Ruminal hydrogenation of cholesterol

J. R. Ashes, S. K. Gulati, L. J. Cook, S. C. Mills, and T. W. Scott

C.S.I.R.O., Division of Animal Production, P.O. Box 239, Blacktown, N.S.W. 2148, Australia

Abstract Cholesterol was hydrogenated by anaerobic incubation with sheep rumen fluid for periods up to 20 hr. The principal product of cholesterol hydrogenation was identified as coprostanol. Cholesterol could be protected against in vitro ruminal hydrogenation by encapsulation in a matrix of formaldehyde-treated casein. Formaldehyde-treated casein–cholesterol preparations were also shown to be protected against hydrogenation in vivo and, when supplements containing 1 g per day of protected or unprotected cholesterol were fed to sheep over a period of 8–9 weeks, there were marked differences in the plasma cholesterol response. The plasma cholesterol of the sheep fed protected cholesterol increased by at least 60%. The plasma cholesterol of the sheep fed unprotected cholesterol also tended to increase during the first 5 weeks of supplementation but thereafter declined to almost control levels at 8 weeks.

Supplementary key words formaldehyde-treated casein · coprostanone, · cholesterol · coprostanol · rumen fluid

Ruminal microorganisms can hydrogenate unsaturated lipids. This hydrogenation has important consequences for the lipid composition of the host animal, together with its metabolism and physiology. Hydrogenation of dietary polyunsaturated fatty acids accounts for the relatively low proportion of these acids in ruminant tissue and milk fats (1, 2); hydrogenation of some compounds, e.g., lasiocarpine, is important in their detoxification (3) while hydrogenation of others, e.g., estrogenic formononetin, actually enhances their deleterious effects (4).

Unsaturated sterols represent a general class of lipids that are subject to ruminal hydrogenation but the nature, extent, and physiological significance of sterol hydrogenation in the rumen has not been extensively studied despite the common occurrence of sterols in the ruminant diet. Ruminant animals normally consume approximately 0.2% of their ration as unsaturated phytosterols, and in some cases may also receive appreciable amounts of cholesterol, an unsaturated sterol (e.g., as meat or blood meal supplement). The metabolism of cholesterol by rumen microbes was reported by Wiggers et al. (5) but these workers did not identify the metabolites. Results of another study also suggested that unsaturated sterols were metabolized by rumen microflora (6) and that the products of this metabolism were the corresponding saturated sterols (6).

The present series of studies was undertaken to further evaluate the nature and the physiological significance of ruminal sterol hydrogenation by utilizing procedures previously developed to protect triacylglycerols against ruminal metabolism (7–9).

MATERIALS AND METHODS

Chemicals

[7α-(n)-^{3}H]Cholesterol (sp act 9.3 mCi/mmol) and [4-^{14}C]cholesterol (sp act 24.6 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England. All unlabeled sterols and solvents were AR grade.

Preparation of sterols for in vitro studies

Unprotected cholesterol. Unprotected cholesterol preparations for in vitro rumen incubation studies were prepared by dissolving the cholesterol in refined sunflower oil containing butylated hydroxyanisole (0.01–0.02% by weight of oil) and emulsifying the cholesterol–oil mixture with a 20% aqueous solution of sodium caseinate.

Protected cholesterol. Protected cholesterol preparations for in vitro studies were prepared using the following stepwise procedure. *a*) One part by weight of cholesterol was dissolved in 2 parts by weight of chloroform; *b*) the chloroform solution of cholesterol was mixed with refined sunflower oil containing BHA (0.01–0.02% by weight of oil) (ratio cholesterol:sunflower oil, 1:3 w/w); *c*) the mixture of sunflower oil, cholesterol, and chloroform was emulsified with sodium caseinate in water (ratio of casein to water, 1:4 (w/w); ratio of casein–sunflower oil–cholesterol, 4:3:1 (w/w/w); *d*) the pH of the emulsion was adjusted to approximately 8.5 with NaOH; *e*) the emulsion was treated with formaldehyde (ca. 3.7 g formaldehyde

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; BHA, butylated hydroxyanisole; Ag^{+}-TLC, argentation thin-layer chromatography.

per 100 g casein); *f*) the resultant gel was comminuted by passage through a 2 mm screen; and *g*) the comminuted gel was dried in a fluid bed drier (air temperature 50°C) until all traces of chloroform and excess formaldehyde were removed.

