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Fate of Radioactive Melengestrol Acetate in the Bovine

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Melengestrol acetate (17-hydroxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione acetate; MGA; The Upjohn Co.) is an effective oral progestational agent. Studies with orally fed MGA, 21 days for tritium and 7 days for carbon-14 labeled, showed that the radioactivity was eliminated from the heifers via the feces and urine in a 6:1 ratio. Fat contained the highest concentration of the "marker compound" MGA (83% of the total radioactivity or 6 ppb) and was established as the "target tissue". Liver, with the highest level of radioactivity, contained only 29% MGA (3.5 ppb of [^{3}H]MGA) or 37% MGA (3 ppb of [^{14}C]MGA). Radioactivity in the muscle was in most cases below the limit of detection, i.e., 0.5 ppb. The non-MGA fraction in both the tritium and carbon-14 studies contained numerous metabolites, all below 1 ppb.

Melengestrol acetate (17-hydroxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione acetate; MGA; The Upjohn Co.) has been shown to be an effective oral progestational agent (Zimbelman and Smith, 1966a,b) when incorporated into the diet of feedlot heifers at levels up to 0.5 mg head^{-1} day^{-1} to increase feed efficiency and rate of gain (Bloss et al., 1966). MGA was approved in Feb 1968 by the Food and Drug Administration (FDA) for use in feedlot heifers (*Fed. Regist.*, 1968, 1969) with a dose range of 0.25–0.50 mg head^{-1} day^{-1}.

Since MGA is administered to food-producing animals for 140–185 days, radioactive studies were carried out to determine the fate of MGA in feedlot heifers. Tracer studies were designed (1) to determine the distribution of radioactive MGA in various tissues, (2) to determine whether MGA was present intact in fat, muscle, kidney, and liver, (3) to determine whether the present assay method (Krzeminski et al. 1976) would extract and quantitate all of the MGA present in tissues, (4) to identify the "marker compound" and "target tissue", and (5) to determine the extent of metabolism in the target tissue.

EXPERIMENTAL SECTION

Radioactive [^{3}H]MGA Dose. Two-dose lots of tritium-labeled MGA (Figure 1) (500 mCi/mM; New England Nuclear) were prepared by dilution with unlabeled MGA. This gave a daily dose for one heifer of 105.6 μCi, 0.508 mg/capsule (sp act. 4.79 × 10^2 dpm/ng), and for the other two heifers a daily dose of 102.2 μCi, 0.558 mg/capsule (sp act. 4.06 × 10^2 cpm/ng). All doses were prepared by pipetting aliquots of labeled MGA in acetone onto sugar contained in gelation capsules.

Radioactive [^{14}C]MGA Dose. Carbon-14-labeled MGA (Figure 1) (55.4 mCi/mM; New England Nuclear) was diluted with unlabeled MGA and the specific activity determined by GLC/EC measurement and scintillation counting. The total amount of labeled [^{14}C]MGA was divided among seven capsules to provide a daily dose of 254 μCi, 0.495 mg/capsule (sp act. 1.14 × 10^2 dpm/ng).

Animal Treatment ([^{3}H]MGA). Three young Angus–Hereford heifers were housed in individual box stalls and fed 8 kg of a complete ground ration (nonmedicated) head^{-1} day^{-1} plus 1 lb of the supplement (medicated, 0.5 mg of MGA/lb) head^{-1} day^{-1} for 4 months. The heifers were moved sequentially into the metabolism stall, acclimated for 1 week, and dosed daily with [^{3}H]MGA capsules for 21 days. While on the [^{3}H]MGA treatment, each heifer was fed 4.0 kg of the complete ration (nonmedicated) head^{-1} twice daily. Water was provided ad libitum. Rectal temperature and general physical condition were monitored daily.

