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Effect of Various Essential Oils Isolated from Douglas Fir Needles upon Sheep and Deer Rumen Microbial Activity

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The effects of essential oils isolated from Douglas fir needles on sheep and deer rumen microbial activity were tested by use of an anaerobic manometric technique. Rumen microorganisms were obtained from a sheep which had been fed mainly on alfalfa hay and dried range grass. One deer used in this study had access to Douglas fir trees the year around, whereas the other deer had no access to Douglas fir. All of the monoterpene hydrocarbons isolated from Douglas fir needles—α-pinene, β-pinene, limonene, myrcene, camphene, Δ^3-carene, and terpinolene—promoted only slightly or had no effect on deer rumen microbial activity, whereas all of them promoted activity in sheep rumen microbes, except Δ^3-carene and terpinolene, which inhibited activity. Of the oxygenated monoterpenes, all monoterpene alcohols—α-terpineol, terpinen-4-ol, linalool, citronellol, and fenchyl alcohol—strongly inhibited the rumen microbial activity of both sheep and deer. Monoterpene esters (bornyl acetate) produced mild inhibition for both sheep and deer microbes, and citronellyl acetate inhibited rumen microbial activity in sheep, whereas it promoted activity in both deer. Monoterpene aldehyde (citronellal) inhibited the activity of rumen microbes from both sheep and deer having no access to Douglas fir from the Hopland Field Station, whereas they produced no effect upon the deer having access to Douglas fir from the Masonite forest. Rumen microbial activity for sheep and deer was promoted slightly with aliphatic ester (ethyl-*n*-caproate). There was a marked difference between sheep and deer rumen microbes as affected by addition of the various essential oils. The monoterpene hydrocarbons promoted activity more on sheep rumen microbes than on deer, and the monoterpene alcohols inhibited sheep rumen microbial activity more than that of deer. Furthermore, the deer rumen microbes from Hopland Field Station were affected more than the deer from Masonite forest.

Although deer browsing of Douglas fir is mainly seasonal and limited to new shoot growth, the financial loss to the timber industry is tremendous. Once the central leaders of young trees are browsed by deer, not only is growth delayed, but the timber may be of lower quality at maturity. Furthermore, browsing sometimes stimulates growth of lateral leaders and gives the tree a dwarfed appearance. Thus, the trees are more accessible to deer when new shoots start to grow the following year. Once the central leader of a young tree grows high enough to escape deer browsing, the tree grows normally even when its new lateral shoots are browsed extensively. The most crucial problem is how to protect the central leaders of young seedlings until they have grown beyond the reach of deer.

Digestion of ingested food in deer depends largely on rumen microbes as is typical of ruminants. The main purpose of this study was to investigate ruminant selectivity of forage plants and digestibility of these plants by rumen microorganisms. This paper involves the effect of various essential oils isolated from Douglas fir needles upon sheep and deer rumen microbial activity.

MATERIAL AND METHODS

Rumen microorganisms. Sheep and deer rumen fluid was obtained by squeezing rumen contents through a layer of cheesecloth. The fluid was immediately gassed with 100% CO_2 while maintained at 39 C in water bath. Permanently fistulated sheep, maintained primarily on alfalfa hay with free access to dried range grass, were used as a source of rumen microorganisms. The deer used in this study were shot at the Hopland Field Station where they had no access to Douglas fir trees, except for one deer which was shot in the Masonite forest where it had had free access to Douglas fir trees the year around.

Essential oil. Volatile essential oils from Douglas fir needles were obtained through steam distillation. Oil was pipetted from an immiscible surface layer of distillate after saturating with sodium chloride. The use of solvents in extracting oil from the distillate was purposely avoided to eliminate possible side effects of any residue of solvent in the rumen microbial assay. The oil thus obtained was further fractionated into three subgroups by temperature programming with a preparative gas chromatograph equipped with an 18 feet (5.5 meters) by 0.25 inch (0.64 cm) stainless-steel column packed with 10% triton X-305 on 60/80 mesh Chromosorb W. The temperature was programmed manually to 200 C from the initial temperature of 60 C and then was run isothermally until all components had emerged. All components of monoterpene hydrocarbons emerged at 125 C, and all components of oxygenated monoterpenes emerged at 192 C. All components collected at 200 C isothermally were designated as sesquiterpenes. The individual compounds were identified by obtaining infrared spectra and by determining relative retention times on several analytical gas chromatographic columns with known compounds.