Preparation of cholesterol for in vivo studies

Unprotected cholesterol. Unprotected cholesterol supplements for use in animal feeding studies were prepared by dissolving 1 part of cholesterol in 3 parts (w/w) of refined sunflower oil containing BHA (0.01–0.02% BHA by weight of sunflower oil) and mixing the cholesterol–oil mixture with equal parts by weight of dry sodium caseinate.

Protected cholesterol. Protected cholesterol supplements for animal feeding studies were prepared as described above for the in vitro studies.

In vitro incubation of cholesterol with rumen fluid

Rumen fluid was obtained from a rumen-fistulated sheep maintained on a ration of chopped alfalfa and oats (1:1, w/w) (800 g per day). On the day of sampling, the daily ration was withheld until after the rumen contents were sampled. Protected and unprotected cholesterol supplements were incubated anaerobically with 10 ml of rumen fluid at 38°C in a shaking water bath (7).

In vivo studies of sterol metabolism

Ruminal hydrogenation of cholesterol. Protected or unprotected cholesterol supplements were mixed with ca. 100 g of crushed oats and fed to abomasally-fistulated sheep. The cholesterol–oats mixture was consumed within 30 min and the balance of the basal ration (800 g chopped alfalfa–oats, 1:1, w/w) was then fed. Abomasal digesta samples (ca. 50 g) were collected at various times during the 24-hr post feeding period.

Feeding of sterol supplements to sheep and plasma cholesterol measurements. Six Border Leicester/Merino sheep (12 months old) were individually fed basal diets of pelleted alfalfa and oats (ca. 800 g per day) for approximately 6 weeks (preliminary period). Three of these sheep were then fed a supplement of protected cholesterol (8 g supplement per day per animal, equivalent to 1 g of cholesterol) mixed with crushed oats as above; three other sheep were fed an equivalent amount of unprotected cholesterol. The cholesterol supplementation was continued with both groups for 8–9 weeks. Blood samples (20 ml) were taken from each sheep at regular intervals throughout the experiment and placed into tubes containing a 10% EDTA solution (0.5 ml). Plasma was obtained after centrifugation. The plasma cholesterol concentrations were measured using the Technicon Autoanalyzer (10).

Extraction of sterols

Rumen reaction mixtures and abomasal digesta samples were saponified as previously described (7) and the nonsaponifiable components were removed by extracting three times with petroleum ether (40–60°C). The extracts were pooled and the solvent was removed by evaporation prior to analysis by thin-layer chromatography or gas–liquid chromatography.

TLC of extracted sterols

Sterols extracted from rumen and abomasal digesta were separated by argentation thin-layer chromatography (silica gel G, 5% $AgNO_3$); the plates were developed in chloroform–acetone 98:2 (v/v). The distribution of separated sterols was determined by spraying the TLC plate with an ethanolic solution of 2,7-dichlorofluorescein and visualizing the separated sterols under a UV light.

Radioactivity of Ag^+-TLC separated sterols

The radioactivity of sterols separated by Ag^+-TLC was measured by either scanning (using a Varian Berthold Radioscanner) or by elution with diethyl ether and assay of the radioactivity by liquid scintillation spectrometry (11).

GLC of extracted sterols

Sterols extracted from in vitro rumen reaction mixtures were separated isothermally at 260°C using a Varian Model No. 1200 gas-liquid chromatograph equipped with a flame ionization detector and a 2M stainless steel column (2 mm OD) packed with 3% OV-17 on GAS-CHROM Q 100/120 mesh (Applied Science Laboratories, State College, PA).

RESULTS

Ruminal metabolism of unprotected cholesterol

In vitro studies. A mixture containing [^{3}H]cholesterol was incubated with rumen fluid for a 20-hr period. A radioscan of nonsaponifiable lipids separated by TLC showed that [^{3}H]cholesterol was extensively converted to less polar components. When these components were eluted from the TLC plate and counted by liquid scintillation spectrometry, it was found that 85% of the original radioactivity was present in metabolites. No conversion was detected when radiolabeled cholesterol was incubated with boiled rumen fluid.

