A METHOD FOR THE *IN VITRO* STUDY OF DRUG TRANSFER ACROSS RUMINAL EPITHELIUM

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SUMMARY

A simple method for the *in vitro* study of drug diffusion across ruminal epithelium is described. The characteristics of the isolated membrane were defined by studies of ketone production from butyrate, histological studies, phenolsulphonthalein penetration and permeability to pentobarbital, antipyrine and tetraethylammonium. The preparation was found to be suitable for studies of less than 12 hours duration; after that time the integrity of the membrane as a barrier was lost due to degenerative changes. The method had a primary advantage in that experimental variables could be rigorously controlled.

INTRODUCTION

The presence of a complex, voluminous, hollow organ such as the reticulo-rumen as a component of the gastrointestinal tract might well be expected to influence the absorption, distribution and excretion of any drug administered by practically any route. In addition, posology in ruminants presents a real problem as up to 20% of the body weight may be attributable to the ruminal contents[35], while the reticulo-rumen may or may not constitute a distribution compartment for a drug. Notwithstanding the importance of these basic considerations, there have been very few studies conducted concerning the absorption of drugs from the reticulo-rumen and the diffusion of drugs from the plasma into the ruminal fluid. This lack has been emphasized by Jones[16], Dobson[7] and Stowe[34].

The physico-chemical factors which govern the passage of drugs across biological membranes have been well delineated[4][5], and the roles which these intrinsic properties play in the diverse ways in which solutes move across membranes have been extensively reviewed by Schanker[28][30]. Moreover, the practical application of these principles to the absorption, distribution and excretion of drugs in monogastric species has been fairly well documented[10][11][26][27][29]. However, once again these basic premises have not been fully evaluated with respect to the reticulo-rumen in which the keratinized stratified squamous epithelium represents a composite series of cellular membranes.

During the last 25 years a wide variety of experimental approaches have been adopted to study absorption from the rumen. Reviews of the methods which have been used to date have been presented by Annison and Lewis[1], Annison[2] and Dobson and Phillipson[8]. Most of these procedures have had both advantages and disadvantages and were employed principally for the study of absorption of nutrients and water from the rumen, and the metabolic functions of the rumen. Notwithstanding these important contributions, there remains a paucity of information associated with drug transfer across this biological barrier. Controlled observations on the effect of concentration gradients, pH differences, plasma-protein binding, and anatomic differences in epithelium from various areas of the rumen on the transfer of drugs have not yet been carried out.

The object of this work was to develop and validate an *in vitro* system which could be used to study distribution of drugs across ruminal epithelium under rigorously controlled experimental conditions.

MATERIALS AND METHODS

Experimental Procedures

The experimental approach was based on utilizing a carefully defined system which permitted the study of the flux of specifically selected compounds across a delineated surface area of ruminal epithelium. The viability and integrity of this biological barrier under the imposed experimental conditions was established to satisfy the necessary criteria[17] for such studies on solute transfer.

Sheep and goat viscera were not readily available on a regular basis in the vicinity of Columbia, whereas cattle were slaughtered daily at a local abattoir. Thus the tissues employed were generally of bovine origin. The unavoidable consequence of this situation was that there was no control over the nutritional status of the donor animals, although it is well known how great a rôle the dietary intake plays in the structure and function of rumen epithelium.

Rumen wall from the latero-dorsal area was collected from a local abattoir. The viscera became available about 10 to 15 min after slaughter and the section of rumen wall which was of interest was then cut free and placed in oxygenated Locke-Ringer's solution at about 311 to 313K (38 to 40°C) for transport to the laboratory within 15 minutes. The whole rumen wall was thoroughly washed in warm Locke-Ringer's solution and was then placed in Krebs-Ringer's phosphate (KRP) solution (at 311 to 312K) through which oxygen was continuously bubbled. The

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ruminal epithelium was carefully dissected free of the underlying muscularis layers while the tissue was bathed in oxygenated KRP. Sections were then cut and affixed to thistle tubes with twine. The total capacity of these thistle tubes was about 30 ml and the mean surface area was 9,01 cm². The stem lengths were cut to about 11cm.

Twenty-five ml of pH 7,4 KRP solution were introduced into the thistle tube. The tube was then placed in 500 ml KRP solution which had been warmed to 311,5K (38,5°C). This solution contained the drug under study at the concentration and pH desired. Thus, the system was based upon the transfer of the compounds studied from a very large fluid compartment, which constituted to some degree an infinite volume and an essentially constant drug concentration, across a fixed surface area of ruminal epithelium into a much smaller fluid compartment. This arrangement greatly facilitated analysis of the results.

