same letter are not significantly different according to Duncan's Multiple Range Test at $P = 0.05$. (B = bacitracin, L = lincomycin, N = neomycin, P = penicillin, SR = Ste. Rosalie, G = Greensboro, S = silica. SD 30 d = 11, 60 d = 8).

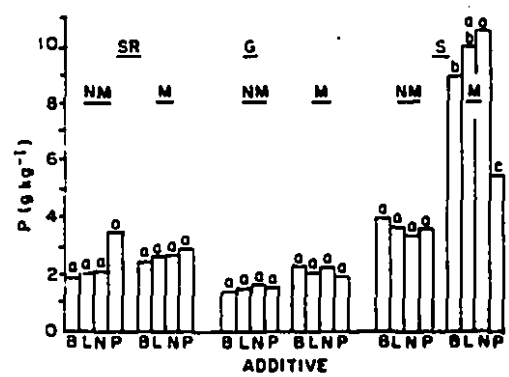

Fig. 4. Additive, manure, and soil effects on cut-1 plant P content at 30 d. Treatments within manure x soil treatments capped by the same letter are not significantly different by Duncan's Multiple Range Test at $P = 0.05$. (SR = Ste. Rosalie, G = Greensboro, S = silica, NM = no manure, M = manure, B = bacitracin, L = lincomycin, N = neomycin, P = penicillin. SD = 1.2 g kg^{-1})

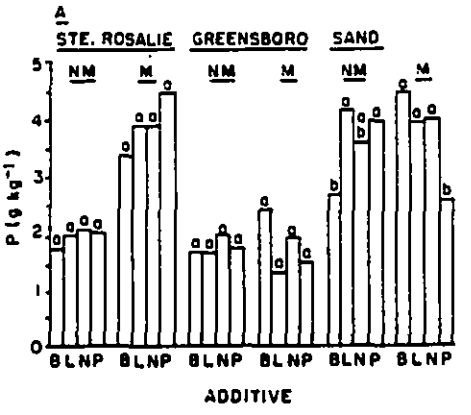

Fig. 5A. Additive, manure, and soil effects on cut-2 plant P concentration. (NM = no manure, M = manure, B = bacitracin, L = lincomycin, N = neomycin, P = Na-penicillin. Columns within manure by soil treatments capped by the same letter are not significantly different by Duncan's Multiple Range Test at $P = 0.05$. SD = 0.8 g kg^{-1} dry matter).

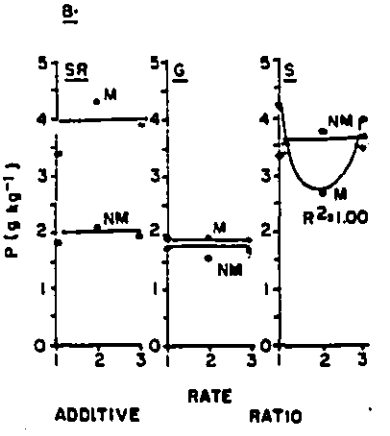

Fig. 5B. Rate, manure, and soil effect on cut-2 plant P concentration. (NM = no manure, M = manure, SR = Ste. Rosalie, G = Greensboro, S = silica. Additive rate ratios are in the order bacitracin, lincomycin, neomycin, and Na-penicillin (μg g^{-1} wet feces) 1 = 10, 2, 60, 10; 2 = 60, 4, 120, 30; 3 = 110, 6, 180, 50. SD = 0.8 g kg^{-1} dry matter).