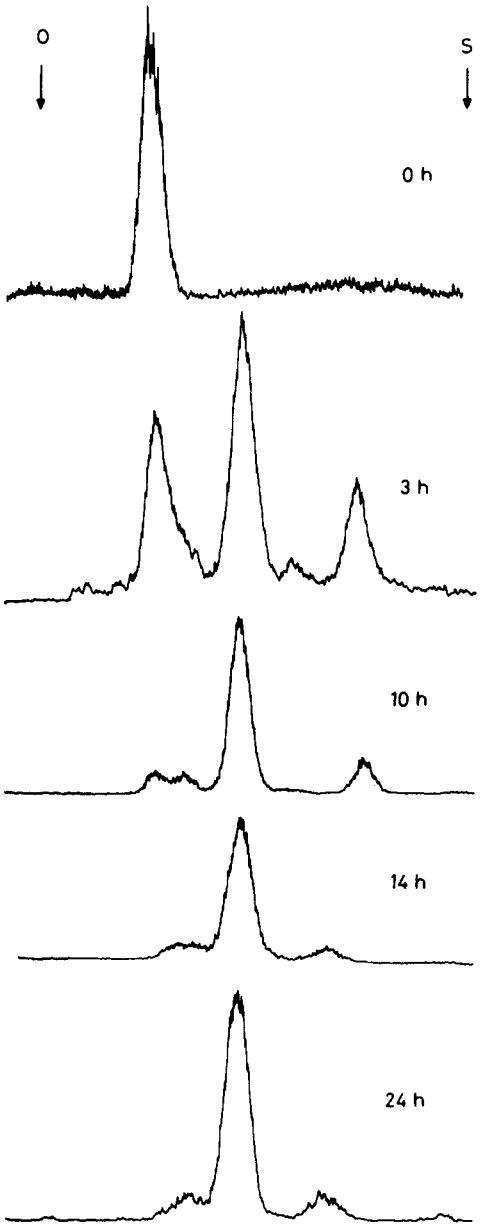

Fig. 1. Effect of period of incubation in the in vitro ruminal metabolism of [^{14}C]cholesterol: radioscans of [^{14}C]cholesterol and ^{14}C-labeled metabolites separated by Ag^+-TLC. The rumen fluid reaction mixtures each contained 10 ml of rumen fluid and 0.4 g of an emulsion prepared by sonicating a mixture of 10 μCi of [4-^{14}C]-cholesterol (sp act 24.6 mCi/mmol), 10 mg of unlabeled cholesterol, 500 mg of sunflower oil (containing 0.02% BHA), 500 mg of sodium caseinate, and 4.2 g of water. h, Hours of incubation; O, origin; S, solvent front.

Fig. 1 shows the effect of period of incubation on the bioconversion of [^{14}C]cholesterol incubated with rumen fluid. The radioscans of the nonsaponifiable material from this study indicate the presence of two major metabolites. The least polar metabolite was present in substantial proportions at 3 hr; however it decreased in amount and was virtually absent at 14 hr. The appearance and disappearance of this component suggests that it may be an intermediate in the synthesis of the principal metabolite. The principal metabolite of [^{14}C]cholesterol had an R_f similar to that of the [^{3}H]cholesterol metabolite.

The bioconversion of cholesterol not only can be detected by using trace amounts of radioactive substrates, but also can be demonstrated by incubating milligram quantities of unlabeled sterol with rumen contents and separating the metabolites on Ag^+-TLC or GLC as shown in **Figs. 2** and **3**, respectively.

The metabolite that appeared in the incubated samples (Fig. 2) had an R_f value of 0.46 which was very close to the principal radioactive metabolite (R_f 0.47) present after incubating [^{14}C]cholesterol for 24 hr (Fig. 1). Furthermore this metabolite corresponded closely in R_f value to that of coprostanol (R_f 0.46) (Fig. 2). The least polar metabolite produced during incubation of the radioactive substrate (see Fig. 1) could not be detected after a 20-hr incubation of the unlabeled cholesterol (Fig. 2) but the R_f value of 0.69 of this metabolite (Fig. 1) corresponded closely to that of coprostanone (R_f 0.70) (Fig. 2).

