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Radioimmunoassay for Diethylstilbestrol and the Monoglucuronide Metabolite in Bovine Liver

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The complex matrix in liver causes difficulties in development of a radioimmunoassay (RIA) to quantify compounds bound in liver. An RIA method was developed for diethylstilbestrol (DES) in bovine liver and employs a purification procedure to circumvent these problems; the procedure includes liquid–liquid partitioning, Sephadex LH-20 chromatography, and quantitative enzymatic hydrolysis of the principal metabolite, DES glucuronide. Assay background due to liver matrix was 0.05 ppb ($1/10^9$). Average recovery of both DES and its monoglucuronide from fortified liver was 43% by RIA. Tritiated DES used as an internal standard had a higher apparent recovery (67.7%) by liquid scintillation counting. With an in vivo contaminated liver, accuracy was confirmed by a gas chromatography–mass spectrometry method. By repeating the extraction and RIA without enzymatic hydrolysis on this same liver, the free DES was calculated to be 7.8%. The limit of determination for this method with 95% confidence limits is 0.3 ppb.

The application of radioimmunoassay (RIA) to drug residue analysis in animal tissues has been limited despite the proven sensitivity and specificity of RIA below the parts per billion (ppb, $1/10^9$) range in biological fluids (Hoffmann, 1978). Complex tissues such as liver necessitate involved purification procedures. Hoffmann (1978) developed such purification procedures with RIA for both natural and synthetic steroids in muscle, liver, kidney, and fat.

Diethylstilbestrol (DES) is difficult to quantitate below 1.0 ppb in bovine liver, which is among the last tissues to contain DES after cattle are withdrawn from the drug (Donoho et al., 1973; Aschbacher, 1976). Methods to measure DES in bovine liver include gas chromatography (GC) (Donoho et al., 1973), GC with mass spectrometry (GC–MS) (Day et al., 1975), and liquid chromatography (Kenyhercz and Kissinger, 1978), but quantitation below 1.0 ppb has not been documented for any of these methods. RIA is capable of greater sensitivity.

Several researchers have developed RIA techniques for DES (Hoffmann, 1978; Gutierrez-Cernosek and Cernosek, 1977; Richoubac et al., 1977), but none was suitable for use with liver. Hoffmann and Laschutza (1980) and Vogt (1980) developed assays with extensive purification procedures for DES in bovine tissues, including liver; Hoffmann and Laschutza used both silica gel chromatography and liquid–liquid partition while Vogt relied entirely on liquid–liquid partition.

In this study, we investigated the use of a simplified purification procedure combined with RIA for detecting and quantifying DES in bovine liver. The method developed was simpler and easier to perform than the above RIA methods.

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EXPERIMENTAL SECTION

Materials. The DES antiserum (No. 40A-16) was obtained from R. M. Gutierrez-Cernosek and S. F. Cernosek and was previously described (Gutierrez-Cernosek and Cernosek, 1977). The antigenic conjugate was prepared by the method of Rombauts et al. (1973) in which one phenolic group is covalently bonded through an ether linkage to the free amines of bovine serum albumin. The second antibody was lyophilized anti-rabbit γ-globulin goat serum obtained from Micromedic Systems, Horsham, PA. Reagent-grade crystalline DES was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ. Monoethyl-tritiated DES ([^{3}H]DES), with a specific activity of 81 Ci/mmol, was purchased from Amersham Corp., Arlington Heights, IL. After storage for more than 1 year at 4 °C in benzene at a concentration of 3 μCi/mL, the [^{3}H]DES required purification by Sephadex LH-20 chromatography, as described below. DES monoglucuronide (DES-MG) was purchased in crystalline form, preweighed in glass ampules, from Aldrich Chemical Co., Milwaukee, WI. β-Glucuronidase from bovine liver was purchased from Calbiochem Corp., San Diego, CA. Absolute ethanol was used, and all other organic solvents were glass distilled. All other chemicals were reagent grade.

