also given for comparison.

*Discussion.* Values for the lysine content of proteins obtained with *S. faecalis* and *L. mesenteroides* are in good agreement with each other and with values cited from the literature, except in the cases of gelatin and silk fibroin. The value obtained with *L. mesenteroides* for gelatin appears to be low, and that for silk fibroin with *S. faecalis* appears to be high when compared with the data cited. The value obtained for the lysine content of horse hemoglobin is in excellent agreement with that obtained by the isotope dilution method.[8]

The single value (2.8%) for the arginine content of horse hemoglobin probably should be disregarded since it is much lower than the values 3.4, reported by McMahan and Snell,[1] and 3.7, reported by Foster.[8] The value (9.4%) for the valine content of horse hemoglobin is somewhat high as compared with the value, 8.8%, reported by McMahan and Snell.[1] Recent values for the histidine content of horse hemoglobin are unavailable. The agreement among other values reported in Table III is excellent.

*Summary.* A method of assay for histidine and lysine employing *Leuconostoc mesenteroides* as test organism has been developed. This method is compared with those of Dunn *et al.* [4,5] A method of assay similar to that of Stokes *et al.*[6] for lysine, arginine, and valine using *Streptococcus faecalis* as test organism has been independently developed and studied.

With few exceptions, the data reported agree closely with those obtained by previous investigators with microbiological or isotope dilution methods.

[8] Foster, G. L., *J. Biol. Chem.*, 1945, **159**, 431.

15259

## The Nature of Circulating Estrogen.*

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In the course of investigations on the physico-chemical nature of circulating estrogen, we have found a large and constant portion of the latter to be closely associated with the blood proteins. Coincidentally, we have observed that blood estrogens will dialyze quantitatively past a collodion membrane.

*Methods.* The methods used for the extraction of blood estrogens will be published in detail elsewhere. In brief, the procedure generally consisted of precipitation of the blood proteins with acetone. The supernatant fraction was combined with the acetone-ether washings of the precipitate, after which the two fractions were treated separately. In all instances, precipitation and washing of the protein was done in the cold.

After evaporation of acetone and ether from the supernatant fraction, the latter was hydrolyzed with 4% $H_2SO_4$. The freed estrogen was then extracted with ether, and the water-washed extract was made up in a small amount of olive oil for subsequent assay.

The previously undenatured protein precipitate was subjected to partial alkaline hydrolysis with 0.1 N NaOH. After neutralization with $CO_2$, this material was extracted with ether and made up in olive oil.

Other methods of estrogen extraction included alcohol-ether precipitation, and ammonium sulfate precipitation followed by prolonged hot alcohol-ether Soxhlet extraction of the protein.

Portions of the same or similar bloods, as well as pure solutions of various natural estrogens, were also subjected to dialysis against distilled water through sausage casing (Visking, 25/32″ diam.) The dialysate

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TABLE I.
Distribution of Blood Estrogen.

| Sample | Method | Volume cc | Fraction | Treatment of protein before ether extraction | Estrogen as μg α-estradiol per 100 cc | | % estrogen in protein fraction |
|---|---|---|---|---|---|---|---|
| | | | | | | Total | |
| Rabbit* Serum | Acetone Pptn. | 72 | Supernatant | | <0.34 | | |
| | | | Protein | | | 0.81 | >58 |
| | | | | Alkaline partial hydrolysis | 0.47 | | |
| Cow* Whole blood† | " " | 100 | Supernatant | — | 0.10 | | |
| | | | Protein | | | 0.30 | 67 |
| | | | | " " | 0.20 | | |
| Pregnant cow (6 mo.) Whole blood† | " " | 100 | Supernatant | — | 0.15 | | |
| | | | Protein | (1) Aqueous extr. | 0 | | |
| | | | | (2) Alk. partial hydr. | 0.23 | 0.38 | 61 |
| | | | | (3) Hydr. at pH 1 | 0 | | |
| Pregnant cow (6 mo.) Whole blood† | Alcohol-ether Pptn. | 65 | Supernatant | — | 0.12 | | |
| | | | Protein | | | 0.36 | 67 |
| | | | | Alk. partial hydr. | 0.24 | | |
| Pregnant human (7 mo.) Whole blood† | $(NH_4)_2SO_4$ Pptn. | 100 | Supernatant | — | 0.20 | | |
| | | | Protein | | | 0.60 | 67 |
| | | | | Soxhlet‡ | 0.40 | | |

* Gonadotrophin treated.
† Preserved with potassium oxalate 0.2%.
‡ Continuous extraction with hot alcohol-ether for 48 hours.

