緬羊第一胃ウレアーゼに及ぼすカプリロヒドロキサム酸の影響

誌名	日本獣醫學雑誌 = The Japanese journal of veterinary science
ISSN	00215295
著者	新林, 恒一 ほか4名,
巻/号	35巻4号
掲載ページ	p. 327-334
発行年月	1973年8月

Jap. J. vet. Sci., **35**, 327~334 (1973)

EFFECT OF CAPRYLOHYDROXAMIC ACID ON RUMINAL UREASE

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(Received for publication January 10, 1973)

The amount of ammonia produced in the rumen is known to be affected by the quantity of the diet given. In ruminal microorganisms whose protein is utilized by a host animal, their own nutrient is also affected by the kind of diet given to the animal. A part of the ammonia produced in the rumen by ruminal microorganisms is absorbed from the rumen into the portal blood stream and converted into the liver. Urea has been used as a source of nitrogen in the diet. It has been thought, however, that employment of urea may be dangerous if the feeding conditions are inadequate. When ingested, urea breaks down rapidly into ammonia in the rumen, and a large amount of ammonia produced escapes necessarily through the ruminal wall. If the reaction prodeeds slowly, no formed ammonia will cause intoxication in the host animal. The reduction of ruminal ureolytic activity to such level as commensurate with microbial ability to synthesize protein from ammonia seems to be useful for increasing the efficiency of urea utilization. ALVARES et al.[1,2] demonstrated that isoniazid, chlortetracyline, copper, and barbituric acid stimulated growth of chicks, lowering gastrointestinal ammonia concentration and ureolytic activity. On the other hand, CHIFFORD et al.[4] examined barbituric acid, copper, and nitrate ion in urea-containing diets for effect upon urea utilization in sheep, but failed to show any noticeable effect.

Hydroxamic acid were found to be highly specific and potent inhibitors by KOBASHI et al.[9] during the cause of experiments on the role of the sulfhydryl groups of urease. GALE and ATKINS[5] assessed a series of 61 hydroxamic acids for inhibitory activity against urease and demonstrated that 21 of these acids appreciably inhibited the cell-free enzyme from the jackbean and *Proteus morganii*. HASE and KOBASHI[8] examined hydroxamic acid for effect on partially purified urease from *Proteus vulgaris* and *Bacillus pasteurii*, and on ureolytic activity of their intact cells. The inhibitory action of aliphatic hydroxamic acids increased with the prolongation of reaction time. It was not affected at all in the presence of SH compounds. On the other hand, the same action of aromatic hydroxamic acids was not related to the time cause, but was influenced by SH compounds. These inhibitory properties of aromatic hydroxamic acids were very similar to those against plant urease. Caprylohydroxamic acid (CHA) was found to be the most effective compound. Recently, BRENT et al.[3] have shown that acetohydroxamic acid had an ability to inhibit the intracellular urease of intact rumen microorganisms.

The studies reported here were undertaken to evaluate caprylohydroxamic acid as a ruminal urease inhibitor.

MATERIALS AND METHODS

Ruminal urease: Rumen content was collected from a donor sheeps fed a commercial formula diet and hay containing 16.9% and 8.2% protein, respectively. It was passed through two layers of gauze, strained through three layers of cheesecloth, and then centrifuged at 300×g for 15 minutes. The supernatant was centrifuged at 15,000×g for 15 minutes. The precipitate was suspended in 0.02 M phosphate buffer and centrifuged at 15,000×g for 15 minutes. The resulting precipitate was resuspended in a small volume of 0.02 M phosphate buffer, pH 7.0, and sonicated in a 10 KC oscillator for 15 minutes. The sonicated suspension was then centrifuged at 30,000 rpm for 3 hours in a type 65 rotor with a Spinco preparative ultracentrifuge. The resulting cell-free supernatant was used for an enzymic experiment. Specific activity was calculated as μ mole ammonia-N/hour/mg protein at 37°C. It was 22.7 for the cell-free extract from intact rumen microorganisms. The highest urease specific activity was found in the precipitate (37.9) obtained by increasing ammonium sulfate saturation from 61% to 72%, though the precipitate was not use for this experiment.

