Influence of Various Sources of Non-Protein Nitrogenous Sources on *In vitro* Fermentation Patterns of Rumen Microbes

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ABSTRACT : The effect of replacement of cotton seed meal (CSM), with various levels and sources of non-protein nitrogenous (NPN), substances on *in vitro* ruminal fermentation were studied. Cotton seed meal, in control ration provided nitrogen equivalent to 12.5 percent crude protein while in experimental rations was replaced at 30, 50 & 70 percent levels with urea, diammonium phosphate (DAP) and biuret, respectively. The results of incubation upto 48 hours indicated an improvement in digestibility by replacement of CSM with urea and biuret upto 50 percent protein equivalent, but not with DAP. Bacterial count from cultures containing 50% nitrogen from biuret was significantly higher than DAP, urea and CSM. Various sources of nitrogen produced NH_3-N in increasing order of CSM, biuret, DAP and urea. Increasing levels of NPN resulted in progressive increase in the levels of NH_3-N. The levels of various NPN sources had no effect on pH. However, the pH values determined for urea and CSM were higher than biuret and DAP.

(**Key Words**: Urea, Biuret, Diammonium Phosphate, *In vitro*, Fermentation)

INTRODUCTION

Ruminants through the participation of various types of bacteria, contained in their rumen, convert the conventional and non-conventional sources of nitrogen into bacterial protein which is ultimately utilized by the host animals. The rumen has excellent environment for the propagation of bacteria.

The available dietary protein sources are not sufficient to fulfil the requirements for animals. There is a need to discover new and feasible protein sources. Microorganisms play essential role to convert cheap nitrogenous materials into valuable proteins in ruminant animals. The ability of rumen microorganisms to utilize NH_3 for protein synthesis permits the replacement of dietary protein with NPN sources.

A little work has been performed on the influence of various NPN sources on *in vitro* patterns of rumen microbes. Certain aspects such as changes in dry matter digestibility rate, liberation of ammonia nitrogen, microbial growth and pH in rumen during *in vitro* fermentation were planned to be investigated in the present study.

MATERIALS AND METHODS

To investigate the effect of replacement of cotton seed meal with various levels of NPN sources on *in vitro* fermentation parameters, four experiments were conducted.

Cotton seed meal (CSM) used in the control experiment provided 2% nitrogen, (12.5% crude protein) and was replaced at 30%, 50% and 70% levels with urea, diammonium phosphate and biuret respectively. The biuret was prepared by heating the urea crystals at boiling temperature for 5 minutes.

EXPERIMENTAL PROCEDURE

Artificial rumen

Artificial rumen as suggested by Johnson (1986) was used with some modifications. Two fistulated buffaloes, maintained at the Nutrition Research Center, University of Agriculture, Faisalabad, served as donors of rumen fluid. The buffaloes were fed green fodder *ad libitum* and 70 g/d of urea intra ruminaly 10 days before start of the experiment and maintained throughout the experiment. The contents from ventral sac of the rumen were collected in a plastic bottle and strained. Artificial saliva was used as buffer mineral solution. The solution was bubbled vigorously with CO_2 until pH was 6.9. Carbonic

atmosphere, pH 6.5-7.0, temperature 39℃ and gentle shaking 2-3 times daily, were the conditions provided for *in vitro* fermentation. Duplicate samples of CSM, urea, DAP and biuret (at various levels of replacement of CSM) were also placed in 250 ml flasks each containing one gram oven dried wheat straw. Both 50 ml of strained rumen fluid and 50 ml of McDougall's (1948) artificial saliva were added to each flask. Immediately after adding all components, the flasks were capped with rubber stoppers equipped with bunsen valve. The valve allowed the gas to escape out but prevented the air from entering the system.

Experiment no. 1

In this experiment, *in vitro* dry matter digestibility was determined by replacing CSM with various levels of NPN sources. After 48 hours incubation, the microorganisms activity was stopped by immediate cooling of flasks in ice cold water. The contents of the flask were strained through 4 layers of sterilized muslin cloth. The filtrate was dried for 24 hours at 70℃ in an oven and the dry matter digestibility was determined by difference method formula (Hopson et al., 1963).

Experiment no 2.