The final arrangement of a single system is illustrated in Figure 1. A thin plastic sheet covering the beaker prevented excessive evaporative loss. A clamp on the stem of the thistle tube allowed adjustment of the level of the inside solution and this precluded hydrostatic pressure differences. An 18 ga.-120 mm filling needle with a connecting tube facilitated sample collections from inside the thistle tube. Thin polyethylene tubing was used to carry the gases into the solutions. Oxygen was continuously bubbled through the inside solution at a fairly rapid rate which allowed oxygenation and agitation of this solution. Nitrogen was bubbled through the outside solution at a slow rate which allowed mixing of the solution. The series of eight sets was placed in a constant temperature water bath at 311,5K (38,5°C). The gases were supplied to each system through two multiple outlet glass manifolds (Fig. 2.)
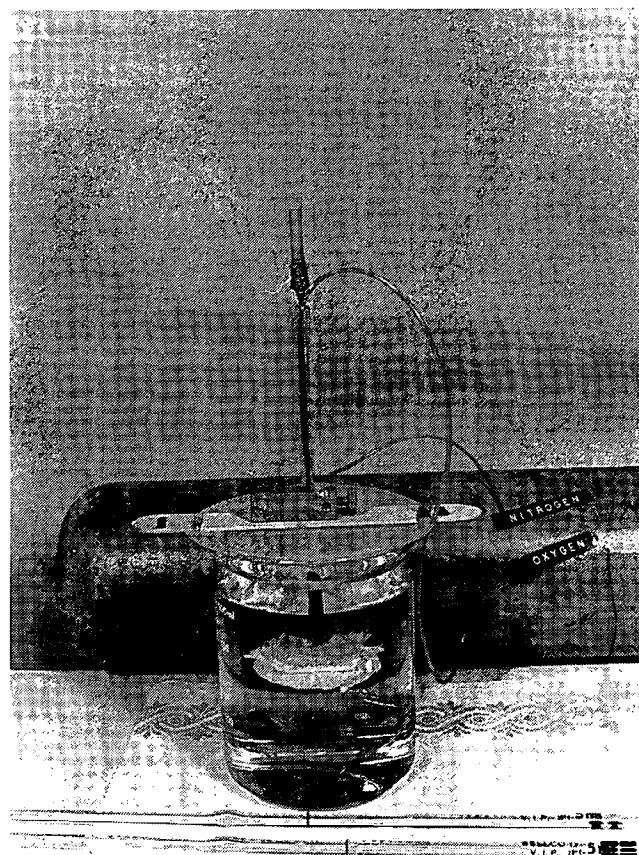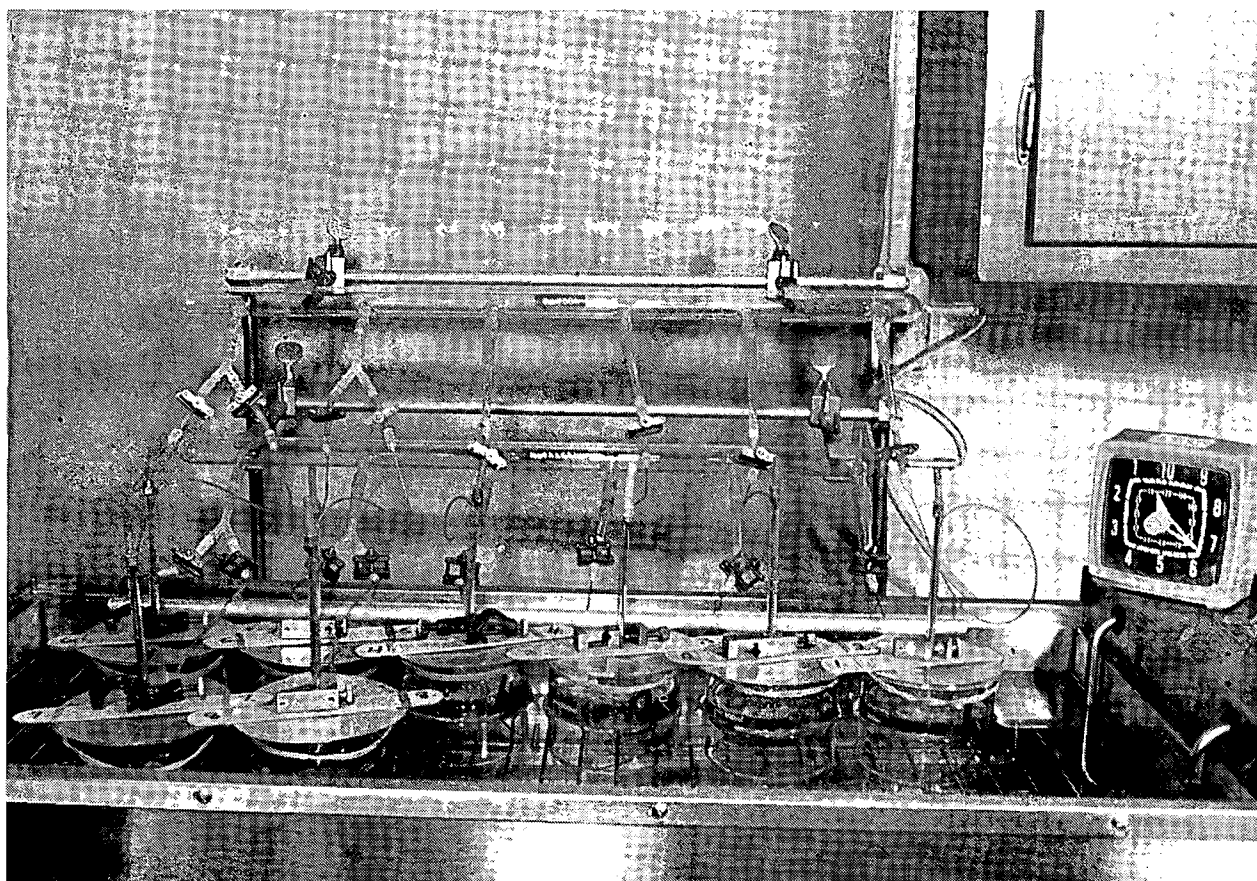

Fig. 1 A single in vitro system.