Assay for urease: Urease activity was assayed by the alkalimetric method of GORIN and CHIN[6] in one case. In this method, it was assayed by adding, in the order listed, 1 ml of 3% urea solution in 0.1 M tris buffer, pH 9.0, and 1 ml of test solution in 0.02 M phosphate buffer, pH 7.0. Incubation was carried out at 37°C for a constant interval and terminated with 2 ml of 0.1 N HCl. Then, the reaction mixture was back-titrated with 0.05 N NaOH, methylorange being used as an indicator.

In another case, urease activity was assayed by adding, in the order listed, 1 ml of 0.5 M phosphate buffer, pH 7.0, 0.5 ml of 1 M urea solution, and 0.5 ml of test solution in Seligson's diffusion flask. Incubation was carried out at 37°C for a constant interval and terminated with 1 ml of 1 M perchloric acid. Then, ammonia produced was determined by the method of SELIGSON and HIRAHARA[13].

Rumen fermentation in vitro: Rumen inoculum was obtained from fistulated sheep maintained on a daily diet composed of 0.3 kg of hay and 0.48 kg of experimental formula feed containing 5% urea. The donor sheep had been fed the same diet for several weeks and was considered to be adapted to urea. Fifteen milliliters of rumen fluid, 0.5 ml of 3% urea solution, and 1.5 ml of 1×10^{-2} M CHA were added to a 50 ml syringe with rubber stopper fitted at the top. The final concentrations of urea and CHA were 1.93×10^{-2} M and 0.9×10^{-3} M, respectively. Incubation was carried out at 38°C for 6 hours under anaerobic conditions of the rumen content.

Total gas production, ammonia, volatile fatty acid (VFAs), and lactate were estimated. On the other hand, time course changes of various compounds in the culture medium were determined. Twenty milliliters of rumen fluid, 20 ml of artificial saliva of McDougall, 300 mg of powdered hay, 480 mg of experimental formula feed, 24 mg of urea, and CHA were added to each 100 ml Erlenmeyer flask with a single hole stopper and valve fitted in such manner as to allow carbon dioxide and other gases to escape and oxygen to be excluded. The flasks were initially filled with carbon dioxide and incubated in water bath at 38°C with constant shaking. The final concentrations of urea and CHA were 1.3×10^{-2} M and 1×10^{-3} M, respectively. The culture media used were analyzed at 0, 60, 180, and 360 minutes for urea, ammonia, VFAs, and free amino acids.

Ammonia and urea were determined by the method of SELIGSON and HIRAHARA[13] and the diacetyl monoxine method of ORMSBY[11], respectively. Lactate and VFAs were determined by the method of Barker and Summerson with modification[12] and by the

