vitamin A levels of animals consuming treated forage drop below control levels. From the 62nd day on, vitamin A levels of animals consuming DDT- or MCPA-treated forage were significantly higher than those of controls, but no differences were observed between the herbicide or pesticide treatment.

Discussion

The environmental stress due to DDT to which these animals were subjected was purposely much greater than would be encountered under normal agricultural practices. Forage was harvested 24 hours after spraying (at 1.5 pounds per acre), so animals were consuming continually over the entire experimental period a diet containing as determined by analysis 40 to 60 p.p.m. of *o,p-*, *p,p-*DDT dry weight. In agreement with previous studies in the rat (*11*), this rate of feeding significantly decreased liver storage of vitamin A and carotenoids. The decreases, however, were not of sufficient magnitude to decrease blood levels of either constituent. The stress of DDT ingestion at high levels did not produce nor approach an avitaminotic state in the steers. It is reasonable to assume that the stress (if any) imposed on bovines by ingestion of DDT-contaminated herbage under practical conditions would not appreciably affect vitamin A utilization and metabolism, and hence should not contribute to increased vitamin A deficiency of beef steers in North America.

Studies conducted by Mitchell, Hodgson, and Gaetjens (*7*) have not demonstrated any harmful effects to sheep or cows grazed on pasturage treated with 2,4-D, or to a cow fed over 5 grams daily of the herbicide. Although there was no gross effect of 2,4-D on performance of the animals, some case reports (*4*) have suggested that vitamin A utilization may be impaired by MCPA in suckling calves. In a previous study with rats, Phillips (*10*) demonstrated that the feeding of relatively large amounts of MCPA did not impair hepatic storage of vitamin A following oral administration of carotene or vitamin A, nor did it alter the rate of decrease of hepatic vitamin A stores. Results of the present experiment with bovines are in agreement with those observed in the rat. Following continual feeding of MCPA-treated forage, there was no evidence that any detrimental or abnormal influence was exerted on the utilization of carotenoids. Serum carotenoids were significantly greater at the termination of the experimental period in the group consuming MCPA-treated forage; however, this did not result from decreased conversion of carotenoids to vitamin A as shown by liver vitamin A concentrations similar to the control group. Hidiroglou and Knutti (*4*) have stated that the indirect hazard to cattle by eating MCPA-sprayed forage in the fall is negligible, because there is a lower proportion of plants absorbing the herbicide and the possible effect of herbicide on carotenoid utilization in older cattle may be less than in calves, because of the vigorous activity of the rumen which rapidly eliminates the plant hormone.

It is concluded that under the conditions of this experiment the feeding of MCPA-treated forages does not influence carotenoid utilization in bovines.

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ANIMAL GROWTH STIMULANTS

Metabolism of Labeled Diethylstilbestrol in Ruminants

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The results of an earlier study on the metabolism of tritium-labeled diethylstilbestrol (DES-T) in lambs and steers have been confirmed and extended. The "free phenolic" material found in the tissues and excreta after DES-T administration was identified by carrier crystallization and chromatographic methods as stilbestrol, and the "conjugated" material excreted in the urine as its glucuronide. It was shown that no other forms of DES are present in appreciable quantities in alcohol-ether extracts of the tissues and excreta. Rumen microflora were shown to be capable of hydrolyzing the glucuronide in vitro and it was proposed that the excretion of the free form in the feces is partially the result of degradation of the conjugate by intestinal bacteria.

Although the administration of diethylstilbestrol (DES) to ruminants as a growth stimulant has become a widespread commercial practice, little is known of the metabolic fate of this compound in these species. Most of the information currently available pertains to monogastric animals, in which the physiological effects of DES (and therefore possibly its catabolism) are known to differ in certain important respects from those in ruminants. The study described here is an extension of an earlier investigation by Mitchell, Neumann, and Draper (*17*) into the metabolism of DES in cattle. It was under-

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taken to obtain additional information regarding the fate of DES administered orally to ruminants and the identity of the main metabolites of this compound formed in the tissues.

In an early study, Stroud (*19*) found that, after injection of DES, rabbits excreted a "free" and "combined" form of the compound in the urine. Mazur and Shorr (*15*) identified the combined form as the monoglucuronide and reported, moreover, that it appeared to be the only metabolite present. The possible occurrence of the ethereal sulfate in rat urine was investigated by Teague (*20*) with essentially negative results, and Malpress (*13*) found that after administration of therapeutic doses of DES to humans only minute amounts of the sulfate were excreted, most of the dose being voided as the glucuronide. Studies of the metabolism of C^{14}-labeled DES by Twombly and Schoenewaldt (*22*) on mice, rabbits, and dogs, and by Hanahan and coworkers (*10*) on rats, indicated that the compound is not metabolized to CO_2. These experiments also indicated that in these species DES is conjugated in the liver and secreted via the bile duct into the intestine as the main excretory pathway. Wilder Smith and Williams (*23*) likewise found that the glucuronide was the only detectable form present in the urine of rabbits treated with DES, but observed that when the conjugate was administered orally or parenterally, it was further metabolized.

