THE urine of fasting animals of several species has been reported to contain a substance(s) which causes increased mobilization of fat (FMS) (Chalmers, 1964). The active constituents isolated from rat urine have been additionally fractionated (Beaton et al., 1964; Beaton, Szlanko and Stevenson, 1964a, 1966) and found to contain several active components.

Sheep have been reported to excrete FMS (Chalmers, 1964) but no purification of the active component(s) has been reported. Since the identity of FMS had not been established and the mode of control of fat mobilization in ruminants is still in question, it was decided to investigate the FMS isolable from sheep urine in order to determine its identity and its possible role in ruminant fat metabolism.

Materials and Methods

Sheep which had previously been fed a mixture of corn, molasses and urea (90:8.5:1.5) ad libitum and .45 kg of grass hay, daily for 1 week were fasted as long as 10 days and their urine collected in benzoic acid. Sodium carbonate extracts were prepared for assay by the method of Chalmers (1964). Assays for adipokinetic activity were performed either by injecting 0.5 ml of Fraction A into fasting male mice (25 to 30 g) and bleeding the mice 1 hr. after injection or by incubation 50 ul of Fraction A with 50 to 55 mg of surviving epididymal fat pad from fasted, decapitated rats, in 1 ml of serum from fasted sheep for 3 hr. and measuring free fatty acids. Four mice or portions of fat pad were used to determine each mean value. Free fatty acids analyses were performed colorimetrically (Itaya and Ui, 1965).

Fractionation and Identification. In each fractionation on Sephadex G-50, pooled sodium carbonate extracts representing 10 sheep days were fractionated, as shown in figure 1, through absorption on Oxycel (Chalmers, 1964) and absorbed substances were eluted with 0.1 N acetic acid (Fraction D). The eluate was then made basic with concentrated ammonium hydroxide and to a final volume of 10 milliliters. Three milliliters of this were eluted from a 2 x 30 cm column of Sephadex G-50 with 0.01 N NH₄OH. The fractions of eluate were assayed for absorbance at 280 µ and ninhydrin determinations (Rosen, 1957) were run on aliquots of each fraction after drying at 70 C to remove the ammonia. Eluted peaks as located by either determination were freeze-dried. The recovered solids of the fractions eluted in the first 200 ml were made up to 5 ml and those eluted after 200 ml were made up to 0.5 ml with distilled water. The fractions eluted in less than 200 ml were assayed in vivo and those eluted between 200 and 400 ml were assayed in vitro due to the extremely small amount of material recovered. Bovine hemoglobin, insulin, Evans blue and bromophenol were eluted from the column under the same conditions to provide an estimate of the elution volumes of their respective molecular sizes.

In a third fractionation, pooled sodium carbonate extracts representing 5 sheep-days which had previously shown adipokinetic activity were reprecipitated at pH 5.3 with 2 volumes of ethanol and the precipitate extracted with 0.1 N sodium hydroxide. The extract was then reprecipitated and dissolved in 10 ml of 0.01 N ammonium hydroxide. Three milliliters were eluted from a 2 x 30 cm column of Sephadex G-25. All of the eluate up to the elution volume of cresolsulphonphthalein (MW 382) was recovered and freeze-dried. The dried solids were made up to a volume of 5 ml with distilled water and assayed in vitro.

Pressor Activity was demonstrated by injections 0.5 ml of Oxycel purified FMS into a pentobarbital anesthetized, vagotomized rat. Blood pressure changes were estimated manometrically.

The Spectrum of sheep FMS was determined on the same preparation which was used for the determination of pressor activity.
URINE
pH 5.3
Benzoic Acid
ETOH

Precipitate
0.1% Na₂CO₃

Precipitate
 Supernatant

Precipitate
 Supernatant (Fraction A)
15 ml/sheep/day
pH 5.3
ETOH

Precipitate
 Supernatant

HCl
ETOH
pH 5.3

Precipitate
 Supernatant

0.1 N NaOH

Precipitate
 Supernatant (B)

pH 7
stirred with oxycel

Elute with 0.1 N NaOH
Elute with 1 N HAC

Fraction C
Fraction D

Figure 1. Initial fractionation procedure for the isolation of sheep FMS.

Chemical Tests. The potassium ferricyanide-formic acid test (Block, 1958) and the test for fluorescense in the presence of ammonia (Block, 1958) were performed on the adipokinetically active fraction removed from Sephadex. Paper chromatographic identification of this peak was attempted. However, following elution, neither ninhydrin, ultraviolet light or potassium ferricyanide revealed the location of the compound.

