Table I. Relative Effects of Temperature and of Wilting on Loss of Ascorbic Acid in Kale during First 2 Days in Storage

Rate of Wilting	Average Loss per Hour,[a] %		
	32° F.	50° F.	70° F.
Slow	0.05	0.32	1.27
Moderate	0.08	0.33	1.45
Rapid	0.11	0.69	1.85

[a] Percentage of ascorbic acid present at time tissue was placed under experimental conditions, calculated for 40-hour storage period.

Each of the 50-gram replicates was extracted with 350 ml. of 0.4% oxalic acid plus 1 ml. of water for each gram of weight lost during the storage period. The 10 replicates were averaged and the ascorbic acid is reported as the percentage lost since the test began. Analysis of variance was used to determine differences required for significance.

Results and Discussion

The effects of wilting and of temperature on the loss of ascorbic acid are shown in Figure 1. Wilting hastened the loss of ascorbic acid in kale, and the differences are statistically highly significant at each of the three temperatures tested. Spinach, collards, turnip greens, and rape were similarly affected and gave curve patterns much like the pattern for kale.

For cabbage the pattern is somewhat different. Normally cabbage loses both ascorbic acid and moisture less rapidly than most leafy vegetables, but when the leaves were separated and the conditions were favorable for wilting, the loss of moisture was fairly rapid and significant differences between treatments were observed. Loss of ascorbic acid was relatively slow, and there was little difference in the rate of loss between the slight-wilting and the moderate-wilting lots. Only in rapid-wilting lots did loss of moisture greatly increase the rate of loss of ascorbic acid.

Snap beans lost moisture rather slowly, and wilting conditions had relatively little effect on the rate of loss of ascorbic acid. Only at 32° F. were there consistent and significant differences in ascorbic acid contents of lots stored under the different wilting conditions; here the differences may have been indirectly influenced by chilling injury, which occurs when snap beans are stored at this temperature.

Wilting thus tends to increase the rate of loss of ascorbic acid in leafy vegetables, but it is of much less importance than unfavorable temperatures (Table I). With kale an increase from little or no visible wilting to a 28% average loss in weight within 2 days resulted in an average increase in loss of ascorbic acid of 60% (from 0.92 to 1.48 mg. per 100 grams per hour). But an increase in temperature from 32° to 70° F. resulted in an average increase from 0.12 to 2.60 mg. per 100 grams per hour, or over 21 times as much. On this basis and with slight to moderate wilting, kale would lose about 40% of its ascorbic acid content if held at 32° F. for 3 weeks, at 50° F. for 4 days, or at 70° F. for 1 day.

Humidity conditions are of greater importance in the preservation of ascorbic acid in vegetables subject to rapid loss of moisture and visible wilting than in those relatively resistant to wilting. As shown in Figure 1, the ascorbic acid content of snap beans was much less affected by humidity conditions than that of green leafy vegetables such as kale. As reported earlier (*4*) humidity conditions caused significant differences in the loss of weight in sweet potatoes, but had little direct effect on the loss of ascorbic acid.

Even though low temperatures are conducive to the preservation of vitamin C in most products, temperatures sufficiently low to cause chilling injury in susceptible products should be avoided as harmful, not only to the product, but also to the vitamin C content.

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ANIMAL GROWTH PROMOTERS

Metabolism of Tritium-Labeled Diethylstilbestrol by Steers

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THE DISCOVERY that adding stilbestrol (α, α′-diethylstilbenediol) to the feed of fattening cattle improves the rate of gain and feed efficiency (*7*) has led to its widespread use in cattle rations. While methods of adapting stilbestrol feeding to various cattle programs have been studied in a multitude of feeding trials, a lack of fundamental knowledge concerning the metabolism of stilbestrol by cattle has made the interpretation of some experimental results difficult.

Considerations of human health have made it necessary to determine whether significant amounts of estrogenic residues are present in the meat of stilbestrol-fed animals. The usefulness of conventional chemical assays in the study of stilbestrol metabolism by ruminants under practical conditions is limited, because of the very small quantities of the estrogen normally administered. However, the high estrogenic activity of stilbestrol has been utilized in biological assays involving in most instances a measurement of the response in weight of the mouse uterus. Bioassays offer a sensitive means of detecting estrogenic activity, but are subject to the effect of other compounds in the tissues which may enhance or depress the response. In the present study the metabolism of stilbestrol by the steer and the occurrence of tissue residues have been studied by tritium labeling. The use of radioactive stilbestrol provides a sensitive tool for the estimation of tissue levels, rates of excretion, and detection of metabolites.

