found at diestrus. 3) The Hx/OHP ratio in the uterus was also cyclical, less during estrus than during diestrus. 4) Concentration and total amount of NCP were greater in the intact animal than in the castrate. 5) Per unit weight (g) and in the total vagina neither OHP nor Hx showed significant alterations during the estrous cycle. 6) The cyclical changes listed above may be related to fluctuations in levels of the natural hormones. 7) Studies of OHP, Hx, total N and NCP were made of the uterus and vagina of the castrate and comparisons were made to the intact animal.

The author wishes to thank Mrs. Jovita Baker for the technical assistance in the chemical analyses.

1. Harkness, M. L. R. Harkness, R. D., *J. Physiol.*, 1954, v123, 492.
2. Woessner, J. F., *Biochem. J.*, 1962, v83, 304.
3. Harkness, M. L. R., Harkness, R. D., Moralee, B. E., *J. Physiol.*, 1957, v135, 270.
4. Morgan, C. F., *Anat. Rec.*, 1961, v139, 257.
5. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v184, 299.
6. Transactions of 1st Conference on *Polysaccharides in Biology*, Josiah Macy, Jr. Foundation, 1955, 31.
7. Boas, N. F., *J. Biol. Chem.*, 1953, v204, 553.
8. Koch, F. C., McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, v46, 2066.
9. Neuman, R. E., Logan, M. A., *J. Biol. Chem.*, 1950, v186, 549.
10. Bowes, J. H., Elliott, R. G., Moss, J. A., *Biochem. J.*, 1955, v61, 143.
11. Robertson, W. van B., *J. Biol. Chem.*, 1952, v196, 403.
12. ———, *ibid.*, 1952, v197, 495.
13. Wolbach, S. B., Bessey, O. A., *Physiol. Revs.*, 1942, v22, 233.
14. Harkness, M. L. R., Harkness, R. D., Moralee, B. E., *Quart. J. Exp. Physiol.*, 1956, v41, 254.
15. Muir, H., *Biochem. J.*, 1958, v69, 195.
16. Sobel, H., Gabay, S., Johnson, C., Hassan, B., *Metab.*, 1959, v8, 180.

Received January 3, 1963. P.S.E.B.M., 1963, v112.

Protein Binding of Estriol Conjugates.* (28143)

JOSEPH C. TOUCHSTONE AND ESTHER CHANG (Introduced by J. J. Christian)
Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia

Data are presented here concerning dialysis of estriol sulfate and glucosiduronate from deproteinized plasma and solutions. Previous experiments in this laboratory suggested that estriol conjugates were bound to plasma proteins. Most reports of protein binding of steroids have been limited to studies of the free compounds primarily because of the unavailability of pure conjugates(1,2). Recently, Puche and Ness described the binding of dehydroepiandrosterone sulfate to serum albumin(3).

Methods. Human serum albumin Fraction V (HSA-5) (Pentex Corp., Kankakee, Ill.), obtained in crystalline form, was made up as 2.5 and 5.0% solutions.

Estriol glucosiduronate was obtained from: Sigma Chemical Co. (mixture), Dr. Philip Katzman (monoglucosiduronate), and Ayerst, McKenna and Harrison Ltd., and estriol sulfate was kindly provided by H. Fex, A. B. Leo, Halsinborg, Sweden.

Heparinized blood was centrifuged within 20 minutes after withdrawal and the plasma separated. Free estrogens were removed by extracting the plasma 3 times with equal volumes of ether leaving the conjugates in solution. Urines preserved with chloroform were kept in an ice box.

Plasma proteins were precipitated by addition of 4 volumes of acetone. The precipitate was centrifuged, washed 3 times with acetone, and the combined acetone supernatant was evaporated *in vacuo*. Dialysis was then carried out on the aqueous residue. The method consisted of 3 steps: I. *Dialyses* were

* This work was supported in part by U. S. Public Health grants.

TABLE I. Dialysis of Estriol Conjugates.