GLC analysis of nonsaponifiables extracted from the rumen reaction mixtures confirmed the results obtained by TLC. Fig. 3 shows the GLC separation of sterols extracted from the same unincubated and

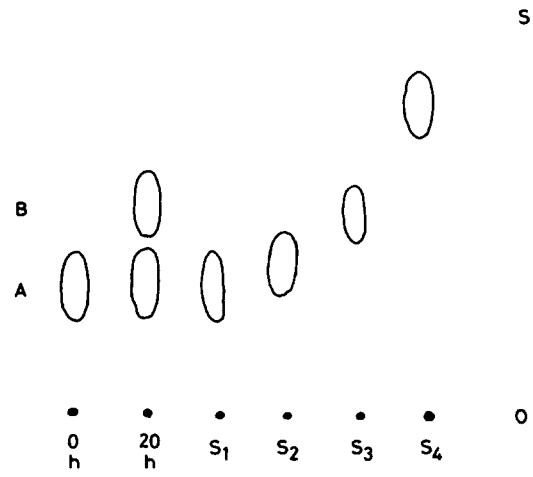

Fig. 2. TLC separation of standard sterols and sterols extracted from in vitro rumen fluid reaction mixtures containing cholesterol. Sterols from incubated (20 hr) and unincubated (0 hr) rumen fluid reaction mixtures were separated by Ag^+-TLC and visualized under UV light after spraying with 2,7-dichlorofluorescein. The incubated and unincubated reaction mixtures each contained 10 ml of rumen fluid and 0.5 g of an emulsion prepared by homogenizing a mixture of cholesterol, sunflower oil, sodium caseinate, and water (1:3:4:20 w/w/w/w). The sunflower oil contained 0.01% by weight of BHA and 2N NaOH was added to the emulsion to raise the pH to ca. 8.5. The incubation was for 20 hr. The sterol standards were S_1, cholesterol; S_2, cholestanol; S_3, coprostanol; S_4, coprostanone; A, cholesterol; B, cholesterol metabolite; O, origin; and S, solvent front.

incubated reaction mixtures as were analyzed in Fig. 2. The major metabolite of cholesterol corresponded in retention time to coprostanol (Fig. 3). Furthermore, when components A and B of the incubated reaction mixtures (Fig. 2) were scraped off the TLC plate, eluted with diethyl ether, and analyzed by GLC there was exact correspondence between the coprostanol standard, the TLC-separated "coprostanol" metabolite, and the GLC-separated "coprostanol" metabolite.

In vivo studies. The TLC distribution of sterols extracted from the abomasal digesta of a sheep fed a single supplement of 6 g of unprotected cholesterol is shown in **Fig. 4**. The abomasal samples were collected at 0, 4, 8, and 24 hr after feeding the cholesterol. Cholesterol appeared in the abomasal digesta at 4 hr postfeeding and the relative intensity of the cholesterol band in the abomasal digesta was maximum at this time. At later times postfeeding the relative intensity of the cholesterol band in the abomasal digesta was progressively reduced and there were corresponding increases in the relative intensities of bands corresponding to less polar compounds. At 24 hr postfeeding very little cholesterol could be detected in the abomasal digesta (Fig. 4).

Ruminal metabolism of protected sterols

In vitro studies. A radioscan of sterols extracted from incubated and unincubated rumen fluid reaction mixtures containing [4-^{14}C]cholesterol encapsulated in a matrix of formaldehyde-treated casein was determined. The [^{14}C]cholesterol was protected against metabolism during the 20-hr incubation in contrast to unprotected radioactive cholesterol (c.f. Fig. 1). In another study, protected [17α-(*n*)-^{3}H]cholesterol was incubated with rumen fluid for 20 hr. After extraction of the sterols and separation by Ag^{+}-TLC, the individual sterol components were eluted and counted by liquid scintillation spectrometry; 95% of the radioactivity was recovered as the unaltered cholesterol.

