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# BATCH-CULTURES OF RUMEN MICROORGANISMS IN VITRO

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**Abstract**

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The modified method of batch-culture of rumen microorganisms in vitro was developed. When checking this method we have found:

- The optimal ratio of urea and glucose in incubation medium was within the range 0.1—0.3 g N-urea/g glucose.
- By adding of 0.003 % amorphous FeS in the inoculated medium more negative redox potential was always observed in the range close to in vivo conditions.
- The optimal microbial protein synthesis was achieved at an osmotic pressure 0.48 MPa. It follows from the results that osmolarity of buffer routine used is low.
- Enhanced utilisation of starch was obviously observed in the presence of maltose.
- By decreasing the glucose concentration to 2 g/l buffer at the beginning of the incubation the lactate production was prevented.
- The isonitrogenic replacement of 25 % urea by amino acids increased the production of protein as well as VFA and $Y_{ATP}$.
- Succinic acid, lactic acid and hydrogen were not present in significant concentrations which correspons to in vivo conditions.

It follows from the results obtained that the developed batch-culture represents a suitable in vitro system for short-term rumen fermentation studies.

*Goats, sheep, fermentation, incubation, medium.*

Rumen microorganisms are able to convert plant forages to nutritionally valuable products. The cellulose degradation is the most important process. Besides this, microorganisms also synthesize most other saccharolytic enzymes. They further synthesize all essential amino acids for the host — in such an amount that under optimal conditions the host is independent of their contents in the food. Besides this they produce sufficient amounts of vitamins of groups B, H and K.

From published results and theoretical studies it follows that the synthetic capacity of rumen microorganisms is utilized only partially. Thus, the research of their metabolism and possibilities of its modification are in the focus of interest in the animal production at the present time.

The rumen fermentation after feeding may be divided depending on the decomposition of saccharides into two parts. At the first stage mono-, disaccharides and alpha-polymers of saccharides are prevalently hydrolyzed and metabolized. The length of this stage is usually of 5 to 10 hrs, depending on the fed substrate. The second stage comes after the first one, and is characterized by a decomposition of beta-polymers, i. e. particularly cellulose. This stage continues up to exhaustion of the substrate or up to next feeding. The two stages affect each other, however, they may be studied separately. Different approaches are used to studying the two stages. For the study of the decomposition of alpha-polymers of saccharides it is possible to use a short-term incubation in vitro. The metabolism of cellulolytic bacteria is more properly studied with the help of the continuous-culture.

Advantages of the batch-culture are particularly simplicity, possibility of analyzing large amounts of samples and easy statistical evaluation of results (Lopper et al. 1966; Barr 1974; Maeng et al. 1976; Chen et al. 1976; Van Nevel and Demeyer 1977 and others). Since the end of the fifth decade, nobody studied in detail the method of batch-cultures or results were not published in spite of the fact that in the meantime many new factors affecting the rumen fermentation revealed. Thus, some authors employed improper conditions of the incubation and particularly

ratios of substrates, which could make the results of the experiment doubtful. When starting with the batch-culture in our laboratory, remarkable spread of results was often encountered and the results were sometimes mutually opposing. Thus, we decided to pay attention to a methodic treatment of the batch-culture of rumen microorganisms.

Table 1

**Comparison of different batch cultures of rumen microorganisms in vitro**

| Reference | Study dealt with | Time of sampling rumen liquor after feeding (h) | Inoculum | Buffer | Anaerobiosis reached by |
|---|---|---|---|---|---|
| LOPER 1966 | amylolytic bacteria | 1–2 | rumen bacteria | McDougall buffer | $CO_2$ |
| BARR 1974 | comparison of Starea and urea | 0 | rumen liquor | own | – |
| SINGH et all. 1975 | $^{14}C$ incorporation into microbial lipids | 0 | rumen liquor | own | – |
| CHEN et all. 1976 | nutrition value of corn | 0 | rumen bacteria | McDougall buffer | – |
| MAENG et all. 1976 | influence of amino acids on fermentation | 2 | rumen bacteria | own | $CO_2$ |
| VAN NEVEL et DEMEYER 1977 | $^{32}P$ incorporation into rumen microorganisms | 0 | rumen liquor | own | $CO_2$ |

In initial experiments we considered a combination of methods summarized in Table 1, where works by Maeng et al. (1976) and Barr (1974) served as a basis for this purpose. These authors were partially interested in the incubation method. A derived method was marked as "method I" (Marounek et al. 1979). When checking this method we used a number of modifications. They led to a modified "method II". Particular modifications and course of the fermentation by the modified method are discussed in the following chapters.

## Materials and methods

### Animals and diets

In the experiments the rumen liquor was used of goats (1 to 2 years, 30 to 45 kg) and sheep (the same age, 50 to 65 kg). The animals were fed once daily by a constant diet consisting of 1 to 2 kg of hay, 0.5 kg of concentrate and 10 g of urea per animal and day. The animals received water ad libitum. All animals were provided with permanent rumen fistulas.

### Sampling of the rumen liquor

The sampling was carried out before feeding, since the microorganisms, except for certain cellulolytic bacteria, are at the stationary stage of the growth that time. However, the cellulolytic bacteria are not remarkably manifested during a short-term incubation in vitro. The rumen liquor was sampled for the experiment from at least two animals by a tube, with the help of a vacuum pump (Doležel et al. 1969).

### Batch-culture method I

100-ml infusion flasks with Bunsen valves in stoppers were used for the incubation. Into each flask 0.1 g cellulose (cotton, ground paper Whatman 2) and 1 g soluble starch for microbiology (Lachema n. p.) or 1 g dried potato flakes were weighed. Further 30 ml buffer I were added, which contained 10 g glucose and 5 g urea per liter unless otherwise indicated. Then the infusion flasks were conditioned to 40 °C. After adding 15 ml of the rumen liquid the flasks were stoppered and placed in the RT-60 water bath (2 cycles/s) at 40 °C. Into the infusion flasks a mixture of gases $CO_2$ and $H_2$ was introduced (20 : 1). Residual amounts of oxygen from technical $CO_2$ were removed by leading the mixture through palladium catalyst. The atmosphere of infusion flasks was washed at a flow rate of 1 ml $CO_2$/s for 30 min to achieve a residual $O_2$ concentration below 0.1 %. Gases produced in the course of the incubation were released through Bunsen valves. The test incubation took 6 hrs.

After completing the incubation, pH and rH were measured (Maluszynska 1973) and the vitality of protozoa was observed. For the determination of proteins (by precipitation with 6 % trichloroacetic acid and kjehldalisation), ammonia (colorimetrically with Nessler agent), urea (Biotest Lachema Brno), or of the incorporation of radioisotopes 10 ml of medium was preserved with 5 ml of 20 % trichloroacetic acid (TCA). The remaining portion was inactivated by adding $HgCl_2$ and used for determining volatile fatty acids (Bartoš et al. 1960) and lactic acid (Marounek 1978). Gas was analyzed on a gas analyzer with a column filled with Porapak Q (3.5 m long and 3 mm in diameter). One infusion flask served for testing one effect in one time interval. For each determination at least four parallel infusion flasks were used. Succinic acid was determined by the one-dimensional paper chromatography. Papers Whatman No. 1 were used. Chromatograms were developed in the mixture of ethanol-ammonia-water. After air-drying at room temperature chromatograms were sprayed with bromthymol blue solution.

Buffers

Buffer I.

| | |
|---|---|
| 8.75 g | $NaHCO_3$ |
| 1.38 g | $NaH_2PO_3 . 2H_2O$ |
| 1.55 g | $KH_2PO_4$ |
| 1.15 g | $MgSO_4 . 7H_2O$ |
| 1.00 ml | mineral solution |

Dissolved in 1 l of distilled $H_2O$. Saturated with $CO_2$. Final pH 6.80.

Buffer II.

| | |
|---|---|
| 13.25 g | $NaHCO_3$ |
| 2.07 g | $NaH_2PO_4 . 2H_2O$ |
| 2.33 g | $KH_2PO_4$ |
| 0,225 g | $MgSO_4 . 7H_2O$ |
| 1.00 ml | mineral solution |

Dissolved in 1 l of distilled $H_2O$. Saturated with $CO_2$. Final pH 6.90.

Artificial Saliva (McDougall 1949)

| | |
|---|---|
| 9.78 g | $NaHCO_3$ |
| 9.28 g | $Na_2HPO_4 . 12H_2O$ |
| 0.60 g | KCl |
| 0.50 g | NaCl |
| 0.04 g | $CaCl_2$ |
| 0.12 g | $MgSO_4 . 7H_2O$ |
| 1.00 ml | mineral solution |

Dissolved in 1 l of distilled $H_2O$. Saturated with $CO_2$. Final pH 6.80.

Mineral solution

| | |
|---|---|
| 0.80 g | $Fe_2(SO_4)_3 . 9H_2O$ |
| 0.40 h | $MnSO_4 . 4H_2O$ |
| 0.40 g | $ZnSO_4 . 7H_2O$ |
| 0.20 g | $CuSO_4 . 5H_2O$ |
| 0.18 g | $CoSO_4 . 6H_2O$ |

Dissolved in 100 ml of water. Stored at 4 °C.

## Results

### Testing and adjustments of batch-culture I

#### 1. The effect of oxidation-reduction potential of the rumen liquor on the fermentation in vitro

During the isolation of rumen microorganisms, where it is not provided that the microorganisms will be able to reduce the medium, a wide spectrum of reducing agents is used, as e. g. cysteine (0.05 %), sodium sulphide (0.025), thiosulphate (0.003 %), thioglycolate (0.05), dithiothreitol (0.001 %) and their combinations. Amorphous ferrous sulphide was also recommended for the cultivation of anaerobic microorganisms (Brock and O'Dea 1977).