Material in bag	N	Time (hr)	Control	Bag	Dialysate	% dialysis
Plasma	3	24	62.7 ± 8.8*	60.0 ± 8.0	3.0 ± 1.7	4.2 ± .8
	2	48	81.0 ± 0	65.5 ± 3.5	12.5 ± .5	15.4 ± .6
Deproteinized plasma:						
HCl hydrolysis	2	24	83.0 ± 2.0	0	80.5 ± .5	97.0 ± 2.0
Enzyme hydrolysis	9	24	76.2 ± 8.0		60.0 ± 11.0†	99.3 ± .1
					10.0 ± 1.4‡	
Urine	8	24	135.6 ± 46.0	33.9 ± 10.5	101.1 ± 39.4	73.5 ± 7.0

* Mean estriol (μg) ± S.E.
† Mylase.
‡ Glucuronidase.

carried out in an ice box at 4°C for 24 hours and in some cases, 48 hours under continuous agitation. Five ml of conjugate solution in the cellulose dialyzer tubing (bag) was dialyzed against 25 ml of water. For reverse dialysis the conjugates were dissolved in 25 ml solvent and dialyzed against 5 ml of HSA-5 in the bag. II. *Hydrolysis, extraction, and column chromatography.* After dialysis the conjugates were hydrolyzed by reflux with 15% by volume of hydrochloric acid for 1 hour. For enzymatic hydrolysis, β-glucuronidase (Sigma) was added to the solution buffered at pH 6.8 or phenolsulfatase (Mylase P) to solutions buffered at pH 6.0. Incubation at 37°C for 24 hours was carried out in the former, while 50°C for 24 hours was used in the latter. Phenolic separation following a modified Brown(4) and Bauld(5) procedure was carried out. Chromatography of the extracts on alumina involved a modification of the procedure described by Eberlein *et al.*(6). III. *Color development and colorimetry.* The Bachman reagent for estriol (7) consisting of 2% p-phenol sulfonic acid in concentrated phosphoric acid was added to the estriol fraction from the alumina column and then heated at 150°C for 10 minutes. Optical density at 545 mμ was determined on a Beckman DU Spectrophotometer. The overall recovery of estriol by this procedure was found to be 80%. In replicate determinations, the standard deviation was 0 in most cases.

All quantitative results reported herein are of the free estrogen determined after acid or enzymatic hydrolysis of the conjugates. Control values represent estriol liberated by acid hydrolysis of conjugates in an equivalent volume of plasma, urine, or aqueous conjugate solution without dialysis.

Results and discussion. Table I shows that little transfer (4%) of estriol conjugates took place after 24 hour dialysis of whole cord blood plasma (5 ml) in the bag against 25 ml of water. Dialysis for 48 hours resulted in an increased amount (15%) of conjugates found in the dialysate. Dialysis of deproteinized cord blood plasma resulted in quantitative transfer of estriol sulfate and glucosiduronate across the membrane. The dialysate of deproteinized plasma when hydrolyzed with β-glucuronidase (Sigma) and Mylase P, gave quantitative recoveries of estriol when compared to the total estriol liberated by acid hydrolysis of the undialyzed control. Control experiments indicated that the amount of estriol found after acid hydrolysis of plasma deproteinized by acetone was more than 90% of that found after acid hydrolysis of whole plasma, none being found in the protein precipitate.

Dialysis of estriol conjugates from 5 ml of pregnancy urine in the bag into 25 ml of water resulted in transfer of as much as 95% of conjugates across the membrane. The completeness of dialysis as shown in Table I varied widely according to the urine specimen. Acid hydrolysis was used for both control and dialysate. These results possibly could be related to the ionic strength and pH of the various urine specimens.

Table II indicates that estriol glucosiduronate dissolved in water and dialyzed against 5 volumes of water for 24 hours did not show complete transfer. Dialysis for 48 hours gave greater recovery of conjugates outside the membrane. Estriol glucosiduronate when

TABLE II. Dialysis of Solutions of Estriol Glucosiduronate.