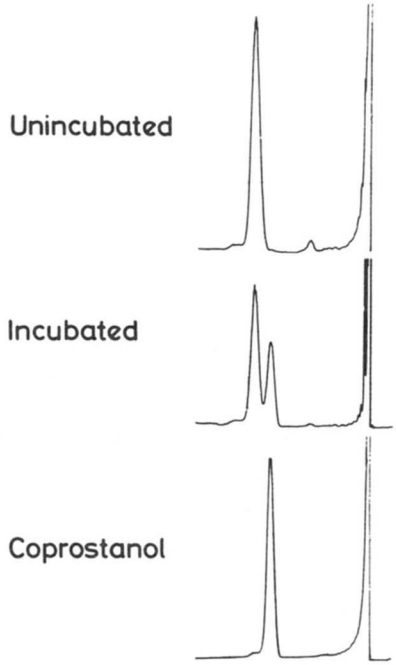

Fig. 3. GLC separation of standard sterols and sterols extracted from in vitro rumen fluid reaction mixtures containing cholesterol. The incubation of rumen fluid with cholesterol was as described in the legend to Fig. 2. The extraction and GLC analysis of sterols was as described in the text.

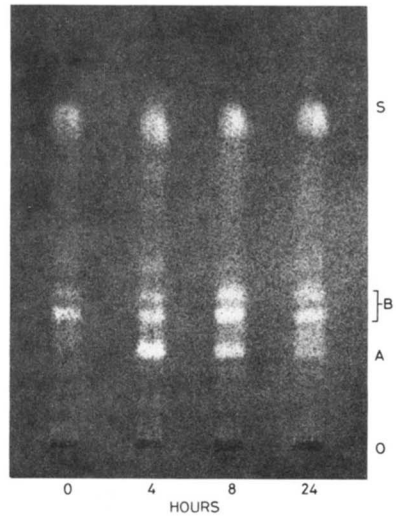

Fig. 4. TLC separation of sterols from abomasal digesta of a sheep fed unprotected cholesterol. Post ruminal digesta was sampled at 0, 4, 8, and 24 hr after the feeding of 6 g of cholesterol. The sterols were separated by Ag^{+}-TLC and visualized using UV light after spraying with 2,7-dichlorofluorescein. O, origin; S, solvent front; A, cholesterol; and B, less polar compounds.

The GLC tracings and Ag^{+}-TLC distribution of sterols extracted from rumen reaction mixtures confirmed that the protected cholesterol was not metabolized to coprostanol during the 20-hr incubation.

In vivo studies. The GLC separation of sterols extracted from the abomasal digesta of a sheep fed protected cholesterol was carried out. The concentration of cholesterol in the abomasal digesta increased with time after feeding and was maximal after 24 hr. The proportion of coprostanol in the abomasal digesta was always very low in contrast to abomasal digesta from sheep fed unprotected cholesterol (Fig. 4).

Effect of cholesterol supplementation on plasma cholesterol of sheep

The plasma cholesterol of sheep fed basal diets of alfalfa and oats supplemented with either protected or unprotected cholesterol (1 g cholesterol per day) is shown in **Fig. 5**. Feeding the unprotected cholesterol caused an increase in the plasma cholesterol concentration during the first 5 weeks of supplementation

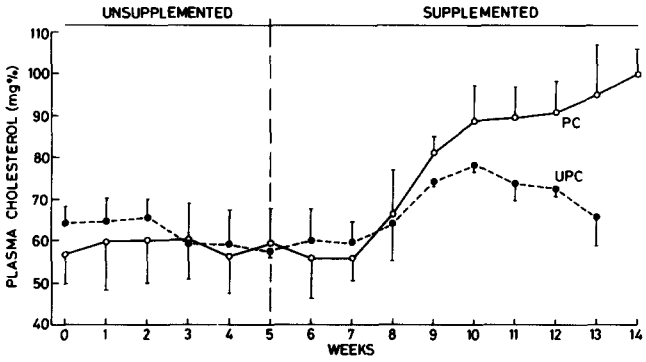

Fig. 5. Effect of cholesterol supplementation on plasma cholesterol of sheep. Each value is the mean and standard error of three sheep. PC, protected cholesterol; UPC, unprotected cholesterol. Sheep were fed 800 g/day of a diet of pelleted alfalfa hay and crushed oats (3:2 w/w) supplemented with 1 g/day of PC or UPC.