Fig. 2. The complete experimental arrangement

Ketone Body Production Studies

The ability of the ruminal epithelium to convert butyric acid to ketone bodies as a function of time was investigated to define the viability of the *in vitro* system.

A series of eight systems was set up as described above with pH 7,4 in both inside and outside compartments. At hourly intervals a system was removed, the epithelial disc excised, blotted with filter paper and then minced with scissors. The minced tissue was placed in a 50 ml beaker containing a marble and 10 ml pH 7,4 KRP solution with 0,02 mole butyric acid. This was incubated in a Dubnoff shaking incubator for 3 hours under an oxygen atmosphere at 39°C and at a rate of 120 cycles per minute. Following incubation 3 ml of the incubation medium were transferred into a Lester and Greenberg microrefluxing still and the total ketone bodies were determined by the method of Procos[23]. Epithelial tissue which had been placed in water at 353K (80°C) for 20 minutes served as the blank. The tissue was removed from the beaker, placed in an oven at 373K (100°C) overnight and weighed 12 hours later. The ketone body production (measured as acetone) by the isolated ruminal epithelium was calculated as u moles/ml fluid/g tissue (dry weight).

Permeability coefficients

Pentobarbital, antipyrine or tetraethylammonium were placed in the outside solutions and their passage into the inside solution was studied over an extended period of time. The permeability coefficients were calculated for each drug using the equation[30].

$$Pt = -\ln \frac{(1 - C1)}{C2R}$$

where C1 represents the drug concentration in the fluid being entered, and C2 that in the outside solution. R is the ratio $C1 \div C2$ at the steady state[6]. On substituting in the equation the observed concentration ratios, and plotting the values of the right side of the equation against time, a straight line is obtained. The permeability coefficient P is given by the slope of the line, $- \ln (1-C1/C2R)/t$.

Drug Metabolism Studies

Since it is more convenient to study radioisotopically-labelled drugs it was necessary to rule out the possibility that the drugs underwent biotranrformation within the system. Accordingly, 4g minced ruminal epithelium or 20 ml ruminal fluid were added to ^{14}C-labelled pentabarbital, salicylate or antipyrine solutions and incubated in a Dubnoff shaking incubator for 3 h at 312K (39°C) and 120 cycles per min. The media were centrifuged and placed in a vacuum oven at 323K (50°C) until the fluid volume was reduced to 0,5 ml. Each solution was then chromatographed on thin-layer plates. The plates were examined under ultra violet light (254 nm) and sprayed with various reagents. The chromatograms were also submitted to radio-assay. In all cases the drugs migrated as a single spot thus demonstrating that they were not transformed by the system.

Phenolsulphonphthalein Studies

In a series of six studies utilizing bovine tissue and four studies with caprine tissue, the passage of phenolsulphonphthalein across ruminal epithelium as a function of time was investigated. The experimental procedure was as described above but the outside solution (pH 7,4 KRP) contained 0,001% (M/V) phenol red. Aliquots were collected from the inside solution every 2 hours up to 24 h in the case of the bovine studies and every hour up to 7 h in the case of caprine tissue. The end point of the experiment was the time of the first appearance of traces of phenol red in the inside solution. The method described by Austin[3] was used for the determination of phenol red concentration.

Histological Studies

To investigate the histological changes which occurred within the ruminal epithelial tissue during the experimental procedure, the tissue was removed at various time intervals, placed in 10% buffered formalin with Bouin's fluid and submitted for histopathological investigation.

RESULTS

Ruminal Epithelium Viability

The production of total ketone bodies (measured as acetone) by isolated ruminal epithelium which had been incubated in pH 7,4 KRP solution containing 0,02 mole butyric acid is depicted in Figure 3. There was a dramatic reduction in activity over the . first two hours followed by a more gradual decline until about 6 h, at which time, the residual activity was barely detectable.

Permeability to Pentobarbital, Antipyrine and Tetraethylammonium as a Function of time

The results of these studies are shown in Figure 4 for pentobarbital (n=8), Figure 5 for antipyrine (n=8) and Figure 6 for TEA (n=4). There was an alteration in the permeability of the ruminal epithelium between 12 and 14 h in each case. The slopes of the lines continued to increase, indicating greater permeability with the passage of time. The increment was greater for TEA than for pentobarbital and antipyrine.

Permeability to Phenolsulphonphthalein

In the case of bovine tissue (n=6) phenol red was detected at 16,7± 0,4 h, while with the goat tissue (n=4) no trace of the dye was detected by 7 h. These results suggested that viable ruminal epithelium was impervious to the strong sulphonic acid phenolsulphonphthalein.