Dietary Energy Density Study. Six wethers were randomly allotted into three groups of two each. They were individually fed grass hay and a concentrate mixture (corn, molasses and urea) at a level calculated to be adequate for maintenance plus 0.045 kg of gain per day. They were fed concentrate to hay ratios of 0, 0.5 or 1.0 to 1.0 for 3 weeks and then fasted. Their urine was collected on days 7, 8 and 9 of the fast in the first collection and on days 3, 4 and 5 in the second collection after a refeeding period of 3 weeks. The urine from each collection was pooled by sheep and sodium carbonate extracts prepared as before. These extracts were assayed in vitro.

Results

Excretion Studies. Figure 2 shows the effect of extracts of urine from two sheep which had been fed a high level of concentrate prior to
fasting. It can be seen that the excretion of the antiadipokinetic substance(s) was maximal on day seven in one sheep and on day 8 of the fast in the other sheep. Figure 3 shows the effect of two doses of another extract upon the blood of FFA of fasting mice. The 1 ml dose depressed the FFA concentration to about the same extent in all cases while there was no significant reduction in the FFA of the mice injected with 0.2 ml of the extracts from days three, four and five of the fast. This was followed by a significant reduction in the blood FFA, of mice injected with extract from day six of the fast. We have interpreted this to mean that the FFA depressing activity was present in the urine on all days tested and that the 1 ml dose caused a maximal response in all cases.

Table 1 shows the results of assay of fractions isolated from sheep urine during a study of the effect of percent concentrate in the diet on the excretion of fat mobilizing substances during the pursuant fast. In collection 1, days 7, 8 and 9 were chosen as the collection days because during this period a net adipokinetic activity was more persistent in previous studies. It can be seen that during collection 1, there was no difference between the zero percent concentrate and control injections and that as the percent concentrate increased extracts from the urine obtained during this period caused a depression of FFA in mice. In the second collection it can be seen that no adipokinetic activity was present in the first four peaks. This is in agreement with the results obtained in another fractionation performed

TABLE 1. BLOOD FFA OF MICE INJECTED WITH EXTRACTS OF URINE FROM SHEEP FED VARIOUS ROUGHAGE: CONCENTRATE RATIOS

<table>
<thead>
<tr>
<th>Percent concentrate</th>
<th>Collection I</th>
<th>Collection II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sheep no.</td>
<td>Sheep no.</td>
</tr>
<tr>
<td></td>
<td>Avg FFA µEq/liter</td>
<td>Avg FFA µEq/liter</td>
</tr>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>940±9.00</td>
<td>1254.0±149.37</td>
</tr>
<tr>
<td>25</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>926±219.54</td>
<td>1030.0±245.98</td>
</tr>
<tr>
<td>50</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>584±64.40</td>
<td>1124.0±133.06</td>
</tr>
<tr>
<td>75</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>904±153.80</td>
<td>885.0±51.00</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>729±65.32</td>
<td>870.0±96.87</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>760±30.25</td>
<td>1122.0±133.24</td>
</tr>
</tbody>
</table>

This study was initiated with the intent of studying diet effects on the excretion of adipokinetic activity. The concentration of a net antiadipokinetic activity in this study was somewhat surprising but bears out the results shown in figures 2 and 3 of the excretion of a net antiadipokinetic or insulin-like activity later in the fast.

Isolation of Adipokinetic Extracts. The peak elution volumes for the standard compounds were hemoglobin, 30 ml; insulin (MW-6000), 44 ml; Evans blue (MW-963), 65 ml; and bromophenol blue (MW-692), 85 milliliters.

Only extracts which had been shown previously to increase FFA release were used to isolate the adipokinetic substance.

The pattern of elution of substances absorbing at 280 mµ and/or which are ninhydrin positive in the fractions from Sephadex G-50 are presented in figures 4 and 5 and the results of the assay of the fractions are presented in tables 2 and 3. It can be seen that no adipokinetic activity was present in the first four peaks. This is in agreement with the results obtained in another fractionation performed

Figure 2. Blood FFA of mice injected with extracts of fasted sheep urine by day of urine collection (mean and SE). On re-assay, in vitro extract of sheep 1, day 8 inhibited lipolysis significantly more than day 6.
Figure 3. Effect of two doses of urine from fasted sheep extract upon FFA of mice (mean and SE).

in this laboratory. The only adipokinetic activity which was recovered from this fraction was eluted well after the elution volume of bromophenol blue (MW-692). The second fractionation on Sephadex G-50 confirmed the finding that adipokinetic substances were eluted in 300 to 400 ml of eluate. The adipokinetic fractions which were found in the second run were eluted in about the same volume as that found in the first run. In both runs, the two fractions which possessed adipokinetic activity were eluted with peaks at about 330 and 370 milliliters. Both fractions were fluorescent in the presence of ammonia. The fraction eluted at 330 ml was positive to the potassium ferricyanide-formic acid test for adrenalin. The quantity of each fraction which was recovered was less than 1 milligram.