but thereafter the cholesterol concentration began to decline and at 8 weeks after supplementation was similar to the preliminary control value. Feeding the protected cholesterol supplement, on the other hand, caused a prolonged increase in the plasma cholesterol concentration and at 8 weeks after supplementation the plasma cholesterol concentration was approximately 60% higher than the initial control values.

DISCUSSION

Ruminants have evolved with a unique capacity for anaerobic fermentation of feedstuffs in the foregut. This microbial fermentation in the rumen facilitates the digestion and utilization of cellulose and nonprotein nitrogen, but the presence of the rumen often modifies the nature and physiological effects of many other dietary constituents.

Earlier studies (5, 6) suggested that unsaturated sterols are also hydrogenated by rumen microflora. We have confirmed that cholesterol was hydrogenated by anaerobic incubation with rumen fluid (Figs. 1, 2, and 3) and the principal metabolite was shown by TLC and GLC separation methods to be coprostanol (Figs. 2 and 3). In a study of the time course of cholesterol hydrogenation in vitro the reaction was virtually complete within 10 hr (Fig. 1) and, at earlier time periods (e.g., 3 hr), the reaction mixture contained appreciable proportions of a metabolite, the R_f value of which resembled that of coprostanone (see Fig. 2). The nature of the principal end product of ruminal cholesterol hydrogenation (coprostanol) and the possible presence of an intermediate (coprostanone) in this reaction suggested that the mechanism for sterol hydrogenation in the rumen is analogous to that in the hind gut of nonruminants (6).

The in vivo ruminal hydrogenation of cholesterol was demonstrated by feeding a sheep unprotected cholesterol in its diet. The initial appearance of cholesterol in abomasal digesta at 4 hr postfeeding (Fig. 4) probably reflects a rapid transport through the rumen of some of the unhydrogenated sterol. At later times postfeeding there was a decline in the relative proportion of cholesterol and an increase in the relative proportion of cholesterol metabolites (Fig. 4). At 24 hr postfeeding there was very little cholesterol in the abomasal digesta (Fig. 4).

Cholesterol was protected against ruminal hydrogenation by encapsulating the sterols in a matrix of formaldehyde-treated casein. In vivo studies on the metabolism of the formaldehyde-treated protein–cholesterol formulations indicated a high degree of protection against ruminal hydrogenation. The concentration of cholesterol in the abomasal digesta was at a maximum at 24 hr postfeeding in contrast to the unprotected cholesterol. These protected sterol preparations appear to be resistant to ruminal metabolism as are other protected lipid formulations (7–9).

The difference between unprotected and protected cholesterol supplementation on plasma cholesterol concentration (Fig. 5) supports previous studies showing that cholesterol is hydrogenated in the rumen and that it can be protected by encapsulating in formaldehyde-treated casein. These data also provide evidence that protected cholesterol is at least partially absorbed from the intestine and incorporated into blood plasma. The rise in the plasma cholesterol concentration after the feeding of unprotected cholesterol supplements is difficult to explain considering the extensive in vitro ruminal hydrogenation of cholesterol (Figs. 1–3). It is possible that the microflora in the rumen of these sheep were initially unable to completely hydrogenate the unprotected cholesterol but with time the microorganisms became better adapted to hydrogenation of the supplemental cholesterol.

In conclusion, the present studies show that cholesterol is extensively hydrogenated by rumen microorganisms and that this hydrogenation can be effectively prevented by coating the sterols with formaldehyde-treated casein. The biological significance of ruminal sterol hydrogenation and the potential effect of protected sterol supplements on carcass and milk constituents has yet to be determined.

We acknowledge the skilled technical assistance of L. Colgan, E. Fleck, E. Dwyer, K. Stephenson, and K. Meers.

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