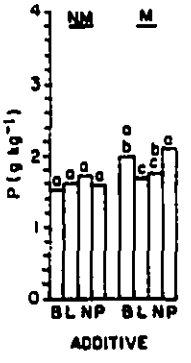

Fig. 6. Additive, manure, and soil effects on barley root P concentration at cut-2. (B = bacitracin, L = lincomycin, N = neomycin, P = penicillin, M = manure, N = no manure. Columns within manure treatment capped by the same letter are not significantly different at $P = 0.05$ by Duncan's Multiple Range Test. SD = 0.4 g kg^{-1})

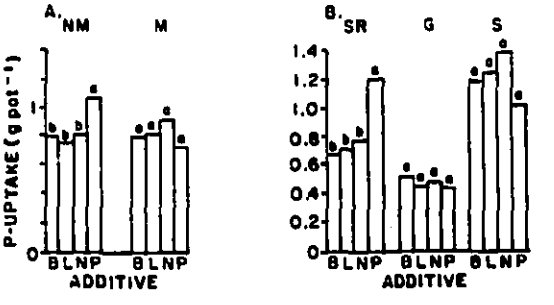

Fig. 7. Additive and manure effects on P uptake by cut-1 barley. (B = bacitracin, L = lincomycin, N = neomycin, P = penicillin, M = manure, NM = no manure, SR = Ste. Rosalie, G = Greensboro, S = silica. Columns within manure (A) or within soil (B) treatment are not significantly different at $P = 0.05$ by Duncan's Multiple Range Test. SD = 0.36 g pot^{-1})

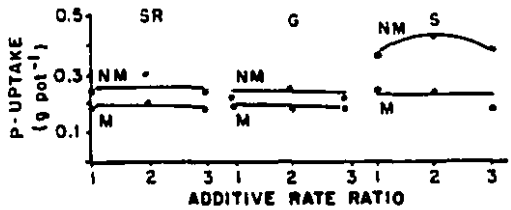

Fig. 8. Additive rate and manure effects on P uptake by barley roots on three media. (SR = Ste. Rosalie. G = Greensboro, S = silica, NM = no manure. M = manure, rate ratios 1 = 10, 2, 60, 10; 2 = 60, 4, 120, 30; 3 = 110, 6, 180, 50 $\mu g\ g^{-1}$(wet feces) of bacitracin, lincomycin, neomycin, and penicillin respectively. SD = 0.18 g pot^{-1})

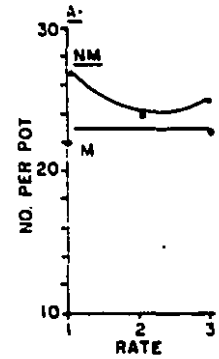

Fig. 9A. Additive and manure effects on cut-1 plant number at thinning. (Rate ratios are, in order bacitracin, lincomycin, neomycin, Na-penicillin ($\mu g\ g^{-1}$ wet feces): 1 = 10, 2, 60, 10; 2 = 60, 4, 120, 30; 3 = 110, 6, 180, 50. NM= no manure, M = manure. SD = 3.1 plants pot^{-1}).

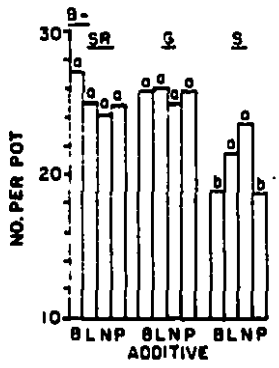

Fig. 9B. Additive and media effects on cut-1 plant number at thinning. SR = Ste. Rosalie, G = Greensboro, S = silica. B = bacitracin, L = lincomycin , N = neomycin, P = Na-penicillin. Columns within soils capped with the same letter are not significantly different by Duncan's Multiple Range Test at $P = 0.05$. SD = 3.1 plants pot^{-1}).

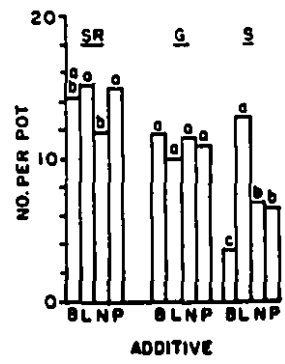

Fig. 10. Additive effect on cut-2 plant number of barley grown on three media. (B = bacitracin, L = lincomycin, N = neomycin, P = penicillin, SR = Ste. Rosalie, G = Greensboro, S = silica. Columns within soils capped by the same letter are not significantly different at $P = 0.05$ by Duncan's Multiple Range Test. SD = 3.0 plants pot^{-1})