Rumen microbial activity. The anaerobic Warburg manometric technique initiated by Hungate et al. (4) was employed to study sheep and deer rumen microbial activity as affected by the addition of various amounts and kinds of essential oils. Rumen microbial activity was measured in terms of total gas production. Either 0.3 or 0.5 g of substrate was placed in the main compartment of the Warburg flask (total volume ranged from 130 to 150 ml). The respective amounts of essential oils were then added with 18 ml of mineral salt solution after equilibrating with 100% CO_2 (*p*H 6.8) and were mixed well with the substrate by shaking. To maintain optimal *p*H for rumen microbial fermentation, double strength Hungate's original salt solution (4) was used. Immediately after 7.0 ml of rumen fluid was placed into the side arm, the flasks were attached to manometers and were placed in the water bath at 39 C. Carbon dioxide (100%) was immediately passed through the flasks. After vigorous gassing for 10 min, the flasks were shaken to insure attainment of equilibrium between the liquid and gas phases, and gassing was continued for 10 more min. At the end of gassing, the stopcocks of the side arms were closed, and carbon dioxide was sucked out from the manometers and flasks with a suction pump until the mercury level in the right arm of the monometers reached 160 mm. After closing the stopcock of the right arm of the manometers and attaining new equi-

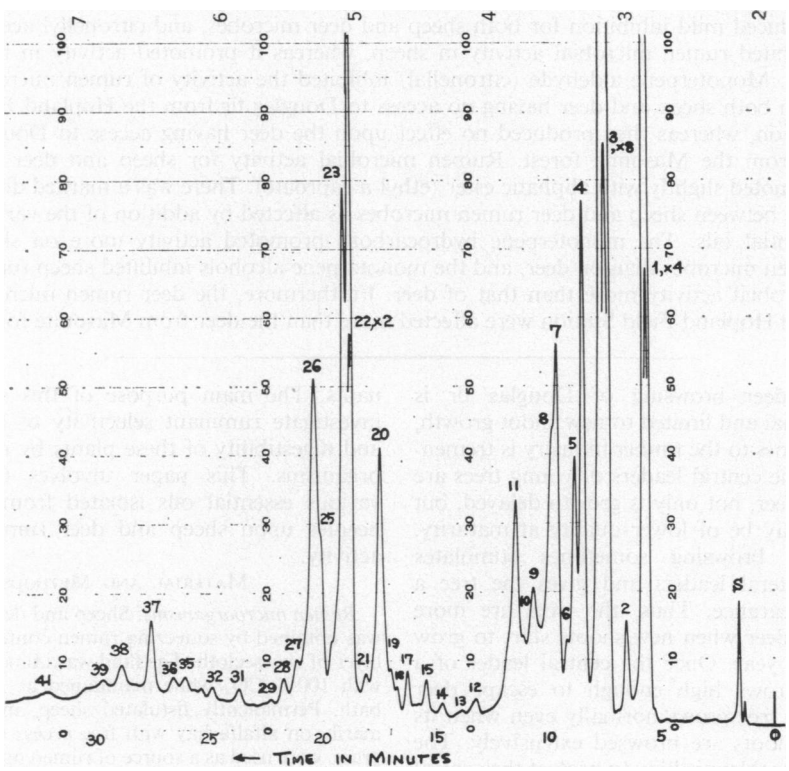

FIG. 1. *Chromatogram of 0.2 μliter of Douglas fir oil from mature needles. Aerograph model 204, flame ionization detection, range 10^2, attenuation 1 except as noted; 10 feet (3.05 meters) by 0.25 inch (0.64 cm) stainless-steel column of 5% carbowax 20M on 60/80 mesh Chromosorb W (HMDS); 30 ml of He per min as carrier gas; isothermal at 75 C for 3 min, programmed at 5 C/min to 200 C, then isothermal.*

