fer" factor previously proposed(9). One could postulate that the proximity of segment 1 to the area of intrinsic factor secretion would make it the logical site of transfer of Vit. B_{12} from dietary proteins to intrinsic factor.

It is of interest that the same anatomical distribution of releasing factor activity in the rat small intestine was observed with 5,6-dimethylbenzimidazolyl coenzyme B_{12} as was found with Vit. B_{12} bound to intrinsic factor. Good quantitative agreement was also found with both cobalamins and the intestinal extracts of segments 1 and 5. This is consistent with the observation that the coenzyme is as well absorbed from the gastrointestinal tract as cyanocobalamin(10).

Summary. The ability of rat intestinal extracts to release Vit. $B_{12}Co^{60}$ bound to rat intrinsic factor extract was studied using equilibrium dialysis. The small intestines of adult rats were divided into 10 equal segments and extracts of each segment were prepared. Releasing factor activity was detected in all the segments but 2 peaks of activity were found in the proximal area (segment 1) and the mid-portion of the small intestine (segments 4, 5 and 6). Releasing factor activity of the proximal and mid-portion was partially inactivated by heat. Sodium fluoride, sodium arsenite, potassium cyanide or 2,4-dinitrophenol at 10^{-2} M did not markedly inhibit the releasing factor activity. The same anatomical distribution of releasing factor activity was observed with 5,6-dimethylbenzimidazolyl cobamide coenzyme bound to rat intrinsic factor. The finding of releasing factor activity in the midgut is consistent with observations on the site of absorption of Vit. B_{12} in the rat.

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Conjugation of C^{14}-Diethylstilbestrol by Chicken Liver *in vitro*.* (28327)

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The role of the liver in the inactivation of estrogens has been extensively investigated as reviewed up to 1950 by Paschkis and Rakoff(1). Zondek *et al.*(2) demonstrated that diethylstilbestrol (DES) could be inactivated *in vitro* by rat liver pulp but to a lesser extent than estrone while prior treatment with DES *in vivo* increased the inactivating influence of the liver. The DES metabolites resulting from liver action were extracted for assay in spayed female mice. Zimmerberg(3) determined that, for rat liver, conjugation was the preferred means of inactivation, oxidation occurring only when the conjugating capacity of the tissue was ex-

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ceeded. Working with aqueous extracts of beef liver Engel and Rosenberg(4) demonstrated a similar DES-inactivating principle while Riegel and Meyer(5) indicated that, for rat liver, the mechanism may be non-enzymatic.

Metabolites arising from DES inactivation were identified as glucuronides after isolation from urine of the rabbit(6,7) and the cat(7). The liver has been shown to be the major organ responsible for conjugation of estrogens and the chief excretory pathway for such water-soluble substances appears to be *via* a hepatic-biliary system.

In the steer Mitchell *et al.*(8) observed that 90% of the radioactivity resulting from injection of tritium-labeled DES present in the bile was conjugated while Hopwood and Gassner(9) determined that 99% of the radioactivity in the bile after treatment of chickens with C^{14}-DES was water soluble. These liver metabolites have not been identified nor has their biological activity been evaluated although it appears to be evident that their biological potency is greatly reduced(10). The use of isotope labeling, coupled with the sensitive bioassay technique introduced by Umberger *et al.* (11) can furnish a means of further evaluating the role of the liver in alteration of estrogens.

Method. In 2 preliminary trials cold DES was incubated with livers from 5 week old White Leghorn cockerels. The tissue was placed on an ice-cold glass plate and macerated by squeezing through surgical gauze with a glass rod. The tissue mince (1 g) was added to 1.6 μg of DES in 10 ml of Ringer-Tyrode buffer at pH 7 and incubated at 37°C in a Dubnoff shaker for either 1 or 2 hours after which times the reaction was stopped by addition of 40 ml of absolute ethanol. Duplicate samples were treated with ethanol without incubation to serve as controls. Both control and incubated tissues were added to mouse feed for determination of estrogenic activity according to Hopwood and Gassner(10) as outlined below.

In 4 experiments, livers were incubated as above with diethyl(ethyl-1-C^{14}) stilbestrol using 0.25 g of tissue, 2 ml of buffer, 1 μg

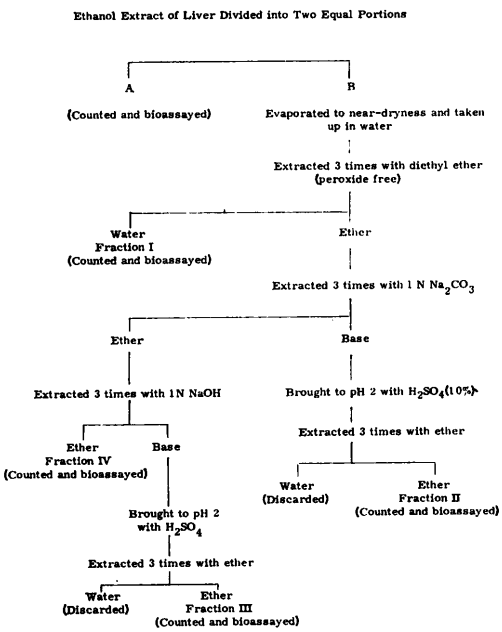

FIG. 1. Fractionation of ethanolic liver extract.