Fig. 1. Time Course of Inhibitory Effect of CHA on Rumen and Jackbean Urease

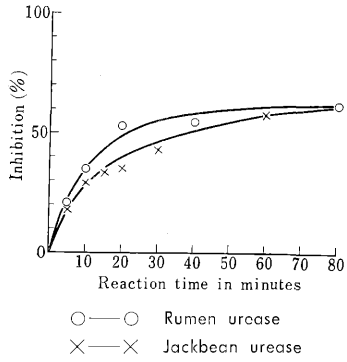


Fig. 2. Inhibitory Effect of CHA on Urease in Rumen Fluid

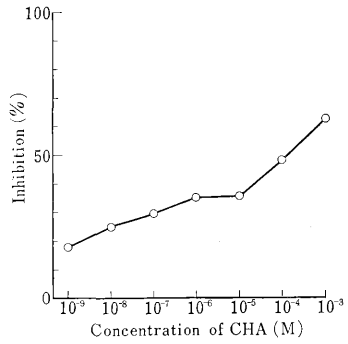


Table 1. Effect of Concentration of Substrate and CHA on Ruminal Urease Activity

Substrate (M)	CHA 0M		CHA 1×10^{-6}M		CHA 1×10^{-5}M		CHA 1×10^{-4}M	
	NH_3 μg/ml	%	NH_3 μg/ml	%	NH_3 μg/ml	%	NH_3 μg/ml	%
0.4×10^{-2}	42.4	100* (62.0)**	40.5	95.5* (68.6)**	24.5	57.8* (38.3)**	3.0	7.1* (7.7)**
0.5×10^{-2}	43.4	100 (70.8)	52.0	107.4 (88.1)	45.5	94.0 (71.1)	4.8	9.9 (12.3)
0.67×10^{-2}	55.9	100 (81.7)	51.5	92.1 (87.3)	53.5	95.7 (83.6)	6.0	10.7 (15.4)
1.0×10^{-2}	58.9	100 (86.1)	58.0	98.5 (98.3)	58.0	98.5 (90.6)	11.9	20.2 (30.5)
2.0×10^{-2}	68.4	100 (100)	59.0	86.3 (100)	64.0	93.5 (100)	39.0	57.0 (100)

* Percentage of urease activity in each CHA concentration to that in the absence of CHA at the same concentration substrate.

** Percentage of urease activity in each concentration of substrate in each concentration of CHA to that in a substrate concentration of 2.0×10^{-2}M.

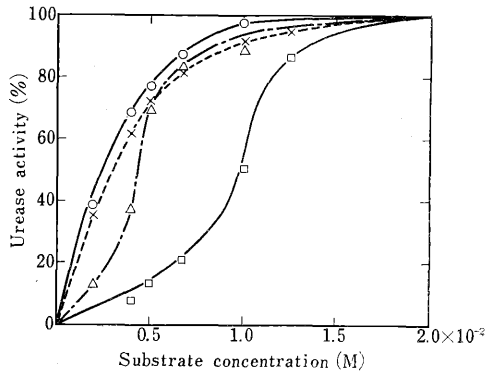
procedure of distillation, respectively. Free amino acids were analyzed by a KLA-3B Hitachi amino acid analyzer originated by SPACKMAN et al.[14] after ultrasonic treatment.

RESULTS AND DISCUSSION

Inhibitory effect of CHA on ruminal urease in cell-free extract: CHA was examined for effect on time course of reaction velocity. The final concentrations of substrate and CHA were 2.3×10^{-1} M and 4.5×10^{-4} M, respectively. The inhibition progressed with the lapse of time. When ruminal urease was compared with jackbean urease, CHA inhibited both enzymes to a very similar degree, as shown in Fig. 1. The reaction velocity was reduced by 40 to 50% at 30 minutes in the presence of this concentration of CHA as in the case of rumen fluid (urease activity of 20 μ moles ammonia-N/hour/ml) filtered through two layers of gauze (Fig. 2).

Km value of ruminal urease: The kinetics of ruminal urease in the cell-free extract was studied. The double reciprocal plot of Line weaver-Burke showed that the Michaelis constant, Km value, was 6.3×10^{-3} M and comparable with the value 3.6×10^{-3} M calculated by BRENT et al.[3] On the other hand, the Km value of jackbean urease was proved

Fig. 3. Effect of CHA on Velocity Curve Following Changes in Substrate Concentration



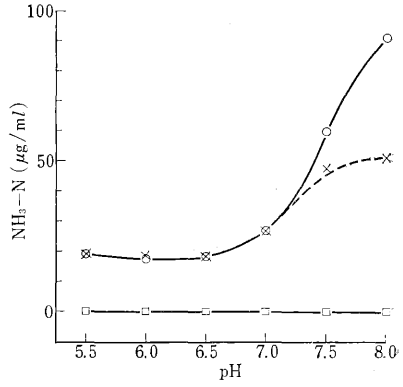
Urease activity was expressed as percentage to that in a substrate concentration of 2.0×10^{-2} M.