librium between the two phases, first readings were taken and the rumen fluid was tipped into the main compartment from the side arm. Subsequent readings were taken at certain intervals for 24 hr and were expressed in millimoles of gas produced per gram (dry weight) of substrate as a criterion of rumen microbial activity. Under anaerobic conditions employing a bicarbonate buffer system, manometric measurements of gas production include CO_2, CH_4, and minor amounts of H_2. Carbon dioxide measured in this system was composed of both metabolic CO_2 from rumen microbial fermentation and CO_2 liberated through production of acids according to the following equation: $H^+ + HCO_3^- \rightleftarrows H_2CO_3 \rightleftarrows H_2O + CO_2$, where H^+ arises from dissociation of acids formed.

RESULTS

Gas chromatogram of essential oil isolated from Douglas fir needles. Figure 1 presents a chromatogram depicting the mixture of components present in the oil isolated from the mature needles of Douglas fir. The detailed operating condition of gas chromatograph and the structure of compounds identified by Kepner's group are specified in the legend of Fig. 1 and in Table 1, respectively. In the fractionation of three subgroups, monoterpene hydrocarbons included peaks 1 to 11, including two nonterpene compounds, namely, 2-hexenal (peak 8) and ethyl-*n*-caproate (peak). Peaks 15 to 26 represent oxygenated monoterpenes, and peaks 27 to 41 were designated as sesquiterpenes.

Effect of increasing amounts of whole essential oil isolated from Douglas fir needles upon deer rumen microbial activity. Lower levels of whole essential oil did not significantly affect rumen microbial activity compared with the reference value, which received alfalfa alone without addition of oil (Table 2). As the level of Douglas fir essential oil increased to 0.3 ml per flask, rumen microbial activity was inhibited and gas production decreased significantly.

TABLE 1. VOLATILE COMPONENTS FROM DOUGLAS FIR NEEDLES

Peak	Compound
1	α-pinene
2	camphene
3	β-pinene
4	3-carene
5	myrcene
7	limonene
8	2-hexenal
9	ethyl caproate
10	γ-terpinene
11	terpinolene
14	ethyl caprylate
15	citronellal
17	linalool
19	fenchyl alcohol
20	bornyl acetate
20	terpinen-4-ol
22	citronellyl acetate
23	α-terpineol
26	citronellol
26	geranyl acetate

TABLE 2. *Rumen microbial activity (Hopland deer) as affected by increasing amounts of steam-distilled whole essential oil from Douglas fir needles*[a]

Addition to alfalfa	Amt of gas/0.30 g of alfalfa[b]	Amt of gas/g of alfalfa	Relative production	Relative effectiveness[d]
	mmoles	*mmoles*	%	%
None	1.16	3.86	100	—
0.10 ml of essential oil	1.21	4.02	104	+4
0.20 ml of essential oil	1.18	3.95	102	+2
0.30 ml of essential oil	0.91[d]	3.03	78	−22

[a] Each flask received 0.3 g of ground alfalfa hay, 18 ml of mineral salt solution equilibrated with 100% CO_2 (*p*H 6.8), the indicated amount of essential oil, and 7.0 ml of strained rumen fluid (*p*H 5.4).

[b] Mean value of two flasks and corrected values against control flask.

[c] Plus or minus means there was promoted or inhibited activity, respectively, compared with reference flask (alfalfa alone).

[d] Significantly different (.05 level) from the alfalfa substrate alone.

Effect of three subgrouped essential oils (monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpenes) from Douglas fir needles upon deer (from Hopland) rumen microbial activity. Monoterpene hydrocarbons and lower levels (0.05 ml per flask) of oxygenated monoterpenes from Douglas fir oil promoted deer rumen microbial activity, whereas oxygenated monoterpenes (0.10 ml per flask) were inhibitory (Table 3). The fraction of sesquiterpenes promoted activity, whereas, as might be expected, a moderate range of inhibition was obtained by the combination of 0.1 ml of monoterpene hydrocarbons and oxygenated monoterpenes.

