pregnancy exhibit mean total DNA of mammary glands of 2.853, 4.118 and 5.969 mg. Thus, estrogen and relaxin stimulated lobule-alveolar growth as indicated by DNA equalled that of 6 days of pregnancy(3).

In the 2 groups ovariectomized, a period of involution of 12 days was allowed. Restimulation with estrogen did not restore the total DNA level to that shown by the intact animals. Neither was estrogen and relaxin able to stimulate total DNA equal to that of the intact groups. This difference is not considered as related to the lack of ovaries or uteri, *per se*, but rather to the effect of gland involution before hormones were begun. Since hysterectomized mice showed slightly greater total DNA than did the corresponding ovariectomized groups, the uterus has no inhibitory action on the effect of relaxin on mammary gland growth.

Summary. Intact, ovariectomized, and ovariectomized-hysterectomized mice were treated with estrogen, estrogen and relaxin, and relaxin alone for periods of 10 days. It was shown that estrogen and higher levels of relaxin synergize in the promotion of lobule-alveolar growth as indicated by total DNA of the glands and by histological examination of whole mounts. Estrogen or relaxin alone did not stimulate such growth. Mean total DNA at optimal levels of relaxin was equal to that observed in mice pregnant 6 days. In the operated groups, reduced total DNA may be due to gland involution of 12 days.

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Method for Determination of 17,21-Dihydroxy-20-Ketosteroids in Urine and Plasma.* (25069)

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The adrenal cortex secretes several steroids directly associated with human metabolism. Metabolism, separation, measurement and identification of these steroids have been the subject of much research. A quick, accurate method of measurement of these steroids is of great value to the clinician for correct diagnosis and treatment. Porter and Silber(1) reported that a solution of phenylhydrazine in sulfuric acid formed a colored product when mixed with 17,21-dihydroxy-20-ketosteroids. Using this color reaction Nelson and Samuels (2) formulated the first practical method for routine quantitative determination of 17-hydroxycorticosteroids in blood. By their procedure the blood extract was purified by solvent partition and chromatography on a synthetic magnesium silicate (Florisil) column. Several other methods for determination of free plasma 17-hydroxycorticosteroids have since been developed. Sweat(3) described a procedure involving chromatographic analysis of pure mixtures of several steroids on a silica gel micro column. Quantitative recovery of steroids was determined by technics employing both fluorescence and reaction with phenylhydrazine. A simple, accurate method for determination of the 17,21-dihydroxy-20-ketosteroids in plasma and urine has now been devised. It consists in a method of extraction similar to that of Paterson(4) and a procedure for chromatographic resolution on silica gel columns similar to the one reported by Sweat (3).

Materials. 1) A 1 ml serologic pipette is used as the column. 2) Glass wool (washed repeatedly with redistilled absolute ethanol)

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is placed into 1 ml serologic pipette and firmly packed until top of glass wool forms a tight, flat pad. 3) Silica gel (Davison, 100-200 mesh size) is washed with redistilled absolute methanol and supernatant is discarded. After washing 11 additional times, silica gel is dried beneath incandescent light (100 watts) overnight and kept in desiccator. 4) Chloroform is redistilled immediately before use. 5) Control samples of plasma came from a group of women and men aged 18 to 35 years. *Chemical assay.* 1) Color development with phenylhydrazine-sulfuric acid solution (6.5 mg phenylhydrazine hydrochloride/10 ml of 7.3 N sulfuric acid) is brought about by incubation in water bath at 38°C for 3 hours. 2) Samples are read in Beckman quartz spectrophotometer at 410 mu in 0.5 ml micro cuvettes. *Extraction.* Plasma (6-20 ml) or urine is adjusted to pH 1, with concentrated hydrochloric acid, extracted 3 times with 2 volumes of freshly distilled chloroform; the combined chloroform extracts are back extracted 2 times with 0.1 volume of 0.1 N NaOH followed by 2 washings of 0.1 volume of distilled water. The chloroform extracts are dried with anhydrous sodium sulfate, evaporated to dryness by distillation *in vacuo,* transferred quantitatively to small test tube and dried with stream of nitrogen. *Chromatography.* 100 mg of silica gel is added to 5 ml of chloroform, placed in serologic pipette with a capillary tube, packed by gentle tapping and washed with additional 8 ml of chloroform. The dried extract of plasma is dissolved in 1 ml of chloroform and placed on silica gel with a capillary tube. The test tube is washed with 8-1 ml portions of chloroform, these washings placed on column and resulting effluent is collected as fraction one. The column is further developed with 3 ml of 0.2% solution of absolute ethanol in chloroform and the effluent is collected as fraction 2. The third and last eluant consists of 5 ml of a 10% solution of absolute ethanol in chloroform. The last 2 fractions are divided into 2 equal aliquots (using aliquot of each fraction as a blank), dried and assayed by procedure previously described.