RIA Buffers. The phosphate-buffered saline (PBS) was that described by England et al. (1974) but containing 0.02% (w/v) merthiolate. The 0.1% gel–PBS included 0.1% (w/v) gelatin, and the concentrated 0.1% gel–PBS was 1.11 times more concentrated. The antiserum buffer was PBS with 0.25% (v/v) normal rabbit serum and 0.05 M disodium ethylenediaminetetraacetate. DES antiserum was diluted 1:12000 with the antiserum buffer. The second antibody was reconstituted with 25 mL of 0.1% gel–PBS. Tracer solution was [^{3}H]DES in 0.1% gel–PBS (3300 dpm/200 μL). Eight DES standards (0.1–2.0 ng of DES/mL) for the RIA standard curve were prepared in 10% ethanol–0.1% gel–PBS.

Sephadex LH-20 Column. The column for purifying DES was packed with 1.00 g (dry weight) of Sephadex

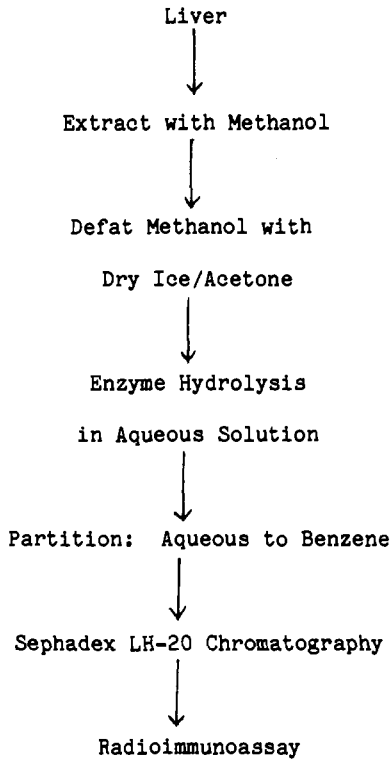

Figure 1. Purification scheme.

LH-20 that had been swollen in the column solvent, chloroform with 5% (v/v) ethanol, for at least 3 h. A glass column, 0.7-cm i.d. and 21 cm from bottom tip (no stopcock) to reservoir, was plugged at the bottom with glass wool and a 0.8-cm filter paper disk. The swollen Sephadex LH-20 was slurried in 15 mL of the column solvent and poured into the column. When the length of the Sephadex LH-20 bed had settled to just under 9 cm, the bed was compressed to between 8.5 cm and 8.0 cm under a 0.8-cm filter paper disk. This disk held down the column bed, which was then washed with 30 mL of column solvent before use. The operating flow rate was between 0.5 and 1.0 mL/min, depending on the height of the column solvent above the bed (not greater than 14 cm). When the column solvent was level with the column bed, the solvent flow stopped. The DES to be purified was dissolved in column solvent and applied to the top of the bed, and the column was eluted with column solvent. The first 15 mL (starting from the application of the DES to the column bed) was discarded. The subsequent 20 mL contained the DES. Columns were not reused.