Animal Treatment ([^{14}C]MGA). One young Angus–Hereford heifer was housed in an individual box stall and

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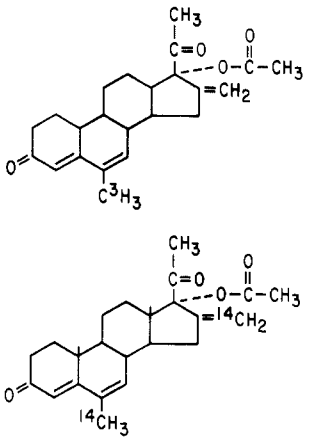

Figure 1. Positions of tritium and carbon-14 labeling in the MGA molecule.

fed 5.0 kg of the complete ground ration (nonmedicated) $head^{-1}$ day^{-1} plus 0.454 kg of the medicated supplement $head^{-1}$ day^{-1} which provided 0.5 mg of MGA $head^{-1}$ day^{-1}. MGA feeding was continuous for 4 months prior to the initiation of the study. The MGA diet was discontinued 15 days prior to [^{14}C]MGA treatment. The selected heifer was moved into the metabolism stall and acclimated for 6 days. Then [^{14}C]MGA was administered daily for 7 days via a gelatin capsule per os. While on the [^{14}C]MGA treatment, the heifer was fed 5.5 kg $head^{-1}$ day^{-1} complete ground ration (nonmedicated). Water was provided ad libitum. Rectal temperature and physical conditions were monitored daily.

Sample Collection. *Urine.* In the tritium study, urine, feces, and stall rinses were collected together until 10 days prior to the scheduled slaughter at which time a Foley-type indwelling rubber catheter was passed into the urocyst (Bordex Foley catheter, size 30 fr, 30-mL balloon, No. 1664V). Extra rubber tubing was spliced into the catheter and the urine collected in an Erlenmeyer flask.

In the carbon-14 study, the catheter was inserted 96 h prior to treatment with [^{14}C]MGA. The urine was collected in an Erlenmeyer flask.

Feces. Daily feces were transferred from the collection trap to a 44-q bucket and weighed. The stall grates were brushed and rinsed, and the rinsings added to the feces. The slurry was weighed and thoroughly mixed with a mixer (Lightnin) for at least 0.5 h, and a 1-qt aliquot removed. This aliquot was homogenized with an Eppenbach mill, and subsamples were weighed out for combustion and counting.

Tissues. Each heifer was slaughtered by electrocution 6 h after the last capsule had been administered. The heifer was processed in an abattoir. The gastrointestinal tract membranes were stripped of fat and rinsed with water. All tissues of large size were ground in a Kitchen Aid meat grinder (Model K-5A) through a face plate with a 4 mm diameter opening. A 10-g aliquot of the ground tissue was homogenized with 50 g of water in the 100-mL glass cup of a Virtis-45 homogenizer.

Determination of Radioactivity. Triplicate homogenates of tissue samples (0.5–0.9 g) were weighed into combustion bags and dried for the Schoniger combustion. In the case of fat and bone marrow samples, 10 g of tissue was homogenized with 50 g of hexane and aliquots were placed in counting vials.

Whole blood, feces, bile, and gastrointestinal contents (0.5–0.9 mL) were pipetted into combustion bags, air-dried, and combusted by the Schoniger procedure. Rumen samples (0.2 g) were digested directly in counting vials with 0.2 mL of 60% perchloric acid and 0.4 mL of 30% hydrogen peroxide at 70 °C for 1 h in tightly stoppered scintillation vials.

All tritium samples were combusted by the Schoniger procedure and counted in Diotol solution. With each day's run, a [^{3}H]MGA standard was counted by combustion and by direct addition to Diotol to determine the efficiency. This factor was used to correct the dpm calculations for combustion losses. All combusted carbon-14 samples were counted in 5:10 phenethylamine-fortified toluene solution (450 mg of POPOP and 22.7 g of PPO per gal of toluene). All urine, aqueous samples, and rumen samples were counted in an Insta Gel emulsifier. The hexane solutions of fat and bone marrow were counted in Diotol. Counting was done in a Packard Tricarb scintillation spectrometer, Model 3375 or Model 3330. Either tritiated water or carbon-14-labeled toluene was used as the internal standard for aqueous or nonaqueous samples, respectively. The samples were first counted for three 10-min intervals, the internal standard was added, and then the samples were recounted for two 1-min intervals. Tissue samples with very low cpm were counted for longer periods (3 × 50 min) on the first three counting cycles.