Effect of various essential oils isolated from Douglas fir needles upon sheep and deer rumen microbial activity. All flasks received 0.075 ml of respective oils per flask including sheep (from Hopland) and both deer (one from Hopland and the other from Masonite) rumen microbial assay, except 0.05 ml of α-pinene and 0.025 ml of β-pinene, myrcene, and terpinolene were used for the sheep rumen microbial study because of shortage of the respective oils.

(*i*) *Effect upon sheep (from Hopland) rumen microbial activity.* A total of 16 compounds—7 monoterpene hydrocarbons (α-pinene, β-pinene, limonene, myrcene, camphene, Δ^3-carene, and terpinolene), and 9 oxygenated monoterpenes (α-terpineol, terpinene-4-ol, linalool, citronellol,

TABLE 3. *Rumen microbial activity (Hopland deer) as affected by the three subgrouped essential oils (monoterpene hydrocarbons, oxygenated monoterpenes, and sequiterpenes) from Douglas fir needles*

Addition to substrate[a]	Amt of gas/0.30 g of substrate[b]	Amt of gas/g of substrate	Relative production	Relative effectiveness[c]
	mmoles	*mmoles*	%	%
None	1.097	3.657	100	—
0.1 ml of monoterpene hydrocarbon	1.262[d]	4.207	115	+15
0.2 ml of monoterpene hydrocarbon	1.323[d]	4.410	121	+21
0.05 ml of oxygenated monoterpene	1.347[d]	4.490	123	+23
0.1 ml of oxygenated monoterpene	0.837[d]	2.790	76	−24
0.1 ml of sesquiterpene	1.284[d]	4.280	117	+17
0.1 ml of hydrocarbon + 0.1 ml of oxygenated monoterpenes	0.919[d]	3.063	84	−16

[a] Substrate consists of 0.15 g of alfalfa hay plus 0.15 g of dried, ground Douglas fir needles after steam distillation.

[b] Mean values of two flasks and corrected values against control flask.

[c] + = promotion compared with reference value (substrate alone); − = inhibition compared with reference value.

[d] Significantly different (.05 level) from substrate alone treatment.

fenchyl alcohol, citronellal, citronellyl acetate, bornyl acetate, and ethyl-*n*-caproate)—were used with sheep rumen microbes. Of the monoterpene hydrocarbons, α-pinene, β-pinene, limonene, myrcene, and camphene promoted activity of sheep rumen microbes, whereas Δ^3-carene and terpinolene inhibited activity (Table 4). Most of the oxygenated monoterpenes isolated from Douglas fir oil strongly inhibited sheep rumen microbial activity. All of the monoterpene alcohols—α-terpineol, terpinen-4-ol, fenchyl alcohol, linalool, and citronellol—inhibited activity most strongly, whereas ethyl-*n*-caproate (aliphatic ester) promoted activity only mildly. Citronellal (monoterpene aldehyde) and citronellyl acetate and bornyl acetate (monoterpene esters) inhibited the activity moderately. Gas production did not commence for several hours after exposing sheep rumen microbes to citronellol, citronellal, and linalool (Fig. 2). There was virtually no gas production when the microbes were exposed to fenchyl alcohol, α-terpineol, and terpinen-4-ol.

(ii) *Effect upon deer (from Hopland Field Station) rumen microbial activity:* The deer used as a source of rumen microorganisms was shot at the Hopland Field Station where deer had no access to Douglas fir trees. Unlike sheep rumen microbes, activity was either promoted or not affected by any of the seven monoterpene hydrocarbons. As seen in Table 4, the relative effective-

TABLE 4. *Effect of various monoterpene hydrocarbons and oxygenated monoterpenes isolated from Douglas fir needles upon sheep and deer rumen microbial activity*