The effect of replacement of CSM with various levels of NPN sources on bacterial counts was studied. The *in vitro* fermentation system was run as in experimen 1. The bacterial counts were determined at prefermentation, 4 & 48 hrs of incubation with modified technique of Knaysi and Ford (1938). The strained rumen fluid was diluted upto 10.2. From the final dilution, 0.01 ml fluid was transferred to a thoroughly cleaned slide, upon which an area of 1 cm^2 had been previously marked.

The sample was spread evenly over the marked area and air-dried and fixed by passing over the flame. It was stained with Gram's Stain (Hucker's modifications) and examined under oil immersion lens. The counts were made from five microscopic fields and calculations were made according to following formula.

- Microscopic factor (MF) = $10^9/11304$
- Average number of organisms per field = (N)
- Number of organisms per ml = MF × N × Dilution factor

Experiment no. 3

Effect of replacement of CSM with NPN sources on release of NH_3-N was studied. The *in vitro* ammonia nitrogen release was studied. The single step fermentation technique was adopted as in experiment No. 1. Eight sets,

each of duplicate flasks, were placed in incubator. Ammonia nitrogen was measurd after 10 min, 20 min, 1 hour, 2 hours, 4 hours, 24 hours and 48 hours of incubation (Nessler, 1976).

The fermented samples were centrifuged for 5 minutes at 4,000 rpm and supernatant was used for NH_3-N measurement. One ml of the supernatant was added to 9 ml of the distilled water, after thorough mixing one ml from this was added to 3.6 ml of distilled water and 0.4 ml of Nessler's reagent was added to each test tube containing the final dilution. Then within 10 minutes the absorbance was measured on Spectronic −21, at wave length of 420nm. The reading for standard was due to 0.01 mg of NH_3-N. The NH_3-N was calculated as below:

$$NH_3 - Nitrogen = \frac{0.01 \times absorbance\ of\ samples}{absorbance\ of\ standard}$$

It was the mg of NH_3-N per 0.1 ml of the contents of flask

Experiment no 4.

The pH changes were measured before and after the *in vitro* fermentation with pH meter. Then pH of all the flasks at 0 times (before incubations) and 48 hours (after incubatino) were measured at temperature of the flask contents.

The results were analyzed statistically by using standard analytical techniques (Steel and Torrie, 1981) by analysis of variances. Significance of means was tested by Duncan's Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Digestibility

The mean results were sub-classified into source, level and interactions. The analysis of variance revealed differences between the nitrogen sources (p < .001) the levels (p < .002) of NPN and interaction between source and level (p < .004).

The results indicated improvement in digestibility by 50% replacement of CSM with NPN sources. Inclusion of NPN beyoud 50% had no significant effect on the digestibility of dry matter (table 1. a).

Interaction between NPN source and its levels indicate that differences between the digestibility obtained for 30, 50 and 70% DAP levels were not statistically different from each other (p > .05). Increase of Urea and biuret from 30 to 50% resulted in maxiumn digestibility of dry matter. However replacement of CSM with NPN at 70% level showed no beneficial effect over lower levels of NPN (figure 1).

Table 1. Dry matter digestibility and Bacterial Count in *in vitro* cultures of rumen microbes as influenced by replacement of CSM with various levels of NPN sources

a) Digestibility(%)

Nitrogen Source[1] (p < .001)		Source[1] × Levels (p < .004)	
Urea	16.80^c	Urea × 30%	13.18^d
DAP	22.09^b	Urea × 50%	18.01^c
BU	28.76^a	Urea × 70%	19.20^{bc}
CSM	12.85^d	DAP × 30%	22.80^b
		DAP × 50%	21.31^b
NPN Levels[1] (p < .002)		DAP × 70%	22.13^b
		BU × 30%	22.32^b
30%	17.39^b	BU × 50%	33.10^a
50%	21.32^a	BU × 70%	30.85^a
70%	21.26^a	CSM	12.85^d

[1]DAP, Diammonium phosphate; BU, Biuret; CSM, Cotton seed meal.
^{a,b,c,d}Means different superscripts differ.