Extraction and Cleanup. The purification scheme is shown in Figure 1. A 10.0-g sample of ground steer liver was extracted twice by blending with 20-mL portions of methanol for 3 min. The extracts were centrifuged and decanted. The combined extracts were defatted by freezing in a dry ice–acetone slurry for 10 min, centrifuging at 2000*g* for 20 min at −10 °C, and decanting the supernatant into a round-bottom flask. Distilled water (10 mL) was then added to the defatted extract, and the volume was reduced to about 5 mL by rotary evaporation at 37 °C to remove the methanol. DES glucuronide in the remaining aqueous solution was enzymatically hydrolyzed to free DES by incubation in the round-bottom flask with 200 Fishman units of β-glucuronidase in 1.0 mL of water and 0.2 mL of 5% (v/v) acetic acid for 15 h at 37 °C. This hydrolysis and an alternate 3-h hydrolysis with 5000 Fishman units of β-glucuronidase were tested by thin-layer chromatography (TLC) (hexane plus ethyl ether, 1:1 v/v) to quantitate the extent of the hydrolysis. Next, 0.2 mL of 5 N HCl was added, and the free DES was extracted 3 times by vortexing with 10 mL of benzene for 1 min. The resulting emulsions were broken by centrifuging at 2000*g* for 20 min at 25 °C, and the benzene extracts were removed with a pipet. **Caution**: Benzene is a carcinogen and should be used only in an operating hood. The combined benzene extracts were evaporated to dryness under a stream of nitrogen and transferred with two 1.0-mL portions of column solvent to a Sephadex LH-20 column constructed as described above. The sides of the column above the bed were then washed twice with 2.0-mL portions of column solvent, and the column was eluted. The DES fraction, collected as described above, was evaporated to dryness under a stream of nitrogen and reconstituted in 0.5 mL of absolute ethanol with 1 min of vortexing. The DES was then diluted with 4.5 mL of concentrated 0.1% gel–PBS and 5.0 mL of 10% ethanol–0.1% gel–PBS and assayed the same day.

Radioimmunoassay. All reagents were initially at room temperature. Aliquots (200 μL) of each extract were transferred with an automatic pipet to triplicate assay tubes (12 × 75 mm) and diluted with 200 μL of tracer solution; 100 μL of diluted DES antiserum and 200 μL of second antibody solution were then added. The tubes were vortexed briefly and incubated 1 h at room temperature. After refrigeration for 15 h at 4 °C, the tubes were centrifuged at 1600*g* for 40 min at 4 °C, the contents were diluted with 3 mL of PBS at 4 °C, and the tubes were recentrifuged at 1600*g* for 30 min at 4 °C. The supernatants were discarded, and the residues were transferred with two 2-mL portions of scintillation fluid to 7-mL minivials for scintillation counting (50 min) to determine percent binding.

The standard curve included eight points (0.1–2.0 ng of DES/mL) in triplicate. Maximum binding with diluted DES antiserum was determined by assaying 10% ethanol–0.1% gel–PBS (blank buffer). Nonspecific binding was estimated for the assay of both blank buffer and extracts by assaying them in the absence of DES antiserum. The amount of binding to the antibody was calculated by subtracting nonspecific binding from total binding. For standards, the value for nonspecific binding for blank buffer was used. For extracts, the value for nonspecific binding for the extract was used.

Validation Study. The method was tested with three control steer livers that had been stored in sealed plastic bags at −20 °C for 1, 1.5, and 5 years and with one steer liver that had been contaminated in vivo and stored at −20 °C for 5 years. The liver with incurred residues had been obtained from a steer 42 h after its withdrawal from 10 days of treatment with 10 mg of DES orally twice per day. None of the livers showed signs of desiccation.

Before extraction, the liver samples were fortified with [^{3}H]DES (25 pg, 16 700 dpm) in 200 μL of methanol, and 1.0-mL aliquots of the final extracts in buffer were counted in 10 mL of scintillation solution to determine the percent recovery. When these extracts were assayed by RIA, the value for total counts from [^{3}H]DES in the assay tubes was corrected by adding the small contribution of the recovery counts from the extract (6% increase).

While the method was tested, eight assays were performed with six to eight liver samples extracted and measured per assay; a total of 62 liver samples was extracted and assayed. RIA parameters were calculated, and the recovery, sensitivity, and selectivity of the method were evaluated.

