on the specific drug sensitivity of the infective organisms, or on particular conditions connected with the cutaneous localization of the infections, remains to be determined. In this respect, it is known that post kala-azar dermal leishmaniasis is insensitive to stilbamidine type drugs, which are highly effective in the corresponding systemic infection.

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Comparative Availability of Phytin and Inorganic Phosphorus to Rumen Microorganisms, *in vitro.**† (27232)

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Numerous studies have shown that when dietary calcium is increased availability of phytin phosphorus is decreased for both man and laboratory animals(1-3). and to a lesser extent for cattle(4,5). Marston(6) has reviewed the early work, and Duncan(7) has discussed and summarized recent studies on the role of phosphorus and calcium in ruminant nutrition. Both *in vitro* and *in vivo* experiments have verified the availability of phytin phosphorus to ruminant animals under natural conditions(4-10), indicating the enzyme phytase, produced by rumen microorganisms, can hydrolyze ingested phytates releasing the available phosphorus for absorption. However, excess calcium ions inhibit phytase(11); and the observed rachitogenic effects of cereal diets have been accredited to the unavailability of phosphorus(12). The subsequent variations in available dietary phosphorus have contributed to existing differences of opinions on the relative effects of amount and ratio of calcium to phosphorus (12,13) upon utilization.

The paucity of information concerning individual contributions of phosphorus and calcium levels and ratios to phytin phosphorus availability in the ruminant animal, and the need for data on the contribution of the rumen to utilization of these phosphorus sources, prompted this study.

Methods and materials. A modification of the artificial rumen technic, washed cell suspension, described by Anderson *et al.*(14) was employed. In each series 1200 ml of rumen liquor were taken from a fistulated cow maintained on a grass hay ration. The microorganisms were washed twice and suspended in 600 ml of basal media, complete with the exception of phosphorus. Five g of purified cellulose and 9 ml of a 3% feather meal hydrolyzate were added and a 24-hour preliminary fermentation period employed to deplete the washed cells of any residual phosphorus. Feather meal hydrolyzate served as a stimulant to increase bacterial activity and, subsequently, achieve maximum phosphorus depletion. The microbial suspension was then made up to 1200 ml with the phosphorus deficient media, and 5 g of purified cellulose were added. Twenty ml of this bacterial suspension were pipetted into sets of 3 individual pyrex digestion tubes containing 0.3 ml feather meal hydrolyzate and 2.068 mg phosphorus as phytin ($Ca_5Mg(C_6H_{12}O_{24}P_6 \cdot 3H_2O)_2$) or as a combination of Na_2HPO_4

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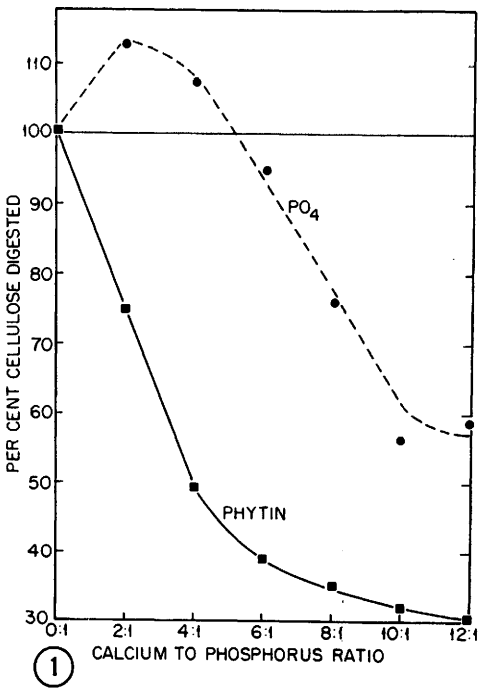

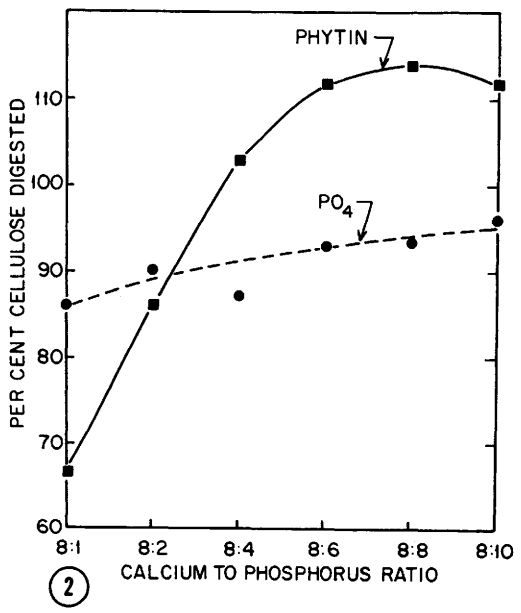

FIG. 1. Effects of increasing calcium level upon phosphorus availability *in vitro* from phytin and inorganic phosphorus (PO_4) (calculations corrected to zero calcium response).