b) Bacterial Count (N × 10^8 ml)

| NPN Source[1] (p < .03)		Source[1] × Levels (p > .05)	
Urea	21.38^b	Urea × 30%	16.61^c
		Urea × 50%	35.30^a
DAP	21.38^b	Urea × 70%	12.23^d
		DAP × 30%	17.53^c
BU	31.16^a	DAP × 50%	24.70^b
		DAP × 70%	23.67^b
CSM	20.67^b	BU × 30%	26.06^b
		BU × 50%	33.09^a
NPN Levels (p < .04)		BU × 70%	32.97^a
		CSM	20.67^c
30%	20.35^b	Source × Time (p < .05)	
50%	28.64^a	Urea × T1	5.93^d
		Urea × T2	37.33^a
70%	22.38^{ab}	Urea × T3	20.88^b
		DAP × T1	7.03^{cd}
Fermentation Time (p < .001)		DAP × T2	38.80^a
		DAP × T3	20.07^b
T1	9.67^c	BU × T1	14.73^c
		BU × T2	42.04^a
T2	34.63^a	BU × T3	36.33^{ab}
		CSM × T1	11.00^c
T3	27.07^b	CSM × T2	20.00^b
		CSM × T3	31.00^b

[1]DAP, Diammonium phosphate; BU, Biuret; CSM, Cotton seed meal.
^{a,b,c,d}Means different superscripts differ.

Bacterial counts

Effect of three sources of NPN i. e. Urea, DAP and biuret on the bacterial conunt were measured from the culture tubes at three time intervals, i. e. 0, 4 and 48 hours post fermentation. The mean values of bacterial counts are presented in (table 1. b). The analysis of variance revealed that various NPN sources (p < .03),

levels of NPN (p < .04) had significant effect on bacterial counts. The interaction between sources × level and sources × time also showed significant difference (p < .05).

Bacterial count (31.16 × 10^8/ml) from cultures containing biuret was significantly higher than DAP, urea and CSM. Among the three levels used 50% replacement

of CSM with NPN source yielded bacterial count of 28.64 × 10⁸/ml which was significantly higher (p > .044) than 20.35 × 10⁸/ml for 30% NPN level. However, increase in NPN upto 70% showed a non-significant declining trend.
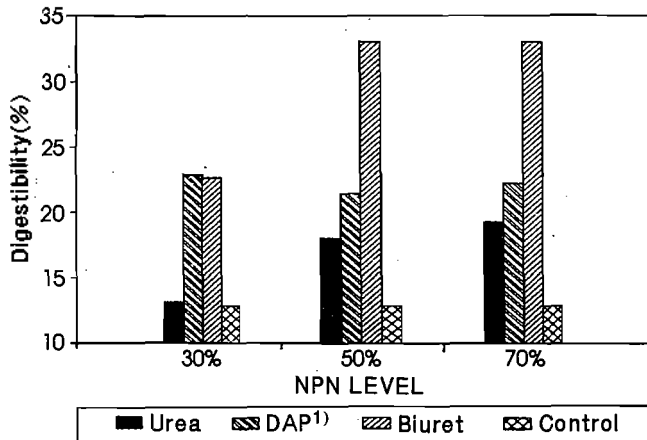
Figure 1. Dry matter digestibility as influenced by various levels of NPN sources.

$^{1)}$DAP : Diammonium phosphate.

Ammonia

The average NH_3-N production due to various nitrogen sources varied significantly (p < .001). The CSM released minimum NH_3-N (8.9 mg/dl), folowed by biuret (13.4 mg/dl), DAP (16.5 mg/dl) and urea (22.4 mg/dl). Regarding the release of NH_3-N the various sources can be ranked as urea > DAP > biuret > CSM.

The analysis of variance revealed that level of NPN had significant (p < .001) effect on rate of NH_3-N release during fermentation. The mean values for the rate of NH_3-N released increased progressively as the level of NPN increased. The mean rate of NH_3-N release for 30, 50 & 70% NPN were 12.88, 14.64, 19.10 mg/dl, respectively. The effect of fermentation on NH_3-N release increased significantly at 40 minutes which further improved at 60 minutes, the differences between 40 and 60 minutes were also significant. The release of NH_3-N from the *in vitro* system at 24 and 48 hours declined significantly (p < .001).