Statistical Methods. A linear response curve was fitted to assay results (in ppb) for fortified and unfortified control

Table I. Cross-Reactivities of Selected Compounds Binding to DES Antibody

compound	cross-reactivity[a] with % of maximum binding of 90	70	50
DES	1.0	1.0	1.0
DES-MG	1.0	1.0	1.0
hexestrol	0.5	0.05	0.005
dienestrol	0.04	0.01	0.006
esterone	0.0002	–[b]	–
estradiol	0.0002	–	–

[a] Cross-reactivity was defined, at a specific percent of maximum binding, as the concentration ratio of DES to interfering compound, causing that percent of maximum binding. [b] Not measured.

liver samples by a reiterated regression weighted by the reciprocal of variance of predicted assay result (Brownlee, 1960; Schwartz, 1979). Heteroscedasticity (lack of uniformity of variance) was demonstrated for the assay results by Bartlett's test. Various transformations (e.g., logarithmic) to make the data homoscedastic were tried. The minimum detectable concentration for the RIA procedure itself was calculated for each assay by the method of Rodbard (1978).

For statistical estimates, 95% confidence limits were used except where noted. Statistical significance was tested by using the two-tailed Student's t distribution with $\alpha = 0.05$.

RESULTS

Cross-Reactivity. The cross-reactivities of selected compounds with the antibody are shown in Table I. At 50% of maximum binding (B_0) only DES-MG cross-reacted, but at 90% of B_0 hexestrol cross-reacted strongly (50%) and dienestrol cross-reacted somewhat (4%). These compounds were not subjected to the purification procedure, so the cross-reactivities reflect only the specificity of the DES antibody.

RIA Background Level. A background of DES-like activity in purified extracts of control livers was detected by RIA. The background for 23 samples averaged 0.05 ppb in DES equivalents with a standard deviation (SD) of 0.02 ppb. RIA measurements were corrected by subtracting this value. Storage time beyond 1 year at −20 °C did not significantly change the background value.

The reagent blank effect was very small. The B_0 value was depressed for test solutions by about 3.3% (SD = 3.0%), resulting in false-positive readings that were less than the minimum detectable concentration (0.03 ppb). No correction was made for the reagent blank. Solvents other than ethanol also improved the solubility of DES in the buffer but gave larger reagent blank values. With dioxane, for instance, the B_0 value for test solutions was reduced by 10–30%, resulting in prohibitively large false-positive assay results.

Fortified Liver Samples. The assay results for in vitro fortified liver samples are shown in Table II. Without enzyme hydrolysis, DES was not formed from DES-MG and recovery was only 1.6%; with enzyme hydrolysis, the DES-MG was hydrolyzed to free DES and the recoveries were improved. When TLC was used to quantitate the extent of hydrolysis, DES-MG was found to be quantitatively converted to free DES under both the 15- and 3-h hydrolysis conditions. Furthermore, the average recoveries of DES and of DES-MG (43.4 and 41.5%, respectively) were not significantly different. Therefore, the assay results for control liver samples fortified with either DES or DES-MG and the assay results for unfortified control livers were combined to calculate the response curve. The standard deviations of assay results were linearly dependent on the assay results ($r^2 = 0.817$). The assay results are significantly heteroscedastic, and no suitable transformation would make them homoscedastic. The estimate of recovery (slope of regression curve) was 43% with a standard error (SE) of 1.5%, and the estimate of background (y intercept of regression curve) was 0.053 ppb (SE = 0.004 ppb). The limit of determination (i.e., the lowest assay result which corrected for background is significantly different from zero) was 0.5 ppb with 99% confidence limits. Although we utilized this method only down to the 0.3-ppb level, extrapolating our results and assuming constant recovery, we calculated a potential limit of determination of 0.2 ppb with 95% confidence limits. The coefficient of variation (CV) for assay results of fortified livers was 24%.

The possibility that the influence of background decreased with the increasing magnitude of assay results was investigated. Analysis with a nonlinear model suggested that recovery of DES and DES-MG was 53%. The linear model was used, however, because calculations were simpler and the differences between the methods were small.