Histological Studies

Over a 14 h period the cellular changes in bovine rumen epithelial tissue specimens were evaluated as follows:

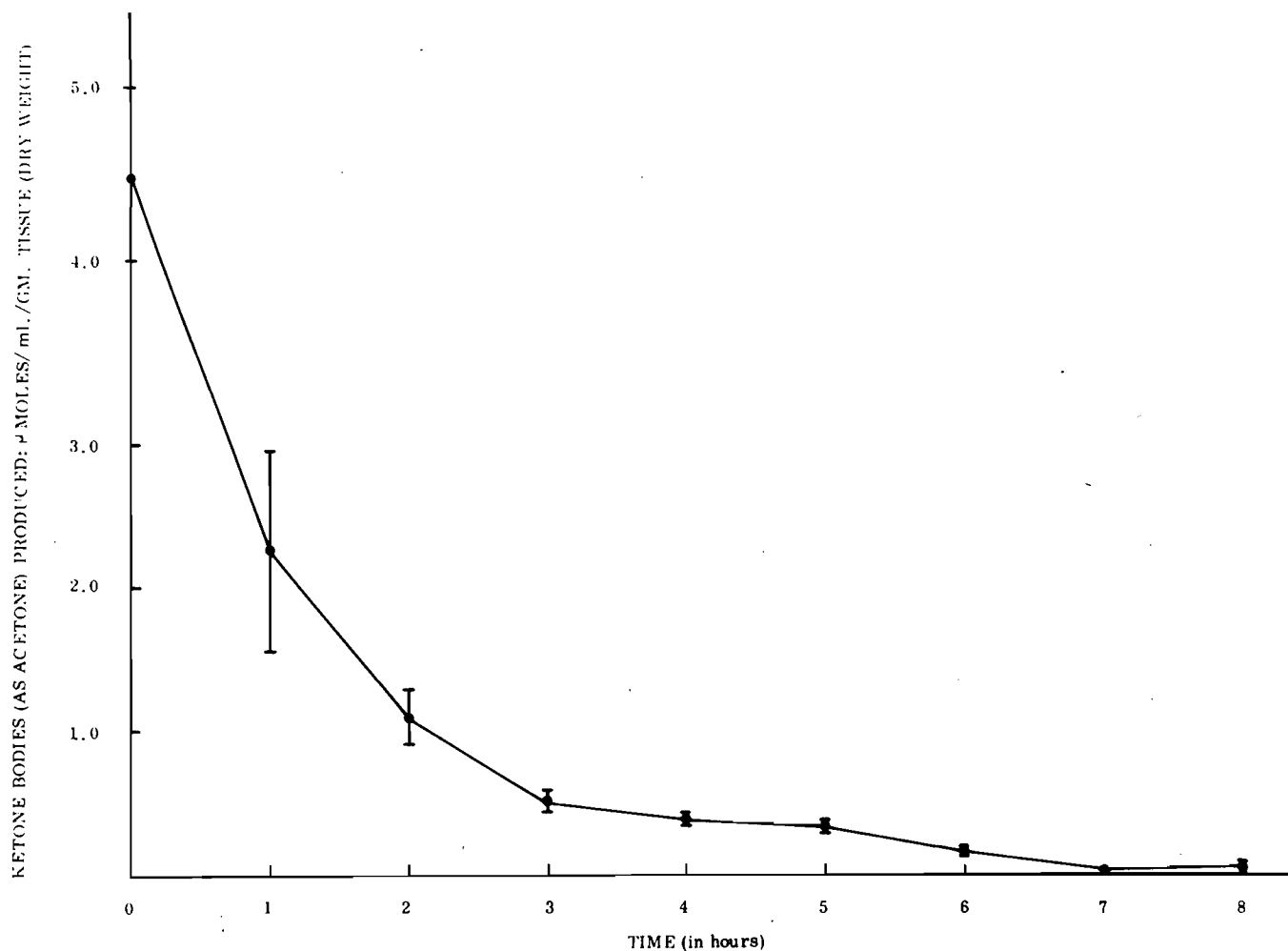0 Hour: The epithelium was approximately 5 to 7