CHA

—×— 0 M —△— 1×10^{-5} M

—○— 1×10^{-6} M —□— 1×10^{-4} M

Fig. 4. Effects of pH and Preincubation on Inhibitory Action of CHA



○---○ Control

×—×— CHA

□—□ CHA–preincubation

Final concentrations of substrate and CHA were 2.3×10^{-1} M and 1×10^{-3} M, respectively. Incubation was carried out at 37°C for 30 minutes.

Table 2. Effect of CHA on Rumen Fermentation

Group	Total gas	CO_2	NH_3	VFAs	Lactate
	ml*	ml*	mg/dl	m mol/l	m mol/l
CHA	12.7±0.7	10.4±0.8	53.4±3.1	116±11	0.90±0.50
Control	12.7±0.2	10.5±0.7	55.5±11.6	117± 9	1.08±0.59

* *Gas volume produced in a syringe containing 15 ml of rumen fluid for a fermentation period of 6 hours.*

to be 3×10^{-3} M by HARMON and NIEMANN[7]. From these results, the Km of ruminal urease is thought to be in the order of 10^{-3} M of urea, and the maximum velocity is 111 μg/30 minutes/total volume of 2.2 ml under the condition used here.

Effects of various concentrations of substrate and CHA on ruminal urease: Changes in the concentrations of substrate and CHA were investigated for effect on ruminal urease. The activity of ruminal urease was stimulated by an increase in substrate concentration. No substrate concentrations inhibited the urea production so long as they were within the range used here. The activity of ruminal urease was slightly inhibited in the presence of the concentration of CHA in the order of 10^{-5} to 10^{-6} M when the substrate concentration was as high as 10^{-2} M in order. The inhibitory effect of CHA, however, was enhanced with an increase in concentration of this substance (Table 1).

Another experiment was performed with rumen fluid filtered through two layers of gauze, as mentioned above. In it, CHA was added to such extent that its final concentration might range from 10^{-9} to 10^{-3} M. As a result, the inhibition percentages were 31.5, 50.7, and 63.9% in the concentrations of 1×10^{-5}, 1×10^{-4}, and 1×10^{-3} M, respectively, when the final concentration of substrate was 2×10^{-1} M (Fig. 2).

Although the substrate concentration was different between both cases, the inhibitory effect of CHA was enhanced promptly in a concentration of 1×10^{-4} M, and very slightly in a concentration within a range 1×10^{-5} to 1×10^{-6} M.

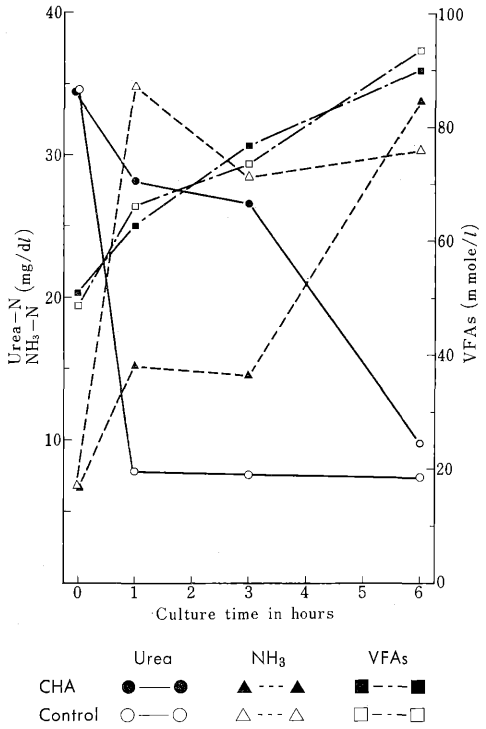
Pattern of the inhibitory effect of CHA on ruminal urease: CHA was investigated for effect on the velocity curve following the change in substrate concentration. The results obtained are shown in Fig. 3. In this figure, the reaction velocity in the presence of each concentration of CHA is expressed as percentage to that in the presence of a substrate concentration of 2×10^{-2} M. When the velocity was plotted against the substrate concentration, a sigmoid saturation curve was obtained, instead of the simple hyperbolic saturation curve observed in the presence of substrate alone. When the substrate concentration was low, the reaction was slowed considerably. The higher the substrate concentration, the smaller became effect of CHA. Finally the pattern of non-competitive inhibition was presented.