In the course of preliminary incubations there was a remarkable spread of the redox potential ($E_h$) in individual infusion flasks under the same conditions of the cultivation. To reduce this spread, we tested the effect of amorphous ferrous sulphide on the course of $E_h$.

After conditioning amorphous ferrous sulphide was added into infusion flasks to achieve the final concentration in the inoculated medium of 0.003 %, and the redox potential ($E_h$) was compared with control samples. For results see Fig. 1. After the inoculation an enhanced redox potential was always observed — in a range of 100 to 200 mV (against hydrogen electrode). In one hour it is reduced by action of microorganisms as much as by 350 mV. At this stage of the fermentation no effect of ferrous sulphide was found. Its effect was obvious only in subsequent intervals. In samples without addition of FeS the redox potential increased after the second hour. By adding FeS a further increase of the redox potential was achieved in 12 hrs. The spread of $E_h$ in measured samples was also reduced.

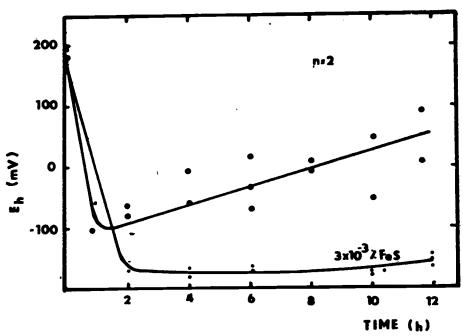



Fig. 1. Effect of amorphous FeS on redox potential during incubation of rumen microorganisms in vitro.

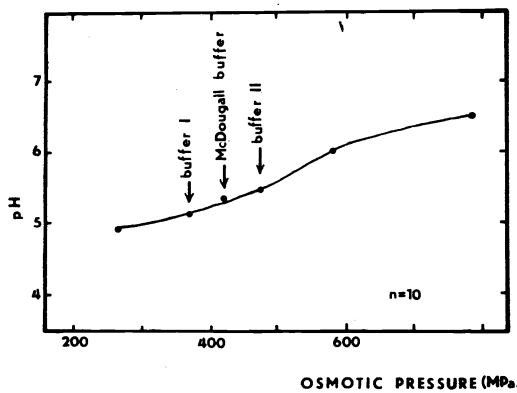



Fig. 2. Effect of osmotic pressure on pH during incubation of rumen microorganisms in vitro.

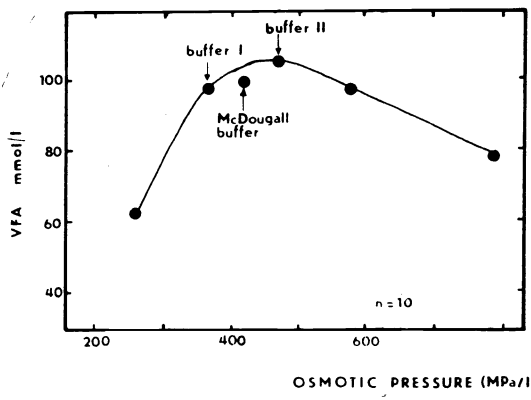



Fig. 3. Effect of osmotic pressure on VFA production during incubation of rumen microorganisms in vitro.

## 2. The effect of the osmotic pressure of incubation medium on the fermentation of rumen microorganisms in vitro

For testing purposes buffer I with the following concentrations was used: diluted 1 : 1 (0.136 MPa/1), non-diluted (0.269 MPa/1), concentrated 1.5× (0.404 MPa/1), 2× (0.538 MPa/1) and 3× (0.808 MPa/1). For a comparison the effect was also established of McDougall artificial saliva (0.336 MPa/1). The osmotic pressure of the rumen liquor varies between 0.56 and 0.62 MPa/1. It follows from the results obtained (Figs. 2 to 4) that the effect of the

buffer osmolarity on the fermentation parameters of interest may be demonstrated. The differences between minimal and maximal values achieve 25 to 40 %. The difference in pH was of 1.5 grades after completing the incubation, the increase being non-linear, since besides the buffering capacity the pH was also affected by the production of volatile fatty acids (Fig. 2).

The production of volatile fatty acids was maximalum at an osmotic pressure of 0.48 MPa/1 (buffer II), which also holds against the McDougall artificial saliva ($P < 0.05$) (Fig. 3).

The protein synthesis was also most remarkable at higher osmotic pressure 0.48 PMa. There was a significant difference between buffers I and II ($P < 0.05$).

To provide optimal conditions for the growth of bacteria and protozoa, only buffer II was used in further incubations.

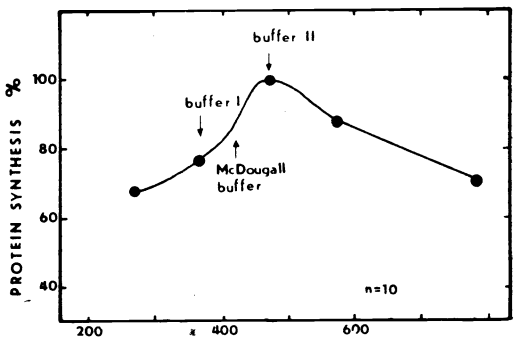



Fig. 4. Effect of osmotic pressure on protein synthesis during incubation of rumen microorganisms in vitro.

Under in vivo conditions the ascertained optimal osmotic pressure of the rumen liquor occurs in animals before feeding. After feeding the osmotic pressure increases and it is likely that, similarly as under in vitro conditions, it leads to a decreased production of volatile fatty acids and proteins. These assumptions were checked indirectly by applying inorganic salts into the food (Barry et al. 1977; Thomson 1978). These authors found, that long-term additions of KCl into food decrease the production of volatile fatty acids and exerts no effect on the ratio of particular volatile fatty acids, i. e. $C_2-C_5$ (Barry et al. 1978). Thomson et al. (1978) found a negative correlation between the production of propionate and diluting rate after the application of 5 to 10 % salt into the diet.

In continuous-culture of rumen liquor a drop of the DNA synthesis was found after the $KClO_3$ application. These nucleic acids correlate with the content of microbial proteins. Simultaneously the production of volatile fatty acids, $CO_2$ and $CH_4$ was reduced. The redox potential of the medium was increased. The authors attributed this effect to a toxic effect of $KClO_3$ (Barry et al. 1978).

To achieve optimal conditions for the growth of rumen microorganisms, in the modified method only buffer II was used (osmotic pressure = 0.4 MPa/1).

3. Optimal ratio of urea and glucose during the incubation in vitro

An optimal course of pH during the incubation can be achieved by suitable buffering capacity of buffer or suitable ratio of N-compounds and saccharides. Since the buffer concentration cannot be varied within wide concentration ranges, it is necessary to search for an optimal ratio of saccharides and nitrogen — containing compounds. The fermentation products of nutrients (particularly ammonia and VFA) have an opposite effect on pH. With respect to the fact that there is a matter of a short-term incubation the largest portion of volatile fatty acids is produced by a decomposition of glucose. Starch participates only partially and celluloses serve for maintaining vital cellulolytic bacteria in medium. The

optimal ratio N : glucose was established in an experiment, where at a constant urea concentration (5 g/l) the glucose level was adjusted to 0, 5, 10, 33 and 66 g glucose/l buffer (Fig. 5). It follows from the graph that optimal results were achieved at glucose concentrations of 10 to 25 g/l buffer, i.e. ratio N : glucose 0.1 to 0.3 Since for in vivo conditions there is a typical drop of pH in 6 hrs, for further work the ratio N : glucose of 0.15 was chosen.

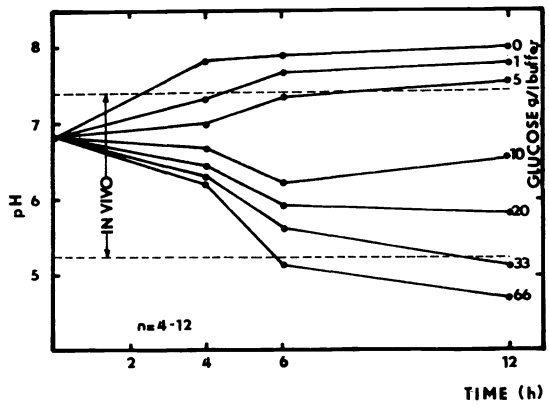



Fig. 5. Effect of glucose on pH during batch-culture of rumen microorganisms in vitro. Dash lines show the limits of pH in vivo conditions.

So, the optimal ratio of urea and glucose was established to 12 g N-urea/100 g glucose. This is in agreement with the used ratio of glucose and urea in certain in vitro methods (Van Nevel and Demeyer 1977; Barr, 1974; Chen et al. 1976). However, these authors did not use in cultures any other saccharides, so that the final ratio N/saccharides was high in their experiments and did not correspond to requirements of rumen microorganisms for their optimal growth. Unfortunately, these authors do not report the final pH during the incubations, so that comparison with our experiments is impossible. Under in vitro conditions the optimal ratio N/saccharides was determined by Zaki El Din and El Shazly (1969) — 2.35 g N/100 g starch. In vivo the optimal value varies about 1.6 to 2.25 g N/100 g DOM (Rofler and Satter 1975; Satter and Slyter 1974). In the case of our method the optimal ratio was established to 1.92 g N/100 g saccharides (glucose, starch and cellulose) which corresponds to an optimal ratio of substrates found in vivo.