Experimental Procedure

Animals. The experimental animals were two 800-pound yearling Hereford steers which had been fed ground shelled corn, soybean oil meal, and alfalfa hay

A study has been made of the distribution of radioactivity in the tissues and excreta of a bile-fistulated steer after a single small oral dose of tritium-labeled stilbestrol, and of an intact steer after stilbestrol administration for 11 days. Approximately 20% of the administered radioactivity was excreted in the urine and 30% was recovered from the feces. Only traces of activity were found in the bile of the fistulated animal. Separation of the radioactivity into free phenolic and conjugated fractions revealed that stilbestrol was present in urine and bile primarily in conjugated form. The radioactive phenolic material was identified as stilbestrol, and its concentration in parts per billion was 0.30 for lean meat, 0.35 for fat, 9.12 for liver, and 4.15 for kidney.

ad libitum. A bile fistula was prepared in one steer while the other was left intact.

Treatments. On the day following the bile-fistula preparation both steers were placed in metabolism stalls in a controlled-temperature room where a temperature of 72° F. was maintained. During a 13-day recovery period, the bile-fistula steer was injected intramuscularly with 3,000,000 units of sodium penicillin daily. Water was offered twice daily, and feed was gradually increased until the daily feed consumption was 5 pounds of alfalfa hay, 4 pounds of ground shelled corn, and 1 pound of soybean oil meal. In order to avoid depletion of bile salts, the bile-fistula steer was drenched daily with 40 grams of commercial bile salts in distilled water.

The stilbestrol was ring-labeled with tritium by an exchange reaction at the carbon ortho to the hydroxyl group and had a specific activity of 33 μc. per mg. This material was weighed to the nearest 0.003 mg. and dissolved in chloroform. Enough chloroform solution to provide 10 mg. of stilbestrol per pound was mixed with soybean oil meal which was subsequently dried at 130° F. The bile-fistula steer received 1 pound of this soybean oil meal at the beginning of the experiment and unsupplemented soybean oil meal during the remainder of the collection period. The intact steer was fed 1 pound of soybean oil meal containing 10 mg. of radioactive stilbestrol daily in two equal feedings over an 11-day period, except for the second day when this animal refused feed and water. Otherwise both steers were fed and watered at 8:00 A.M. and 5:00 P.M. daily. The intact steer was slaughtered at the end of the twelfth day after feed had been withheld for 27 hours.

Collection and Storage of Samples. Urine was collected with an adaptation of the harness described by Horn, Ray, and Neumann (*7*) at 6-hour intervals for the first 48 hours and then at 12-hour intervals until the conclusion of the test. After each collection period the urine was measured, placed in a brown glass bottle, and stored at 0° F. It was later prepared for analysis by thawing and filtering through glass wool.

Bile was collected in a plastic test tube taped to the collection harness. The collection periods for bile were 3 hours each for the first 48 hours and 6 hours each for the remainder of the experiment. Bile samples were stored at 0° F.

The feces were collected directly during 6-hour periods for the first 48 hours and during 12-hour periods thereafter. Fecal samples were weighed, dried at 130° F., ground in a Wiley mill, and stored at room temperature in brown glass bottles.

The longissimus dorsi muscle (lean meat), liver, heart, kidneys, and samples of internal fat, blood, and bile were taken from the intact steer at the time of slaughter. The solid tissues were finely ground and placed in glass bottles. All samples were stored at 0° F. until the time of extraction. Aliquots of the contents of the rumen, small intestine, and large intestine were also taken and handled in the same manner as the fecal samples.

Extraction Methods. The total activity of each urine and bile sample was determined by direct plating. The free and conjugated forms of stilbestrol were separated by the method of Teague and Brown (*12*), in which the glucuronide is removed from an ether extract of the sample using bicarbonate solution and is then recovered in ether following acidification. The radioactivity present in each fraction was determined by plating aliquots of the three most active samples of urine. Bile taken from the gall bladder of the intact steer at the time of slaughter was also fractionated. No attempt was made to fractionate the small amount of radioactivity collected in the bile of the fistulated steer.