Essential oils[a]	Relative effectiveness (%)[b] upon rumen microorganisms from		
	Hopland sheep	Hopland deer	Masonite deer
Reference flask	2.98[c]	5.27[c]	4.61[c]
Monoterpene hydrocarbons			
α-Pinene	+25[d]	+6	+4
β-Pinene	+21[e]	+4	+3
Limonene	+18	+5	−3
Myrcene	+14[e]	+6	—[f]
Camphene	+8	+5	+3
Δ^3-Carene	−25	+3	+1
Terpinolene	−58[e]	+1	—[f]
Oxygenated monoterpenes			
α-Terpineol	−96	−48	−56
Terpinen-4-ol	−97	−38	−11
Linalool	−55	−10	+2
Citronellol	−37	−22	−19
Fenchyl alcohol	−97	−74	−45
Bornyl acetate	−27	−5	−1
Citronellyl acetate	−13	+2	+7
Citronellal	−38	−33	+2
Ethyl-*n*-caproate	+6	+3	+7

[a] Except for the reference flasks which used only substrate, oil was added in the amount of 0.075 ml per flask.

[b] + or − refers to stimulatory or inhibitory effect of each compound compared with the reference flasks.

[c] Each flask received 0.5 g of ground Douglas fir needles which had been steam-distilled for essential oils. Results are expressed in millimoles of gas per gram of substrate.

[d] Placed only 0.05 ml of α-pinene per flask because of shortage of oil.

[e] Placed only 0.025 ml of respective oils per flask because of shortage of oils.

[f] No determination because oil supplies were exhausted.

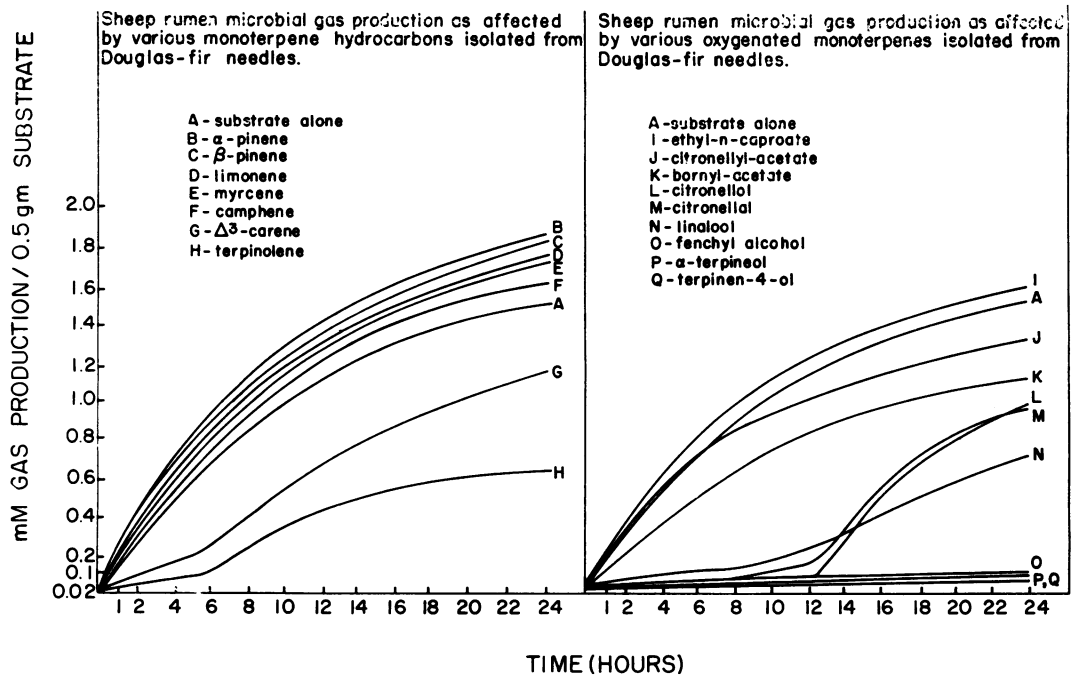

FIG. 2. *Sheep (from Hopland) rumen microbial gas production as affected by various monoterpene hydrocarbons or oxygenated monoterpenes isolated from Douglas fir needles.*