The work of Mitchell, Neumann, and Draper (*17*) suggested that the metabolism of DES follows a similar pathway in ruminants. After oral administration of tritium-labeled DES (DES-T) to steers, the compound was found to be rapidly eliminated in the urine and feces, mainly in a conjugated form in the former and in a "free" form in the latter. The fact that the material secreted in the bile was largely conjugated indicated that the bound form underwent hydrolysis in the intestinal tract prior to its excretion in the feces. A small residue of "free phenolic material," considered to be DES, was found in the tissue 24 hours after oral administration.

In the present study, the free phenolic material found in the liver was firmly identified as DES by carrier crystallization and chromatographic methods, and the conjugated form was identified as the glucuronide. It was also found that the conjugate is readily hydrolyzed by bacterial glucuronidases and is the only significant metabolite present in the urine.

Experimental Procedure

Labeled Compounds. The DES used in these experiments (except where otherwise noted) was ring-labeled with tritium in the position ortho to the hydroxyl group and had a specific activity of 33 microcuries per milligram. The conjugated form was obtained from the urine of the steers used by Mitchell and coworkers (*17*), as well as from the urine of a lamb after injection with DES-T. In each case the conjugated and free phenolic compounds were separated by a modification of the extraction and fractionation procedures described by Teague and Brown (*21*).

The radioactivity in most of the samples was estimated using a Packard Tri-Carb liquid scintillation spectrometer. Samples containing DES-T were counted in 14 ml. of redistilled toluene containing 2,5-diphenyloxazole and 1,4-bis-2(5-phenyloxazolyl)benzene (3 grams and 50 mg. per liter, respectively). Conjugate fractions were dissolved in 1 ml. of absolute ethanol before the toluene solution was added. All samples were counted in triplicate to give a probable counting error of 5% or less, and corrections were made for counter efficiency and quenching. In the case of highly colored samples, solid counting using a windowless gas flow counter was employed, as described by Mitchell *et al.* (*17*). Paper chromatograms were either scanned in a windowless Vanguard Model 880 Autoscanner (using 4π geometry) to locate radioactive spots or were cut into pieces, eluted, and counted by liquid scintillation.

Experimental Animals. Tissues and excreta from two steers and two lambs were used as sources of labeled metabolites. The two steers were those described by Mitchell *et al.* (*17*), one of which had received a 10-mg. oral dose of DES-T daily for 11 days and the other a single oral dose of the same size.

Lamb 1, a wether weighing 69 pounds, was used to investigate the extent of salivary recycling of DES as well as to provide additional urinary metabolites. This animal, having been maintained for 8 weeks on a hay and grain ration, was injected intravenously with 2.7 mg. of DES-T. The dose was administered in 5 ml. of propylene glycol by means of a 1.5-mm. polyethylene cannula which was inserted about 6 inches into the right jugular through a No. 10 hypodermic needle. For the next 2 hours, the animal was maintained under light Nembutal anesthesia (administered through the cannula) and saliva was collected in 100-ml. fractions as it dripped from the mouth. The animal's jaws were opened slightly by a cork placed between the teeth. At the completion of the collection the cannula was removed, the lamb was given access to a natural ration, and urine was collected in three time periods over the next 40 hours. All samples were stored at −15° C. until extracted.

As the amount of radioactivity recovered from the internal organs of these animals was too small to permit identification of the labeled compounds, lamb 2, a wether weighing 75 pounds, was given an intravenous dose of 1.8 mg. of uniformly labeled DES-T having a specific activity of 1.1 mc. per mg. (obtained from Volk Radiochemical Co.). After 6.5 hours this animal was killed, and the liver was removed for extraction.

Extraction of Samples. Urine samples were thawed, acidified, and extracted three times with diethyl ether. The "conjugate fraction" was obtained by extracting the ether solution with saturated $NaHCO_3$ solution, as described by Teague and Brown (*21*), and the radioactivity was recovered in ether after acidification of the aqueous extract. The possibility that ether extraction failed to remove some conjugated forms of DES—e.g., the ethereal sulfate—was tested by acid hydrolysis and re-extraction with ether, after the procedure of Simpson and Wilder Smith (*18*). Fifty milliliters of the extracted aqueous solution were refluxed with 0.5 ml. of concentrated HCl for 1 hour and extracted three times with 0.25 volume of ether. No radioactivity was found in the combined extracts.