4. Effect of the formulation of saccharides on rumen microorganisms in vitro

Starch is a basic substrate during short-term incubation of the rumen liquor. Experiences of authors (Durand et al. 1976; Minato and Suto 1976) however, indicate that there are rather large differences between different types of starch. Thus, we compared soluble starch, potato starch and corn starch in two experiments, where these starches served as the only one source of saccharides. The finding of volatile fatty acids at the end of the incubation was the highest in the case of the soluble starch, next to it there is the corn starch and the potato starch was manifested as the worst. (Expressed relatively: 100—92.4—82.4 %). Thus for the experiments we recommend soluble starch and, by contrast to this, we do not recommend potato starch. Besides this we searched for a suitable mono- or disaccharide in a mixture with starch, through the decomposition of which the rumen microorganisms could gain energy from the very beginning of the incubation. Glucose, maltose, cellobiose and equimolar mixture maltose + cellobiose were tested.

In this experimental series the urea concentration was reduced to 0.5 g/l and starch was added in an amount of 0.5 g/infusion flask. For concentrations of the other saccharides see Tables 2 and 9. The $^{32}P$ concentration was of 2.44 KBq/ml medium.

In the experiments no significant differences in the production of volatile fatty acids and pH values were ascertained, however, the samples containing maltose exerted a higher production of volatile fatty acids and reduction of pH connected with this (Table 2). The protein production, as determined by the $^{32}P$ incorporation, was also higher after adding maltose. The effect of the other saccharides on the $^{32}P$ incorporation was insignificant. In a mixed sample of cellobiose and maltose the effect of disaccharides was not additive. A drop was found of the $^{32}P$ incorporation as well as of the volatile fatty acid production.

Table 2
**Effect of saccharides on starch fermentation by rumen microorganisms in vitro**
Values are means of four observations.

| Substrate | pH | VFA (mmol/l) | Protein synthesis (mg N × 6.25/l) |
|---|---|---|---|
| Starch 10 g/l | 6.32 ± 0.08 | 59.7 | 2249 ± 180 |
| Starch 10 g/l + glucose 1 g/l | 6.35 ± 0.02 | 87.9 | 2473 ± 163 |
| Starch 10 g/l + xylan 1 g/l | 6.22 ± 0.03 | 94,9 | 2676 ± 148*) |
| Starch 10 g/l + cellobiose 0.5 g/l + malbose 0.5 g/l | 6.22 ± 0.02 | 88.3 | 2676 ± 91*) |

*) $P < 0.05$

Most authors use glucose as a basic saccharide for in vitro cultures (Maeng et al. 1976; Chen et al. 1976; Owens and Issacson 1977 and others). However, there was an objection (Van Nevel and Demeyer 1977) that an addition of glucose can result in an increased production of lactic acid on account of volatile fatty acids as observed by Maeng and Baldwin (1976). In the elaborated method the increased production of lactate (20 to 50 mmol/l) was often found in experiments, where the initial glucose concentration was higher than 3 g/l buffer and always when pH of medium dropped below pH = 5.2 (Marounek 1978, 1980). By decreasing the glucose concentration to 2g/l buffer, at the beginning of the incubation the lactate accumulation was prevented and pH was not decreased below 5.5.

In further experiments the effect of xylane (ICN Pharmaceuticals, Inc.), glucose and equimolar mixture cellobiose + maltose was observed. As compared to starch, after adding other saccharide an enhanced production of volatile fatty

Table 3
**Effect of mono- and disaccharides on starch fermentation by rumen microorganisms in vitro**
Values are means of ten observations.

| Substrate | pH | VFA (mmol/l) | $^{32}P$ incorporations (Bq/l × $10^{-6}$) |
|---|---|---|---|
| Starch 10 g/l + glucose 2 g/l | 6.63 ± 0.07 | 50.8 ± 2.4 | 1.29 ± 0.08 |
| Starch 10 g/l + cellobiose 2 g/l | 6.64 ± 0.05 | 51.2 ± 0.2 | 1.54 ± 0.12 |
| Starch 10 g/l + maltose 2 g/l | 6.55 ± 0.06 | 54.6 ± 2.0 | 1.94 ± 0.07*) |
| Starch 10 g/l + maltose 1 g/l + cellobiose 1 g/l | 6.55 ± 0.05 | 55.1 ± 2.8 | 1.44 ± 0.03 |

* $P < 0.05$

acids was observed. There were no significant differences in the protein production, however, an enhanced proteosynthesis was observed in samples with additions of xylane and mixture cellobiose + maltose (Table 3).

Cellobiose with maltose are main products of the hydrolysis of cellulose and starch. Their effect was followed in vitro in the absence of further saccharides according to the following schematic diagram:

| Sample No. | g maltose/1 buffer | g cellobiose/1 buffer |
|---|---|---|
| 1 | 0 | 0 |
| 2 | 0 | 1 |
| 3 | 0 | 5 |
| 4 | 1 | 0 |
| 5 | 1 | 1 |
| 6 | 1 | 5 |
| 7 | 5 | 0 |
| 8 | 5 | 1 |
| 9 | 5 | 5 |

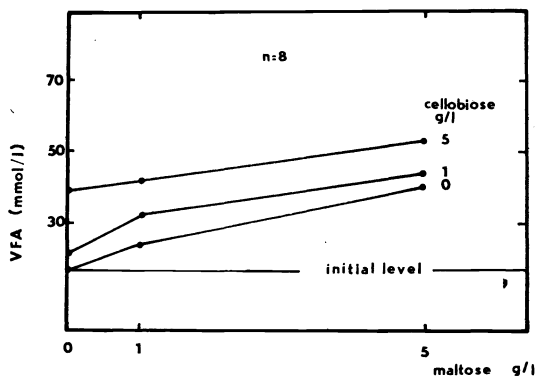



Fig. 6. Effect of cellobiose and maltose on VFA production during incubation of rumen microorganisms in vitro.

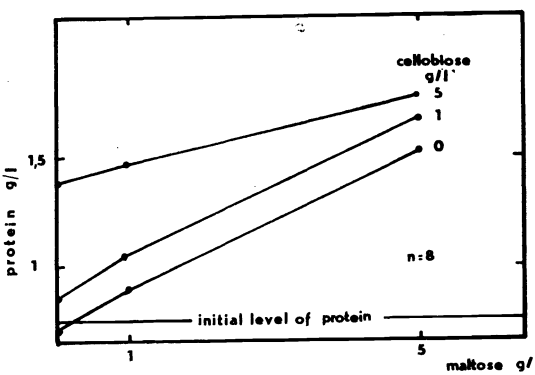



Fig. 7. Effect of cellobiose and maltose on protein synthesis during incubation of rumen microorganisms in vitro

For results see Figs. 6 and 7. The mixture of both disaccharides does not affect the volatile fatty acids production (Fig. 8). By contrast to this, the protein synthesis is affected (Fig. 9).

The results of experiments testing the effect of disaccharides and starch on the growth of rumen microorganisms are in agreement with those by Barr (1974), who demonstrated a relative possibility of replacing usual monosaccharides when affecting the proteosynthesis. Enhanced proteosynthesis in the presence of maltose obviously resulted probably from a high selection of amylolytic bacteria during the incubation. An addition of xylane makes a growth of a wider spectrum of bacteria possible, thus favourably affecting the utilisation of available substrates (Williams et al. 1969; Belasco 1956).

The last experiments were supposed to elucidate a mutual effect of cellobiose and maltose on the growth of microorganisms. Besides glucose, this is a matter

of main intermediate products of the polysaccharide decomposition. The ascertained effect of an addition of cellobiose to maltose in medium corresponds to results of a study by Russel et al. (1979). These authors, when using five pure cultures of bacteria, also ascertained an inhibition effect of cellobiose in the presence of maltose on the bacteria growth.

Henderickx and Martin (1963) by contrast to this report found that the proteosynthesis of rumen microorganisms is higher in the presence of cellobiose than that in the presence of maltose. In all probability, the effect of cellobiose is one of control mechanisms between amylolytic and cellulolytic bacteria in the rumen.

By addition of cellobiose to maltose the proteosynthesis of microorganisms was reduced, however, the production of volatile fatty acids was unaffected. From this it results that the utilisation of ATP is different when combining both substrates:

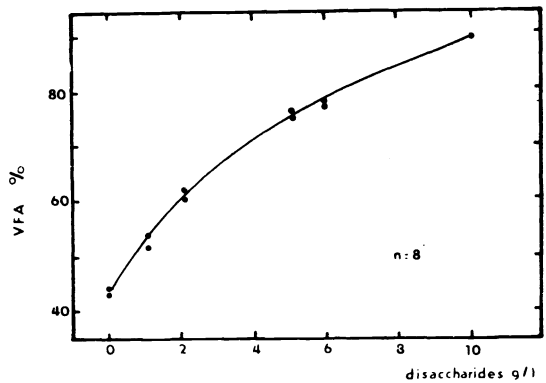



Fig. 8. Effect of mixture of cellobiose and maltose on VFA production during in vitro incubation.

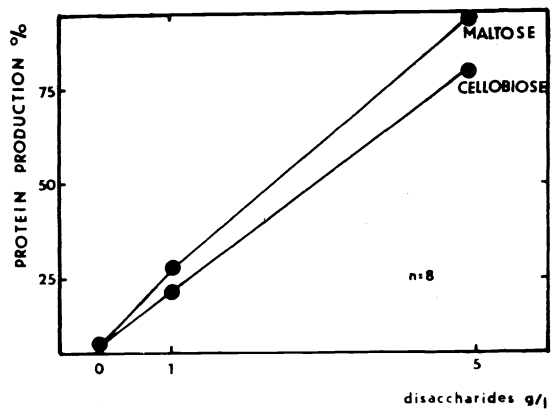



Fig. 9. Effect of cellobiose and maltose on microbial protein production during in vitro incubation.

| | content in medium (g/l) | $Y_{ATP}$ (g d. l./mol ATP) |
|---|---|---|
| cellobiose | 5 | 7.72 |
| maltose | 5 | 9.14 |
| cellobiose + maltose | 5 +5 | 8.74 |

It can be seen from the table that through the decomposition of cellobiose the microorganisms gain less ATP than through the decomposition of maltose. In a mixture of both disaccharides, the inhibition effect of cellobiose on the maltose utilisation was manifested again. It would be suitable to check the utilisation of ATP under these conditions on pure cultures of bacteria and to establish in which bacteria the $Y_{ATP}$ is affected.