Recovery of [^{3}H]DES. The recovery of [^{3}H]DES, as determined by liquid scintillation counting of tritium from liver samples fortified with [^{3}H]DES plus either DES or DES-MG, was 67.7% (SD = 4.7%; CV = 6.9%). This value for recovery of [^{3}H]DES was significantly different from the recovery of DES and DES-MG determined by RIA, regardless of whether the linear or nonlinear analysis of the RIA data was used. Over the range of concentrations of DES or DES-MG tested in the fortified liver samples, no change in recovery of [^{3}H]DES was detected.

In Vivo Contaminated Liver. An unfortified liver sample, which by the GC–MS method of Day et al. (1975) contained 5.6–8.0 ppb of DES (corrected for recovery), was diluted with control liver by a factor of 4 so that it contained 1.4–2.0 ppb of DES. The concentration of DES determined by our RIA method with enzyme hydrolysis

Table II. Recovery of DES and DES-MG from Fortified Liver Samples by RIA

fortification of liver samples: DES, ppb	DES-MG, ppb[a]	no. samples	RIA response: mean corrected for background, ppb[b]	SD, ppb	recovery as % of fortification: mean	SD
1.3		3	0.61	0.10	47	8
	1.0	4	0.52	0.05	52	5
0.8		4	0.32	0.08	40	10
0.5		4	0.22	0.05	44	10
	0.3	4	0.09	0.03	30	10
	10.0[c]	2	0.16[c]	0.014	1.6[c]	0.1

[a] The amount of DES-MG is expressed as an equivalent amount of DES. [b] Background was 0.05 ppb. [c] The method was performed without enzymatic hydrolysis.

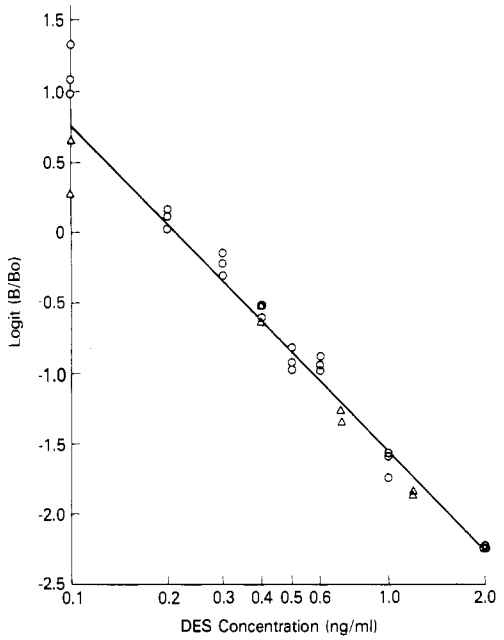

Figure 2. Typical standard curve for RIA of DES. Agreement is shown of standard curves with and without extract: (O) without extract; (Δ) with extract. Because purification did not concentrate DES, RIA measurements in nanograms of DES per milliliter equaled liver concentrations in parts per billion.

was 1.8 ppb of DES (SE = 0.1 ppb) after correction of the results for background (0.05 ppb) and recovery (43%). The percentage of free DES in the undiluted in vivo contaminated liver was measured by taking advantage of the poor recovery of DES-MG without enzyme hydrolysis (see Table II). The undiluted liver was assayed without hydrolysis, and 0.40 ppb of DES (SE = 0.01 ppb) was found when values were corrected only for background (0.05 ppb). After considering the total DES estimated for the undiluted liver (7.2 ppb) and correcting, with simultaneous equations, for the small contribution of DES-MG, the free DES in the liver was calculated to be 7.8% of the total DES.

Liver-Matrix Effects. A standard curve with extract closely paralleled a standard curve with buffer (Figure 2). In addition, dilution did not significantly affect assay results (Figure 3). Nonspecific binding in extracts was significantly different from nonspecific binding in buffer in only 25% of assays and averaged only 9.3% deviation from nonspecific binding in buffer. The average intraassay CV for nonspecific binding was 8.0%. No significant deviation was detected for diluted buffers.