Tritiated Water Determination. Twenty-five grams of each tissue (muscle, liver, and kidney) was mixed with 25 g of water and homogenized to a uniform consistency in a Waring Blendor. Approximately 2 g (accurately weighed) was placed in a 20-mL distillation flask. The sample was frozen onto the inside surface of the flask with the aid of a solid CO_2–alcohol bath. Then a 50-mL round-bottom flask was attached to the distillation flask, and the flask immersed in another solid CO_2–alcohol bath and vacuum applied. The distillation flask was allowed to warm up at air temperature, and the water distilled over into the cold receiver flask. The frozen distillate was thawed and quantitatively transferred to a scintillation vial, and the radioactivity determined. For fat, the addition of water was omitted; instead the solid ground fat (2 g accurately weighed) was placed directly into the distillation flask and distilled as above. For urine, 2 mL was pipetted directly into the distillation flask, frozen, and distilled as above. For rumen fluid, 1.0-mL aliquots were used.

Fat Extraction. Twenty-five grams of fat was extracted with hot hexane and partitioned between hexane and acetonitrile. The MGA fraction was isolated on a Florisil column by elution with 8:2 hexane–acetone and quantitated by electron capture gas chromatography (Krzeminski et al., 1976).

Extraction of Liver. Twenty-five grams of frozen liver was placed into a Waring Blendor jar and allowed to thaw for 10–15 min, then 150 mL of 2:1 methanol–chloroform added, and the mixture blended for 3 min. The mixture was centrifued and the liquid decanted. The solids were reextracted 2 more times and then dried for combustion. The extract was dried on an all-glass rotary evaporation unit (Buchi). The condensate was counted for radioactivity; the solid residue from the methanol–chloroform fraction was extracted sequentially with hexane, acetonitrile, water, and methanol. The hexane fraction was partitioned between acetonitrile and the lower acetonitrile phase dried on a rotary evaporator. One aliquot was chromatographed on a 400 × 19 mm i.d. Florisil column by the MGA procedure to obtain the 8:2 hexane–acetone eluate. The column was then eluted with 200 mL of water, followed by 200 mL of 1:1 methanol–water. Another aliquot was dissolved in 95:5 hexane–acetone, then methyl red and isatin were added, and the mixture was chroma-

tographed on an LH-20 column.

Seventy-five to one hundred grams of LH-20 was slurried in a 85:15 benzene–methanol mixture and allowed to stand for 1 h. It was then packed in a glass column (SR 25/45), allowed to settle, and washed with 500 mL of the 85:15 benzene–methanol mixture (Drewes and Kowaski, 1974). The sample was introduced via a three-way valve at the bottom of the column. A Milton Roy (Model No. 396-31) pump was set at 1 mL/min, and 100-drop fractions were collected.

Thin-Layer Chromatography. Aliquots of each fraction were placed on a silica gel TLC plate (250 μm), and 10 μL of a MGA standard (1 μg/mL) was placed on top of the spot as the reference for UV visualization. The plates were placed in a tank saturated with 1:1 methanol–chloroform and developed until the solvent front had migrated 1 cm above the origin. The plates were then removed and allowed to dry for 15.0 min and developed for a total of 14 cm in a tank saturated with 75:25:5 benzene–ethyl acetate–methanol (solvent system I) or methanol (solvent system II), or 65:20:10 benzene–ethyl acetate–methanol (solvent system III). The plates were then dried. One-centimeter sections were scraped from the plate and placed in counting vials, and 0.2 mL of water and 15 mL of Diotol added and counted to obtain DPM. A computer program was used to generate histograms. Autoradiograms were obtained by placing the dried TLC plates on no-screen X-ray film for 80–100 days, and the images intensified by photography on high-contrast film.

Calculations. Assuming under dynamic equilibrium conditions that the heifer has two doses in its metabolic pool and that the hot dose ([^{3}H]MGA) is constant, the following expression defines the amount of cold MGA present after and k number of doses, i.e., the number of hot doses to displace the cold to the Y level.

$$Y = (\text{cold}/\text{total})^k \times \text{cold}$$

Y = amount of cold remaining in the pool at the kth dose, k = number of times hot was added, cold : amounts of cold MGA in the pool at the time the hot treatment is started (assumed to be 2 times the dose), and total = amount of cold plus [^{3}H]MGA at the time the hot treatment is started (3 times the dose). If Y = 1 ng, the dose = 0.5 mg of MGA head^{-1} day^{-1}; therefore k = 17 doses.