The liver from lamb 2, weighing 409 grams, was ground, placed in two Soxhlet extractors, and successively extracted for a total of 120 hours with ethanol and diethyl ether as previously described (*17*). The combined extracts were fractionated into free phenolic and conjugate moieties by a modification of the procedure of Merker and coworkers (*16*, *17*).

Identification of DES-T. The residue from the phenolic fraction of the liver of lamb 2, plus 236.9 mg. of pure DES carrier, was dissolved in a minimum volume of diethyl ether, and 5 volumes of benzene were added with warming. The DES was recrystallized at −15° C. until constant specific activity was recorded. Aliquots of this fraction were also chromatographed, as described below, against pure DES in several paper systems. A carrier crystallization was carried out in a like manner on a sample of the phenolic fraction from steer urine.

Identification of Glucuronide. Samples of the total tritium-labeled material present in steer and lamb urine, as well as that portion recovered in the conjugate fraction, were subjected to enzymatic hydrolysis with β-glucuronidase. This enzyme affords a specific method for identification of glucuronides and for distinguishing them from other conjugated forms such as ethereal sulfates (*6*). The method employed was that of Beer and Gallagher (*3*). One hundred and twenty-five milliliters of steer urine were divided into five 25-ml. samples. After acidification to pH 5 with 1*N* H_2SO_4, 2.5 ml. of pH 5 acetate buffer and 7500 units of a commercial preparation of salt-free β-glucuronidase (Nutritional Biochemicals Corp.) were added. The system was incubated at 37° C. for 5 days, then extracted and fractionated into free and conjugate moieties. Duplicate samples and enzyme blanks were used, and a separate urine sample was fractionated to obtain "zero time" values.

A sample of the conjugate fraction of urine from lamb 1 was also subjected to enzymatic hydrolysis. The sample was placed in the reaction vessels after being dissolved in 3 ml. of diethyl ether, which was subsequently removed by means of a nitrogen sweep. Twenty-five milliliters of distilled H_2O (acidified to pH

5 with 9N H_3PO_4), 2.5 ml. of pH 5 acetate buffer, and 20,000 units of enzyme were added, after which the contents were incubated at 37° C. for 5 days. Duplicate samples and enzyme blanks were employed. At the end of the incubation the contents were extracted with ether, and the extract was fractionated into free and conjugate portions, which were assayed for radioactivity.

Paper and Thin-Layer Chromatography. Portions of the phenolic extract of steer urine (*17*) and of the liver of lamb 2 were chromatographed in several paper systems against pure DES or DES-T standards. The systems (solid and mobile phases, respectively) found to be most satisfactory were the following: paraffin-coated paper, 75% ethanol (*8*); silicone-coated paper, 60% acetonitrile (*4*); Whatman No. 1 paper, toluene–*tert*-butylalcohol–acetic acid–water (85:15:30:70 v./v.) (*5*); zinc carbonate–impregnated paper, cyclohexane (*9*); and Whatman No. 1 paper, absolute methanol. The extract from liver was also purified on a silica gel thin-layer chromatogram using pyridine-water (1:1) as the mobile phase.

The labeled compounds were located either by scanning or by eluting and liquid scintillation counting, and their R_f values were compared with those observed after spraying the strips with iodine solution or with specific color reagents. The spot tests found most useful were those based on the reaction between DES and $SbCl_5$, described by Axelrod (*2*), and on the reaction with $FeCl_3$–$K_4Fe(CN)_6$ reagent, described by Jellinek (*11*).

Artificial Rumen Study. The observation that DES-T was secreted in the bile of a steer mainly as a conjugate but was present in the feces in the free form (*17*) indicated that deconjugation had occurred as the result of bacterial action in the rumen or the intestine. Certain bacteria are known to contain powerful β-glucuronidases. To test this hypothesis, samples of the conjugated material recovered from urine were incubated with rumen fluid in vitro; after incubation, the mixture was examined for the presence of free DES.