RIA Characteristics. A graph of a typical RIA standard curve for DES is shown in Figure 2. A final antiserum dilution of 1:84000 in the absence of DES bound 52% (SD = 8%) of the [^{3}H]DES. Nonspecific binding averaged 4.5% (SD = 0.8%). The minimum detectable concentration of the RIA ranged from 0.02 to 0.03 ng of DES/mL. The concentration of DES required to reduce binding to half of B_0 was 0.23 ng of DES/mL (SD = 0.02 ng of DES/mL). The absence of an extra incubation period between addition of the first antibody and addition of the second antibody did not affect RIA parameters. The room temperature incubation after the second antibody was required for maximum binding.

DISCUSSION

Accuracy and Specificity. This assay method measures the total of free DES and its glucuronides in a liver sample regardless of their relative concentrations because recoveries of both were not significantly different as determined by RIA. This lack of difference in recovery is due to the quantitative enzymatic hydrolysis of the glucuronides, since DES-MG was not extractable without hydrolysis. The measurement of free DES as well as its glucuronides is important because most of the total DES is bound as glucuronides, as was found in this present work as well as by Hoffmann and Laschutza (1980), Rumsey et al. (1975), and Aschbacher et al. (1975). In spite of this, neither Hoffmann and Laschutza (1980) nor Vogt (1980) documented, for their methods, equal recovery and measurement of free DES and its glucuronides; both reports only coincidently describe provisions for measurement of the glucuronides.

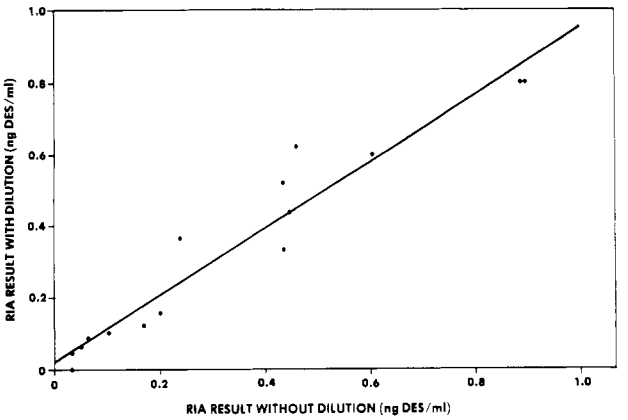

Figure 3. Agreement of RIA results with and without dilution. The fitted linear curve (r^2 = 0.95) (y = 0.94x + 0.013) was not significantly different from $y = x$, for x = RIA result without dilution and y = RIA result with dilution.

The use of [^{3}H]DES as an internal standard for this method is problematic in view of the significant difference in recovery between [^{3}H]DES as determined by liquid scintillation counting and DES and DES-MG as determined by RIA. The difference may be due to in vitro metabolism of the fortified DES and [^{3}H]DES, a possibility demonstrated by Masaracchia (1969), or to degradation of the fortified DES and [^{3}H]DES during the purification procedure. Apparent differences in recovery would result if metabolites or degradation products of DES and [^{3}H]-DES survived the purification procedure and if the tritium was detected by liquid scintillation counting but the metabolites or degradation products were detected poorly or not at all by the RIA. Feil et al. (1977) and Tennent et al. (1976) demonstrated that DES degradation products and other impurities associated with DES are often difficult to separate from DES.

The method is selective, as demonstrated by its resistance to the liver-matrix influence. The background for this method (0.05 ppb) was similar to that reported by Hoffmann and Laschutza (1980) and Vogt (1980). Compared with the GC method of Donoho et al. (1973), upon which other GC methods for DES are based, all these RIA methods have superior specificity and lower background (0.01–0.05 vs. 0.4 ppb).