RESULTS AND DISCUSSION

Excretion. The studies were carried out with four heifers, three on tritiated MGA and one on carbon-14-labeled MGA. All animals were primed on cold MGA for 4 months prior to the administration of radioactive MGA. The radioactivity excreted after the administration of ~0.5 mg head^{-1} day^{-1} tritiated MGA ([^{3}H]MGA) to three heifers for 21 days was ~72% via the feces and urine. The low accountability in the tritium studies was later ascribed to the air drying of excrement samples for Schoniger combustion and loss of tritiated water.

A similar excretion pattern was obtained for the single heifer on the [^{14}C]MGA. Catherization of the heifers for the last 10 days of the tritium study and for all 7 days of the carbon-14 study showed that 10.1% (tritium) and 8.3% (carbon-14) of the radioactivity was excreted via the urine. Analysis of the feces taken from a group of feedlot heifers fed 0.5 mg head^{-1} day^{-1} *unlabeled* MGA showed that ~10–17% of the administered dose passed through the gastrointestinal tract unabsorbed (Davis, 1973).

Radioactive Residues. Heifers slaughtered 6 h posttreatment showed that the highest levels of radioactivity were observed in the bile (Table I): 75.1–142.5 ppb of MGA equiv for the tritium study and 97.6 ppb for the carbon-14 study. Previous bile cannulation studies showed bile to be a primary excretion route which closely paralleled the total daily fecal output (Neff, 1964).

Table I. Concentration of Apparent MGA in Tissues and Organs[a]

	^{3}H heifer				^{14}C heifer
	ppb	ppb	ppb	av ppb	ppb
organs					
liver	12.0	15.4	9.0	12.1	8.2
tongue	2.0	2.1	1.2		1.0
kidney	1.7	1.8	1.2	1.6	1.3
spinal cord	1.6	1.2	0.7		NS[b]
heart	1.5	1.0	0.8		NS
posterior hypothalmus	1.1	1.0	–		NS
lung	1.1	0.9	0.5		NS
anterior hypothalmus	0.9	0.4	–		NS
brain	0.9	0.7	0.6		NS
spleen	0.8	0.9	0.6		NS
eye	0.7	1.2	0.1		NS
bladder	0.3	0.5	–		NS
oviduct	4.2	3.8	3.0		1.4
cervix	1.5	1.4	0.9		0.7
uterus	1.4	4.6	0.7		1.1
vagina	1.1	0.8	2.0		–
ovaries	1.0	0.6	0.5		–
glands					
thymus	4.5	1.5	2.5		0.9
adrenal	3.0	4.3	0.1		1.7
salivary	2.6	4.7	1.6		1.0
pancreas	1.9	1.6	1.3		1.0
lymph	1.5	1.1	0.8		NS
thyroid	1.3	0.8	2.0		1.1
mammary	1.0	3.8	2.2		3.2
pituitary	0.9	0.1	1.1		NS
tissues					
fatty bone morrow	7.9	9.0	7.6		2.1
perirenal fat	7.5	7.7	8.0	7.7	3.6
visceral fat	7.5	7.5	7.7		2.9
auxiliary fat	6.0	6.6	7.9		2.9
red bone marrow	6.0	2.7	2.4		1.9
muscle	0.6	1.0	0.5		NS
digestive tract contents					
rumen-reticulum	7.5	9.3	8.4		14.6
omasum	5.2	14.4	5.3		39.0
abomasum	8.8	10.7	6.1		16.8
duodenum	0.5	6.0	1.2		4.1
jejunum-ileum	17.4	37.3	15.6		44.8
large intestine	24.1	38.2	10.2		60.7
bile	142.5	120.7	75.1	112.8	97.6
digestive tract membranes					
rumen-reticulum	5.8	3.5	3.5		1.8
omasum	6.2	2.9	–		2.8
abomasum	3.9	2.6	2.8		1.3
duodenum	–	8.8	2.0		4.9
jejunum-ileum	5.4	4.6	3.2		6.4
large intestine	11.1	8.2	4.5		7.0

[a] Radioactivity was calculated as MGA equivalence from the original specific activity. [b] NS = not significant (when cpm values were less than background plus 3 SD).