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Fig. 3. Total Ketone Bodies (as acetone) produced by isolated ruminal epithelium as a function of time. $N = \frac{2}{3}$*
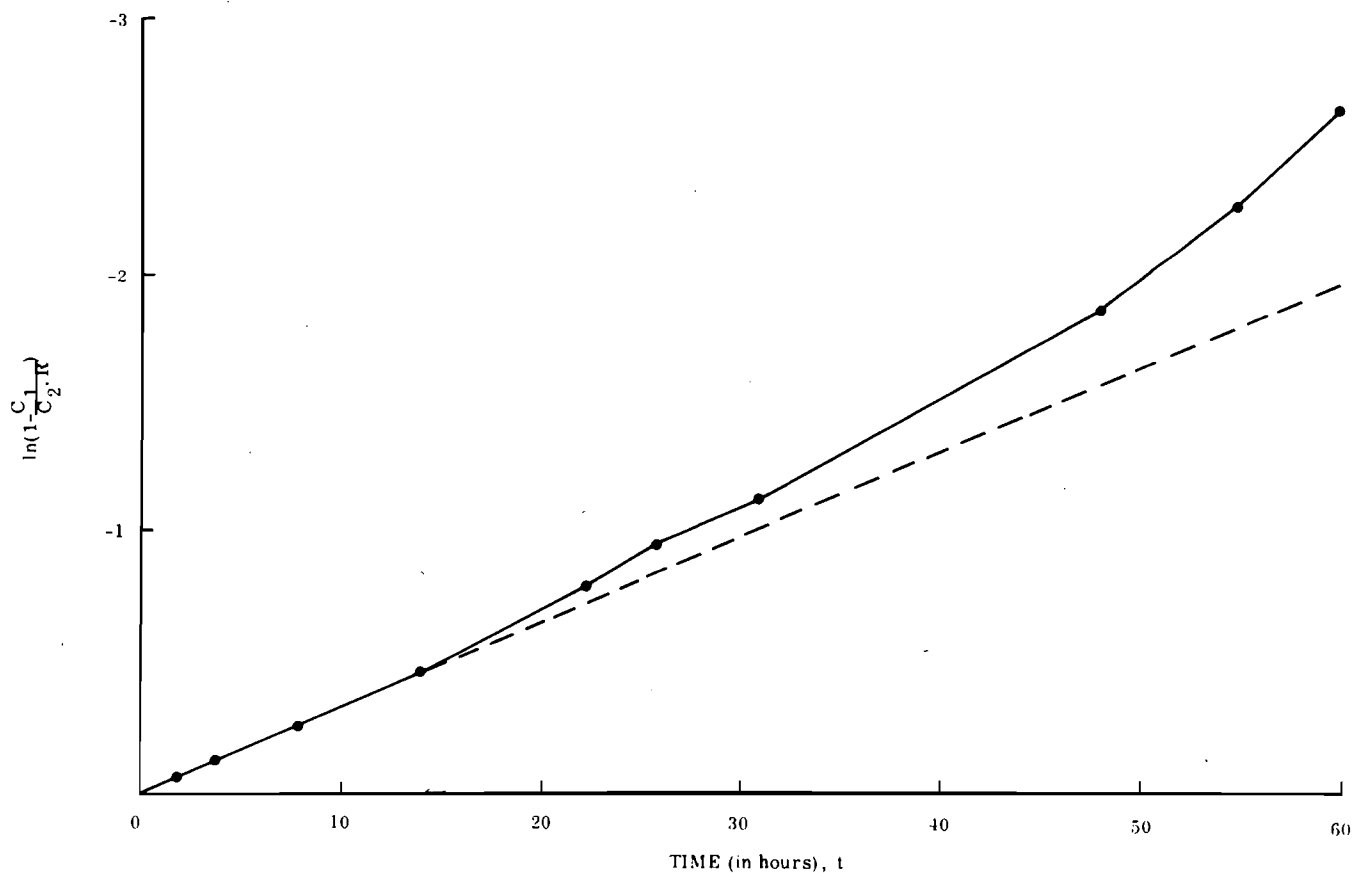

Fig. 4. Permeability of ruminal epithelium to pentobarbital.
$C1$= concentration in inside solution, $C2$= concentration in outside solution, $R = C1/C2$ at steady state. Slope of line, $-\ln(1-C1/C2R)t$, represents the permeability coefficient. Note increasing permeability after 12 hours.

cells in thickness. There was a discontinuous layer of stratum corneum on the surface. Below this layer, the stratum lucidum (also discontinuous) was approximately 1 to 2 cells thick. For the most part these cells were swollen and contained a clear, non-stainable, cytoplasm with fairly well defined nuclei. The lamina propria consisted of a fine network of collagenous fibres. The submucosa was loose and contained open and collapsed vascular channels with distinct endothelium (Fig. 7A).

1 Hour: The morphology was essentially the same. However, the superficial half of the squamous
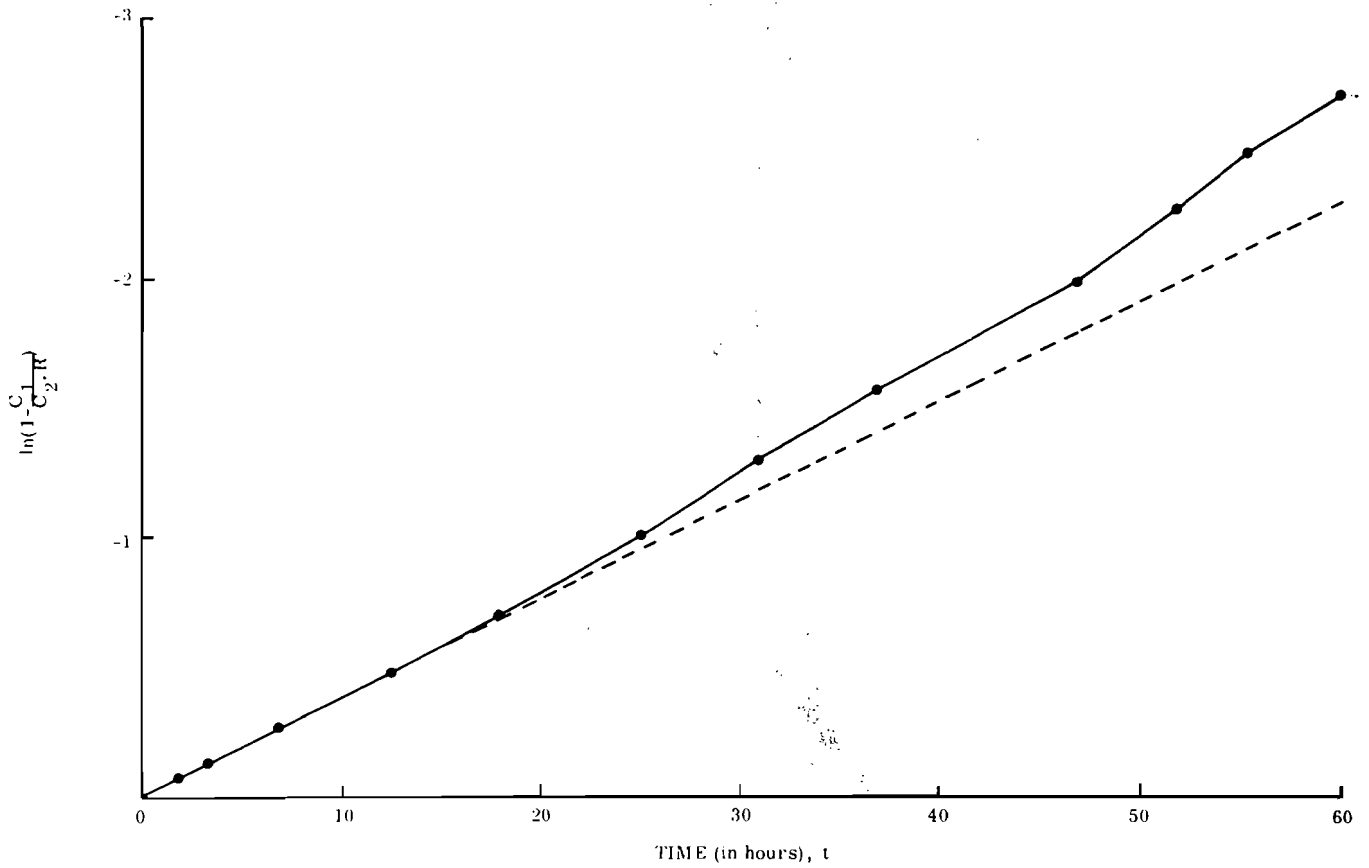