In 25% of our assays, nonspecific binding was slightly affected by the extracts, but the problem was minor and easily corrected. In each assay, a single measurement of nonspecific binding in a composite of extracts would ensure accuracy.

The specificity of this method is dependent on the antiserum in the RIA, which was prepared from the same antigenic conjugate as that of Hoffmann and Laschutza (1980). Our cross-reactivity data are in agreement with the data of Gutierrez-Cernosek and Cernosek (1977) and Hoffmann and Laschutza (1980). Hoffmann and Las-

chutza (1980) tested various steroids but found none that cross-reacted more than 1%.

When corrected for background and recovery, this assay method is accurate as was confirmed by its agreement with the GC–MS method of Day et al. (1975).

Practicability. The methods of Hoffmann and Laschutza (1980) and of Vogt (1980) are more complex and less practicable than the presented method since they involve more purification steps, including many liquid–liquid partition steps, some with narrow pH limits requiring a pH measurement. The presented method, in contrast, is simpler, with a single fat freeze-out step, a single liquid–liquid partition step not requiring exact pH control, and a simple chromatographic step. With adequate preparation (reagents, columns, and equipment) and by use of 3-h enzyme hydrolysis, 20 samples could be extracted and purified and the RIA procedure initiated in 12 h. The procedure could be completed in less than 48 h.

Precision. Variability of results and heteroscedasticity are problems inherent to RIA and were problems in this method. Tembo et al. (1976) described the problem of heteroscedasticity in RIA. The CV of the RIA response (24%) is apparently mainly due to variability of the RIA since the recovery CV for [^{3}H]DES was only 6.9%. The high variability is typical of analytical methods in this low concentration range (Horwitz et al., 1980). The CVs for RIA results of Hoffmann and Laschutza (1980) and Vogt (1980) are comparable.

Sensitivity. RIA tends to be more sensitive than most physical methods. Donoho et al. (1973) encountered problems with quantitation below 2.0 ppb with GC, and Day et al. (1975) were unable to document sensitivity below 1.0 ppb with GC–MS. We have employed our method down to 0.3 ppb. A detection limit of 0.09 ppb was calculated for our method by the formula of background plus 2 times the SD in order to make a comparison with the methods of Vogt (1980) and Hoffman and Laschutza (1980), who used this same formula. It compares satisfactorily with the values they reported of 0.03 and 0.06 ppb, respectively. These authors did not, however, report limits of determination.

Difficulties Overcome. Interference of liver matrix in the RIA was a major problem that may have been due to nonspecific interference of lipids and fatty compounds as reported by Shaw et al. (1976) and Rash et al. (1980). Because the interference is caused by an undefined source, a more specific antibody would probably not correct the interference problem. Attempts to prepare a more specific antibody were unsuccessful and emphasis therefore was placed on finding satisfactory purification procedures to eliminate the interference. A purely liquid–liquid partition procedure similar to that used by Donoho et al. (1973) was unsatisfactory, as was silica gel chromatography. A combination of the benzene extraction, which eliminated polar interference, and Sephadex LH-20 chromatography, which eliminated nonpolar interference, was found to be effective and reproducible. The fat freeze-out procedure eliminated a considerable portion of the interfering lipids.

Another major problem was the lack of solubility of DES in aqueous solution. This is a significant problem at very low levels of analyte (Kushinsky and Anderson, 1974) and was solved by first dissolving DES in ethanol and then diluting with buffer to 10% ethanol. The order of solvents was important because buffer containing 10% ethanol was ineffective in dissolving DES.

DES in Other Bovine Tissues. Rumsey et al. (1975) have shown that in bovines withdrawn from DES treatment, DES decreases rapidly in muscle and serum, less rapidly in feces and urine, and most slowly in bile, kidney, and liver. Our method may be adaptable to measuring DES in these tissues as well as in liver. Because of high proportions of free DES in feces (Aschbacher et al., 1975), the method might be implemented for use with feces without enzymatic hydrolysis.

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