Rumen fluid drawn from a wether lamb by means of a fistula was strained through eight layers of cheesecloth and placed in 90-ml. centrifuge tubes fitted with CO_2 input and exhaust tubing. The tubes were charged at least 1 hour in advance with 7 ml. of a salts solution (*1*), 23 ml. of water, and a 300- to 400-mg. sample of cotton on which the radioactive material had been deposited. After addition of 20 ml. of rumen fluid, the mixture (total volume 50 ml.) was incubated under CO_2 in a water bath at 38° C. Duplicate samples of pure DES-T and of conjugate, obtained from the urine of lamb 1, were tested in the presence and absence of rumen fluid. After incubation for 48 hours (during which time the cotton was completely digested) the mixture was strained through cheesecloth, the filtrate and residue were extracted with diethyl ether, and the relative proportions of free and bound compounds were determined.

Results and Discussion

Identification of DES-T in Lamb Liver. Recrystallization of DES added to the free phenolic fraction, obtained from the liver of lamb 2, yielded a product which exhibited constant specific activity after the fifth crystallization. The values (d.p.m. per mg.) for the fourth through seventh crystallizations were: 759, 594, 586, and 587.

Cochromatography of aliquots of this fraction with pure DES in several paper systems yielded in each case a single radioactive spot which coincided with that of the carrier. The R_f values recorded for the labeled compound and carrier DES on paraffin-coated and silicone-coated paper, respectively, were 0.92 and 0.95. The silica gel thin-layer chromatogram was divided into seven sections, which were eluted individually with diethyl ether. When the eluates were counted, it was found that 93% of the counts were present in the section opposite the pure DES standard (R_f 0.65).

The above findings demonstrated that DES-T was the only compound present in significant quantities in the free phenolic fraction of liver. Accordingly, the concentration of DES in the tissues may be calculated from the amount of radioactivity in this fraction and the specific activity of the DES-T employed, as done by Mitchell and coworkers (*17*). On this basis, the concentration of DES in the liver of lamb 2 (killed 6.5 hours after intravenous injection of 1.8 mg. of DES) was calculated to be 12.7 parts per billion. This value compares with that of 9.1 parts per billion calculated previously (*17*) for the concentration of DES in the liver of a steer 24 hours after discontinuing oral administration of DES-T at the level commonly employed in commercial practice (10 mg. per day).

Identification of DES-T in Steer Urine. Aliquots of the radioactive material in the free fraction of steer urine were chromatographed on paper in four of the systems described above. In each case a single radioactive spot was observed which corresponded to the R_f value for a pure DES standard. The R_f values observed in the first four systems described previously were 0.92, 0.95, 0.92, and 0.92, respectively.

The labeled compound was located in this instance by cutting the paper strips into small pieces, eluting with toluene, and counting, while the position of the standard was determined by staining with $SbCl_5$ or $FeCl_3$–$K_4Fe(CN)_6$ reagent. The colors were developed by dipping the strips in the reagent solution. Color reactions were observed on the sample strips at an R_f which corresponded to that of the radioactive peak and to that of DES on the standard strips. In the case of paraffin- and silicone-coated paper, $SbCl_5$ treatment resulted in blackening which obscured the location of the compounds.

Carrier crystallization was applied to a sample of the radioactive material recovered in the free fraction of steer urine after oral administration of DES-T (*17*). Two hundred milligrams of pure DES were combined with 3.6×10^6 d.p.m. of radioactivity, and crystallization was carried out from benzene at −15° C.

The crystals obtained were heavily contaminated with urinary pigments, and attempts at purification, including chromatography and solvent fractionation, were only partially successful. However, in the final product, similar recoveries of the added carrier and of sample radioactivity were obtained (24.4 and 20.4%, respectively). This finding confirmed the results of paper chromatography in showing that a large proportion, if not all, of the radioactivity in the free phenolic fraction of urine was attributable to DES-T.

Identification of Monoglucuronide in Urine. The effect of incubating the urine of a DES-T–treated steer with β-glucuronidase on the distribution of radioactivity between free and conjugate fractions is illustrated in Table I. The action of this enzyme resulted in almost complete conversion of the conjugated material to the free form and negligible amounts of additional radioactivity were released by subsequent acid hydrolysis. The results of this experiment are in agreement with the conclusions of Teague (*20*), Dodgson and coworkers (*7*), and Simpson and Wilder Smith (*18*) that the ethereal sulfate is of little or no quantitative significance as a urinary metabolite of DES. It is noteworthy that the proportion of counts in the conjugate fraction of urine was observed to decrease with storage and handling, indicating a gradual hydrolysis to the free form. Examination of fresh urine samples indicated that a high proportion (perhaps all) of the DES-T was present in conjugated form at the time of excretion.