With respect to the results mentioned it was decided to use glucose in in vitro cultures up to the final elucidation of the interaction of cellobiose and maltose on the fermentation.

5. Urea concentration in the course of the in vitro incubation

The original concentration of urea in medium was high and ammonia released by the hydrolysis achieved concentrations which are not typical under in vivo conditions. Thus the in vivo fermentation was followed at an urea concentration of 0.5 g/l buffer. At this concentration an equilibrium is established in the incubated medium (Fig. 10) where the released ammonia is proportional to the consumption for the synthesis of microbial proteins, i. e. 100 to 300 mg $N-NH_2/l$ and its concentration varies within physiological ranges. Simultaneously with the adjustment of the initial urea concentration the glucose concentration was reduced in such a way that the optimal ratio N/saccharides was adhered to. By this decrease a possibility was almost precluded of increasing the lactate production. The inhibitory effect of high ammonia concentration was also prevented, which is indicated by Owens and Isaacson (1977). The reduced concentration of nitrogen sources is lower than that used by the other authors (Table 7), however, it yielded good results in cultures.

6. Choice of nitrogen source for the fermentation

The fermentation activity of in vitro system expressed by the volatile fatty acid production is affected not only by the amount and type of supplied saccharides, but also by the source of N. In a further experiment we compared as sources of nitrogen urea (35 mg N/45 ml) and isonitrogenic amount of an ammonium salt ($NH_4Cl$), casein hydrolysate, yeast autolysate and combination of urea with yeast autolysate (17.5 mg N-urea + + 17.5 mg N-yeast autolysate/45 ml). The amount of saccharides (starch, cellulose, glucose in usual concentrations) was identical in all cases. Regarding the volatile fatty acid production as well as protein production in the incubation medium ammonium chloride is a least suitable source of nitrogen ($52.2 \pm 4.2$ mmol VFA/1). Urea is insignificantly better ($56.9 \pm$ $\pm 2.1$). Amino acids in the form of casein hydrolysate and yeast autolysate stimulate the fermentation ($61.4 \pm 6.5$ and $64.5 \pm 8.1$ mmol VFA/1, respecti vely). The differences as against $NH_4Cl$ are significant. The combination of urea and yeast autolysate ($67.5 \pm 3.5$ mmol/1) was shown to be the best. The proteosynthesis follows a similar trend. A reason for this is obviously in a dependence of certain bacteria on amino acids — peptides, demonstrated in a number of papers.

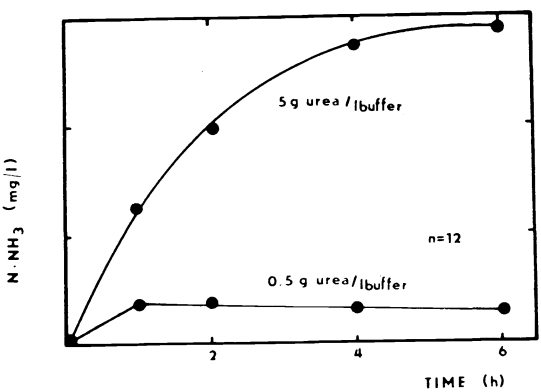



Fig. 10. Effect of urea on ammonia level during incubation of rumen microorganisms in vitro.

An addition of urea to the source of amino acids and peptides is, however, advantageous from the standpoint of a rapid delivery of ammonia into the culture medium. It removes the dependence of bacteria requiring ammonia as a source of nitrogen on bacteria deaminating amino acids.

7. The effect of peptides and amino acids on the fermentation in vitro

In short-term cultures of the rumen liquor, the amylolytic bacteria, protozoa and bacteria utilizing intermediate metabolic products contribute essentially to the fermentation. They are dependent on the amount of amino acids in medium more than other microorganisms. Thus, the isonitrogenic replacement of 25 % urea by casein hydrolysate or potato flakes which served as a natural source of plant proteins was tested. The casein hydrolysate is advantageous in that it is not precipitated with 6.5 % TCA and that it does not affect the determination of the proteosynthesis.

One liter of buffer contained 0.5 g urea and 0.36 g casein hydrolysate (Imuna n. p. Šarišské Michaľany) and control flasks contained buffer with 0.65 g urea/l. The glucose level was the same in both cases — 2 g/l buffer. In isotope experiments the $^{32}P$ activity was of 0.925 MBq/45 ml medium, (i. e. infusion flask). The same amounts of starch and potato flakes were weighed — 1 g/45 ml medium. The potato flakes in the infusion flask contained 30 mg amino acids.

For results of the experiment see Figs. 11—14. An addition of amino acids as well replacement of starch by potato flakes increases the production of protein- as well as incorporation of $^{14}C$-acetate into lipids and incorporation of $^{32}P$-phosphate into microorganism biomass. The increase of proteosynthesis determined by the kjeldahlisation and incorporation of $^{14}C$-acetate was significant. The spread of results during the determination of the proteosynthesis with the help of the $^{32}P$-phosphate incorporation disturbed the significance, although the trend was the same as in preceding experiments. An addition of amino acids to potato flakes reduced (insignificantly) the proteosynthesis. From this it follows that further increases of the amino acid concentration are useless. The effects of the amino acid addition on the pH and volatile fatty acid production are summarized in Table 4. Samples with amino acids yield a higher production of volatile fatty acids with simultaneously reduced pH.

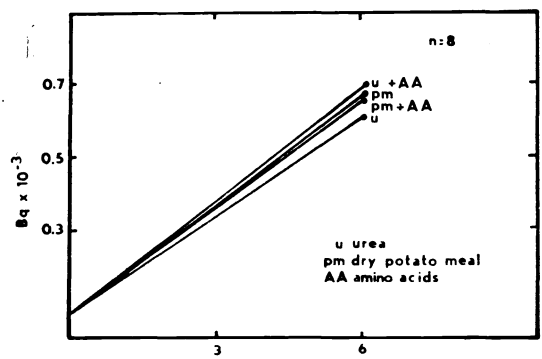



Fig. 11. Effect of amino acids on $^{32}P$ incorporation into rumen microorganisms during incubation in vitro.

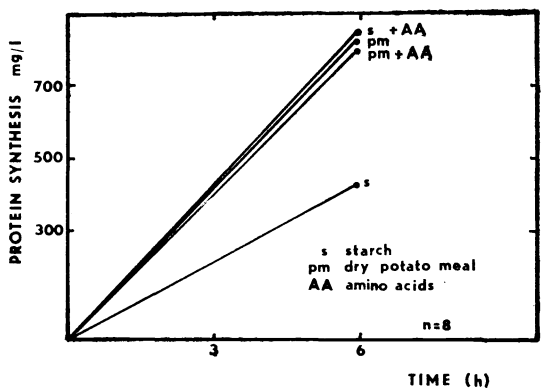



Fig. 12. Effect of amino acids on protein synthesis of rumen microorganisms in vitro.

Most authors using batch cultures do not add amino acids into incubation medium (Lopper et al. 1966; Barr 1974; Chen et al. 1976, Van Nevel and Demeyer 1977). So, they intentionally prefer bacteria, which utilize ammonia as a single

Table 4

**Effect of amino acids on pH and VFA production during incubation of rumen microorganisms in vitro**

a) pH (average of 6 observation)

| Time (h) | Starch | Starch + amino acids | Potato meal | Potato meal + amino acids |
|---|---|---|---|---|
| 0 | 6.91 ± 0.06 | 6.90 ±0.05 | 6.90 ± 0.05 | 6.90 ± 0.03 |
| 6 | 6.71 ± 0.11 | 6.46 ± 0.15*) | 5.92 ± 0.18 | 5.45 ± 0.28*) |

b VFA production in mmol/l (average of 4 observation)

| Time (h) | Starch | Starch + amino acids | Potato meal | Potato meal + amino acids |
|---|---|---|---|---|
| 0 | 63.7 ± 3.4 | 63.7 ± 3.4 | 62.1 ± 3.7 | 62.1 ± 3.7 |
| 6 | 97.9 ± 4.0 | 113.2 ± 10.3*) | 117.3 ± 2.3 | 128.1 ± 1.3*) |

*) $P < 0.01$

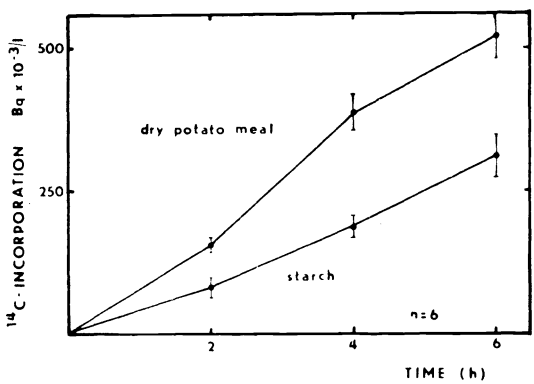



Fig. 13. Effect of starch and dry potato meal on $^{14}C$ incorporation from acetate to lipids on rumen microorganisms in vitro.

