activity of sheep livers is actually lower than that observed for rats.

Summary. Rat, sheep, and dog liver homogenates and microsomal preparations were assayed for glucuronosyl transferase activity and ability to regenerate UDPGA from glucose. Sheep liver displayed a greater capacity to conjugate H_4E, p-nitrophenol, and phenolphthalein than did similar preparations from dogs and rats. This enhanced ability was attributed to an increase of glucuronosyl transferase activity and the ability to regenerate UDPGA from glucose. In addition, glucose was observed to be a better precursor for regeneration of UDPGA than galactose with sheep homogenate preparations. Dog liver was found to conjugate H_4E poorly, but was able to conjugate p-nitrophenol and phenolphthalein.

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Δ^4-Reductase and Sulfokinase Activities of Rat and Sheep Liver Preparations.* (29321)

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It has been pointed out by other workers (1,2,3) that ruminant animals are normally presented with large amounts of ionone derivatives of dietary origin. These compounds react with many of the colorimetric reagents in tests for steroids(2,4,5) and are known to

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be conjugated and excreted along with steroids by these animals(3,5). Furthermore, ruminant animals have long been noted for the low levels of steroids in their blood, urine, and feces(6). It has previously been shown that the reduction of the Δ^4-3-ketone group of the steroid molecule is the rate-limiting step in the overall process of steroid elimination via excretion in the urine and feces(7,8). It also has been shown that reduction of the Δ^4-unsaturation in ring A of the steroid molecule precedes the reduction of the 3-ketone group and that both must be reduced prior to conjugation of most steroid molecules(8). In addition, some ionone derivatives have a grouping similar to the Δ^4-3-ketone group of steroids(1) which could be reduced to the corresponding tetrahydro derivatives by either the microbial population of the rumen or the liver of these animals.

Since it is possible that ingestion of ionone derivatives from the diet may influence the rate limiting steps in the process of steroid inactivation and conjugation in the liver of these animals, the purpose of this investigation was to see whether either the Δ^4-reductase or the sulfate conjugation mechanism of the liver of sheep differs from that of a non-ruminant, the rat.

Materials and methods. Five western type, female sheep weighing 30-45 kg were maintained on pasture and a prairie hay supplement with water given *ad libitum*. Five mature, female, Holtzman rats weighing 300-360 g, were maintained on laboratory chow and water *ad libitum*. Livers obtained from rats after decapitation and from sheep after electrocution, were immediately chilled, weighed, minced with scissors, and homogenized with Potter-Elvehjem homogenizers. A 30% homogenate (W/V) in 0.15 M KCl containing 0.001 M EDTA, pH 7.0, was prepared for the sulfokinase studies. The temperatures of all preparations were kept between 0-2°C during subsequent steps in the purification of the enzyme. The homogenate was centrifuged for 30 minutes at 105,000 $\times$ *g* in a Spinco model L preparative centrifuge to remove the particulate matter. The supernatant solution thus obtained was subjected to an ammonium sulfate fractionation according to the method of Roy(9). The fraction between 1.7 M and 2.3 M ammonium sulfate was found to contain the bulk of the enzyme activity in both species. The precipitate was dissolved in glass-distilled water at a proportion of 0.7 ml of water per gram of liver. The purified enzyme, if not immediately used, was stored at —20°C until assayed. At such time, the rat liver preparation was diluted one to four(10) with glass-distilled water while the sheep liver preparation was diluted one to six. The assay mixture of Roy(9) was used with an incubation time of 30 minutes for p-nitrophenol and one hour for DHEA‡(10).

Following incubation, the mixture was acidified with 0.25 ml of 0.5 M acetate buffer, pH 4.5, and the total volume brought to 1.5 ml with glass-distilled water. The mixture was boiled for 5 minutes and then cooled. One ml of methylene blue reagent(10) was added and the sulfate-methylene blue complex was extracted with chloroform. The chloroform extract was dried with anhydrous sodium sulfate(9), and the optical densities of the colored solutions were read in a Beckman model DU spectrophotometer. The optical densities at 700 mμ were converted to mμmoles of substrate conjugated by comparing them with appropriate standard curves. All cofactors, substrates, and standards used in the incubations and the preparation of standard curves were obtained from Sigma Chemical Corp.

Enzymic reduction of the Δ^4-grouping of steroid molecules was assayed according to the method of Yates *et al*(11). The enzyme was prepared from the previously described liver samples. Ten grams of liver were homogenized in 75 ml of cold 0.25 M sucrose in glass-distilled water. The mitochondria and cell debris were removed from the suspension by centrifuging the mixture at 10,000 $\times$ *g* for 10 minutes in a refrigerated centrifuge. Following incubation under a nitrogen atmos-

‡ The following abbreviations are used in this paper: DHEA, 5-androsten-3β-ol-17-one; UDPGA, uridine diphosphoglucuronic acid; cortisol, 4-pregnene-11β, 17α,21-triol-3,20-dione; cortisone, 4-pregnene-17α,21-diol-3,11,20-trione; corticosterone, 4-pregnene-11β,20-diol-3,20-dione; EDTA, ethylenediamine tetraacetic acid.