The interaction for sources of NPN × time and level × time of incubation revealed no differences therefore data are not presented. The data on effects of replacemnt of CSM with different levels of Urea, DAP and biuret are shown in fig 3-4; (table 2. a. i).

pH

The mean values for pH are presented in table 2. The

Table 2. Rumen ammonia nitrogen and pH values in *in vitro* cultures of rumen microbes as influenced by replacement of CSM with various levels of NPN sources

a) Rumen Ammonia-N (mg/dl)

(i) Nitrogen Source$^{1)}$ (p < .001)	
Urea	22.40^{a}
DAP	16.51^{b}
BU	14.32^{c}
CSM	8.94^{d}

(ii) Fermenation Time (p < .001)	
10 Minutes – T1	14.06^{d}
20 Minutes – T2	14.83^{cd}
40 Minutes – T3	15.67^{b}
One hour – T4	16.62^{a}
Two hours – T5	16.62^{a}
Four hours – T6	16.90^{a}
24 hours – T7	15.12^{bc}
48 hours – T8	14.50^{c}

(iii) NPN Levels (p < .001)	
30%	12.88^{c}
50%	14.64^{b}
70%	19.10^{a}

$^{1)}$DAP, Diammonium phosphate; BU, Biuret; CSM, Cotton seed meal.
^{a,b,c,d}Means different superscripts differ.

b) pH Values

(iv) Source$^{1)}$ × Levels (p < .00)			
Urea × 30% 16.44^{c}	BU × 30% 11.50^{e}
Urea × 50% 18.31^{b}	BU × 50% 15.06^{d}
Urea × 70% 32.44^{a}	BU × 70% 16.39^{c}
DAP × 30% 14.66^{d}	CSM × 30% 8.94^{f}
DAP × 50% 16.25^{c}	CSM × 50% 8.94^{f}
DAP × 70% 18.62^{b}	CSM × 70% 8.94^{f}

Nitrogen Source$^{1)}$ (p > .05)	
Urea	7.217
DAP	7.150
BU	7.133
CSM	7.200

(c) NPN Levels (p > .05)	
30%	7.150
50%	7.188
70%	7.187

(d) Fermentation Time (p > 0.0001)	
0 hour – T1	7.075^{a}
48 hour – T2	7.275^{b}

$^{1)}$DAP, Diammonium phosphate; BU, Biuret; CSM, Cotton seed meal.
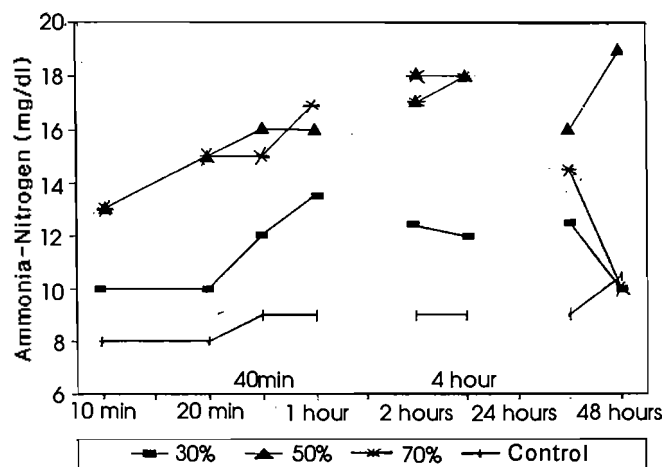^{a,b,c,d}Means different superscripts differ.

Figure 2. Effect of replacement of CSM with different levels of biuret on *in vitro* release of $NH_3 - N$ (mg/dl).
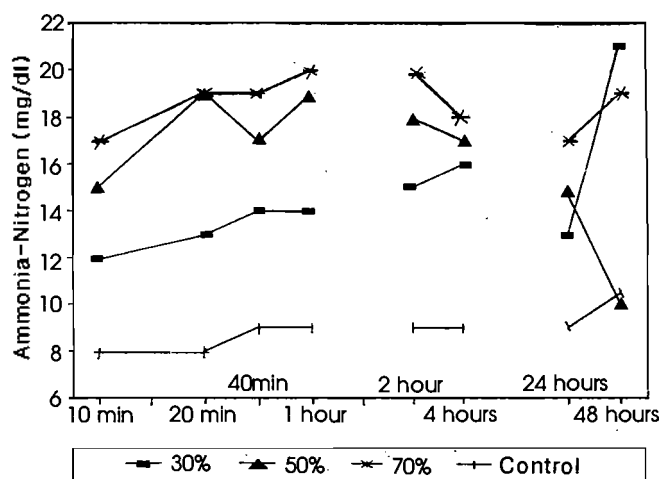