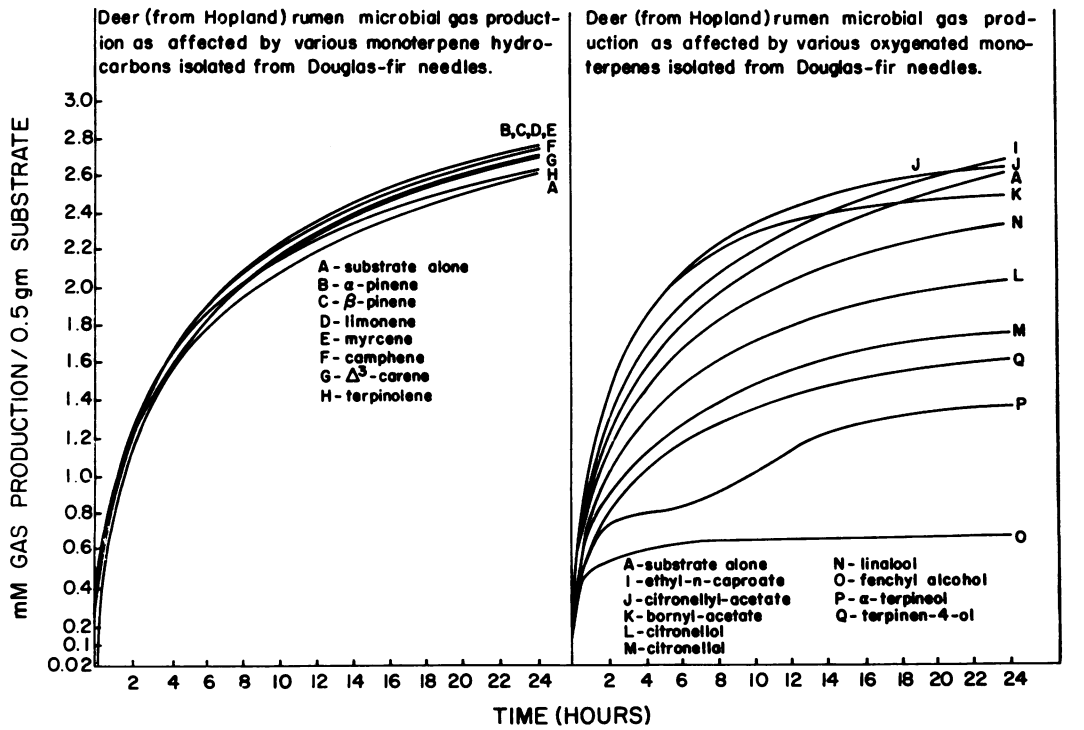

FIG. 3. *Deer (from Hopland) rumen microbial gas production as affected by various monoterpene hydrocarbons or oxygenated monoterpenes isolated from Douglas fir needles.*