Conjugate solution in bag	N	Time (hr)	Control	Bag	Dialysate	% dialyzed
Male plasma (ppt) solution						
Ayerst*	4	24	14.7 ± .4†	0	14.7 ± .4	100.0 ± 0
Saline solution						
Ayerst*	3	24	12.5 ± 2.5	4.0 ± 2.0	8.5 ± .5	70.0 ± 10.0
Aqueous solutions						
Ayerst*	2	24	11.5 ± 3.5	9.0 ± 3.0	2.0 ± 0	20.0 ± 6.0
Sigma*	2	24	5.5 ± .5	3.5 ± .5	2.0 ± 1.0	35.0 ± 15.0
	2	48	4.0 ± 2.0	0	4.0 ± 2.0	100.0 ± 0
Katzman*	2	24	5.5 ± .5	4.0 ± 0	1.5 ± .5	26.5 ± 6.5
	3	48	15.0 ± 5.0	.5 ± .5	14.5 ± 4.5	98.0 ± 2.0

* Source of estriol glucosiduronate. † Mean estriol (μg) ± S.E.

TABLE III. Reverse Dialysis of Estriol Conjugates.

Solution outside bag	N	Time hr	% HSA in bag	Control	Outside	In bag	% dialysis
Cord blood:							
Deproteinized	3	24	2.5	9.8 ± .6*	2.0 ± .1	7.6 ± .2	77.0 ± 3.3
	3	24	5.0	9.8 ± .6	.9 ± .1	8.9 ± .1	91.0 ± 1.0
Pregnancy urine	2	24	5.0	30.5 ± .5	13.0 ± .5	17.0 ± 0	55.5 ± .5
" diluted 1:5	2	24	5.0	41.0 ± 10.0	16.0 ± 3.0	24.5 ± .5	60.0 ± 3.0
Estriol glucosiduronate:							
Aqueous solution	2	24	2.5	10.5 ± .5	3.0 ± 1.0	6.5 ± .5	62.0 ± 2.0
	2	24	5.0	11.0 ± 0	2.5 ± .5	7.0 ± 1.0	64.0 ± 9.0
	2	48	5.0	11.0 ± 0	1.0 ± 1.0	10.0 ± 1.0	91.0 ± 9.0
Saline solution	4	24	5.0	8.0 ± 1.1	2.0 ± 0	5.0 ± .6	63.0 ± 1.7
Estriol sulfate:							
Aqueous solution	2	24	2.5	13.0 ± 0	10.0 ± 0	2.0 ± 0	76.0 ± 0
	3	24	5.0	13.0 ± 0	9.0 ± 0	3.0 ± 0	76.0 ± 0
	3	48	5.0	15.0 ± 0	14.0 ± 0	1.0 ± 0	93.0 ± 0

* Mean estriol (μg) ± S.E.

dissolved in deproteinized male blood plasma and dialyzed against water showed a greater transfer than was shown when water or saline was used as the solvent.

Transfer of estriol conjugates from deproteinized cord blood plasma into 5% HSA-5 in saline in the bag was complete after 24 hours dialysis (Table III). However, reverse dialysis of human pregnancy urine for 24 hours was not as complete. Slaunwhite and Sandberg(8) in their experiments changed the protein solutions several times during 72 hours dialysis in order to effect more complete recovery of conjugates from urine.

The binding force of human serum albumin for estriol conjugates is illustrated in the reverse dialyses listed in Table III. Dialysis of 2.5 or 5% HSA-5 against 5 volumes of aqueous solutions of estriol glucosiduronate or estriol sulfate resulted in transfer of the conjugate to the plasma side of the membrane. Most complete transfer was found with 5% HSA-5 after 48 hours dialysis. When saline was used as the solvent for the conjugate less transfer occurred.

These data suggest that both estriol sulfate and glucosiduronate are attracted to plasma protein. Purdy *et al.*(9) have shown that estrone sulfate was found in the supernatant of the Cohn Fraction IV of maternal blood proteins. Further studies are required to obtain insight on the mode of binding of the conjugates to protein.