Feces were assayed by placing 50-gram aliquots of the dried, ground feces in a Soxhlet extractor and extracting for 24 hours with absolute ethyl alcohol, then for 48 hours with ethyl ether. These extracts were stored at 0° F. Later, the alcohol and ether extracts were warmed to room temperature, suction-filtered with ether washing, combined, and concentrated under vacuum. The concentrate was plated directly to determine total activity. When radioactive stilbestrol was added to two portions of inactive feces, which were handled in the same manner as experimental samples, 73 and 83% of the added radioactivity was recovered by this procedure. Separation of the free and conjugated material was accomplished by the method used for urine, beginning with the initial extraction with saturated sodium bicarbonate solution.

The samples of lean meat, liver, heart, and kidneys were assayed by a modification of the method described by Merker, Edwards, Andrews, and Christian (*9*). After the ground tissues were thawed, 250-gram samples were placed in Soxhlet extractors and extracted for 48 hours with absolute ethyl alcohol and then for 72 hours with ethyl ether. These extracts were stored at 0° F. until analysis. The alcohol extract was warmed to room temperature and extracted five times with one volume of ether. The ether-insoluble material then was mixed with 2 volumes of water and 10 ml. of 85% phosphoric acid and filtered under vacuum. The acid filtrate was extracted once with 0.4 volume and twice with 0.2 volume of ether. The ether extracts were combined and added to the ether extract of the original tissue. The conjugated fraction was removed from the ether extracts by the method used for urine. The free phenolic material then was removed by extracting twice with 0.1 volume and once with 0.05 volume of 2*N* sodium hydroxide. These extracts were combined, made acid to Congo red with 9*N* phosphoric acid, and extracted three times with 0.4 volume of ether. The ether was pooled, concentrated under vacuum, and transferred to a volumetric flask prior to plating and counting. Determinations were made on two samples of lean meat and one sample of each of the other tissues. When radioactive stilbestrol was added to meat which was handled and stored in the same manner as the experimental samples, the above procedure yielded 98% of the added radioactivity in the phenolic fraction.

A 250-gram sample of fat was dissolved in ether, filtered, and assayed in the same manner as the ether extract of lean meat; 100 ml. of blood from the intact steer was analyzed by the method described by Teague and Brown (*12*).

Counting Method. Radioactivity was measured by solid counting using a Tracerlab SC-16 windowless Q-gas flow counter and a Nuclear Model 163 scaling unit. Three aliquots from each sample or extract were plated on previously weighed stainless steel planchets. The counting time for each planchet was sufficient to give a probable counting error of less than 5% (*3*). Unless variation

Table I. Recovery of Radioactivity from the Feces, Urine, and Bile of a Bile-Fistula Steer Given a 10-Mg. Oral Dose of Tritium-Labeled Stilbestrol

Collection Period, Hours	% of Dose[a]		
	Feces	Urine	Bile
0– 6	0	0.2	0
6– 12	0.3	3.1	0
12– 18	1.1	2.1	0
18– 24	2.2	2.0	0
24– 30	4.1	2.9	0
30– 36	3.0	2.1	0.03
36– 42	3.6	3.8	0.03
42– 48	2.8	0.8	Trace
48– 60	7.1	2.7	Trace
60– 72	2.7	2.0	Trace
72– 84	0.7	0	Trace
84– 96	1.1	0	0
96–108	0.2	0	0
108–120	0.3	0	0
120–132	0.3	0	0
132–144	0	0	0
Total	29.3	21.8	..

[a] 698.369,000 disintegrations per minute.

among triplicates was less than 10%, the series was discarded and three new aliquots were plated. Some difficulty in obtaining reproducible counts was encountered early in the experiment, because of a tendency of the counting efficiency to decline in the latter portions of long counting periods. This problem was alleviated by adequately grounding the counter, counting in 5-minute increments, and grounding the planchet after each increment. The difficulty is believed to have been due to the accumulation of static electricity on the surface of the samples during long counting periods, which interfered with the counting of the low-energy emissions of the isotope.

Separate self-absorption curves were constructed for urine, bile, feces extract, meat extract, and crystalline stilbestrol in chloroform. The curves for urine and feces extract were different from the stilbestrol curve and from each other. The curves for meat extract and for stilbestrol were the same. Counting efficiency varied from 1.9 to 23% depending on the nature and thickness of the material being counted.

Results and Discussion

The recovery of radioactivity from the feces, urine, and bile of a bile-fistula steer fed a single 10-mg. dose of radioactive stilbestrol is shown in Table I.