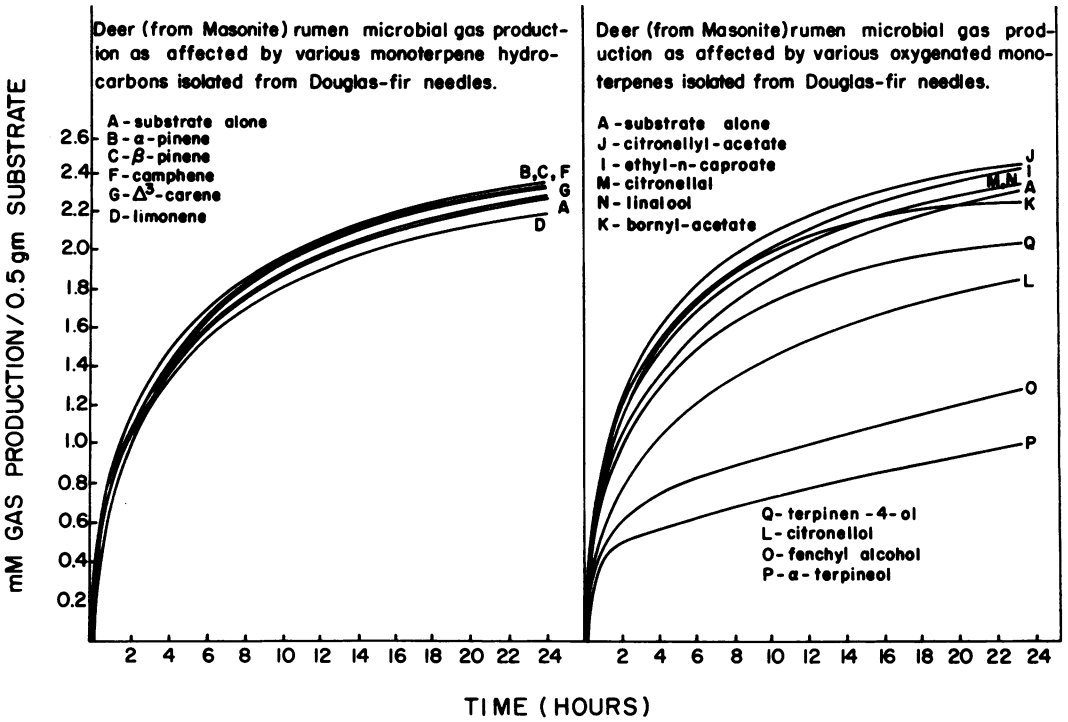

FIG. 4. *Deer (from Masonite) rumen microbial gas production as affected by various monoterpene hydrocarbons or oxygenated monoterpenes isolated from Douglas fir needles.*

ness ranged from +1 to +6%, whereas the range was from −58 to +25% for sheep rumen microbes even though lower levels (0.025 to 0.05 ml per flask) of some of the oils were used. As in the sheep rumen microbial trial, all five monoterpene alcohols displayed an inhibitory effect on deer rumen microbial activity. Fenchyl alcohol exhibited the most potent inhibitory effect upon activity among the monoterpene alcohols. Little effect was demonstrated by citronellyl acetate, bronyl acetate, and ethyl-*n*-caproate, whereas moderate inhibition was obtained with monoterpene aldehyde, citronellal. Gas production commenced immediately after rumen microbes were exposed to all compounds (Fig. 3).

(iii) *Effect upon deer (from Masonite forest) rumen microbial activity:* Data obtained with the microbes from the Masonite forest deer, which had access to Douglas fir, are presented in Table 4 and Fig. 4. Overall effect of rumen microbial activity in this trial was milder than that of the previous trial with deer from the Hopland Field Station. All five monoterpene hydrocarbons increased activity only slightly or had no effect as with monoterpene aldehyde, esters, and aliphatic ester (ethyl-*n*-caproate). The relative effectiveness of these compounds ranged from −1 to +7%. As in the previous trials with deer and sheep from Hopland, monoterpene alcohols were consistently inhibitory upon activity with the exception of linalool (+2%). There was a marked difference between deer and sheep rumen microbial activity as affected by the various essential oils isolated from Douglas fir oil. The seven monoterpene hydrocarbons promoted activity more on sheep rumen microbes, even though lower levels of these compounds were added to the sheep rumen microbial assay. The five monoterpene alcohols which were added at the same level inhibited sheep rumen microbial activity more than deer.

DISCUSSION

Douglas fir was one of the least palatable species among the browse plants tested from the Hopland Field Station area (*unpublished data*). However, nearer the coast in the Douglas fir-redwood region such as on the Masonite property, the deer have much less choice and they browse Douglas fir quite heavily. This browsing is mainly on new shoots of the central and lateral leaders during the growing season. Like monogastric animals, ruminants may select their food for chemical compounds such as protein, carbohydrate, fat, vitamins, minerals, nonprotein nitrogenous compounds, or volatile flavor compounds which in turn affect the animals' taste, smell,

touch, vision, etc. directly. Balch and Campling (1) have shown that the voluntary intake of forage by sheep is determined by the bulkiness of digesta and their rate of disappearance from the reticulo-rumen. Since digestibility of the ingested feed in ruminants depends on the microbial action in the reticulo-rumen, it is apparent that feed intake is governed by the rumen microbial activity which is directly affected by the chemical constituents of ingested feed. Reduced rumen microbial digestion resulting from antibacterial substances present in the ingesta would result in prolonged reticulo-rumen retention of ingesta, distention of the rumen, and consequently reduction of feed intake. Thus, when forage plants contain aromatic volatile compounds, which inhibit rumen microbial activity, it is logical to expect that ruminants' feed intake or selectivity would in turn be affected by those aromatic volatile compounds in both ways, olfactory and antirumen microbial action.