Effects of pH and preincubation on the inhibitory action of CHA: The pKa of CHA was not measured in this experiment. It was noted by Kobashi, however, that the smaller the pKa of hydroxamic acid, the more intensely the inhibitory action of urease was stimulated, and that long-chain aliphatic hydroxamic acid showed a faster reaction velocity than short-chain aliphatic hydroxamic acid. Ferthermore, it was suggested by KOBASHI[10] that the pH curve of inhibition by hydroxamic acid might be parallel to the pH curve of urease activity and that the higher the activity of urease, the more intensely this substance might be inhibited. The reaction to urease was usually carried out at pH 8.6. Since the rumen is usually neutral or slightly acidic, the pH of reaction mixture varied between 5.4 and 8.0. The final concentration of substrate and CHA were 2.3×10^{-2} M and 1×10^{-3} M, respectively. Incubation was done at 37°C for 30 minutes. Consequently, it was found that the activity was enhanced remarkably as pH was elevated from 7 to 8, that 20% and 50% inhibition took place at pH 7.5 and 8.5, respectively, and that inhibition was hardly observed at a pH between 5.5 and 7.0. Since its range of pH was similar to that of rumen content, the reaction was allowed to take place under the same condition after the preincubation of CHA with the enzyme for 5 to 10 minutes, so that the enzyme activity might be inhibited almost completely (Fig. 4). The effect of preincubation on the inhibitory action of CHA was also observed in the case of jackbean urease.

Effect of CHA on rumen fermentation: The results obtained are summerized in Table 2. There were no significant differences in quantities of total gas, ammonia, VFAs, and lactate between the CHA-free group and the CHA group when the determination was made at 6 hours of incubation. In this experiment, the final concentration of urea was presumed to be nearly the same as the concentration of this substance in the rumen of an animal fed a ration containing 5% urea. Why was these no marked difference in urea concentration? In order to clarify the cause, the time course changes of various compounds were measured in culture medium.

The composition of the culture medium used was similar to that of the content of the syringe mentioned above. In the CHA-free group, urea was decomposed rapidly within 3 hours, and after that the urea concentration remained within a range of 5 to 10 mg/dl. In the CHA group, urea was decomposed gradually until 3 hours and rapidly after that (Fig. 5). It was assumed that the amount of ammonia produced might have changed with the laps of time in reverse proportion to the decomposition of urea. The amount of ammonia produced was almost the same in both groups when estimated 6 hours after the beginning of incubation. These results were similar to those obtained from the syringe experiment. As a reason why urea was decomposed rapidly after 3 hours in the

Fig. 5. Effect of CHA on Time Course of Rumen Fermentation



CHA group, it is suggested that the multiplication of microorganisms may be active at about 3 hours of incubation, and that then the activity of urease may increase or concentration of CHA decrease with the lapse of time. The production of VFAs was not affected from the initial stage by the addition of CHA. No values of pH were affected either by the addition of CHA, except a slightly higher value noticed in the CHA group between 1 and 3 hours of incubation.