TABLE I. Effect of Dilution on Sulfate Conjugation of Rat and Sheep Liver Preparations.

Dilution	mμmoles of p-nitrophenol conjugated/2 hr	
	Rat	Sheep
0	39	38
1:1	75	84
1:2	118	138
1:3	188	181
1:4	167	**241**
1:5	156	278
1:6	—	258

phere for 6 minutes, the protein was precipitated by the addition of 20 ml of cold dichloromethane (—20°C). The steroids were exhaustively extracted with additional aliquots of dichloromethane and the resulting extract purified with 0.1 N HCl. The dichloromethane was blown off with a stream of nitrogen and the residue taken up in 15 ml of redistilled methanol. Additional tubes similarly prepared were kept at zero degrees during the incubation period and served as controls. The amount of steroid disappearing during incubation was obtained by difference. Optical density readings at 240 mμ wavelength were obtained from the samples with a Beckman DU spectrophotometer.

Statistical comparisons were made with Student's "t" test(12).

Results. The results from the incubation of p-nitrophenol and dehydroepiandrosterone with rat and sheep liver preparations are shown in Tables I and II. Prior to the acceptance of these two substrates and the previously described assay for routine analysis, incubation times, enzyme dilutions, and the stability of the enzyme preparations were checked. Through a series of dilutions (Table I) of the respective enzyme preparations of rats and sheep, an optimal dilution of one part diluted enzyme plus 3 parts glass-distilled water was chosen for the rat, while one part of diluted enzyme plus 5 parts of glass-distilled water was chosen for sheep. In this manner, the inhibitor, presumably ammonium sulfate, was kept at a minimum. The enzyme preparations of both species were assayed before and after storage at —20°C with the storage time kept constant at one week. A small consistent increase in enzyme activity of preparations was observed following freezing; the difference approached significance ($P>0.20$).

From the data in Tables II and III, it can be seen that sheep have a greater ability to conjugate p-nitrophenol than dehydroepiandrosterone. Conversely, rats have a greater ability to conjugate dehydroepiandrosterone. The difference in the ability of the two species to conjugate p-nitrophenol, approached significance at the 90% level. A greater individual variation was observed for the ability of sheep to conjugate p-nitrophenol than was observed for dehydroepiandrosterone. In this respect, several sheep gave very positive assays while others gave only slightly positive results.

TABLE III. Statistical Inferences from the Data in Table I.

Item	Comparison	Statistical probability
Sheep	p-nitrophenol vs DHEA	$P<.010$
Rat	" " "	$P<.001$
p-nitrophenol	Sheep vs rat	$P>.100$
DHEA	" " "	$P<.001$
Liver/body wt ratios	" " "	$P<.001$

When similar liver preparations from rats and sheep were assayed for their ability to reduce the double bond between carbons 4 and 5 of the steroid nucleus (Tables IV and V), it was observed that the Δ^4-reductase activities of these two species paralleled each other (*i.e.*, the rat had the greatest activity

TABLE II. Sulfate Conjugation of p-nitrophenol and Dehydroepiandrosterone by Rat and Sheep Liver Preparations.

Animal	No. of animals	p-nitrophenol*	Dehydroepiandrosterone	Liver/body wt ratios
Sheep	5	114.4 ± 59.6†	21.18 ± 16.47	.0164 ± .0040
Rat	5	61.3 ± 24.7	334.8 ± 28.9	.0302 ± .0020

* mμmoles/hour/0.4 ml of enzyme preparation.
† Mean ± standard deviations.

TABLE IV. Δ^4-3-Keto Reductase Activity of Rat and Sheep Liver Preparations.

Animal	No. of animals	Cortisol*	Cortisone	Corticosterone	Liver/body wt ratios
Sheep	5	14 ± 2.06†	23 ± 7.91	25 ± 7.89	.0164 ±.0020
Rat	5	159 ±24.39	152 ±24.48	180 ±26.40	.0379 ±.0184

* Activity expressed in terms of mμmoles of substrate disappearing per 6 min per unit of enzyme used.

† Mean ± standard deviations.

for conjugation of dehydroepiandrosterone and for formation of the dihydroderivatives of the 3 steroids tested).