CHAPTER 8

GENERAL CONCLUSIONS

Organic P (P_o) in fresh, uncontaminated commercial farm animal feces constituted 37% to 53% of total fecal P (P_t). Of P_t, generally less than 2% was in the phospholipid form (P_l). Most P_o was in the residual (P_r) (nucleic acid) form which varied from 11% of P_t in broiler feces to 41% in finisher cattle feces and in the acid-soluble organic (P_{aso}) form which varied from 8% of P_t in dairy cattle feces to 53% of P_t in broiler feces. Therefore present-day fresh, farm animal feces contain significant levels of P_o. Concentrations of inorganic (P_i) and P_o forms are however, often significantly different from older published values.

There was some indication that diet manipulation could influence the concentrations of some of these P forms. Regrowth forage caused calves to produce feces higher in P_i and P_l than when fed first-harvest material. This effect was independent of species or cultivar. Layer hens produced feces with lower concentrations of P_{aso} than broilers and may be related to the fact that layers had about twice the amount of wheat in their diet as did the broilers. Considerable potential therefore exists for the manipulation of P forms in animal feces.

The Association of Official Analytical Chemists (AOAC) method for lipid extraction from flour was useful to extract phospholipid from animal feces. The method produced less variation in results, required less time, and was less dangerous than the McAuliffe and Peech method. Phospholipids were removed in a single extraction with the AOAC method.

Antibiotic feed additives affected the mineralizing activities of phytase and alkaline phosphatase in aqueous solution. The effects for most additives were small and could be positive or negative. However, phytase activity was enhanced by bacitracin above, and by neomycin at feed additive rates. Alkaline phosphatase activity was depressed by bacitracin at feed additive rates. Therefore it would be expected that most feed additives at low rates would have little negative impact in a farm situation on these two enzymes, except possibly bacitracin on alkaline phosphatase. The hypothesis that feed additives negatively affect enzyme activities was shown to be true in some cases but was not universal and therefore can't be generalized.

The antibiotic feed additives bacitracin, lincomycin, neomycin, and Na-penicillin initially depressed fecal phosphatase activity but this changed rapidly upon incubation. The negative effects disappeared for lincomycin and neomycin, increased for bacitracin and changed direction for Na-penicillin. Therefore the hypothesis presented in Chapter 2, that feed additives depress phosphatase activity was shown to be true initially but upon incubation no longer was universal. Since additives had no effect on P_i and P_r, the hypothesis broached in Chapter 2 that fecal P availability to plants would be reduced by feed additives, was not true, at least within the context of this experiment. Similarly, fecal phosphatase activity, although sensitive to additives, was not a good predictor of additive effects on fecal P_i. However, the experimental period may have been too short and secondly, the initial P_i levels too high for measurable differences in P_i to occur. Furthermore animals on high-quality diets, may not excrete a high proportion of P_o. Gas production, which was sometimes stimulated by additives (ex. lincomycin and penicillin) when fecal phosphatase activity was unaffected or depressed, may mean that internal microbial phosphatases are unaffected by additives. Nevertheless, there was some evidence that the mineralization of P_{aso} might be retarded by increasing feed additive rates.

Soil phosphatase activity was generally negatively affected by feed additives when effects occurred but the presence of manure could eliminate the effect as it did for bacitracin or depress it as occurred for penicillin. Therefore the hypothesis that soil phosphatase activity is depressed by feed additives was found to be true for some additives but was not universal. Furthermore these depressive effects on soil phosphatase activity did not influence plant P concentration on the loam or clay soils. By contrast, no additive effects were produced on the silica phosphatase activity but depressive effects, especially by penicillin and bacitracin, occurred on plant P concentration for barley grown in this media. Penicillin which depressed soil phosphatase activity on the clay soil, increased plant P uptake. Soil phosphatase activity although sometimes sensitive to feed additives was not a good predictor of P availability to plants. As for the manure, added and soil P_i levels

may have hidden any effect of P_o mineralization, although the media were low in available P. Although plant number was depressed by additives in the absence of manure there was no effect with manure and thus would not be a concern at the farm level. Similarly, most plant P and number effects occurred on the silica and not on the two soils.