TABLE I. Mean Percentage of Recovery with Varying Amounts of Hydrocortisone Added to Plasma (5 Exp.).

No. of samples	Added hydrocortisone (μg)	% recovery (mean)	% recovery (range)
3	.60	80	78-82
3	1.17	83	80-86
6	3.53	85	83-88
3	3.30	89	87-92
4	4.25	92	90-95

Vol of plasma used in extraction, 10 ml.

TABLE II. Recoveries of Hydrocortisone from Plasma in 11 Tubes.

Exp.	Added hydrocortisone (μg)	P-S* chromogens (μg)	Amt recovered (μg)	% recovery
1	6.62	7.69	5.99	90
2	"	7.77	6.07	92
3	"	7.44	5.74	87
4	8.50	9.68	7.98	94
5	"	9.36	7.66	90
6	0	1.58		
7	0	1.78		
8	0	1.73		
9	0	1.65		
10	0	1.70		
11	0	1.60		

Vol of plasma used in extraction, 20 ml.

Exp. 1-5: reproducibility.
6-8: non-partitioned extract.
9-11: partitioned " .

* 17,21-dihydroxy-20-ketosteroids.

Results. Chromatography of hydrocortisone, cortisone and Reichstein's compound S resulted in recoveries of 90 to 96%. When the compounds were added to plasma, extracted and chromatographed, recoveries ranged from 80 to 94%.

Table I shows mean percentage of recovery in control experiments in which different amounts of hydrocortisone were added to pooled plasma. Recoveries were higher with larger amounts of hydrocortisone. Lines 1 and 2 illustrate recovery that may be expected from normal plasma by this method, since our values in 19 normal subjects ranged from 6 to 13 μg/100 ml of plasma.

Table II contains recovery data of hydrocortisone and results of representative experiments in which determinations of 17,21-dihydroxy-20-ketosteroid group of pooled plasma were made before and after partition of chloroform extracts between a 70% solution of aqueous ethanol and low boiling petroleum ether. The values for Exp. 1 through 5 illustrate the

TABLE III. Urinary and Blood Values of 3 Patients with Cushing's Disease and Adrenal Hyperplasia.

Urinary K-S	Urinary formald.	Urinary P-S chromogens	Plasma P-S chromogens	Plasma P-S chromogens after ACTH
mg/24 hr			μg/100 ml	
23.70	1.68	3.07	30	
12.80	2.34	2.88	43	67
39.50	11.95	53.60	81	96

reproducibility of this procedure. Exp. 6 through 8 represent initial amounts of 17,21-dihydroxy-20-ketosteroids in plasma. Exp. 9 through 11 represent samples that were partitioned between low boiling petroleum ether and a 70% solution of aqueous ethanol. All chloroform extracts gave positive reactions to Tollen and blue tetrazolium tests. The reactive material migrated at the same rate as hydrocortisone on paper chromatograms(5).

Table III contains results obtained in 3 patients with diagnoses of Cushing's disease and adrenal hyperplasia subsequently confirmed at operation. Column 4 gives initial high values of plasma Porter-Silber chromogens with increase after administration of ACTH (column 5).

When urinary extracts obtained from 20 ml of normal urine were chromatographed according to method described, the values were approximately 13 to 20% less than those of corresponding unchromatographed samples. This is probably due to removal of impurities which tend to give a false high value for the dihydroxy-acetone group since chromatographic recovery experiments show a loss of only 4 to 7%. Recoveries of tetrahydrocortisone added to urine ranged from 87 to 102%.

Fig. 1 illustrates relative values obtained when formaldehydogenic and Porter-Silber determinations are performed on the same urinary extract. The values are below 1 mg/24 hr in most instances.

Discussion. Partial purification of plasma and urinary extracts is sometimes a desirable preliminary step for determination of 17-hydroxy-corticosteroids, although it can be time-consuming. A quick, accurate procedure for their determination is highly desirable. By applying our procedure to plasma and urine samples, 6 to 8 determinations are completed in a day without difficulty. Using twice the volume of chloroform shortens the time needed for extraction and results in few instances of emulsion formation.

Thirty μg of steroid could be chromatographed on silica gel columns described. When mixtures of cortisone, hydrocortisone and Reichstein's compound S (10 μg each) were chromatographed, there was only partial resolution of the compounds.