Similar results were obtained by treating a sample of the conjugated material obtained from lamb urine (Table II). This experiment showed that the glucuronide was the only conjugated form excreted in significant amounts in the urine of lamb 1 and, together with the results of the experiment on steer urine, indicates that the metabolism of DES in ruminants (except for the enhanced role of bacteria in the intestinal tract) is similar to that in nonruminants.

Action of Rumen Microorganisms on Free and Conjugated DES. The data

Table I. Effect of β-Glucuronidase Treatment on "Conjugated" DES-T of Steer and Lamb Urine

	% of Counts Recovered as		
	Free phenol[a]	Conjugate	Acid hydrolyzate[b]
Unfractionated steer urine			
Zero-time values	66.5	33.0	0.5
Complete system[c]	99.0	1.0	0.0
Enzyme blank	63.3	35.0	1.7
Conjugate fraction of lamb urine			
Zero-time values	0.0	100.0	...
Complete system[c]	97.2	2.8	0.0
Enzyme blank	0.8	99.2	0.0

[a] All data are for average of two duplicates.
[b] Represents counts recoverable by acid treatment and ether extraction after prior extraction for removal of free phenolic and conjugated material.
[c] See text for composition.

Table II. Effect of in Vitro Incubation of Free and Conjugated DES-T with Rumen Fluid

Incubation System			% of Counts Recovered as	
Rumen fluid	DES[a]	Conjugate[a]	DES[b]	Conjugate
+	+	−	96.9	3.1
+	−	+	97.7	2.3
−	−	+	1.1	98.9

[a] 640 × 10^3 DPM as pure DES and 1104 × 10^3 DPM as conjugate extracted from urine of lamb 1. See text for composition of incubation medium.
[b] All data are for average of two duplicates.

presented in Table II show that incubating the glucuronide of DES with rumen fluid resulted in essentially complete hydrolysis to the free form. This observation indicates the presence of bacterial β-glucuronidase activity and is in accord with the reports of Marsh, Alexander, and Levvy (*14*) and Karunairatnam and Levvy (*12*) that this enzyme is present in the microflora of the rumen and intestine. As Marsh and coworkers have reported that the enzyme is intracellular, the present experiment suggests that the glucuronide entered the bacterial cells during incubation. The small percentage of radioactivity recovered in the conjugate fraction after incubation suggests that the separation of the two forms was incomplete. Recoveries of radioactivity were in some instances quantitative, indicating that no degradation of stilbestrol occurred during incubation.

Although DES secreted in conjugated form in the bile is not likely to be returned to the rumen, the fact that rumen microorganisms are capable of hydrolyzing it strongly implies that a similar degradation probably occurs as a result of bacterial action in the intestine. This would account for the previous observation (*17*) that, whereas DES-T was excreted primarily in the bound form in urine, it was present predominantly in the free form in the feces.

The results of this study and of the previous experiments by Mitchell and coworkers (*17*) provide a fairly comprehensive picture of the over-all metabolism of DES in ruminants. It is apparent from the short biological half life and mode of excretion that the catabolism of this compound proceeds by a detoxification mechanism—i.e., that DES is metabolized as a foreign compound. Conjugation with glucuronic acid is a well known biological mechanism for detoxification of phenolic compounds. Thus, the monoglucuronide has been shown to be the predominant form in urine and bile, although the material secreted through the bile is restored to the free form prior to elimination in the feces, apparently as the result of bacterial action.

It has been confirmed that, although the amounts are very small, there is a residue of stilbestrol present in the tissues 24 hours after cessation of oral administration at the usual level used for growth stimulation (*17*). This would be anticipated from what is known of the time intervals required for rumen clearance. The results of the in vitro experiments and of the analysis of the feces (*17*) indicate that DES is stable to the action of anaerobic bacteria in the digestive tract of ruminants. While the use of radioactive DES in these studies has made it possible to demonstrate the presence of the compound in certain tissues with a specificity and sensitivity which surpass those of conventional bioassay techniques, the concentrations found are of doubtful significance from the standpoint of possible physiological reactions resulting from human consumption of these tissues.

Although the free form and the glucuronide were shown by Mitchell and coworkers (*17*) to be the only compounds present in significant amounts in the alcohol-ether extracts of the tissues and excreta of steers, the over-all recovery of DES-T was only about 50%. Much of the unrecovered radioactivity was apparently present in the feces. Similar recoveries from biological materials have been reported by other workers. It is apparent that a substantial fraction of the DES in the tissues is present in a form resistant to solvent extraction, possibly bound to proteins, and until this portion is accounted for it cannot be concluded that no additional forms exist in vivo.

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