Free amino acids in the culture medium were determined in both groups at the same intervals as the other compounds. An aliquot of the culture medium was sonicated in a 10 KC oscillator for 15 minutes. An amino acid sample for the automatic amino acids analyzer was prepared from the sonicated medium. The results obtained are summarized in Table 3. The highest percentage to total free amino acids was shown by aspartic acid, glutamic acid, proline, glycine, and alanine at 3 hours of incubation, whereas histidine, valine, isoleucine, leucine, and tyrosine decreased in percentage extremely at that time. Probably, microbial protein may be synthesized more actively at 3 hours of incubation, when the total amount free amino acids decreased extremely and the so-called essential amino acids were utilized more actively than the so-called non-essential amino acids. No significant effect of CHA was observed on the concentration of free amino acids.

From these results, CHA is thought to be a potent inhibitor for ruminal urease. The compound may make it possible for the ruminant to utilize urea by reducing the ureolytic activity in the rumen. The results of examination of CHA for inhibitory effect upon sheep and rats will be reported in a paper to come.

Table 3. Time Course Changes in Proportion of Free Amino Acids during Fermentation*

Amino acid	Control				CHA-supplemented			
	0	60	180	360	0	60	180	360 (min.)
Lys	14.5	16.5	13.4	12.0	15.3	19.4	24.1	13.2
His	1.5	0.9	0.6	1.1	1.8	0.7	0.4	1.0
Arg	1.4	1.6	1.9	1.6	1.8	2.2	1.7	2.3
Asp	7.8	8.5	16.5	9.9	7.8	5.0	11.5	8.9
Thr	4.9	3.9	2.8	5.2	5.0	3.1	3.2	4.5
Ser	3.9	3.6	2.4	4.7	3.8	2.5	2.4	4.1
Glu	13.5	11.9	22.8	14.5	16.0	11.1	20.8	14.1
Pro	5.1	4.2	9.8	4.9	4.3	3.2	7.1	5.9
Cit	4.3	3.9	3.2	3.4	3.5	5.1	5.5	4.1
Gly	4.1	2.4	3.3	3.0	4.4	4.4	3.7	3.4
Ala	10.3	11.0	16.5	10.5	9.1	10.3	10.3	10.5
Val	5.8	6.3	1.5	5.5	5.3	7.5	2.0	5.3
Met	3.2	4.2	2.4	4.0	2.5	4.4	3.1	4.3
Ile	5.3	4.7	1.0	4.7	5.3	4.9	0.7	4.4
Leu	7.2	7.9	1.1	7.4	6.8	6.8	1.2	6.9
Tyr	3.7	4.8	0.6	3.3	3.4	5.1	1.5	3.3
Phe	3.7	3.8	0.2	4.2	4.0	4.4	0.9	3.9
Total amino acids** (mg/dl)	31.1	29.6	9.3	21.5	27.0	21.9	9.3	17.4

* Expressed as weight percent of free amino acids recovered.

** Sum of free amino acids described in the table.

SUMMARY

A cell-free extract was prepared from the rumen fluid by ultracentrifugation after ultrasonic treatment. It was used as an enzyme specimen for the enzymic experiment. It was found that ruminal urease had some similar properties to those of plant urease. The Km value of ruminal urease was 6.3×10^{-3} M, which was in the same order as that of plant urease. The inhibitory effect of caprylohydroxamic acid (CHA) was enhanced promptly in the concentration of 1×10^{-4} M in both cell-free extract and rumen fluid, whereas it was very slight in the concentration of 1×10^{-5} to 1×10^{-6} M.

When the velocity was plotted against the substrate concentration, instead of simple hyperbolic saturation curve obtained in the presence of the substrate alon, a sigmoid saturation curve was obtained. At low substrate concentrations, the reaction was slowed considerably. The higher the substrate concentration, the lower became the effect of CHA, and a pattern of noncompetitive inhibition was shown.

CHA was investigated for effect on rumen fermentation. Neither production of volatile fatty acids nor pH was affected in the culture medium by addition of CHA, but the decomposition of urea accompanied by production of ammonia was affected significantly by the added CHA until 3 hours of incubation.