(1,500,000 cpm) of the labeled estrogen and 3 μg of cold DES. The reaction was stopped with 10 ml of absolute ethanol after which the control (not incubated) and incubated tissues were extracted continuously with 95% ethanol and the extracts treated as shown in Fig. 1.

Radioactivity of each fraction, as indicated, was determined by use of an automatic Packard Tri-Carb Liquid Scintillation Spectrophotometer according to the procedures described earlier(10) using Cab-O-Sil for the suspending gel. The estrogenic activities of these fractions were then determined by bioassay.

Immature female mice at 21 days of age weighing between 8 and 10 g were randomly divided into groups of 7-8 animals each and were fed and watered *ad lib.* Consumption of feed was recorded. After 7 days the mice were killed, weighed, and their uteri were removed and placed in Bouin's fluid. After fixation these were trimmed free of excess tissue, blotted on filter paper, and weighed individually to the nearest 0.1 mg. For statistical evaluation, the mean and its standard error were determined for each treatment group. In addition, groups of mice were fed

TABLE I. Effect of Chicken Liver *in vitro* on Biological Activity of Cold DES as Determined by Uterine Weight Assay in the Immature Mouse.

Treatment	Incubation time, min	No. mice	Body wt, g	Uterine wt, mg	DES ppb recovered	% inhibition
Basal feed	0	8	16.3 ± .2*	11.5 ± .7*	—	—
DES 1.6 μg + 1 g liver	0	7	17.3 ±1.1	31.8 ± 3.2	8.8	—
Idem	60	7	16.3 ± .5	14.2 ± .7	4.4	50
Basal feed	0	8	13.0 ± .5	8.2 ± .6	—	—
DES 1.6 μg + 1 g liver	0	7	14.9 ± .8	18.6 ± 1.4	6.1	—
Idem	120	7	14.0 ± .6	12.5 ± 1.4	3.1	49

* ± S.E.

with the basal diet or with feed to which 2, 4, 8 or 12 ppb (μg per kg) of DES were added. The equation for the best-fitting straight line was calculated, based on the log of the uterine weight response in mg *vs* the dosage of DES in ppb. By use of this equation the uterotropic response of each group fed with liver or liver fractions was expressed in terms of DES activity.

To determine the nature of the metabolites 0.25 g of liver, 1 μc of C^{14}-DES (1,200,000 cpm) and 3 μg of cold DES were added to 20 ml of Ringer-Tyrode buffer in duplicate. One of these was at once treated with absolute ethanol to prevent reaction; the other was incubated for 2 hours at 37° and then treated with ethanol. The control and incubated sample were extracted for 12 hours with 95% ethanol and radioactivities of the residue and extract determined. The ethanol extract was evaporated to near dryness and taken up in water and partitioned between ether and water.

The aqueous extract resulting from incubation was treated for 72 hours in acetate buffer at pH 4.5 with β-glucuronidase (Ketodase, Warner-Chilcott) at 500 Fishman units per ml and extracted with ether. The ether solution was concentrated and chromatographed on Whatman No. 2 paper in a system of benzene-Skellysolve (1:1) *vs* 80% methanol. The developed chromatogram was dried and scanned for radioactivity in a Vanguard Model 800 Autoscanner. A quantitative measurement was obtained by integrating the areas under the curves by planimetry.

Chromatography of an aqueous extract not subjected to hydrolysis gave radioactive areas that, on treatment with aniline hydrogen phthalate and heating at 100°C, were positive for carbohydrate. Such areas disappeared after hydrolysis with β-glucuronidase to yield DES. Turnbull's reagent applied to the conjugated material isolated by chromatography yielded a blue color indicating the phenolic nature of the sugar-positive area.

Results. Table I shows the deactivation of DES by chicken liver *in vitro* in the preliminary experiments. Activity based on uterotropic response in immature mice was reduced by 49-50% in the separate experiments after incubation for either 1 or 2 hours. When C^{14}-DES was incubated with chicken liver (Table II), 94.6% was extracted with ethanol from the control tissue while but 82.6% could be removed after incubation. Only 70% of the total counts from control tissue were in the water solution and were not extractable with ether while incubation caused 74% of the C^{14} to remain in the aqueous phase.