Fig. 5 Permeability of ruminal epithelium to antipyrine. Terms are same as described in legend for Fig. 4.
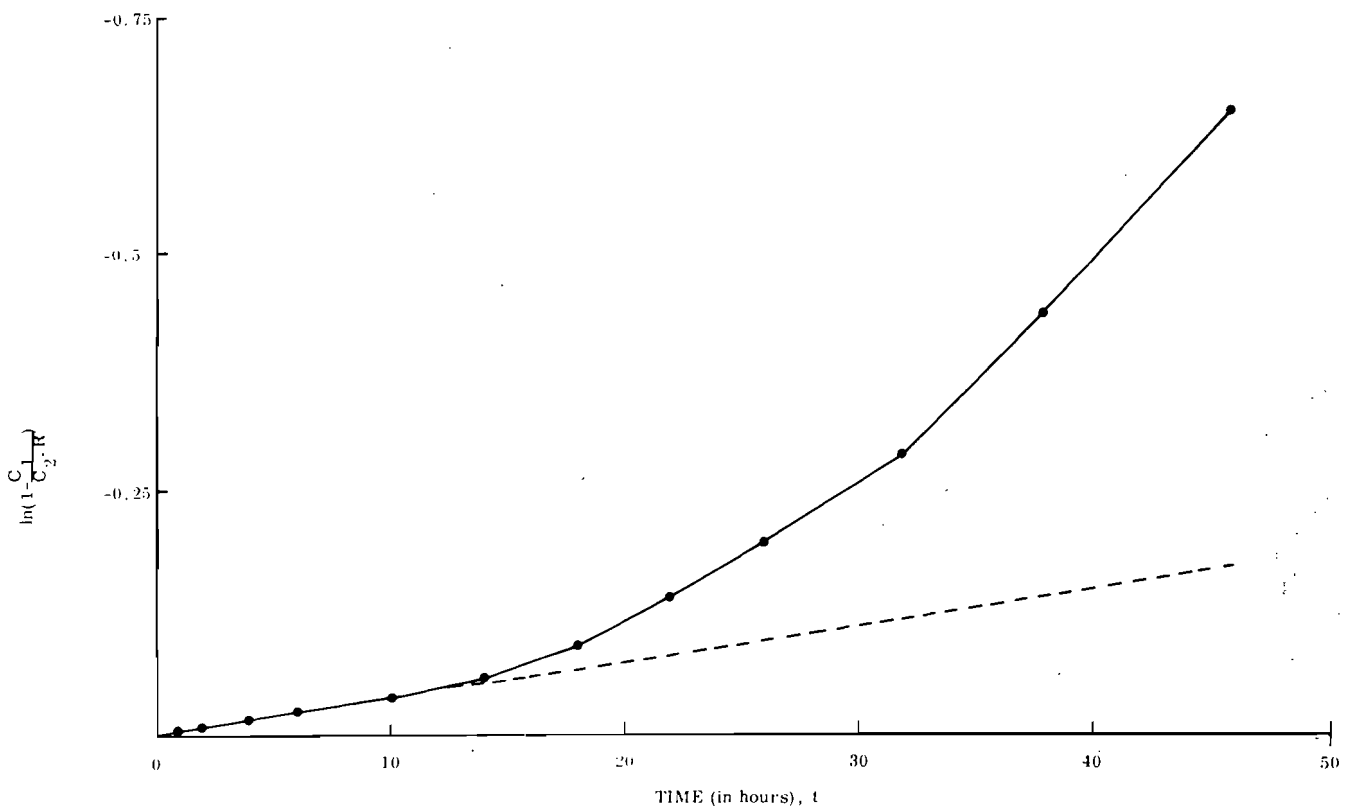

Fig. 6. Permeability of ruminal epithelium to tetraethylammonium. Terms are same as described in legend for Fig. 4.

layer revealed hydropic degeneration. Between this hydropic area and the subadjacent deeper staining layers there was a sharp line of demarcation.