In routine analysis of 24 hour urine collections for formaldehydogenic compounds and 17,21-dihydroxy-20-ketosteroids determinations are carried out on the same chloroform extract. When urinary extracts are chromatographed and 17,21-dihydroxy-20-ketosteroids are determined quantitatively by Porter-Silber procedure, results are invariably lower than formaldehydogenic results. This is explained by the fact that only corticosteroids having 17,21-dihydroxy-20-keto side chain will give a positive reaction to the Porter-Silber test, whereas all types of 21-hydroxy corticoids will give a positive formaldehyde test.

Summary. A simple, accurate method for determination of the 17,21-dihydroxy-20-ketosteroids in plasma and urine has been described. The chloroform extract is dissolved in chloroform and chromatographed on silica gel in a 1 ml serologic pipette. The 17,21-

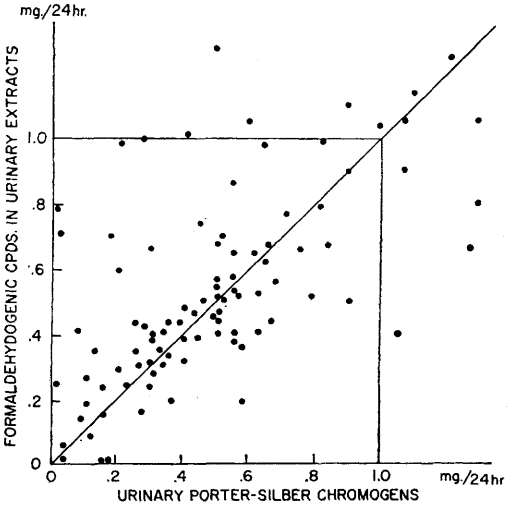

FIG. 1. Urinary Porter-Silber chromogens *vs* formaldehydogenic compounds in urinary extracts.

dihydroxy-20-ketosteroids are eluted with ethanol-chloroform mixtures and assayed by a micro Porter-Silber procedure.

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Inosine Effects on Plasma Inorganic Phosphate Movements in Fresh and Cold-Stored Blood.* (25070)

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Our purpose was to determine changes in phosphate movement in fresh and cold-stored blood in the presence of inosine, a substance currently used in clinical preservation of whole blood by cold storage. Influx of phosphate from plasma to the red cell has been studied several times in both fresh and cold-stored blood(1,2,3) but the efflux of phosphate from the cellular phase to plasma has been studied only in fresh blood(4,5,6). Inosine and other purine ribosides are metabolized by human erythrocytes(7,8,9) and this is associated with an increase in intracellular phosphorylated compounds. These and other intracellular metabolic changes doubtless influence the movements of phosphate between plasma and cells. We studied movements into and out of red cells by following changes in 1) plasma inorganic phosphate and 2) P^{32} in plasma and cells. To limit the number of variables, we added only anticoagulant and the reagents under study.

Methods. Blood from adult male donors was handled as described by Kahn and Acheson(10) except that glucose was omitted. At least 3 experiments were performed for each treatment. I. One hundred ml of whole blood either fresh or cold-stored was divided in half, and Sodium Radiophosphate (Abbott Labs) was added to one-half of the blood. Both samples of blood were incubated in constant temperature oven at 37°C for 75 minutes with intermittent gentle agitation. The 2 samples were then centrifuged at 0-4°C and the plasmas removed and saved. The cells were washed once with cold saline to remove most of trapped plasma. After centrifugation of the resuspended cells, the saline was discarded and the cells with P^{32} were added to the plasma without P^{32}, while the cells without P^{32} were added to the plasma with P^{32}. After the plasmas were exchanged, blood was added to glass-stoppered Erlenmeyer flasks containing 0.1 volume of either saline (control), or 40 mM inosine. The flasks were then shaken 3 hours at 37°C in a Dubnoff shaker. Samples were taken at different intervals and measurements of plasma radioactivity and inorganic phosphate were made. Zero time was the time the reconstituted blood was added to the flasks. II. In other experiments fresh blood was preincubated with P^{32} in the manner just described, but both samples (radioactive and non-radioactive) were stored in the cold room for 6 days before centrifugation, exchange of plasmas and 37°C incubation. A Beckman Model G pH meter measured pH of whole blood. Both fresh and cold-stored blood, containing inosine or saline, measured before, during and after the experiment, had pH's between 7.6 and 7.7. Hematocrits were measured in duplicate by the technic of Guest and Siler(11). When standard errors of the mean or probabilities were determined, the "t" test

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