Of the four tissues of concern (fat, liver, muscle, and kidney), liver had the highest level of total radioactivity, i.e., MGA and its metabolites (12.1 ppb of [^{3}H]MGA equiv) (Table I). Fat was next with 7.7 ppb and then kidney with 1.6 ppb. Muscle, the most important from the consumer standpoint, was very low with 0.7 ppb. Both the tritium and carbon-14 data were in good agreement for the other tissues and organs.

MGA Analysis. For determination of whether all of the radioactivity in fat, liver, kidney, and muscle was due to MGA, the tissues were subjected to the AOAC procedure (Krzeminski, et al., 1976) (Figure 2). The results

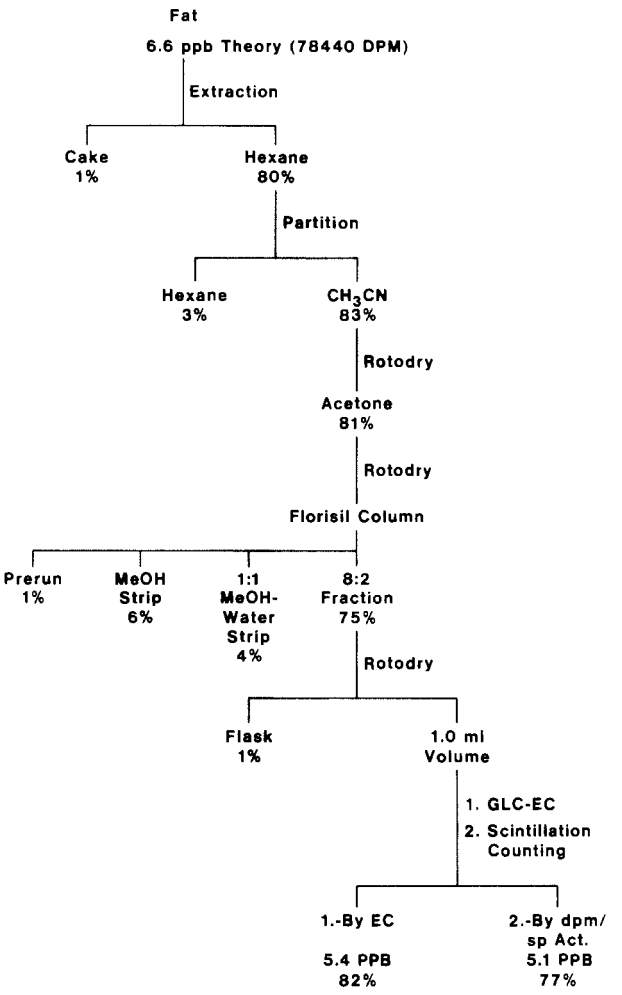

Figure 2. Distribution of radioactivity in the various steps of the MGA method for fat.

Table II. Percent Radioactive MGA in Tissues[a]

	[^{3}H]MGA, %			av, %	[^{14}C]MGA, %
fat	78	86	94	86	75
liver	30	30	28	29	37
muscle	31	72	40	48	45
kidney	24	34	126[b]	29	30

[a] Amount in the 8:2 hexane–acetone fraction from Florisil columns (MGA method) as determined by scintillation counting. [b] Not used in calculation of the average.

showed fat had the highest percentage of intact [^{3}H]MGA (86%) while liver had less (29%) (Table II). Muscle and kidney had 48 and 29%, respectively. On the basis of the data from Table I and II, fat contained 6.6 ppb of [^{3}H]-MGA while liver contained 3.5 ppb of [^{3}H]MGA.