It is noteworthy that radioactivity appeared in the feces within 12 hours after administration of the dose and was detectable until 132 hours. The terminal excretion times for radioactivity in bile and urine indicate that the period required for elimination of stilbestrol from the tissues of steers is about 72 hours.

Table II. Recovery of Radioactivity from Feces and Urine of Normal Steer Fed Radioactive Stilbestrol[a]

Collection Period, Hours	Feces, DPM[b] $\times 10^{-3}$	Urine, DPM $\times 10^{-3}$
0– 6	0	5,993
6– 12	0	7,335
12– 18	0	14,204
18– 24	8,170	13,856
24– 30	11,515	11,088
30– 36	No defecation	10,580
36– 42	7,842	15,168
42– 48	9,304	111,809
48– 60	7,456	7,569
60– 72	9,428	55,637
72– 84	5,388	67,273
84– 96	20,699	105,409
96–108	26,349	88,829
108–120	52,594	50,507
120–132	34,960	76,917
132–144	33,695	68,744
144–156	204,374	67,677
156–168	33,225	81,224
168–180	99,169	76,186
180–192	120,825	73,566
192–204	138,973	54,221
204–216	193,620	54,598
216–228	152,252	59,879
228–240	200,056	35,271
240–252	67,092	46,056
252–264	125,307	53,449
264–279	106,626	56,416
Rumen contents	199,268	...
Contents of small intestine	33,642	...
Contents of large intestine	54,697	...
Total	1,956,526	1,269,461
% of total dose	28.1	18.2

[a] A total of 6,957,896,000 disintegrations per minute in 20 equal doses over an 11-day period (100 mg. of stilbestrol). Final dose given at 252 hours.

[b] Disintegrations per minute.

This observation may be of practical importance with respect to the time before slaughter at which stilbestrol feeding should be discontinued in order to obtain minimal residues in the carcass.

The recovery of radioactivity from the feces and urine of an intact steer fed 100 mg. of radioactive stilbestrol during an 11-day period is presented in Table II.

The interval between the initial feeding of radioactive stilbestrol and the first detection of radioactivity in the feces was somewhat longer than for the bile-fistula steer. This later appearance of radioactivity in the feces probably reflects the relatively constipated condition of the intact steer. Total activity recovered from the feces and intestinal contents of the two animals was comparable (28.1 and 29.3%).

When the irregular feed intake of the intact steer early in the experiment is considered, the pattern of urinary excretion agrees closely with that observed for the bile-fistula steer. As little radioactivity was removed in the bile, this agreement should be expected. The sharp increase in urinary excretion after 36 to 42 hours may reflect the attainment of a threshold level in tissue retention or the accumulation of stilbestrol in the rumen early in the experiment.

Table III. Radioactivity Recovered from Tissues of a Steer Fed 100 Mg. of Radioactive Stilbestrol over an 11-Day Period[a]

Tissue	DPM[b]	P.P.B.[c] Stilbestrol
Lean meat	5,293	0.30
Internal fat	6,106	0.35
Liver	159,086	9.12
Kidney	72,499	4.15[d]
Heart	0	0
Blood	0	0

[a] A total of 6,957,896,000 disintegrations per minute fed in 20 equal doses over an 11-day period, and steer slaughtered 27 hours after the last feeding.

[b] Disintegrations per minute per 250-gram sample.

[c] Parts per billion, calculated from specific activity of dose (33 μc. per mg.).

[d] 33% in conjugated form.

Table IV. Fractionation of Radioactivity Recovered from Tissues and Excreta of Steer Fed Tritium-Labeled Stilbestrol

	Free Stilbestrol, %	Conjugated Fraction, %
Bile	10	90
Urine	27	73
Feces	98	2
Kidney	67	33
Liver, lean meat, fat	100	..

The average excretion of 20% of the oral dose in the urine of the intact and bile-fistula steers is approximately ten times the fraction of injected doses recovered in the urine of bile-fistula rats by Daskalakis (*4*). It is also from two to four times the urinary fraction reported for normal rats.

Table III presents the results of the tissue analyses. The heart and blood were the only tissues analyzed in which no radioactivity was detected. The method was sensitive to 0.17 p.p.b. of stilbestrol in the heart or 0.008 γ of stilbestrol per 100 ml. of blood.