Figure 3. Effect of replacement of CSM wih different levels of diammonium phosphate on *in vitro* release of $NH_3 - N$ (mg/dl).

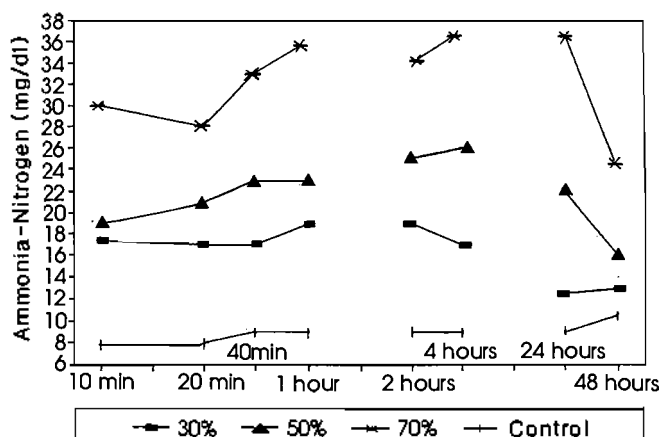**Figure 4.** Effect of replacement of CSM with different levels of urea *in vitro* release of $NH_3 - N$ (mg/dl).

analysis of variance revealed that levels had no sighificant effect on variation of pH. However, the sources of NPN ($p < .07$), time of fermentation ($p < .0001$) had definite effect on the pH. The application of DMR-Test to observe the effect of sources revealed that pH of 7.21 and 7.20 for urea and CSM were significantly higher than 7.13 for biuret. However, the pH 7.15 for DAP was significantly lower than pH 7.21 for urea. The LSD also indicated that pH value of measured at 0 hours (7.07) was significantly lower than pH at 48 hours (7.27) of fermentation (table 2. c).

DISCUSSION

Digestibility

The DMD of treatment containing biuret was maximum (28.76%) followed by DAP (22.09%) and Urea (16.8%). The better digestibility with NPN sources, as compared to the CSM indicated that the bacterial types obtained from the animals were predominantly of ureolytic type and this population seems to be maintained during *in vitro* fermentation. The high ureolytic bacterial count is the possible explantion for poorer DMD (12.85%) in cultures containing CSM. However, the lower digestibility obtained by Urea containing cultures as compared to biuret at all levels also gave an indication that higher ammonia content may be inhibitory for ureolytic bacteria. These results agree to those of Church and Santoes (1981) who reported that NPN reduced digestibilites of dry matter in animals fed molasses base diet. The urease activity might have also suppressed the digestion of urea containing culture as indicated in earlier report (Slyter et al., 1971), that urease activity in the rumen bacteria was reduced in steers fed urea. It was observed that when NPN level was raised from 50% to 70% of CSM-N, there was no statistical difference between two levels. However, the difference between 30% with that of higher levels of NPN were significant ($p < .002$). Interaction between all sources of NPN × 50% level, the DMD was optimum and replacement of NPN beyond 50% of CSM-N was not feasible in *in vitro* system. However *in vitro* digestion gives no information on residence time in the rumen and therefore provide no direct estimate of nutrient availability (Nocek and Ruesall, 1988).

Bacterial counts

The replacement of CSM with NPN sources revealed that only biuret significantly increased the bacterial count whereas the Urea and DAP had no effect on this parameter. These results disagree with earlier reports of

Mir et al. (1980) and Agha et al. (1983) who found that buffalo bulls fed rations containing urea and urea phosphate produced higher ruminal bacterial counts at 4 hours post feeding as compared to control rations. This variation may be attributed to the fact that present work was conducted in *in vitro* system whereas earlier reports were based on *in vivo* system. During *in vitro* studies, the accumulation of fermentation end products (gases & VFA) might have inhibitory effect on bacterial activity.