Thus, it would not be expected that manure contaminated with the antibiotics evaluated in this research, when applied at feed additive rates, would have a negative impact on plant P nutrition and crop growth when the manure is applied as a fertilizer to agricultural soils.

CHAPTER 9

CONTRIBUTIONS TO KNOWLEDGE

Historical Statement

It has been found that agrochemicals such as insecticides and herbicides can affect the activity of dephosphorylating enzymes in pure solution, in plants, and in insects.

The principal objective of the research conducted for this thesis was to examine the effects of some feed additives on the mineralization of fecal organic P in the context of using animal wastes as phosphorous fertilizers. This research resulted in the following contributions to knowledge:

1. An Association of Official Analytical Chemists lipid extraction method developed for flour, when adapted for fecal lipid extraction, was found to remove more lipid P, produced less variability in the phospholipid P results, and was less dangerous than the McAuliffe and Peech method for phospholipid extraction.
2. Concentrations of inorganic and organic phosphorous fractions in current farm animal fecal material often differed significantly from older, published values, probably because of higher quality feedstuffs.
3. The proportion of broiler fecal acid-soluble organic P was higher than that of layers which may be related to the fact that there was about half as much wheat on a per ton basis in broiler diets.
4. Although the presence of antibiotic feed additives sometimes affected the mineralization activities of phytase and alkaline phosphatase in aqueous solution, the effects could not be generalized because they depended on the additive and additive rate and could be positive or negative. The major effects involved phytase activity which was increased by bacitracin (a polypeptide) only at concentrations above feed additive rates and by neomycin (amino glycoside) which increased activity at feed additive rates but depressed it noncompetitively at higher rates. Alkaline phosphatase activity was depressed by bacitracin competitively at feed additive rates but increased at

higher concentrations. Other antibiotic additives produced effects on the activity of these enzymes but were small in magnitude. Since neomycin, which affected phytase in solution, generally had no effect on p-nitrophenyl phosphatase activity soil or decomposing feces, and bacitracin which increased alkaline phosphatase activity at high concentrations in aqueous solution generally depressed p-nitrophenyl phosphatase activity in soil or in decomposing feces, results obtained in aqueous solution are not reliable indicators of effects in other environments.

5. The p-nitrophenyl phosphatase method used to evaluate soil phosphatase activity was useful to detect antibiotic feed additive effects on animal fecal phosphatase activity. Fecal phosphatase activity was minimum at pH 6.5 and there was no maximum even at pH 12.
6. Antibiotic feed additives in decomposing dairy cattle feces had no effect on fecal inorganic P concentration, and therefore fecal p-nitrophenyl phosphatase activity, which was affected by some feed additives, was not a good predictor of fecal organic P mineralization, at least in the short term with the relatively high P_i feces. Furthermore since antibiotic feed additives affected gas production and fecal p-nitrophenyl phosphatase activity differently in decomposing dairy cattle wastes, the latter is not a reliable predictor of other activities in decomposing feces.
7. Antibiotic feed additives applied to clay and loam soils with or without manure had no effect on plant P concentration, and therefore soil p-nitrophenyl phosphatase activity which was affected by some additives, was not closely related to P availability to plants within the conditions of this experimental work where manure and soil P_i levels may have been too high and obscured mineralization and availability effects. Furthermore, this was also substantiated by the fact that P uptake was increased by penicillin in one instance at the same time soil phosphatase activity was depressed.
8. Since bacitracin and Na-penicillin produced effects more frequently on p-nitrophenyl phosphatase activity in soil and in decomposing feces, manure containing polypeptide and β-lactamine antibiotic feed additives, may be of greater interest and concern regarding enzyme activity in those media.

APPENDIX

A graphical presentation of the data in Tables 1, 2, and 3 of chapter 5 is found in Fig. A1, A2, and A3 respectively.