Discussion. The data (Tables II and III) indicate that sheep liver has a greater ability to conjugate p-nitrophenol than DHEA. This is the opposite of what was found for these two substances with rat liver. Furthermore, rat liver had a greater capacity to conjugate DHEA than did sheep liver. Although conjugation of p-nitrophenol by sheep and rat liver preparations approached statistical significance (Table III), it is felt that this difference is probably real, since those animals displaying the lowest phenol sulfokinase activity were subjected to a change in diet. This change in diet occurred as the spring grass began to grow with a consequent decrease in consumption of prairie hay.

These data are similar to what has been found for glucuronide conjugation by sheep, dog, and rat livers(3). The authors showed that sheep liver preparations have a greater capacity to conjugate some steroids and phenols (phenophthalein and p-nitrophenol) with glucuronic acid than do dog or rat preparations. This increased ability to conjugate various substrates was attributed to increased amounts of glucuronosyl transferase activity and to a more efficient UDPGA generating mechanism. This species difference was thought to be due to the presence of large quantities of ionone derivatives of dietary origin in the ruminant diet. The greater glucuronide conjugation for both steroids and phenols in sheep(3) and the elevated sulfate conjugation of p-nitrophenol in this investigation can partially be explained on the basis of separate sulfokinases which have been noted for steroids and phenols(13). Although the membrane-bound glucuronosyl transferase enzyme(s) has been liberated by digitonide treatment(14), no one has demonstrated the presence of substrate specific enzymes in this fraction. To further substantiate these claims, the data (Tables IV and V) show that rat liver has much more Δ^4-reductase activity than does sheep liver. Thus, it appears that ionone derivatives ingested by ruminant animals might increase the ability of the liver of these animals to conjugate these materials by increasing the glucuronosyl transferase activity and the ability of this organ to synthesize UDPGA. Likewise, there is an increase in phenol sulfokinase activity but not steroid sulfokinase activity. It is speculated that the low Δ^4-reductase activity observed for sheep could be maintained at a low level to prevent the flushing of the steroids from the blood stream in the case of those steroids which are conjugated with glucuronic acid. In this respect, ruminant animals have long been known for the low levels of steroids in their blood(6). This is especially significant since hepatic Δ^4-3-keto reductase activity is considered to be an important factor regulating adrenal cortical function(15,16).

Other factors also could be responsible for

TABLE V. Statistical Inferences from the Data in Table IV.

Item	Comparison	Statistical probability
Cortisol	Sheep *vs* rat	$P<.001$
Cortisone	*Idem*	$P<.001$
Corticosterone	"	$P<.001$
Liver/body wt ratio	"	$P<.050$
Sheep	Cortisol *vs* corticosterone	$P<.025$
"	Cortisol *vs* cortisone	$P<.050$
"	Cortisone *vs* corticosterone	$P>.500$
Rat	Cortisol *vs* corticosterone	$P<.300$
"	Cortisol *vs* cortisone	$P>.500$
"	Cortisone *vs* corticosterone	$P<.200$

the lower Δ^4-reductase activity observed in sheep livers. In this respect, L. S. Pope (personal communication) has observed a condition in ruminants which were fed diets high in certain grains. These animals showed local foci of bacterial abscesses in their livers. In this respect, Δ^4-reductase is reportedly localized in the reticuloendothelial cells while glucuronosyl transferase is localized in the parenchymal cells(17,18). Therefore, since ruminant animals produce large quantities of small chain fatty acids and absorb very little glucose from the alimentary canal(19), adrenal secretion in these animals may be lowered due to a decreased need for adrenal cortical steroids. Furthermore, the fetal lamb has been reported as having considerable hexose requirements(19,20). Therefore, it is conceivable that these conditions might in some way give rise to a lowered reticuloendothelial content of liver which is reflected in the Δ^4-reductase activity as well as the susceptibility to localized foci of bacterial abscesses in the livers of these animals.

Although a 2-fold greater liver/body weight ratio was observed for rats, this weight difference should be minimized since ruminant animals have a larger alimentary tract than do rats. This difference in weight would almost account for the liver/body weight ratios observed in this investigation.

Summary. Sheep liver preparations demonstrated a greater capacity to conjugate p-nitrophenol than dehydroepinandrosterone based on an equivalent unit of liver. The opposite was observed for rat liver. Rat liver was also able to conjugate more dehydroepiandrosterone than was sheep liver. This species difference in the ability to conjugate various substrates was attributed to the presence of ionone derivatives in the feed of ruminant animals. Rat liver displayed a greater Δ^4-reductase activity than did sheep liver for cortisol, cortisone, and corticosterone. This species difference was attributed to dietary differences plus other metabolic differences known to exist in ruminant animals. The results of this investigation were discussed in terms of what has been previously observed for glucuronosyl transferase activity for sheep, dog, and rat.

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