The assay of the fraction of the sodium hydroxide soluble extract which was of a molecular size greater than or equal to the molecular size of cresolsulphonphthalein (MW-382) indicated that this fraction was not adipokinetic (the treatment FFA were 95.9±5.6% of the controls). This confirmed results of the other fractionations where no adipokinetic activity could be found of a molecular size large enough to be a peptide.

When an oxyceel purified preparation of sheep FMS was injected into a rat preparation the blood pressure was increased from 74 and 59 mm of Hg to 87 and 67 mm of Hg, respectively, in two successive injections. The injection of saline did not increase the blood pressure.

The spectra of FMS, epinephrine and norepinephrine are compared in figure 6. It can be seen that, in spite of the heterogenous nature of the FMS preparation, the spectrum of FMS compares rather well with what might be expected of a mixture of these two catecholamines.

Fractionation of Antiadipokinetic Extracts.
The fractions which were obtained from a pool of extracts from sheep fed a high level of concentrate and eluted from Sephadex G-50 and their relative effects upon adipokinesis are presented in figure 7. While all of the extracts compared caused a slight decrease in the blood FFA in mice, peak 2 which contained the smallest amount of solid, depressed the FFA concentration slightly more than did the other extracts.

Discussion

This project was initiated to study adipokinetic substances present in the urine of
FAT MOBILIZATION IN SHEEP

Figure 5. Elution pattern of extracts of urine from fasted sheep from Sephadex G-50 (ninhydrin).

fasting sheep. However, it was more common to find a net antiadipokinetic activity in the urine. The presence of activity of either kind appeared to be dependent upon the prior feeding of a fairly high level of concentrate. No activity of either kind was found in the urine of sheep fed only roughage prior to the fast by the extraction procedure used in these experiments.

In contrast to early reports, no activity of a molecular size large enough to be a peptide containing one each of the amino acids previously reported to be present (Chalmers, 1964) could be isolated. The only fat mobilizing substance present was of a molecular size less than that of cresol sulphophthalein, positive to chemical tests for epinephrine, and had pressor activity.

The antiadipokinetic active substance was eluted from Sephadex G-50 after the elution volume of crystalline insulin standard and, hence, could not be insulin by virtue of its lower molecular weight. The physical characteristics of this substance may be summarized as following: (1) insoluble in acid

TABLE 2. ASSAYS OF FRACTIONS ELUTED FROM SEPHADEX G-50 (TRIAL 1)

<table>
<thead>
<tr>
<th>Fraction (ml)</th>
<th>FFA uEq/liter</th>
<th>Fraction (ml)</th>
<th>FFA uEq/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo assay</td>
<td>In vitro assay</td>
<td>In vivo assay</td>
<td>In vitro assay</td>
</tr>
<tr>
<td>11–50</td>
<td>895±22.6</td>
<td>251–260</td>
<td>983±37.7</td>
</tr>
<tr>
<td>71–100</td>
<td>1035±106.0</td>
<td>301–310</td>
<td>833±23.4</td>
</tr>
<tr>
<td>101–120</td>
<td>1231±88.3</td>
<td>321–330</td>
<td>1208±19.3</td>
</tr>
<tr>
<td>151–180</td>
<td>1050±72.9</td>
<td>361–370</td>
<td>1044±51.2</td>
</tr>
<tr>
<td>Control</td>
<td>1154±218.4</td>
<td>Control</td>
<td>961±15.6</td>
</tr>
</tbody>
</table>

Figure 6. Spectra of FMS extracted from sheep urine, epinephrine, and norepinephrine. All were dissolved in 0.1 N sodium hydroxide saturated by oxycel.