FIG. 2. Effects of increasing phosphorus levels upon phytin and inorganic phosphorus availability *in vitro* when calcium is maintained high (corrected to maximum response for each source of phosphorus).

and KH_2PO_4. In both the tubes containing inorganic phosphorus and those containing phytin, calcium to phosphorus ratios were adjusted with calcium chloride to 0:1, 2:1, 4:1, 6:1, 8:1, 10:1 and 12:1, and incubated for 24 hours; the undigested cellulose in each tube was measured by conventional procedures(14).

In the second phase of this study the above experimental procedure employed was similar, but modified to maintain the high calcium level (0.0165 g) of the 8:1 calcium to phosphorus ratio tubes and to add phosphorus as phytin or inorganic phosphorus to give ratios of 8:1 to 8:10.

Results. Added calcium and phosphorus utilization. The effects of increasing calcium levels upon phosphorus availability *in vitro* from phytin and inorganic phosphorus are shown in Fig. 1. For convenience of presentation all data were calculated and corrected to zero calcium, considering cellulose digestibility at the Ca/P ratio 0:1 as 100% for both forms of phosphorus studied.

When phosphorus level was maintained at 0.0021 g from phytin or inorganic salts and Ca/P ratios were increased by added increments of calcium, it was observed that: (a) the inhibitory effects of added calcium upon cellulose digestibility were significantly greater ($P<0.01$) upon phytin phosphorus than upon inorganic phosphorus; (b) effect on phytin appeared to be direct, while effect on inorganic phosphorus represented a delayed action that was preceded by an active increase in cellulose digestion at the lower levels of added calcium; (c) at the 2:1 Ca/P ratio, cellulose digestion in the phytin tubes dropped 25% below their zero calcium control, while this same decrease was not approached in the inorganic phosphorus tubes until a ratio of 8:1 was reached; (d) at the 4:1 ratio, cellulose digestion in the phytin tubes had fallen to one-half, while in the inorganic phosphorus tubes, the added calcium ions appeared to have but little inhibitory effect upon availability for bacterial activity; (e) ratios above 6:1 depressed rumen microorganisms' ability to digest cellulose *in vitro*.

These data permitted calculation of the comparative availability to rumen microor-

ganisms of phytin phosphorus relative to inorganic phosphorus. Considering cellulose digestion in those tubes receiving inorganic phosphorus as 100% for each calcium level, it was shown that, in the absence of added calcium, phytin phosphorus was only slightly less available to rumen microorganisms than inorganic phosphorus. However, when calcium was increased 2-fold, at the 2:1 ratio, cellulose digestion was 37% greater for inorganic phosphorus; and at the 6:1 ratio digestion was maintained at 61% over that of phytin phosphorus ($P<0.01$).

Added phosphorus levels with high calcium. The effect of increasing phosphorus levels as phytin or as the inorganic salt upon cellulose digestion by microorganisms *in vitro*, when calcium level was maintained high, is shown in Fig. 2. For clarity and convenience of data presentation the Ca/P ratios demonstrating maximum response for each source of phosphorus in the previous calcium series of trials were considered as 100%. The ratio was 2:1 for inorganic phosphorus and 0:1 for the phytin phosphorus source. When calcium was maintained at 0.0165 g and phosphorus added as phytin or inorganic phosphorus to give ratios of 8:1 to 8:10, cellulose digestion was not significantly affected by the added inorganic phosphorus. However, with increased levels of phytin phosphorus, cellulose digestion increased to equal that of the zero calcium control values and continued to increase up to the 8:8 observed Ca/P ratio. Data indicate the independent relationship of level and ratio and verify the significance of limiting factors on response of rumen microorganisms in their ability to digest cellulose.