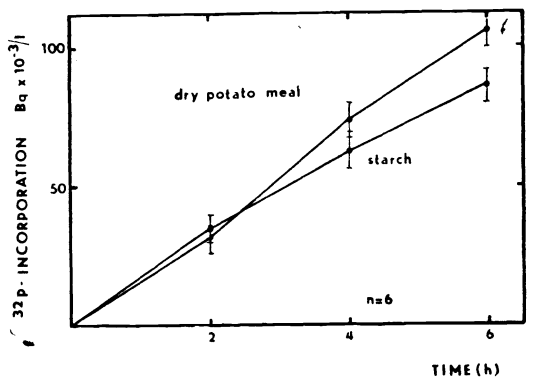



Fig. 14. Effect of starch and dry potato meal on $^{32}P$ incorporation from phosphate into rumen microorganisms in vitro.

source of nitrogen. These extreme conditions do not occur in in vivo processes. Even animals on a synthetic diet are passaging mucins of saliva into the rumen and also the desquamation of rumen epithelium serves as a source of proteins for rumen microbes. It was demonstrated in our experiments, that a 25 % replacement of urea by amino acids or proteins is sufficient for providing an optimal growth of microorganisms as Maeng and Baldwin (1976). However, these authors found that an addition of amino acids had no effect on the volatile fatty acid production. This may be explained by that in their experiments there was an enhanced production of lactate, which sometimes exceeded the production of the other volatile fatty acids (Maeng and Baldwin 1976c). In our experiments the production of lactate was low (3—8 mmol/l/6 hrs) and amino

acids exerted an effect on the production of volatile fatty acids. The addition of amino acids increased significantly the fermentation rate, which is in agreement with the found nutritional requirements of amylolytic bacteria (Bryant and Robinson 1961, 1962, 1963).

The utilisation of ATP for the synthesis was also increased by adding amino acids. As far as urea served as a single source of nitrogen, $Y_{ATP}$ was of 11.46 g b. s./mol ATP. The addition of amino acids resulted in an increase to 15.75 g b. s./mol ATP, i. e. by 37 %. A similar result was obtained by Maeng and Baldwin (1976). In the absence of starch in the medium amino acids increased $Y_{ATP}$ by 33 %.

It follows from the results presented, that in short-term incubations, where a maximal growth of microorganisms is required, it is necessary to supply a part of the nitrogen source in the form of amino acids.

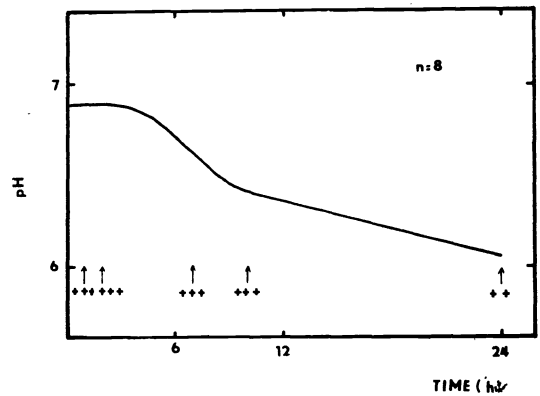



Fig. 15. Changes of pH during incubation of rumen microorganisms in vitro.
Rumen protozoa: +++ abundant and vital
++ vital
+ non-vital

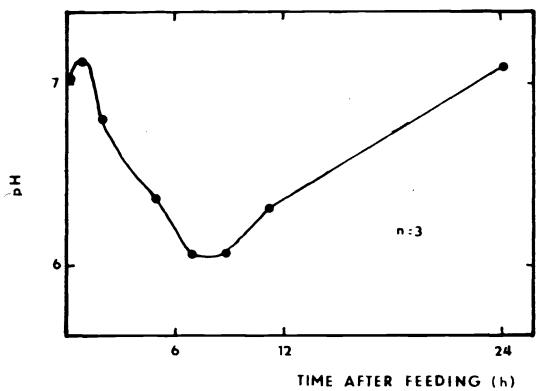



Fig. 16. Changes of pH in sheep rumen liquor after feeding.
Feed ration consisted of:
1 kg hay
0.5 kg concentrate
10 g urea

## 8. The effect of light on the in vitro fermentation

This effect was tested in one experiment. One half of incubation flasks were painted black to prevent the admitance of light to the incubated rumen liquor. The concentrations of glucose, urea and casein hydrolysate were of 2, 0.5, and 0.36 g/l buffer, respectively.

Due to a small spread the differences were significant ($P < 0.05$). Changes of pH and production of volatile fatty acids and protein were followed. In a 6-hr incubation the effect of light was manifested as an inhibition of the production of protein as well as fatty volatile acid:

| light | present | absent |
|---|---|---|
| pH | 5.13 ± 0.02 | 5.05 ± 0.03 |
| VFA (mmol/l) | 66.7 ± 0.04 | 69.1 ± 1.4 |
| protein (mg/l) | 2946 ± 22 | 3170 ± 66 |

The light exerts bactericidal or bacteriostatic effects on most heterotrophic bacteria, particularly the pathogenic ones (Hampl 1964). In our experiments it was demonstrated that it also acted on rumen microorganisms. The ascertained inhibition effect on the fermentation was small (about 5 %) however for establishing optimal conditions it is more suitable to eliminate the effect of the light in the course of the incubation.

## Modified Method of the Incubation of Rumen Microorganisms in Vitro

The modified method was checked by a 24-hrs incubation of the rumen liquor. Sampling as well as inoculation of the rumen liquor were performed similarly as in the original method. Into an infusion flask 0.5 g of soluble starch (Lachema Brno) and 0.1 g cellulose (cotton or ground paper Whatman No. 2) were weighed. Then 30 ml buffer II were added and the flasks were heated for 15 min to 40 °C. The glucose concentration was of 2 g/l buffer, urea concentration 0.5 g/l buffer and casein hydrolysate concentration 0.36 g/l buffer. After heating amorphous FeS was added, whose final concentration in medium was of 0.003 %. The incubation time was 24 hrs.

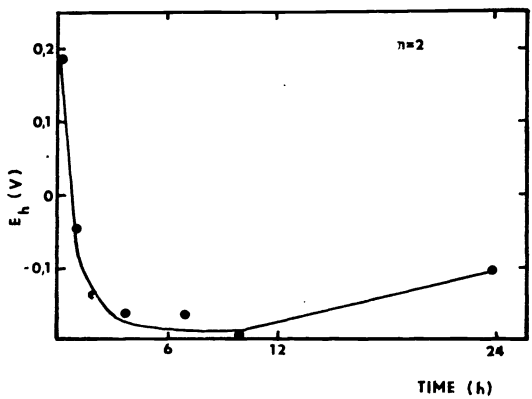



Fig. 17. Changes of $E_h$ during incubation of rumen microorganisms in vitro.

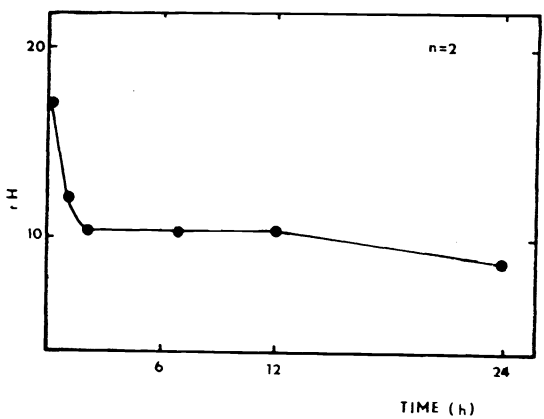



Fig. 18. Changes of rH during incubation of rumen microorganisms in vitro.

For the course of pH see Fig. 15. For a comparison a course is presented of the pH of rumen liquid of rams, from which the inoculum was obtained (Fig. 16). The drop of pH in initial hours under in vivo conditions is more rapid than that during the incubation. In both cases the course is in the same range of pH. The decrease of pH after the 12th hr of the incubation under in vitro conditions results from the volatile fatty acid accumulation. These acids are resorbed in the rumen. The vitality of protozoa was followed microscopically during the in vitro incubation. It is indicated in the graphs. A decrease of the protozoa vitality occurs only in the second half of the incubations. In 6 hrs, i. e. time of the incubation, the vitality of protozoa remained unaltered.

The course of the oxidation-reduction potential of the incubation medium can be seen in

Fig. 17. From the initial value of 180 mV it decreases in one hr to −74 mV, up to the second hr it drops to −160 mV and it remains constant up to 12th hr. Then there is a moderate increase of $E_h$ to −100 mV in 24 hrs. The measured values were calculated with respect to rH units (Fig. 18). Even here the course was similar. The final drop of rH after 24 hrs of incubation results from a decrease of pH.

The volatile fatty acid production may be controlled by an amount of added saccharides. Under mentioned conditions their production is shown in a graph (Fig. 19). In first 10 hrs the volatile fatty acids originate from the decomposition of glucose (at most 17 mmol/l) and starch (at most 160 mmol/l). The decreased production of fatty volatile acids in an interval of 12 to 24 hrs results from decreasing the number of microorganisms (Fig. 24). The maximal rate of the volatile fatty acid production is of about 12.6 mmol/hr/l medium. The concentration of individual volatile fatty acids in the course of the incubation is in Fig. 20 and besides this, in Fig. 21 there is a production of individual volatile fatty acids. In first 10 hrs of the incubation the rate of the propionate production is the same as that of the acetate production. The molar ratio of individual volatile fatty acids in the course of the incubation was as follows:

| Incubation time (hrs) | acetate (mol %) | propionate (mol %) | butyrate (mol %) |
|---|---|---|---|
| 0 | 80.95 | 14.97 | 4.08 |
| 1, 15 min | 72.22 | 23.08 | 4.70 |
| 2 | 74.73 | 19.75 | 5.52 |
| 4, 30 min | 64.91 | 29.24 | 5.85 |
| 7 | 60.00 | 35.08 | 4.92 |
| 10 | 57.45 | 38.87 | 4.68 |
| 12 | 60.83 | 34.12 | 5.05 |
| 24 | 61.73 | 33.96 | 4.31 |

The lactic acid concentration in the medium remained unaltered in 7 hrs of the incubation:

| Incubation time (hrs) | lactic acid (mmol/l) |
|---|---|
| 0 | $4.58 \pm 2.86$ |
| 7 | $2.93 \pm 1.63$ |

This means that under mentioned conditions no lactic acid accumulation occurs, which also corresponds to the course of pH.