3 Hour: There were foci in which the lamina propria blended imperceptibility into the epithelial layers making it difficult to discern the basalar layer of the epithelium in these foci. In other areas the basalar layer was still well delineated and there was a faint line of demarcation between the epithelium and the lamina propria.

4 Hour: Appearance was very similar to the 3 h specimen (Fig. 7B).

8 Hour: The washed out appearance of the epithelium had become more pronounced. There were more foci in which the lamina propria and epithelium seemed to blend one into the other. For the most part the basalar layer of the epithelium was the only layer which still retained good hematoxylin differentiation in most areas. The lamina propria was still quite clear (Fig. 7C).
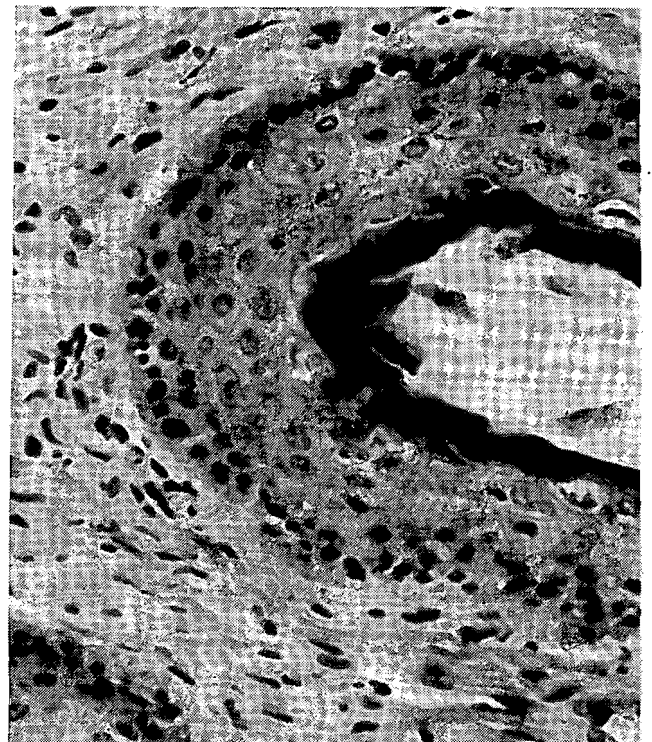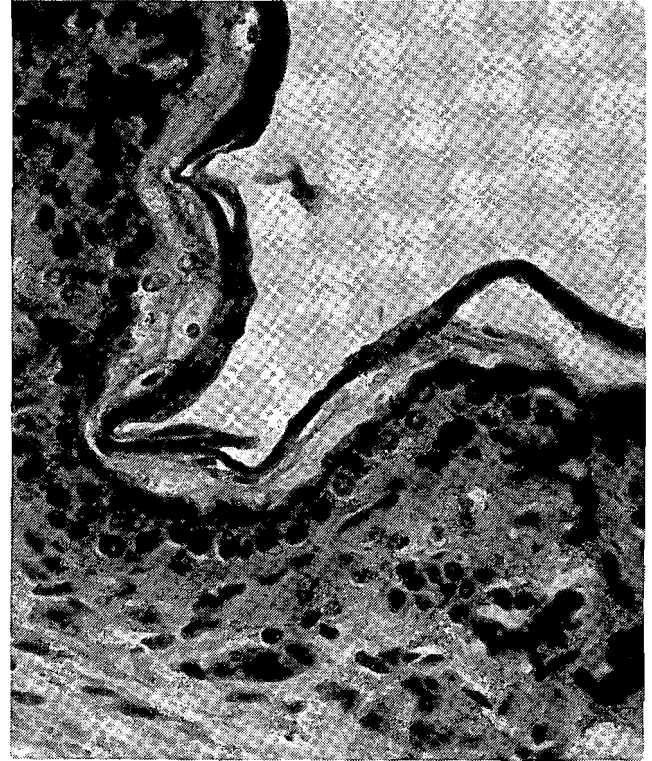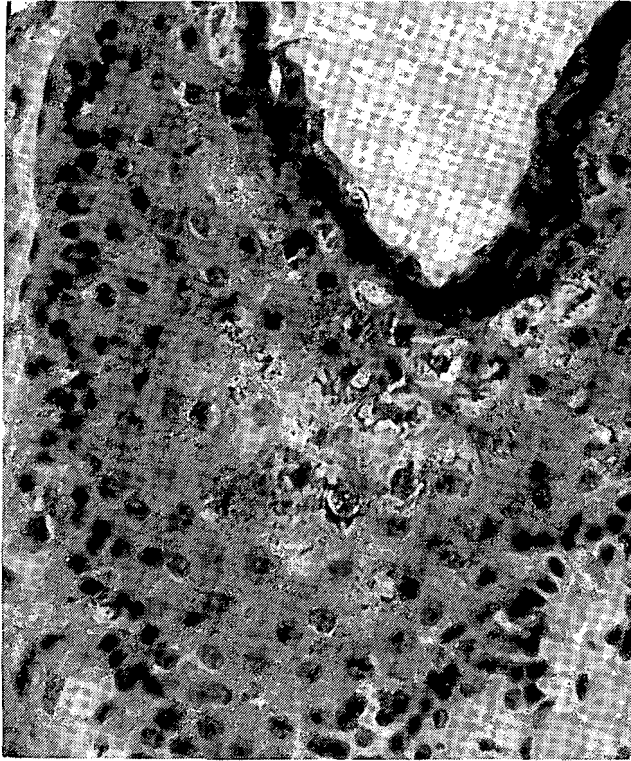
Fig. 7. Histological appearances of ruminal epithelium at time of removal from the *in vitro* system.
A. 0 Hour   B. 4 Hours   C. 8 Hours   D. 14 Hours.