Discussion. The initial increase in cellulose digestion observed for inorganic phosphorus with low levels of added calcium (Fig. 1) gave indications of a calcium requirement by rumen microorganisms. Since calcium was not included in the basal medium, it is probable that the residual calcium, as well as the phosphorus, was depleted during the preliminary 24-hour fermentation period. This supports the earlier report of Hubbert *et al.*(15) demonstrating that rumen microorganisms have a definite calcium requirement. The highly significant decrease in cellulose digestibility with increasing Ca/P ratios demonstrated with phytin phosphorus follows the patterns observed in man and laboratory animals(1-3). These data support earlier evidence that Ca/P ratios can be critical in ruminant rations(7,13). When adequate phosphorus was maintained, Ca/P ratios above 4:1 depressed availability; and at ratios above 6:1 the ability of rumen microorganisms to digest cellulose was significantly inhibited on either phosphorus source.

In the absence of added calcium, however, phytin phosphorus was only slightly less available for use by rumen microorganisms than inorganic phosphorus(6,7). The observed sharp increase in cellulose digestion with increasing phytin phosphorus at the high calcium level (Fig. 2) gave evidence that phytase activity was not inhibited by high calcium(5,6). However, the decreased phytin phosphorus availability to rumen microorganisms in the presence of increased calcium (Fig. 1) appeared to be more likely related to a chemical reaction between phytin and/or its hydrolytic products than to a direct inhibition by excess calcium of the enzyme phytase itself(2,5,12).

Summary. Results of cellulose digestibility in a series of studies utilizing calcium and phosphorus depleted suspensions of rumen microorganisms confirm the calcium requirements of rumen microorganisms, and indicate phytin phosphorus utilization to be significantly more sensitive than inorganic phosphorus to calcium levels and ratios. Data suggest decreased phytin phosphorus utilization at the wider Ca/P ratios to be more directly related to a chemical effect than to enzyme inhibition, and provide basic evidence of the critical need for careful consideration of levels as well as ratios in the adjustment of calcium to phosphorus ratios for ruminant animals on high phytin rations.

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Insulin Content of Pancreas after Sodium Fluoroacetate-Induced Hyperglycemia.* (27233)

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Studies of the basic metabolic disturbance induced by sodium fluoroacetate (SFA) suggest that it undergoes a "lethal synthesis" forming fluorocitrate which by interfering with the action of aconitase blocks the Krebs cycle(1,2). Citrate then accumulates in all tissues. Elliott and Phillips(3) and Engel *et al.*(4) noted that rats given SFA *in vivo* also developed hyperglycemia and ketosis, "SFA diabetes." It was not known whether the diabetes was due to a general block of the Krebs cycle resulting in secondary hyperglycemia, or a specific inhibition in the mitochondria of beta cells of the pancreatic islets, causing a deficiency of insulin production. While much of the data correlated better with a general peripheral block of the Krebs cycle, persistent diabetes in one rat after an injection of SFA(5) left open the possibility of specific damage to insulin-producing cells.

To investigate the action of SFA in rats, we applied a recently developed method for the immunochemical assay of insulin in pancreatic tissue(6).

Methods. Twenty female Long-Evans rats weighing 100-170 g were divided into 3 groups as follows: 1) 5 normal controls were fasted for 12 hr, then decapitated. 2) Five rats received rapid infusions of alloxan (Eastman Organic Chemicals, Rochester, N. Y.), 50 mg/kg, in the tail vein after a 72-hr fast. Diabetes was established by urinary glucose level of 2-9 g per day while rats were fed *ad libitum*. On the third day after alloxan administration the animals were killed as above after a 12-hr fast. 3) Ten rats were given an intraperitoneal injection of 0.6 mg per 100 g body weight freshly dissolved SFA, 0.06% in saline. Three animals died within 6 hr of the injection. The 7 surviving rats were fed *ad libitum* for 12 hr after SFA injection, then fasted for 12-18 hr and decapitated. Within 6 hr after receiving SFA, all animals manifested glycosuria and ketonuria as measured with Clinistix and Acetest tablets (Ames Co., Elkhart, Ind.). Jugular vein blood was collected at decapitation and fasting blood glucose levels were measured by the Somogyi method(7).

Insulin assay. The tail and body of the pancreas were immediately removed, blotted, weighed and placed in 10% trichloroacetic acid. After homogenization with mortar and pestle and centrifugation, the insulin was extracted from the precipitated protein with acid alcohol (15 ml of 12 N HCl diluted to 1 L with 75% ethanol) and further purified by the method of Grodsky and Tarver(8).

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