The production of gas ($CO_2$ and $CH_4$) was measured only for 7 hrs.

| Incubation time (hrs) | gas production (ml/45 ml medium/hr) |
|---|---|
| 0 | 0 |
| 2 | $1.16 \pm 0.27$ |
| 4.30 min | $8.67 \pm 1.05$ |
| 7 | $17.60 \pm 1.10$ |

After the incubation (7 hrs) the residual atmosphere was washed with 40 % NaOH and so $CO_2$ was removed. The remaining gases ($CH_2$ and $H_2$) represented 50 to 10 % of the original gas volume.

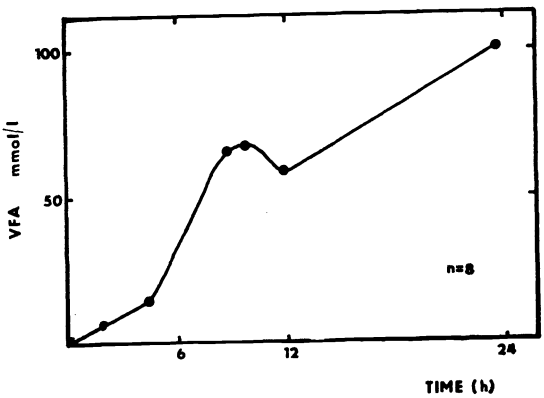



Fig. 19. VFA production during incubation of rumen microorganisms in vitro.

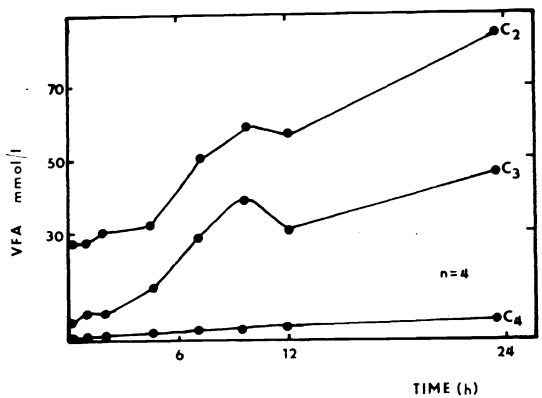
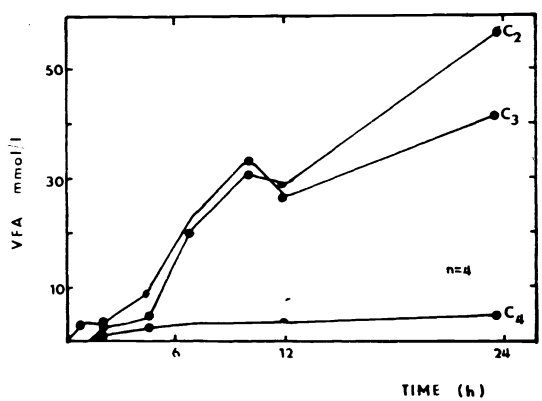



Fig. 20. Concentration of some VFA during incubation of rumen microorganisms in vitro.
$C_2$ — acetate
$C_3$ — propionate
$C_4$ — butyrate




The gas chromatographic analysis of a sample of gas from the end of the incubation presented the following result:

826 $\mu$l $CO_2$/1 ml
131 $\mu$l $CH_4$/1 ml
4 $\mu$l $H_2$/1 ml
20 $\mu$l $N_2(+ O_2)$/1 ml

On Porapak Q nitrogen and oxygen are eluted in only one peak and thus, it is impossible to decide whether this is a matter of nitrogen or nitrogen with residual amounts of oxygen. A low value of redox potential and high value of methane in gas (product of methanobacteria sensitive to oxygen) however, provide sufficiently good anaerobiosis of the methodic experiment. $CO_2$ originating from the acid decomposition of sodium hydrocarbonate contained in the buffer represents the main component of the gas. Hydrogen, considered as an atypical fermentation product is present only in a low amount. As far as succinic acid is concerned its level did not exceed 0.08 mmol/l.

The rate of the urea hydrolysis varied about 500 mg N-urea/hr/l medium during the in vitro incubation (Fig. 22). In one hour 98 % urea was decomposed to ammonia and $CO_2$. These results also correspond to the dynamics of ammonia in the medium incubated (Fig. 23). In the first hour of the incubation there was a maximum concentration of ammonia — 295 mg $N-NH_3$/l.

Fig. 21. VFA production during incubation of rumen microorganisms in vitro.
$C_2$ — acetate
$C_3$ — propionate
$C_4$ — butyrate

It was continuously consumed for the synthesis of microbial proteins. After 8th hour of incubation the ammonia level is already decreasing below the concentration optimal for the proteosynthesis. This decrease also resulted in decreasing the production of volatile fatty acids and proteins. After 10th hour of incubation there is a moderate increase of the ammonia concentration. This increase obviously results from the decomposition of microbial proteins, amino acids or amines. By suitable choice of initial concentration of urea and amino acids an equilibrated dynamics was achieved.

The synthesis of total proteins was followed in a time interval of 0 to 24 hrs (Fig. 24). In first two hours the production of proteins stagnated. This corresponds to a latent stage of the growth of microorganisms. From the second to seventh hour the exponential stage was observed. In intervals 2 to 4 hrs and 4 to 7 hrs the rate of the protein synthesis varied about 48.3 and 51.3 mg N-proteins/hr/1 medium, respectively. After the seventh hour the protein synthesis was stopped. The decreasing level of ammonia exerted the main effect on the limitation of the proteosynthesis. In 7 hrs the protein concentration in medium reached 85 % of the initial concentration in the rumen liquor.

The utilisation of ATP by microorganisms in medium was as follows (Fig. 25). In the initial hour the utilisation of ATP is concentrated to the adaptation to new environment and with respect to the decrease of protein the $Y_{ATP}$ is of zero value. Between the first and second hours the microorganisms start

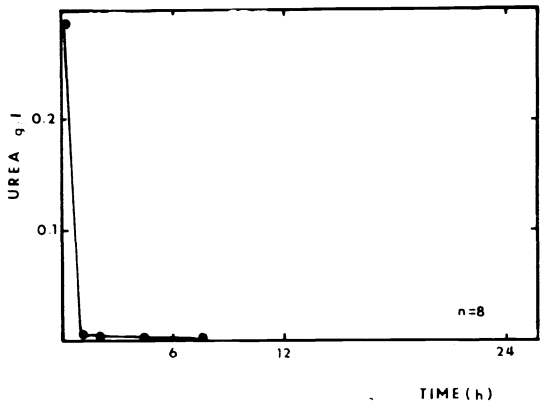



Fig. 22. Urea hydrolysis in in vitro system with rumen microorganisms.

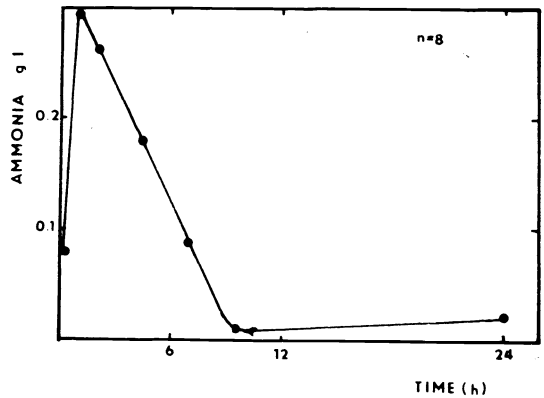



Fig. 23. Ammonia concentration during incubation of rumen microorganisms in vitro.

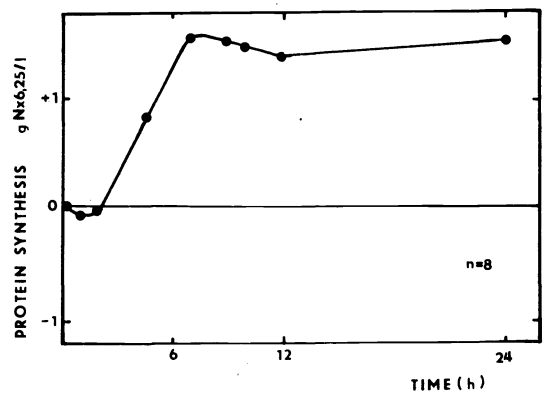



Fig. 24. Crude protein synthesis in in vitro incubation of rumen microorganisms.