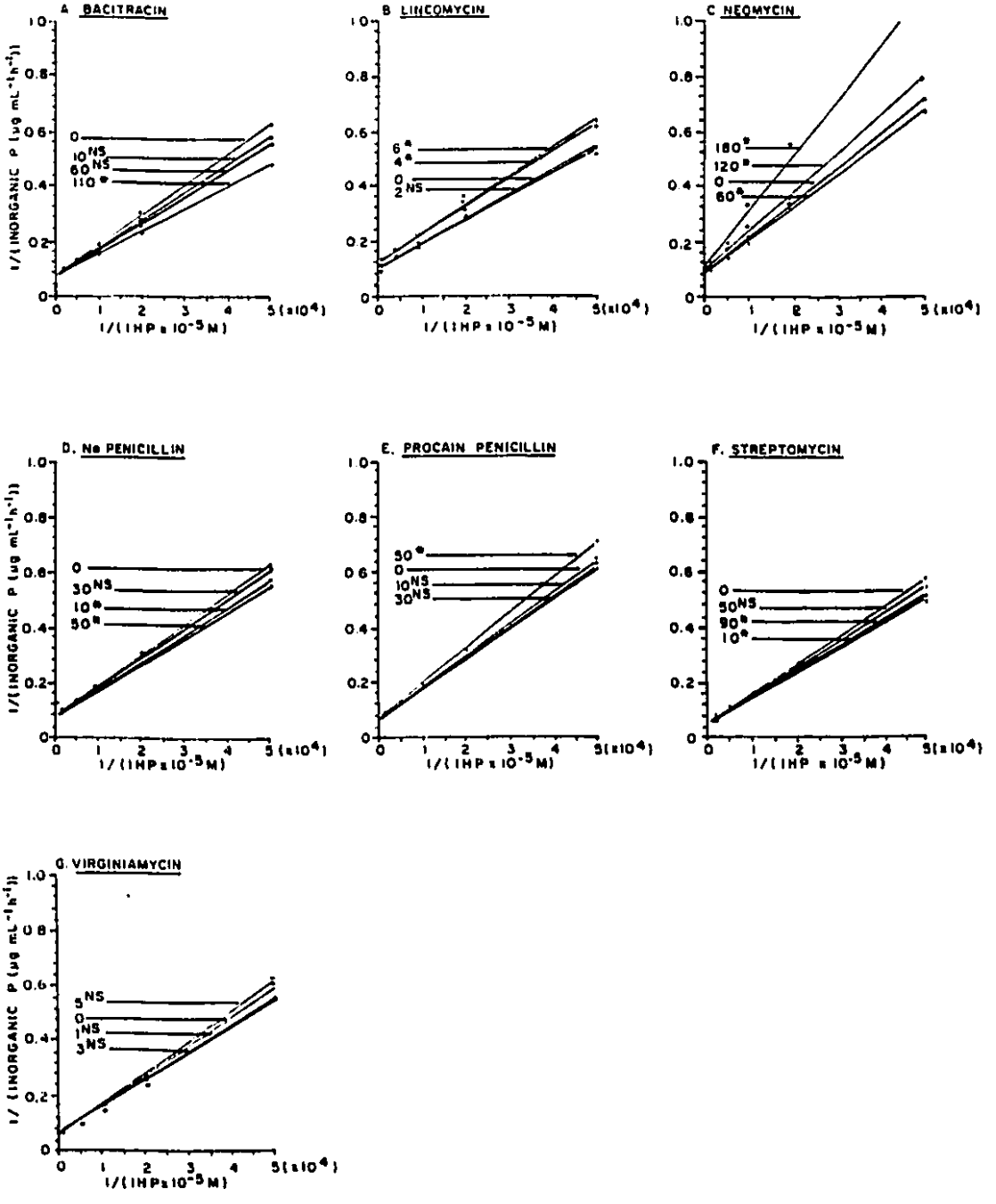

Fig. A1. Lineweaver-Burk plots of feed additive effects on phytase mineralization of inositol hexaphosphate. (*, NS = outside and inside 95% confidence limits for one or both regression coefficients for the "0" feed additive rate).