TABLE 3. IN VITRO ASSAY OF FRACTIONS ELUTED FROM SEPHADEX G-50 (TRIAL 2)

<table>
<thead>
<tr>
<th>Fraction (ml)</th>
<th>FFA uEq/liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>326–345</td>
<td>1028±12.8</td>
</tr>
<tr>
<td>351–360</td>
<td>850±12.2</td>
</tr>
<tr>
<td>371–375</td>
<td>917±8.0</td>
</tr>
<tr>
<td>376–395</td>
<td>838±18.6</td>
</tr>
<tr>
<td>Control</td>
<td>858±13.8</td>
</tr>
</tbody>
</table>
alcohol, (2) ninhydrin positive and (3) 6000 > molecular weight > 1000. The dietary conditions requisite for excretion include that a minimum of 50% of the ration be concentrate, and that it be fed for 1 or more weeks prior to the fast.

The above observations that a high concentrate diet prior to fasting is requisite for the excretion of either adipokinetic or anti-adipokinetic activity focus upon the concept that the roughage fed ruminant is quite similar to the fasting ruminant and superficially to the fasting monogastric. The substrates absorbed by the roughage fed ruminant are quite similar to those which are catabolized during fasting. Therefore, the transition from the roughage fed state is minor relative to the change from the concentrate fed to the fasting state which appears to represent a severe stress situation to the ruminant.

The concept of an adipokinetic hormone secretion during fasting, by monogastrics, has become more or less accepted. Growth hormone (GH) and glucagon have been suggested (among others) in monogastrics as possible adipokinetic peptide hormones since their concentration has been shown to be increased under conditions where the concentration of blood FFA are known to be increased (Roth et al., 1963; Ungar, Eisentraut and Madison, 1963). However, circulating immunoreactive GH levels did not change during fasting in the sheep although immunoreactive insulin levels did decrease (Machlin et al., 1967).

Whether increases in adipokinesis in ruminants during fasting principally represents a stimulation (by hormonal or nervous factors) or a decreased inhibition due to decreased levels of antiadipokinetic hormones is still an inadequately answered question. The presence of large amounts of FFA depressing substances in the urine of fasting sheep which were previously fed concentrate, in contrast to an easily demonstrated net adipokinetic activity in the urine of fasting monogastrics, might be interpreted to mean that inhibition of an existing adipokinetic potential is the principal control over the rate of adipokinosis in the ruminant. The hormones stimulating gluconeogenesis, i.e., glucagon and glucocorticoids, which are adipokinetic, alone and in combination with growth hormone, respectively, may provide a continuing lipolytic potential not present in monogastrics.

Insulin per se may not decrease adipokinesis. Lindsay (1961) demonstrated that the injection of insulin (40 units) into a fasting sheep caused an increase in the plasma FFA which occurred subsequent to hypoglycemia and that only insulin plus glucose depressed plasma FFA concentrations. Similarly, it has been shown that insulin increases plasma FFA in the chicken (Lepkovsky, 1967) and causes an increased release of FFA by perfused rat parametrial fat (Scow, 1967). The great differences between plasma insulin levels as measured by radio immunoassay (10 to 30 µ units/ml) and by the rat diaphragm (10 to 200 µ units/ml) or adipose tissue (50 to 800 µ units/ml) bioassays indicate that substances other than insulin may be involved (Batchelor, 1967) in the limitation of fat mobilization. Insulin-like fractions have been reported to be present in bovine serum albumin and to obscure the effects of known adipokinetic agents in in vitro studies (R. Scow, personal communication). The data presented in figures 1 and 2 show consistency relative to time of maximal excretion. Therefore, it is reasonable to state that this activity is a physiological phenomenon and not an artifact of the isolation procedure.

The observation of Lindsay (1961) that
increases in FFA occur subsequent to insulin induced hypoglycemia suggests that insulin, in ruminants, is dependent upon its glucose effect to affect an antidiopokinetic action rather than possessing an inherent independent "antilypolytic action". Therefore, other substances which are "insulin-like" in adipose tissue could be important to the economy of the ruminant in maintaining energy homeostasis. Additional studies will have to be conducted in greater depth to determine the significance of such a mechanism and to elucidate it completely.

Summary

Studies of the urinary excretion of substances which affect fat mobilization were conducted. The only adipokinetic active substance was of a low molecular weight (~385) and reacted positively to qualitative chemical tests for catecholamines. A net antidiopokinetic or insulin-like activity was found more frequently in the urine. The presence of this activity was dependent upon a prior feeding of a diet containing 50% concentrate. The antidiopokinetic substance(s) were of a molecular size smaller than insulin and larger than Evans blue (985). The significance of these observations is discussed.

Literature Cited