In conclusion, CHA was expected to be applicable to the ruminant fed a ration containing urea.

REFERENCES

1) ALVARES, A. P., HARBERS, L. H. and VISEK, W. J. (1964): *J. Nutr.*, **82,** 93～98.
2) ALVARES, A. P., HENDRICKSON, R. F. and VISEK, W. J. (1964): *J. Nutr.*, **83,** 79～84.
3) BRENT, B. E., ADEPOJU, A. and PORTELA, F. (1970): *J. anim. Sci.*, **32,** 794～798.
4) CLIFFORD, A. J., BOURDETTE, J. R. and TILLMAN, A. D. (1968): *J. anim. Sci.*, **27,** 1073～1080.
5) GALE, G. R. and ATKINS, L. M. (1969): *Arch. int. Pharmacodyn.*, **180,** 289～298.
6) GORIN, G. and CHIN, C. (1966): *Analyt. Biochem.*, **17,** 49～59.
7) HARMON, K. M. and NIEMANN, C. (1949): *J. biol. Chem.*, **177,** 601～610.
8) HASE, J. and KOBASHI, K. (1967): *J. Biochem.*, **62,** 293～299.
9) KOBASHI, K., HASE, J. and UEHARA, K. (1962): *Biochem. Biophys. Acta*, **65,** 380～383.
10) KOBASHI, K. (1972): *Seikagaku*, **44,** 187～204.
11) ORMSBY, A. A. (1942): *J. biol. Chem.*, **146,** 595～604.
12) PENNINGTON, R. J. and SUTHERLAND, T. M. (1956): *Biochem. J.*, **63,** 353～361.
13) SELINGSON, D. and HIRAHARA, K. (1957): *J. lab. clin. Med.*, **49,** 962～974.
14) SPACKMAN, D. H., STEIN, W. H. and MOORE, S. (1958): *Analyt. Chem.*, **30,** 1190～1206.

緬羊第一胃ウレアーゼに及ぼすカプリロヒドロキサム酸の影響

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反芻動物に対する尿素の飼料化は，わが国においても普及の段階にある．尿素の利用を安全かつ効果的に行なわせるため，ここに基礎的実験を行なった．小橋らによって発見された抗ウレアーゼ剤としてのヒドロキサム酸のうち，とくに強力であるカプリロヒドロキサム酸（CHA）のルーメンウレアーゼに及ぼす影響を，この実験で検討した．

ルーメン液を超音波処理した後，超遠心分離して得た上清を，無細胞抽出粗酵素標品として用いた．二三の酵素学的性質を調べた結果，ルーメンウレアーゼは植物由来のウレアーゼと似た性質を持つことを知った．反応時間がたつにつれて，CHA の阻害が強くあらわれ，CHA の最終濃度が 10^{-4}M 以上になると，急に阻害が強くなることは，ルーメン液そのものを酵素液として用いた場合と同様であった．CHA の阻害形式を CHA および基質濃度を変えて検討した結果，基質濃度が高く，CHA 濃度が低いときは，単純な非拮抗阻害がみられ，基質濃度が低く，CHA 濃度が高いときは，反応速度曲線が S 字状の複雑な様相を呈した．

ルーメン醗酵に及ぼす CHA の影響を，発酵管法と培養法について検討した．その結果，ルーメン液の始めの尿素および CHA 濃度が約 1～2×10^{-2}M と 1×10^{-3}M であるとき，NH_3，尿素，揮発性脂肪酸（VFAs），乳酸濃度は 6 時間目にはほぼ同じであったが，経時的には，3 時間まで尿素の分解が阻害され NH_3 の発生も低かった．しかし VFAs 産生には影響しなかった．

以上のことから，CHA はルーメンウレアーゼに影響を与え，尿素飼料給与時に利用し得ることが示唆される．