The effect of replacement of CSM with various levels of NPN revealed that cultures contatining 50% NPN had stimulatory effect and 70% NPN had inhibitory effect on bacterial counts. The interaction of Urea × 50% and Biuret × 50% were pronounced as compared to that of DAP × 50%.

This indicated that the optimum level of NPN source to replace CSM-N was 50%. Therefore for economical use of protein sources 50% replacement with cheaper NPN sources particularly urea and biuret shall be considered in livestcok production systems. Production trials with larger number of animals are suggested to evaluate this hypothesis. Paul et al. (1975), compared the metabolism of urea and biuret in ruminants. They reported that biuret was comparatively slowly hydrolyzed, therefore, it could be used at higher levels as a protein supplement or in complete diets. Satter and Slyter (1974) reported that concentration of 5 mg of NH_3-N/dl of rumen fluid was sufficient for microbial growth. However, in present study NH_3-N concentration of 14.32 mg/dl rumen fluid for biuret was significantly lower as compared to DAP and urea, though it is about three times higher than that reported by Satter and Slyter (1974). The results indicated that 14.32 mg NH_3-N was not toxic for the bacterial growth.

Ammonia nitrogen concentration

Clements and Johnson (1973) reported that animals fed biuret for more than four days consecutively are well adopted because of the development of biureolytic bacteria. In the present experiment, the donor animals were adopted with urea and maximum liberation of NH_3-N by the bacteria was obtained from these animals. This indicated that urease producing strains were dominating in the inoculum. The cultures containing 70% N from biuret released 16.39 mg/dl NH_3-N which was similar to those of urea-N × 30% and DAP-N 50%.

This indicated that at higher levels, biuret can be as a NPN source without much losses of NH_3-N as compared to that of urea and DAP. Slyter et al. (1971) observed that during *in vitro* studies, NH_3-N concentration of rumen culture contents was higher when urea was used as

NPN source as compared to that of biuret. Paul et al. (1975) also reported that biuret released NH_3-N slowly as compared to that of urea. They also indicated that biuret was comparatively non toxic and could be used at higher levels in protein supplements in complete diets. This agrees with the results of the present study that biuret can replace 70% of the dietry protein with comparatively lesser nitrogen losses than that of urea and DAP.

The effect of fermentation time indicated that NH_3-N concentration of 16.63 mg/dl achieved after 1 hour was not significantly different (p > .05) from those achieved after 2 and 4 hours (table 2).

There was progressive increase in NH_3-N liberation as observed after 10 minutes, 20 minutes and 60 minutes (1 hour) of fermentation (figure. 2-4) showing that the *in vitro* system used in the present study worked efficiently and resulted in significant growth of bacterial activity thus hydrolyzing the NPN sources. The interaction between sources and time indicated that all the three NPN sources achieved their peaks after 1 hour which were maintained till about 4 hours. The pattern of NH_3-N released for CSM as the only nitrogen source showed that releases of NH_3-N was slow and uniform throughout the fermentation period.

pH

The highest pH because of the urea containing cultures can be due to maxium NH_3-N concentration during incubation. However, higher pH of cultures containing CSM needs explanation. The lower pH in cultures containing DAP and biuret was also in agreement with the values of NH_3-N as discussed in previous sections. These results supported the findings of Sial et àl. (1980), who reported that urea levels higher than 1% of the diect resulted in increased pH of the rumen content. However, they reported that pH measured at different time intervals in urea fed animals were not significantly different. This can be explained by the fact that in the present study a closed *in vitro* system was used where NH_3-N was not absorbed. Therefore, the accumulation of NH_3-N in the *in vitro* system may be the possible cause for higher pH after 48 hours of incubation in this experiment. Mir et al. (1980) and Agha et al. (1983) also reported increase in ruminal pH when urea was fed to the animals. The pH values obtained in the *in votro* systems can not be compared with that of the *in vivo* system because the later system is dynamic in its nature. A continuous flow of saliva rich in buffer as well as absorption of NH_3-N in the blood do not allow appreciable changes in ruminal pH particularly when the NPN levels are not very high.

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