TLC of the 8:2 hexane–acetone eluates and radioautography for fat, liver, and muscle samples from the [^{14}C]MGA study showed a single band at the R_f of the [^{14}C]MGA standard for these tissues; kidney had an additional band at an R_f lower than that of the MGA standard.

Metabolites. The non-MGA fraction routinely discarded in the MGA procedure was investigated by TLC techniques to determine the number and amount of radioactive metabolites. Liver from the tritium study was extracted with chloroform–methanol to remove polar metabolites and the processed as in the MGA procedure (Figure 3). The 8:2 hexane–acetone and methanol eluate fractions from Florisil were then chromatographed on silica gel plates. TLC-generated histograms showed [^{3}H]MGA (31%) to be the only radioactive peak in the 8:2 fraction.

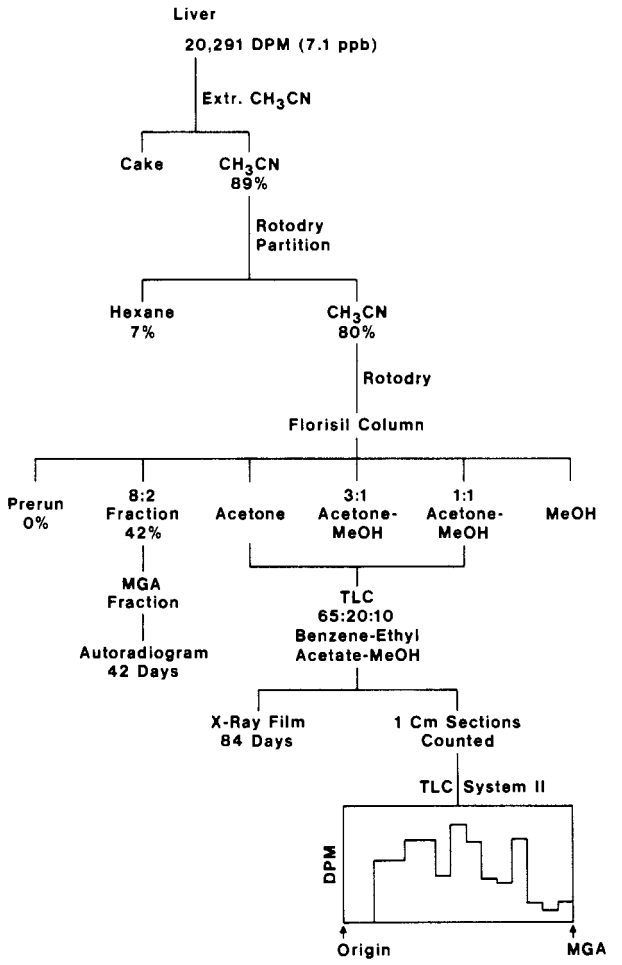

Figure 3. Fractionation of liver tissue from a heifer treated with [^{3}H]MGA.

The TLC histogram of the methanol fraction contained three major peaks, none of which exceeded 1 ppb. Column chromatography of the acetonitrile fraction on an alternate LH-20 column, although it separated [^{3}H]MGA (32%) from the polar metabolites, did not resolve the polar metabolites. Use of dye markers on the LH-20 column suggested the metabolites may be of the dihydroxy type.

Liver from the carbon-14 study was fractionated according to the MGA procedure (Figure 3). Histograms of the TLC plate showed three major metabolite fractions similar to those found in the histograms from the tritium study. Autoradiograms of the TLC plate showed three radioactive bands for metabolites more polar than MGA (Figure 3); all three bands were less than 1 ppb of [^{14}C]-MGA equiv. Levels of metabolites at the 1-ppb level or lower were quantitatively too small to characterize further.

Previous studies with tritiated MGA in women and rabbits (Cooper and Kellie, 1964; Cooper, 1967) showed that MGA was metabolized to 22 metabolites, 1 of which was identified as the 6-hydroxymethyl metabolite. Consequently, a metabolic loss of the tritium from the 6-methyl position was not unexpected in our heifer studies. Tritiated water was found in the various tissues, organs, and fluids of the heifer treated 21 days and slaughtered 6 h posttreatment (Table III). The data showed that there was a loss of tritium from the 6-methyl position with the production of 5.6% tritiated water in liver, 3.4% in the kidney, 51.3% in the muscle, 8.4% in the urine, and 28.5% in the rumen fluid. Although the relative amounts of tritiated water varied due to the other metabolites present in the samples, the tritiated water was uniformly distributed in all tissues, except fat, at ~300 dpm/ng of tissue.