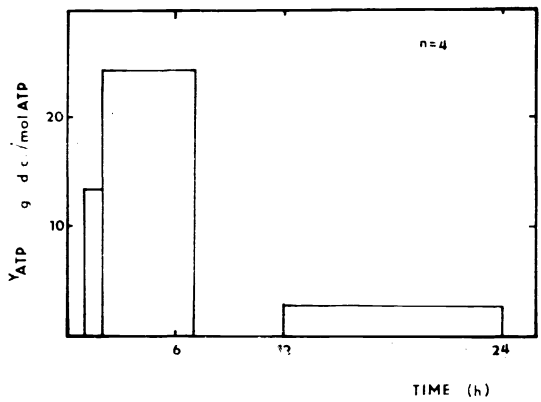



Fig. 25. Growth efficiency of rumen microorganisms expressed as g dry weight of cells per mol ATP (g d. c./mol ATP) in incubation in vitro.

to grow and $Y_{ATP}$ increased to 13.4 g d. b./mol ATP. In the exponential stage of the growth the $Y_{ATP}$ increases to 21.8. Then the concentration of protein is decreased. The growth of bacteria after the 12th hour is limited by the substrate and this also corresponds to a low $Y_{ATP}$ — — 2.5 g d. b./mol ATP.

In our experiments, numbers of bacteria in the course of 6-hrs incubation were established. The increase in three groups of bacteria — amylolytic, proteolytic and cellulolytic — was also determined. For the cultivation of amylolytic bacteria medium according to Bryant and Burkey (1953) was used, the group of proteolytic bacteria was cultivated in medium according to Fulghum and Moore (1963) and the cellulolytic group in medium Hungate (1950).

These groups of bacteria were cultivated in solid agar medium on Petri dishes. The dishes were put into an anaerostat, where the anaerobic atmosphere was formed by a mixture of gases (95 % $CO_2 + H_2$).

The determination of the number of the grown colonies was performed after 5 days in amylolytic and proteolytic bacteria and after 7 days in the cellulolytic group.

| Group | Number of bacteria before incubation | after 6-hr incubation |
|---|---|---|
| Amylolytic | $5.0 \pm 1.0 \times 10^5$ | $3.7 \pm 1.3 \times 10^8$ |
| Proteolytic | $4.2 \pm 0.6 \times 10^5$ | $1.5 \pm 0.3 \times 10^7$ |
| Cellulolytic | $7.1 \pm 2.0 \times 10^6$ | $2.4 \pm 0.9 \times 10^9$ |

The rumen liquor was taken from experimental animals just before feeding. This corresponds to the initial representation of individual groups: the largest increase in biomass was found in cellulolytic bacteria, smaller increase was observed in amylolytic and proteolytic bacteria. In six hours of the incubation there was the most remarkable increase in the amylolytic group, about twice as high as that in the cellulolytic group. The smallest growth was noted in proteolytic bacteria.

## Discussion

By adjusting the "method I" conditions were achieved, under which the in vitro fermentation yields results comparable to those obtained during an in vivo fermentation.

Besides the first hour, the oxidation-reduction potential in the course of the incubation is within physiological ranges. The initial increase obviously results from the contamination with oxygen and from sudden addition of a large amount of the substrate. The redox potencial was tentatively expressed in terms of rH. However, some authors do not recommend this expression for anaerobic

media (Jacob 1970). The expression of redox potential in rH units presented in our case smaller spread of results than that in $E_h$ units. Similar results were obtained by Barry et al. (1977) and Marounek (1980). For this reason, during the incubation of the rumen liquid in vitro it is possible to recommend the expression of the redox potential with the help of rH.

The results of the dependence of rH on time also show a physiological course. They differ from Broberg's (1957) results by about two units of rH. In spite of this increase there is no obvious disturbance of the activity of rumen microorganisms. The vitality of metanogens, i. e. bacteria which are most sensitive to the increase in rH was demonstrated in the course of the in vitro incubation.

The production of volatile fatty acids in the followed experiments is similar to that under in vivo conditions. The ascertained production rate of fatty volatile acids — 12.6 mmol/hr/l medium — corresponds to the rate of the production in the rumen — 5 to 18 fatty volatile acid/hr/l rumen liquor (Van Der Walt and Briel 1976; Marounek 1978). The increased production of propionate is characteristic for concentrate feeding (Ryan 1964; Church 1970). For a given composition of substrates the production of particular fatty volatile acids is normal. The dynamics of the production of fatty volatile acids in the course of the incubation did not correlate with the protein production and so it cannot be recommended as an indicator of the bacteria growth.

In the course of the incubation no lactate production was found. From this it is possible to consider that neither *Streptococcus bovis* nor other bacteria producing lactate are selected as in the case of a method used by Maeng and Baldwin (1976).

The maximal rate of the hydrolysis of urea in the experiments performed varied about 500 mg N-urea/hr/l medium. This corresponds to the hydrolysis rate in the rumen (Sharma and Shukla 1973; Henderickx 1976; Mahadevan et al. 1976, 1977). The ammonia concentration in medium (at most 300 mg $N-NH_3$l) cannot moderate the growth of microorganisms (Owens and Isaacson 1977) and its level is the same to the 8th hour of incubation as that in the rumen (Church 1970).

The synthesis of the microbial protein was satisfactory in the incubated medium. The initial stagnation represents the lag-phase of the microorganism growth. It is obviously affected by a change of the environment and initial high redox potential of the medium (Málek 1953). A similar stagnation was also observed by Maeng and Baldwin (1976) who sampled rumen microorganisms for the incubation in log-phase of the growth, i. e. 2 hrs after feeding. With respect to the adaptation of microorganisms to a new environment, it is unsuitable to use the batch-cultures for following the proteosynthesis of microorganisms in a time interval shorter than 2 hrs (Barr 1974). The rate of the growth of microorganisms in the log-stage is in an agreement with the found rate of the growth in vivo (Walker et al. 1975; Church 1970) as well as in vitro (Nikolič et al. 1975; Henderickx 1976).

There was a high utilisation of ATP for the growth during the incubation. Some authors refuse the batch-culture just for low utilisation of ATP for synthetic processes, or for high consumption of ATP in the basal metabolism (Hespell 1979). The consumption of the basal energy — $M_e$ — is to the largest extent dependent on the diluting capacity of the medium. With respect to the fact that the batch culture has continuously a zero diluting rate, the growth of microorganisms depends on the initial dilution of the inoculum. In our case the rumen

liquor was diluted during inoculation three times which, when related to 10-hrs interval of incubation, represents 20 % dilution/hr.

In the exponential stage the value $Y_{ATP}$ achieves the growth of 21.8 g d. b./mol ATP. However this was not achieved in all the incubations. The $Y_{ATP}$ values usually vary between 13 and 23 g d. b./mol ATP, which are results comparable with those obtained in continuous incubations (Hespell and Bryant 1979). Similar $Y_{ATP}$ values with the batch culture were also obtained by Maeng and Baldwin (1976).

Our results indicate that the obtained parameters of the batch fermentation in vitro in 6 hrs are comparable with parameters of the fermentation obtained in vivo. It is obvious, that in 6 hrs particularly amylolytic organisms and organisms utilizing intermediate products contribute to the fermentation. This fact should always be considered when evaluating the incubation results.

## Vsádková kultivace bachorových mikroorganismů in vitro

Byla vyvinuta modifikovaná metoda vsádkové kultivace bachorových mikroorganismů in vitro. Při jejím ověřování bylo zjištěno, že:
Optimální poměr močoviny a glukosy v inkubačním médiu se pohybuje v intervalu 0,1—0,3 g N-močoviny/g glukosy.
Po přidání 0,003 % amorfního FeS je redox potenciál stabilnější a přibližuje se hodnotám zjištěným in vivo.
Optimální osmotický tlak inkubačního média pro mikrobiální proteosyntézu je 0,480 MPa/l. Z toho vyplývá, že koncentrace doposud používaných pufrů je nízká.
Maltosa stimuluje utilizaci škrobu v bachorovém prostředí.
Snížením počáteční koncentrace glukosy pod 2 g/l pufru nedochází k tvorbě kyseliny mléčné.
Isonitrogenní záměna 25 % N-močoviny aminokyselinami zvyšuje produkci proteinů, TMK i $Y_{ATP}$.
V inkubačním médiu nebyla zjištěna přítomnost kyseliny jantarové, mléčné ani vodíku, což odpovídá podmínkám fermentace in vivo.

Hlavní ukazatele fermentace v prvých 8 hodinách inkubace jsou srovnatelné s nálezy zjištěnými in vivo. Ověřená metoda inkubace in vitro poskytuje schůdnou cestu pro studium metabolismu bachorových mikroorganismů.

## Культивация микроорганизмов рубца загрузкой в пробирке

Был разработан модифицированный метод культивации загрузкой в пробирке микроорганизмов рубца. В ходе его проверки было установлено, что:
— Оптимальное соотношение мочевины и глюкозы в инкубационной среде достигает пределов 0,1—0,3 г Н-мочевины/г глюкозы.
— После добавления 0,003 % аморфного ЯеС окислительно-восстановительный потенциал является более стабильным и приближается величинам, выявленным в живом организме.
— Оптимальное осмотическое давление инкубационной среды для микробиального протеосинтеза — 0,480 МПа/л. Из этого вытекает, что концентрация используемых до сих пор буферных растворов является низкой.
— Мальтоза стимулирует утилизацию крахмала в среде рубца.

— Понижением начальной концентрации глюкозы ниже 2 г/л буферного раствора не происходит образование молочной кислоты.
— Изонитрогенное замещение 25 % Н-мочевины аминокислотами повышает продукцию протеинов, ТМК и УАТР.
— В инкубационном растворе не было выявлено присутствие янтарной, молочной кислот, ни водорода, что соответствует условиям ферментации в живом организме.

Основные показатели брожения в течение первых 8 часов инкубации сравнимы с результатами, установленными в живом организме. Проверенный метод инкубации в пробирке является приемлемым направлением в процессе изучения метаболизма микроорганизмов рубца.

## References


BARR, G.: In vitro rumen fermentation techniques for studying urea utilization in the bovine rumen. PhD thesis State University in Kansas, Manhattan, 1974.