Summary. Estriol sulfate and estriol glucosiduronate present in aqueous solutions, urine or in deproteinized cord blood plasma dialyze through a cellulose membrane. Reverse dialysis of estriol sulfate or glucosiduronate from urine, protein free plasma, or aqueous solutions against human serum albumin Fraction V was accomplished readily.

1. Westphal, U., in *Mechanism of Action of Ster-*

oid Hormones, C. A. Villee and L. L. Engel, Ed., Pergamon Press, New York, 1961, p33.

2. Daughaday, W. H., *Physiol. Rev.,* 1959, v39, 885.

3. Puche, R. C., Ness, W. R., *Endocrinology,* 1962, v70, 857.

4. Brown, J. B., *Biochem. J.,* 1955, v60, 185.

5. Bauld, W. S., *ibid.,* 1956, v63, 488.

6. Eberlein, W. R., Bongiovanni, A. M., Francis, C. M., *J. Clin. Endocrin. Metab.,* 1958, v18, 1274.

7. Bachman, C., *J. Biol. Chem.,* 1939, v131, 463.

8. Slaunwhite, W. R., Sandberg, A. A., *Endocrinology,* 1958, v62, 283.

9. Purdy, R. H., Engel, L. L., Oncley, J. L., *J. Biol. Chem.,* 1961, v236, 1943.

Received December 26, 1962. P.S.E.B.M., 1963, v112.

Enhanced Proteolytic Activity at pH 2.0 in Lungs of Infants Dying from Hyaline Membrane Disease (HMD).* (28144)

KURT BENIRSCHKE, ARTHUR J. SAMUELS† AND ERIC MOORE
(Introduced by R. E. Gosselin)
Department of Pathology, Dartmouth Medical School, Hanover, N. H.

Hyaline membrane disease of the newborn infant (HMD) represents a fairly circumscribed pathologic entity. A major point of disagreement remaining is whether the "idiopathic respiratory distress syndrome of the newborn" without the typical hyaline membranes on histologic study has the same pathogenetic mechanism(1). It is agreed that the membrane formation is a postnatal event (2) and that the membranes are largely composed of fibrin, thus representing in part at least an exudative phenomenon(3). The etiology of the disease is not understood; however, various investigations indicate an associated deficiency of lung-lining-film substance(4), a pulmonary deficiency of fibrinolytic activity at pH 7.4-8.0(5) and degenerative and regenerative phenomena of the respiratory and bronchiolar epithelium, recently reviewed by Boss and Craig(6).

The clinical course of most of these infants, the presence of hyaline membranes in adults with a history of aspiration of vomitus(7) and the ability to produce similar lesions in animals by tracheal instillation of hydrochloric acid(8) have led one of us (K.B.) to postulate that the disease may be caused by inhalation of acid gastric juice at time of birth. This suggestion is not new(9,10) but no laboratory support has been forthcoming and this etiologic possibility is not seriously considered by most workers. In adults, gastric aspiration is traceable not only by the clinical event, but by histologic identification of food particles in the lung in which a severe chemical and bacterial pneumonitis usually ensues. In the hope of finding evidence for the possible aspiration of gastric content it was conjectured that pepsin, because it is the most active gastric enzyme, might remain active in the lung long enough after death to be detected at autopsy, essentially serving as a tracer.

Two methods of testing this hypothesis are available: a) the immunologic, in which fluorescein-conjugated antibody to pepsin might be used to label any pepsin still contained in the lungs and b) direct enzymological determination of pepsin activity. Because of the costliness of preparing antibody, the uncertainty involved in species crossreaction, and the probable lack of specific localization of the pepsin, it was decided to postpone the immunologic method and attempt the enzyme analysis. It was recognized, however, that finding a proteolytic activity even at pH 2.0 would not constitute conclusive evidence for the presence of pepsin. It has been shown previously that newborn infants of varying gestational ages may have considerable quantities of acid gastric juice at birth(11) and that pepsin secretion is also in progress at this time(12).

* This work was supported by a grant from Nat. Inst. Health, U.S.P.H.S.

† Established Investigator, Am. Heart Assn.