The concentration of radiostilbestrol in lean meat and internal fat was 0.30 and 0.35 p.p.b., respectively. On the basis of the concentration found in meat, approximately 7610 pounds would have to be consumed in order to provide 1 mg. of stilbestrol. Six or seven times the level found would be needed to provide a measurable response in biological assays with a sensitivity of 2 p.p.b. Stob, Perry, Andrews, and Beeson (*11*) have reported the presence of estrogenic residues, detected by a mouse uterine assay, in the muscle, kidney fat, liver, intestine, and kidney of steers fed 10 mg. of stilbestrol daily. No indication is given of time lapse between cessation of treatment

and slaughter, which probably exerts an important influence on the amount of residue in the tissues.

The detection of 9.12 p.p.b. of labeled stilbestrol in the liver is in disagreement with the failure of Preston *et al.* (*10*) to detect as much as 2 p.p.b. in the liver but is in general agreement with the results of Gossett, Smith, and Downing (*5*), who found a low level of estrogenic activity in the liver of a steer fed 10 mg. of stilbestrol per day and slaughtered 24 hours after treatment was discontinued.

The recovery of 4.15 p.p.b. of stilbestrol from the kidneys is in close agreement with the 4 p.p.b. reported by Turner (*13*), who measured the estrogenic activity of the tissues of steers slaughtered 48 hours after treatment.

The results of separating the labeled compounds in the extracts of tissues and excreta taken from the intact steer into free phenolic and conjugated fractions are presented in Table IV. As anticipated from the position of the label in the ring portion of the stilbestrol molecule, no evidence of hydrogen exchange was observed, and the compound was recoverable in the phenolic fraction when added to tissue samples. The radioactive material in the free phenolic fraction of urine was identified as stilbestrol by chromatography and by carrier crystallization from benzene (*6*). Consequently, the radioactivity in this fraction has been calculated as stilbestrol. Almost 100% of stilbestrol administered to laboratory animals can be recovered as the free phenol and the glucuronide (*4*, *14*) and no metabolite other than the glucuronide has been reported in the literature. With the exception of 33% of the radioactivity found in the kidney, all of the radioactivity recovered from the tissues was in the free phenolic fraction.

Ninety per cent of the radioactivity found in the bile and 73% of that found in the urine appeared in the conjugated fraction. This indicates that a major pathway of metabolism of stilbestrol in the steer is the conversion of the free phenol to the water-soluble conjugated form(s).

Only 2% of the radioactivity in the fecal extract from the intact steer was in the conjugated fraction. When the fecal extract from the bile-fistula steer was fractionated, none of the radioactivity was found in this fraction. If bile were the only source of conjugated material, no radioactivity would be expected to appear in this form in the feces of the bile-fistula steer. Likewise, because the bile activity was predominantly in the conjugated fraction, the amount of radioactivity in this fraction of the fecal extract from the intact steer might indicate the amount of stilbestrol secreted in the bile, and the radioactivity in the free fecal fraction might be used to measure unabsorbed stilbestrol. Both the small amount of radioactivity recovered in the bile of the fistulated steer and the preponderance of free phenol in the feces of the intact steer suggest that the bile was a minor pathway of excretion. However, the volume of bile collected from the fistula (343 ml. in 144 hours) was much smaller than anticipated from literature values. Further, the possibility exists that the smaller fraction of fecal activity recovered as the conjugate may be due to the release of free phenol by microbial action. Callow, Callow, Emmens, and Stroud (*2*) observed that putrefaction in urine converts the conjugate to the free form, and Marsh (*8*) has demonstrated the presence of a true glucuronidase in rumen microorganisms. Therefore, the quantity of stilbestrol normally secreted in the bile of stilbestrol-treated steers cannot be accurately estimated from the data obtained in this study. This problem is under further investigation.

Approximately one half of the radioactivity administered to each steer was recovered. The urine, bile, and feces of the bile-fistula steer accounted for 51.1% of the administered activity. Similarly, 46.3% of the administered radioactivity was recovered in the urine and feces of the intact steer. The levels of radioactivity detected in the tissues were not high enough to contribute appreciably to the total recovery. Because the urine was plated directly, no problem in extraction was involved. Hence the feces appear to be the most important site of unrecovered radioactivity. The feces extraction procedure recovered from 73 to 83% of free stilbestrol added to moist feces. Correction for this loss accounts for approximately 20% of the missing activity. Daskalakis (*4*) compared ether extraction with combustion of feces from rats fed stilbestrol labeled with carbon-14 and found that only 35 to 50% of the total radioactivity in the feces was ether extractable. Unfortunately, combustion of fecal samples for tritium counting was not feasible in this experiment.

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