Table III. Tritiated Water in the Heifer at Slaughter

tissue	dpm/g of wet tissue[a] combustion	^3H_2O	total[b]	% ^3H_2O of total radioact
liver	5140	303	5443	5.6
kidney	601	329	930	35.4
muscle	288	303	591	51.3
fat	3158	4	3162	0.1
urine	–	353	4186	8.4
rumen	–	228	803	28.5

[a] Average of three heifers; 6 h posttreatment. [b] Schoniger value (after drying and combustion) plus ^3H_2O obtained by distillation of ^3H_2O from frozen samples. Urine was counted directly in Diotol. Rumen fluid was digested by the perchloric acid technique.

SUMMARY AND CONCLUSION

Tissues from heifers fed nonradioactive MGA for several months (~0.5 mg head^{-1} day^{-1}) followed by radioactive MGA and then slaughtered on-treatment contained varying amounts of radioactivity (MGA and metabolites). The highest level of radioactivity was found in liver. The levels in liver were based on three 21-dose tritium studies (12.1 ppb of MGA equiv) and on one 7-dose carbon-14 study (8.2 ppb of MGA equiv). The average MGA per se was 29% for the tritium study (3.5 ppb) and 37% for the carbon-14 study (3.0 ppb).

Peripheral fat contained an average of 7.7 and 3.6 ppb of MGA equiv of radioactivity for the tritium and carbon-14, respectively, of which nearly all was intact MGA. The average MGA per se level in fat was 86% (6.6 ppb) and 75% (3.0 ppb) for tritium and carbon studies, respectively. The higher level of MGA in fat relative to liver established fat as the "target tissue".

The non-MGA fraction in liver was separated on thin-layer plates of silica gel and found to be composed of three or more metabolites, none of which were greater than 1 ppb or 10% of the total radioactivity in the sample.

These data established MGA as the "marker compound" and demonstrated that fat, because of its high concentration of MGA, was the "target tissue". Currently, this method is applicable for the analysis of MGA in fat, liver, kidney, and muscle that is sensitive to 10 ppb which has AOAC final action status. Since there were no residues of metabolites greater than 1 ppb, no other residue methods were required.

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Preparation and Characterization of $FeH_3(PO_4)_2 \cdot 2CO(NH_2)_2$

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TVA has been investigating the production of urea phosphate in a crystallization process. A contaminating precipitate that was detected in the product was identified as poorly characterized crystals of the compound $FeH_3(PO_4)_2 \cdot 2CO(NH_2)_2$. Homogeneous crystals of the compound were prepared and its properties were characterized. These fine-grained crystals precipitate when the iron content of the recycle urea phosphate mother liquor increases to ~0.7% iron (1.00% Fe_2O_3) and are retained in the purified urea phosphate product, thus limiting the effectiveness of the process. Tests have shown that increasing the acidity of the urea phosphate process with either phosphoric or sulfuric acid will increase the solubility of the iron salt, decrease the solubility of urea phosphate, and extend the useful life of the urea phosphate recycle liquor. Studies on the spent liquor from urea phosphate purification have shown that ammoniation to pH 6 will allow recovery of a highly purified liquid fertilizer of 14–12–0 grade and a solid fraction containing the impurities, including uranium and heavy metals, at a concentration level suitable for separation and purification of the individual components.

Precipitated Solids in Recycled Mother Liquor from Urea Phosphate Process. A poorly characterized crystalline material, first detected as a solid component in the urea phosphate purification process, has been prepared as a homogeneous product from simulated urea phosphate mother liquor (Tennessee Valley Authority, 1978) to characterize its precise chemical and physical properties.

The original solids as precipitated from recycled mother liquor from the urea phosphate process were filtered, washed with acetone, and air-dried at ambient temperatures. The chemical analyses of solids from three different mother liquors made from commercial acids are presented

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