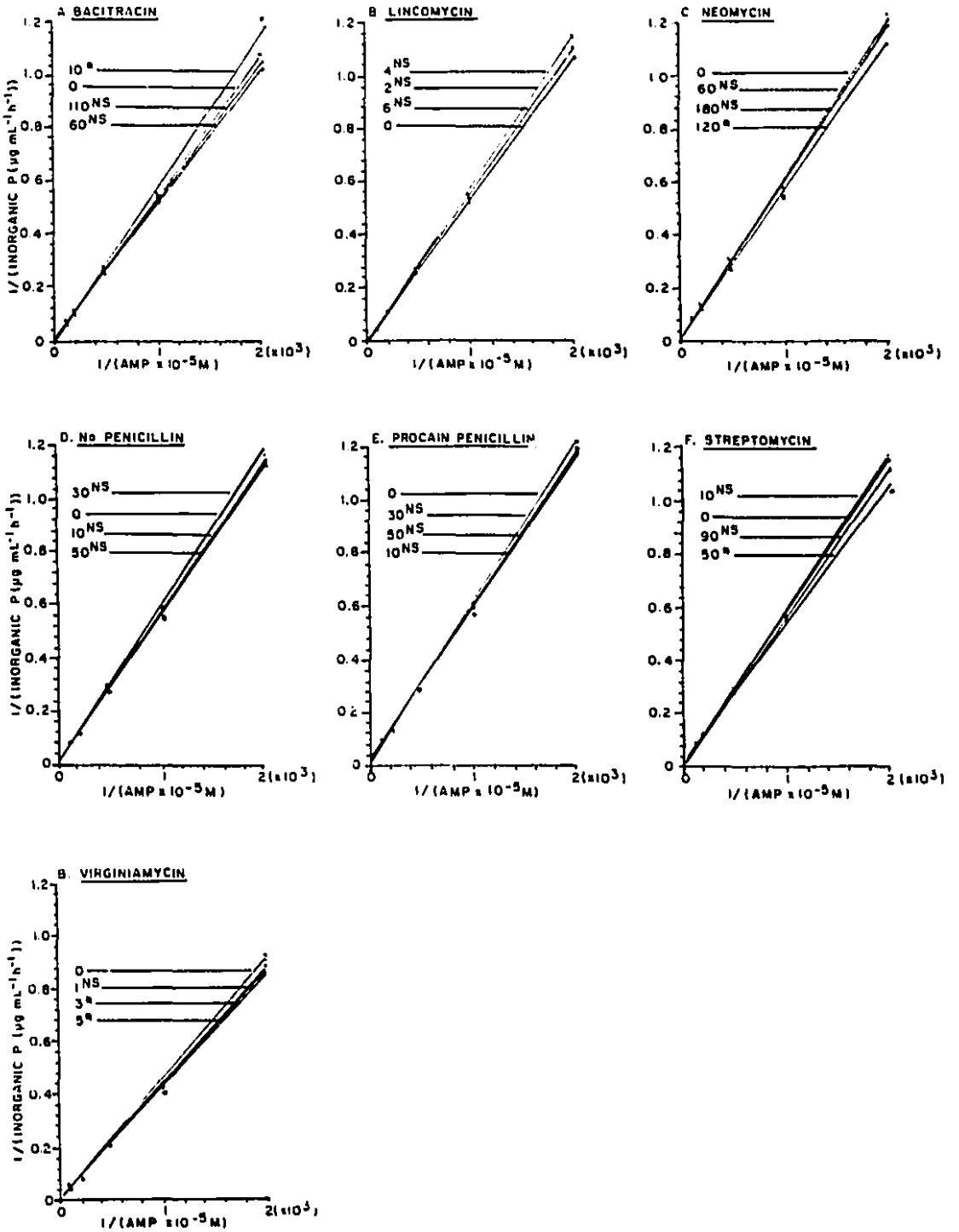

Fig. A2. Lineweaver-Burk plots of adenosine 5'-monophosphate mineralization by alkaline phosphatase in the presence and absence of feed additives - low rates. (*, NS = outside and inside 95% confidence limits for one or both regression coefficients for the "0" feed additive rates).