Without incubation, 87% of the total counts could be extracted from ether with NaOH, characteristic of the phenolic nature of DES. After incubation but 23% of the counts were removed by the base. The few counts extractable from ether with sodium carbonate were alike in quantity both from control and incubated tissues. Counts remaining in ether after treatment with carbonate and hydroxide were, likewise, low and were not influenced by incubation with liver.

The biological activity of fractions isolated after incubation of DES with chicken liver is shown in Table III. There was good agreement between biological- and radioactivity of the ethanol extract from unincubated tissue; however, after incubation a like

TABLE II. Percentage of Total Counts of C^{14}-DES Extracted from Liver Before and After Incubation and Their Distribution After Solvent Fractionation.

Treatment		Exp 1	Exp 2	Exp 3	Exp 4	Avg
Ethanol (Extract A)	Control	98.4	93.0	96.2	90.9	94.6
	After incubation	92.4	80.0	81.1	77.0	82.6
Water (Fraction I)	Control	12.5	.3	11.0	4.7	7.1
	After incubation	83.0	72.1	70.9	71.8	74.5
NaOH (Fraction III)	Control	72.1	94.0	86.9	95.3	87.1
	After incubation	16.8	26.0	22.8	28.0	23.4
Na_2CO_3 (Fraction II)	Control	3.2	—	—	—	—
	After incubation	4.1	—	—	—	—
Ether (Residue IV)	Control	1.3	3.5	1.5	—	2.1
	After incubation	2.0	2.4	1.2	—	1.9

0.25 g liver incubated with 1 μg (1,500,000 cpm) of diethyl(ethyl-1-C^{14}) stilbestrol (C^{14}-DES) plus 3 μg of cold DES for 1 hr at 37°C. Fractions were derived as shown in Fig. 1.

number of counts had uterotropic activity that was but 8% of the value expected from the radioactivity. Without incubation the water-soluble, ether-insoluble material (Fraction I) contained little of the total radioactivity of the added DES, but the estrogenic activity of this quantity was relatively high. Counts in this fraction increased markedly by incubation, but the biological activity, expected from the quantity of C^{14} present, decreased to 9% of the original potency.

In the control and incubated tissue material removed from ether by NaOH (Fraction II) was as biologically active as free DES although these counts in the first experiment were too low after incubation for adequate biological estimation and could not be considered. Fractions II and IV had few counts and negligible biological activity.

Determination of the nature of the liver metabolites is shown in Table IV. The behavior of the DES on incubation was as previously observed. After fractionation between ether and water and subsequent chromatography of these extracts 16.4% of the material in ether was conjugated and the re-

TABLE III. Biological Activity (Mouse Uterine Weight Assay) of Fractions After Incubation of DES with Chicken Liver.

	Cpm added to feed		ppb DES expected		ppb DES actual response		% of theoretical response	
Treatment	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2	Exp 1	Exp 2
C^{14}-DES standard	397,000	205,666	13.0	8.1	12.9	8.1	99	100
Ethanol (Extract A)								
Control	382,000	280,449	12.9	11.0	12.4	11.8	96	107
After incubation	322,000	316,600	10.4	12.5	1.1	.6	11	5
Water (Fraction I)								
Control	125,700	52,408	4.0	2.1	3.9	.7	97	33
After incubation	627,800	479,800	24.7	18.9	1.6	2.2	6	12
NaOH (Fraction III)								
Control	552,000	267,400	17.9	10.5	15.6	11.3	87	108
After incubation	17,440	251,000	.6	9.9	0	9.3	—	94
Na_2CO_3 (Fraction II)								
Control	6,400	—	.2	—	0	—	—	
After incubation	44,800	—	1.4	—	.8	—	—	
Ether (Residue IV)								
Control	17,400	—	.6	—	0	—	—	
After incubation	10,850	—	.3	—	2.1	—	—	

0.25 g liver incubated with 1 μg (1,500,000 cpm) of diethyl(ethyl-1-C^{14}) stilbestrol (C^{14}-DES) plus 3 μg of cold DES for 1 hr at 37°C. Fractions derived as shown in Fig. 1. Biological assay conducted as shown in procedure.

TABLE IV. Percentage of Metabolites Obtained After Incubation of DES with Chicken Liver Followed by Extraction, Fractionation, and Hydrolysis of the Conjugate with β-glucuronidase.

Fraction	% of DES metabolites on chromatogram			
	Conjugated DES	Free DES	More polar than DES	Less polar than DES
Ether soluble (577,000 cpm)	16.4 (94,628 cpm)	83.6 (482,372 cpm)	0	0
Water soluble (591,000 cpm)	93.5 (552,585 cpm)	6.5 (38,415 cpm)	0	0
Conjugate after hydrolysis (552,585 cpm)	0	89.8 (496,221 cpm)	6.7 (37,023 cpm)	3.5 (19,404 cpm)

mainder was free DES. No other radioactive areas were observed. Chromatography of the aqueous extract gave 93.5% of the counts in the area occupied by the conjugate while a small portion was free DES. After elution of the conjugate area and hydrolysis with β-glucuronidase 89.8% of the material was free DES but a small quantity of material more polar (6.7%) and less polar (3.5%) than DES was detected on the scanned chromatogram.