14 Hour: The cells of the epithelium presented hydropic degeneration to the depth of the basalar layer. There were keratinohyaline granules in these cells to this depth. In addition, there was a considerable increase in the size of the cells of the stratum germinativum. In focal areas the basalar columnar cells were undergoing pyknosis of the nuclei and vesiculation of the cytoplasm. Evidence of focal subepithelial oedema was also present and the collagen fibres of the lamina propria were forced aside by fluids. The collagen fibres were becoming indistinct at this stage (Fig. 7D).

DISCUSSION

The definition of the *in vitro* system by the variety of approaches used, fulfilled to a large extent the criteria for solute transfer across biological membranes proposed by Levine and Pelikan[17].

The ability of the ruminal epithelium to convert butyric acid to ketone bodies, first described by Pennington[22], proved to be a fairly useful measure of the viability of the biological membrane under the conditions of study. There was measurable enzymatic activity present up to 6 h, although about 80% of the activity disappeared within the first 2 to 3 hours. This demonstration of metabolic activity did not really represent a true reflection of the functional capacity and integrity of the cellular membranes within the epithelium. This is especially true as the site of B-hydroxybutyrate dehydrogenase activity has been localised in the mitochondria[13].

A better criterion might have been the ability of the membrane to transport sodium against an electrochemical gradient[8]. Stevens[31] showed that the tissue potential decreased with time and that this was due to a decreasing tissue current, which in turn was associated with a decrease in net sodium transport. This change occurred over about 7 h from the initial collection. Similar isolated ruminal epithelium systems have been used with success by a number of workers while investigating transport and metabolism of volatile fatty acids[12 32 33]. Thus by observation and inference, it seemed that the ruminal epithelial cells remained metabolically functional for about 6 h under the applied experimental conditions although there was a continual decrement of activity with the passage of time.

The measurement of the period of time during which the biological integrity of the ruminal epithelium could be regarded as being intact was carried out by evaluating the permeability coefficients for pentobarbital, antipyrine and tetraethylammonium. It was evident from these results that the permeability of the epithelium changed between 12 and 14 h. After 14 h the permeability coefficient increased gradually with time for the nonionized lipid-soluble compounds, pentobarbital and antipyrine, which would be expected to cross the epithelium by lipid diffusion.

A far greater increment occurred in the case of TEA, a relatively small (MM 130 daltons and molecular diameter 0,68nm) and highly polar molecule, which would be expected to traverse a lipoid-sieve barrier by diffusion through aqueous pores. It was concluded from these studies that the lipoproteinaceous architecture of the cell membranes became disrupted at about 12 h and that the structure of the aqueous channels seemed to degenerate somewhat faster than the lipid and protein components.

Additional evidence for loss of membrane integrity at about this same time interval was provided by the results of the studies on the permeability of ruminal epithelium to phenolsulphonphthalein. Phenolsulphonphthalein is a strong sulphonic acid with a pKa less than 1,0 and molecular mass of 354. This molecule is completely ionized at all physiological hydrogen ion concentrations. Phenol red has been shown to cross most biological membranes to a very limited extent and the percentage absorption from the gastrointestinal tract has varied between 0 and 2 per cent[4 5 19 21 24 25 26]. Austin[3] achieved quantitative recovery of phenol red from the reticulo-rumen of calves but Williams and Mackenzie[36], using acute preparations, found some absorption from the rumen in sheep. Utilizing our *in vitro* system we observed that the mean time for the first appearance of traces of phenol red was 16,7 h. This finding once again suggested an alteration in permeability due to architectural collapse of the membrane following which the diffusion of a relatively large polar molecule became possible.

The histological appearance of the ruminal epithelium initially conformed very closely with the descriptions of Dobson *et al.* [9]. Moreover, the pattern of cellular degeneration which occurred with the passage of time was anticipated[13]. It does seem that if the previous findings concerning the definition of the *in vitro* system are taken into account, the critical difference between the 8 hour and 14 hour specimen was that the progressive hydropic cellular degeneration finally involved the basalar layer of columnar cells during this time interval. This may represent the initiation of the degeneration of the most important area of the epithelium as regards its efficacy as a barrier. Studies of the ultrastructure of ruminal epithelium have revealed the presence of intercellular spaces throughout the epithelium and penetrating down to the basal cell borders[15 18]. This may be the explanation for the permeability alterations at about 12 h.

The results of the drug metabolism studies using both epithelial mince and rumen liquor revealed that no biotransformation of pentobarbital or antipyrine occurred. This finding validated the *in vitro* results obtained with the ^{14}C-labelled compounds. TEA has been shown not to be metabolized by any species or in any biological system[20].

The *in vitro* experimental system proved to be a very useful model for evaluating the transfer of drugs across the ruminal epithelium over periods of less than 12 h. Due to the simplicity of the system it is possible to define rigorously the variables which control the rate of transfer of solutes across this membrane. There are some rather obvious disadvantages to the system such as absence of blood flow and inability to control the actual surface area exposed to the solution due to varying degrees of papillation.

ACKNOWLEDGEMENTS

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