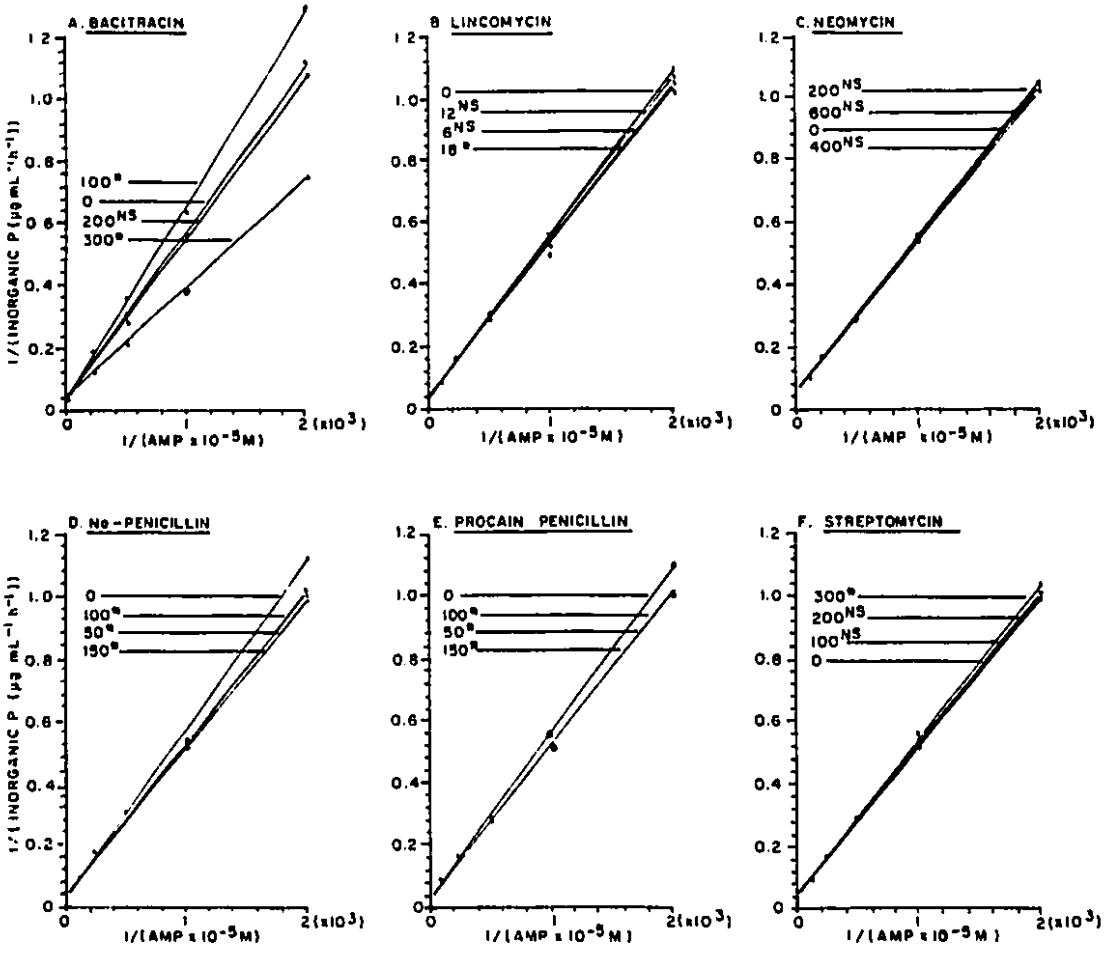

Fig. A3. Lineweaver-Burk plots of adenosine 5'-monophosphate mineralization by alkaline phosphatase in the presence and absence of antibiotics - high rates. (*, NS = outside and inside 95% confidence limits for one or both regression coefficient for the "0" feed additive rates).