Most of the least palatable forage plants for ruminants studied to date at the Hopland Field Station contain volatile compounds which have an inherent characteristic odor and different degrees of antirumen microbial action. Douglas fir is a typical species in this category.

Through gas chromatography, it is obvious that more than 40 different compounds contained in whole essential oils of Douglas fir oil represent the characteristic odor of that species. Investigation of the effect of whole essential oil distilled from Douglas fir upon selectivity and voluntary intake of feed with sheep is underway at the present time. The increased rumen microbial activity obtained by low levels of essential oil and the inhibitory effect produced by high levels can be attributed to the promoting or inhibiting effect of the three subgrouped oils and their concentration ratios. Of the three subgroups, monoterpene hydrocarbons and sesquiterpenes promoted sheep and deer rumen microbial activity, whereas oxygenated monoterpenes inhibited the activity. The concentration ratios of the monoterpene hydrocarbons (promoting group), oxygenated monoterpenes (inhibiting group), and sesquiterpenes (promoting group) were approximately 5:1:0.05, respectively. These ratios are of primary importance in determining the net effect on microbial activity as oil levels are varied. At the lowest level of oil (0.10 ml per flask), the concentration of oxygenated monoterpenes (inhibiting group) is not high enough to offset the promoting effect of monoterpene hydrocarbons and sesquiterpenes. At the higher level (0.3 ml per flask), however, the inhibiting group (oxygenated monoterpenes) was sufficiently high that the net effect became negative. No doubt different components of oil produce selective promotion or inhibition on the various species of rumen microorganisms.

The effect of individual essential oils upon sheep and deer rumen microbial activity agreed with the results obtained from the three subgrouped oils. As expected, among those individual essential oils grouped as monoterpene hydrocarbons, α-pinene, β-pinene, limonene, myrcene, and camphene promoted activity of sheep and deer rumen microorganisms, whereas Δ^3-carene and terpinolene inhibited activity. Likewise, all of the monoterpene alcohols grouped as oxygenated monoterpenes (inhibiting group)—α-terpineol, terpinen-4-ol, fenchyl alcohol, linalool and citronellol—inhibited consistently both for sheep and deer rumen microbes. The remaining oxygenated monoterpenes—citronellal, citronellyl acetate, and bornyl acetate—varied the effectiveness according to the sources of rumen microorganisms. Bryant and Burkey (3) and Warner (6) reported that predominant bacterial species and their populations in the rumen were dependent upon their dietary regime. Bauman and Foster (2) and Maki and Foster (5) also reported that rumen bacteria from an alfalfa hay-fed cow and high grain-fed cow were different not only in predominant form but in end products of their fermentation as well. Based on the above results, it is clear why such a marked difference between sheep and deer rumen microbes resulted from individual compounds isolated from Douglas fir needles. It is assumed that the predominant species and the number of sheep and deer rumen microbes differ according to their habitat. When the same levels of monoterpene alcohols were added to the rumen fluid from Hopland sheep and deer and Masonite deer, the greatest inhibitory effect was obtained with rumen microbes of sheep which had had no previous access to monoterpene alcohols. Intermediate inhibition was obtained with the deer from the Hopland Field Station which had no access to Douglas fir. However, the browse species utilized by deer in this area contain a variety of essential oils. A mild inhibition resulted from the rumen microbes obtained from the Masonite forest deer which had access to Douglas fir trees year around. It is assumed that the deer rumen microbes in this area had become adapted to such inhibitory essential oils.

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