BARRY, T. N. — THOMPSON, A. — ARMSTRONG, D. G.: Rumen fermentation studies on two contrasting diets. J. Agric. Sci., Camb., **89,** 1977: 183—195.

BARRY, T. N., — HARTE, F. J. — PERRY, B. N. — ARMSTRONG, D. G.: Some effect of pottasium chlorate administration on in vitro and in vivo rumen fermentation. J. Agric. Sci. Camb., **90,** 1978: 345—353.

BARTOŠ, S. — HATLEOVÁ, A. — HALAŠOVÁ, E.: Metoda stanovení těkavých mastných kyselin v krvi skotu. Sborník ČSAZV, Živ. výr., **5,** 1960: 857—862.

BELASCO, I. J.: The role of carbohydrates in urea utilization cellulose digestion and fatty acid formation. J. Anim. Sci., **15,** 1956: 496—502.

BROBERG, G,: Measurements of the redox potencial in rumen content: in vitro measurements of healthy and sick animals. Nordisk Veterinaermedicin, **9,** 1957: 918—942.

BROCK, T. D. — O'DEA, K.: Amorphus ferrous sulphide as a reducing agent for culture of anaerobes. Appl. Environ. Microb., **33,** 1977: 254—256.

BRYANT, M. P. — BURKEY, L. A.: Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. J. Dairy Sci., **36,** 1953: 218—224.

BRYANT, M. P. — ROBINSON, I. M.: Studies on the nutrients requirements of some ruminal cellulolytic bacteria. Appl. Microbiol., **9,** 1961: 96—102.

BRYANT, M. P. — ROBINSON, I. M.: Some nutritional characteristics of predominant culturable ruminal bacteria. J. Bact., **84,** 1962: 605—611.

BRYANT, M. P. — ROBINSON, I. M.: Apparent incorporation of ammonia and amino acid carbon during growth of selected species of rumen bacteria. J. Dairy Sci., **46,** 1963: 150—159.

DOLEŽEL, F. — BARTOŠ, S. — JUKL, A.: Zařízení pro odběr a filtraci bachorové tekutiny za anaerobních podmínek. Vet. med., **14,** 1969: 545—548.

DURAND, M. — DUMAY, Ch. — BEAMOTIN, Ph. — KUMAERSAN, A. — VAN NEVEL, C. J. — TASSENCOURT, L.: In vitro studies on non-protein nitrogen utilization by rumen microflora. In: Tracer studies on non-protein nitrogen for ruminants III. International Atomic Energy Agency Vienna, 1976: 41—48.

FRIEDMAN, T. E. — GRASSER, J. B.: The determination of lactic acid. J. Biol. Chem., **100,** 1933: 291—308; In. Marounek, Thesis, Praha 1978.

FULGHUM, R. S. — MOORE, W. E. C.: Isolation, enumeration and characteristic of proteolytic ruminal bacteria. J. Bacteriol., **85,** 1963: 808—815.

HAMPL, B.: Mikrobiologie. SNTL Praha, 1964.

HENDERICKX, H. K.: Quantitative aspects of the use of non-protein nitrogen in ruminant feeding. Cuban J. Agric. Sci., **10,** 1976: 1—18.

HENDERICKX, H. — MARTIN, J.: In Hungate 1966, Compt. Rend. Rech. Inst. Rech. Sci., Ind. Agr. Busech, **31,** 1963: 110.

HESPELL, R. B.: Efficiency of growth by ruminal bacteria. Fed. Proc., **38,** 1979: 2707—2712.

HESPELL, R. B. — BRYANT, M. P.: Efficiency of rumen microbial growht: influence of some theoretical and experimental factors on $Y_{ATP}$. J. Anim. Sci., **49,** 1979: 1640—1659.

HUNGATE, R. E.: The anaerobic mesophilic cellulolytic bacteria. Bact. Rev., **14,** 1950: 1—49.

CHEN, M. C. — BEESON, W. M. — PERRY, T. W.: In vitro studies on the effect of SDS and CDS on cellulose digestion and microbial protein synthesis. J. Anim. Sci., **43,** 1976: 1280—1285.

CHURCH, D. C.: Digestive physiology and nutrition of ruminants. Volume 1 and 2, CORVALLIS, 1970.
JACOB, H. E.: Redox potential, in: Method in microbiology, Vol. 2, Academic Press, Inc., London, 1970.
LOPPER, D. C. — LITTLE, C. O., — MITCHEL, G. E.: In vitro procedure for studying starch digestion by rumen microorganisms J. Anim. Sci., **25,** 1966: 128—131.
MAENG, W. J. — BALDWIN, R. L.: Factors influencing rumen microbial growth rates and yields: Effect of urea and amino acids over time. J. Dairy Sci., **59,** 1976: 643—647.
MAENG, W. J. — BALDWIN, R. L.: Factors influencing rumen microbial growth rates and yields: Effect of amino acids additions to purified diet with nitrogen from urea. J. Dairy Sci., **59,** 1976: 648—655.
MAENG, W. J. — VAN NEVEL, C. J. — BALDWIN, R. L. — MORRIS, J. G.: Rumen microbial growth rates and yields: Effect of amino acids and protein. J. Dairy Sci., **59,** 1976: 68—79.
MAHADEVAN, S. — SAUER, F. D. — ERFLE, J. D.: Studies on bovine rumen bacterial urease. J. Anim. Sci., **42,** 1976: 745—753.
MAHADEVAN, S. — SAUER, F. D. — ERFLE, J. D.: Purification and properties of urease from bovine rumen. Biochem. J., **163,** 1977: 495—501.
MÁLEK, I.: Obecná mikrobiologie lékařská. SZdN, Praha 1953.
MALUSZYNSKA, G. M.: Wplyw potencjalu oksydoreduksyjnego oraz uboezny wplyw zviazkow stosowanych do obnizanija potencjalu na vzorst bakterii zwaezowach i rozklad celulozy. PhD. thesis. Jastrzebies, 1973.
MAROUNEK, M.: Studium látkové přeměny v bachoru se zaměřením na metabolismus těkavých mastných kyselin, lipidů, sloučenin dusíku a fosforu. PhD. Thesis, Praha, 1978.
MAROUNEK, M.: nepublikovaná data, 1980.
MAROUNEK, M. — KOPEČNÝ, J. — BARTOŠ, S.: Srovnání močoviny ureafosfátu a diamonium fosfátu z hlediska vlivu na bachorovou fermentaci. 10. Dny fysiologie hospodářských zvířat, Liblice, 1979.
MINATO, H. — SUTO, T.: Technique for fraction of bacteria in rumen microbial ecosystem. I. Attachment of rumen bacteria to starch granules and elution of bacteria attached to them. J. Gen. Appl. Microbiol. **22,** 1976: 259—276.
NIKOLIĆ, J. A. — JOVANOVIĆ, M. — FILIPOVIĆ, R.: Microbial protein synthesis by bovine rumen content in relation to ammonia concentration. Studies on Nonprotein Nitrogen for Ruminants II, FAO/IAEA, Wien, 1975.
OWENS, F. N. — ISAACSON, H. R.: Ruminal microbial yields: factors influencing synthesis and by pass. Fed. Proc., **36,** 1977: 198—202.
ROFFLER, R. E. — SATTER, L. D.: Relationship between ruminal ammonia and non-protein nitrogen utilization by ruminants II. Aplication of published evidence to the development of a theoretical model for predicting non-protein utilization. J. Dairy Sci., **58,** 1975: 1889—1898.
RUSSEL, J. B. — DELFINO, F. J. — BALDWIN, R. L.: Effects of combination of substrates on maximum growth rates of several rumen bacteria. Appl. Envir. Microb., **37,** 1979: 544—549.
RYAN, R. K.: Concentration of glucose and low-molecular weight acids in the rumen of sheep changed gradually from a hay to a hay plus grain diet. Am. J. Vet. Res., **25,** 1964: 653—659.
SATTER, L. D. — SLYTER, L. L.: Effect of ammonia concentration on rumen microbial protein production in vitro. Br. J. Nutr., **32,** 1974: 199—208.
SHARMA. R. S. — SHUKLA, P. C.: Effect of dietary protein and non protein nitrogen on rumen proteolytic and ureolytic activities. Ind. J. Nutr. Dietet., **10,** 1973: 299—302.
THOMSON, D. J. — BEEVER, D. E. — LATHAM, M. J. — SHARØPE, M. E. — TERRY, R. A.: The effect of infusion of mineral salts in the diet on dilution rate, the pattern of rumen fermentation and the composition of the rumen microflora. J. Agric. Sci. Camb., **91,** 1978: 1—7.
VAN DER WALT, P. — BRIEL, K.: Volatile fatty acid metabolism in sheep. Onderste-poort J. Vet. Res., **43,** 1976: 11—22.
VAN NEVEL, C. J. — DEMEYER, D. I.: Determination of rumen microbial growth in vitro from 32-P-labeled phosphate incorporation. Br. J. Nutr., **38,** 1977: 101—114.
WALKER, D. J. — EGAN, A. R. — NADER, C. J. — ULYATT, M. J. — STORER, G. B.: Rumen microbial protein synthesis and proportions of microbial and non-microbial nitrogen flowing to the intestines of sheep. Aust. J. Agric. Res., **26,** 1975: 699—708.
WILLIAMS, D. L. — MOORE, J. D. — MARTIN, L. C. — TILLAMN, A. D.: Studies on liquid hemicellulose and cane molasses as carbohydrate sources in urea containing diets of sheep. J. Anim. Sci., **28,** 1969: 667—674.
ZAKI EL-DIN, M. — EL-SHAZLY, K.: Some factors affecting fermentation capacity and net growth of rumen microorganisms. Appl. Microbiol., **18,** 1969: 313—317.