Discussion. It is interesting to observe that significantly fewer counts were extracted from liver after incubation than from controls, indicating a binding of the estrogen to liver. No volatile metabolite such as CO_2 was evolved during incubation since all the counts remaining in the residue after extraction accounted for all the radioactivity applied to the tissue. In addition we have observed that $C^{14}O_2$ was not evolved up to 6 hours after administration of C^{14}-DES to chickens. This observation differs from that reported in the rat by Twombley and Schoenwaldt(12) and Gawienowski *et al.*(13).

The results indicate that *in vitro* only a conjugation reaction is of consequence since after incubation with liver C^{14}-DES was distributed either free or as a water-soluble conjugate; however, the formation of 2 other metabolites at very low concentrations presents the possibility that these could have been formed prior to conjugation. It also must be considered that the hydrolysis reaction could have created an alteration of the DES molecule since these were found after hydrolysis.

The biological potency of the conjugate fraction is in agreement with that reported by Simpson and Wilder-Smith(14) who found the glucuronide of DES to be but 5 to 10% as active as the free estrogen.

We have extracted a water-soluble fraction from bile of chickens implanted with C^{14}-DES and have observed that only DES was released after hydrolysis with β-glucuronidase. Incubation of the conjugate with ingesta or with homogenates of small intestine of chickens did not release DES. Furthermore, we have been unable to obtain a reaction with intestine homogenates similar to that with liver.

Summary. Chicken liver was incubated with C^{14}-labeled diethylstilbestrol (C^{14}-DES). Without incubation 94.6% of the radioactivity was extracted with ethanol while after incubation only 82.6% was removed from the tissue. Partition of the radioactivity between water and ether at pH 7 gave 7% of the counts in water from unincubated tissue and 75% of the counts in water after incubation. Hydrolysis of the aqueous portion with β-glucuronidase and paper chromatography established that the material was conjugated DES. Assay of the extracts for uterotropic activity using immature mice indicated that the conjugate was but 8% as potent as the free compound although incubation caused a 50% loss of estrogenic potency.

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Antibody Response in Rabbits to Adenovirus Type 12*† (28328)

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The report by Trentin and associates(1) on the oncogenic properties of Adenovirus Type 12 has been confirmed by Huebner *et al.*(2) who have further demonstrated the production of tumors in hamsters with Adenovirus Type 18. These studies suggest a need for an *in vitro* test to differentiate strains of a particular Adenovirus type. The following pilot study on antibody production to Adenovirus in rabbits was initiated with the ultimate goal of demonstrating immunological differences among Adenovirus Type 12 strains. Previous reports(3-7) involved hyperimmunization with multiple doses of large amounts of infectious virus. As here shown, satisfactory antibody titers have been obtained following a single injection of relatively small amounts of viral antigen.

Methods. New Zealand rabbits, 3.5 to 4.5 kg, were bled immediately prior to inoculation, and at 1, 7, 15, 21, 28, 30, 35 and 42 days thereafter. Sera were clarified by light centrifugation and stored at –20°C until tested.

Adenovirus Type 12 antigen was prepared by making 3 serial passages in KB tissue culture of the American Type Culture Collection prototype strain (Huie). Infected cells and fluid from the third passage harvest were freeze-thawed 3 times and lightly centrifuged to remove cellular debris. The supernatant virus fluid represented the antigen used in these studies. This pool‡ has since been demonstrated to be oncogenic by Huebner *et al.* (2). Infectivity titrations in HeLa cells have shown the virus pool to titer $10^{3.5}$/0.1 ml after 7 days. Considerably higher titers up to $10^{6.3}$/0.1 ml were obtained when the virus was titrated for 14 days in primary human embryonic kidney (HEK) tissue culture. All virus titers listed in the following discussion and tables are based on titrations in HEK.

Three groups of 2 rabbits each were inoculated intravenously with 1½ ml of Adenovirus Type 12 with 30,000,000 tissue culture infective doses — 50% ($TCID_{50}$); 300,000 $TCID_{50}$; and 3,000 $TCID_{50}$. Rabbits #3012, #3014 and #3016 were given a booster inoculation of 300,000 $TCID_{50}$ intravenously on day 28.

Serum neutralization tests were carried out in 24-hour-old HeLa tube cultures. Cells were maintained for the duration of the test on Eagle's Basal Medium(8) in Earle's Balanced Salt Solution (BSS) containing 5% Agamma Calf Serum.§ All media were supple-

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