TABLE II.
Dialysis of Pure Estrogens

| Estrogen | μg inside before dialysis | Form | Hrs. of dialysis | μg found in | μg found out | % recovery | % dialysis* |
|---|---|---|---|---|---|---|---|
| α-Estradiol | 10 | Na Salt | 24 | 4.0 | 6.4 | 104 | 74 |
| ,, | 10 | ,, | 48 | 1.0 | 8.0 | 90 | 94 |
| ,, | 1.0 | ,, | 48 | — | 0.9 | — | 105 |
| ,, | 9.5 | Free | 48 | 4.5 | 4.0 | 90 | 35 |
| ,, | 2.0 | ,, | 48 | — | 0.4 | — | 25 |
| β-Estradiol | 10 | Na Salt | 48 | — | 8.3 | — | 100 |
| Estrone | 2.0 | ,, | 48 | — | 1.8 | — | 106 |
| Estriol | 30 | ,, | 48 | — | 24.8 | — | 98 |

* Based on theoretical considerations with respect to volume ratios across the collodion membrane.

was ether-extracted before and after acid hydrolysis and prepared for injection in olive oil. The material inside the cellophane bag was often analyzed for estrogen coincidentally; in the case of blood, the acetone procedure outlined above was employed. In all instances, dialysis was carried out in the cold (2°C) in a mechanical shaking device.

Biological assay for estrogenic activity was by a modification[1] of the 6-hour uterine-weight method of Astwood.[2] The index employed was the increase in the ratio of uterine weight to body weight in immature female rats. Most of the results were represented arbitrarily in terms of alpha-estradiol equivalents.

*Results.* Blood and sera obtained from normal and pregnant cows, normal and gonadotrophin-injected rabbits, and pregnant women were analyzed for estrogen.

It was uniformly found that only one-third of the total estrogen was present in the acetone supernatant fraction from which the protein had been removed without denaturation (Table I). This portion was apparently almost entirely present in a conjugated form (probably as an ester), since it could be extracted with ether only after acid hydrolysis. The remainder, or two-thirds, existed associated with the protein in a form liberated by weak alkaline hydrolysis. Subsequent hydrolysis at pH 1 did not increase the yield. It may be noted that only very small amounts of estrogen were present in all instances.

In the course of related studies it was found that pure estrogens will dialyze quantitatively past a collodion membrane. This is illustrated in Table II. Among the estrogens studied, free estradiol (which we have not found to any significant degree in blood) dialyzes too slowly to reach equilibrium in a reasonable length of time. This may be due to its hydrophobic nature in a salt-free environment. Otherwise, dialysis appeared to reach theoretical completion in about 48 hours. Dialysis was not complete in 24 to 26 hours. This is illustrated by the incomplete dialysis of the sodium salt of alpha-estradiol after 24 hours (Table II) and of pregnant cow plasma and laked cells after 26 hours (Table III). Similar samples had dialyzed completely after 36 to 48 hours.

Table III shows that the amount of estrogen obtained from a 36 to 72 hour dialysate of blood was approximately equivalent to that found by exhaustive chemical extraction procedures (Table I). The optimal volume ratio of material inside the collodion bag to that outside was found to be about 1:5. Dialysis was incomplete where this condition was not met, as in the case of the whole blood sample from the gonadotrophin-injected cow (Table III). In this case, the volume ratio was only 1:2.

*Discussion.* Previous studies on natural blood steroids having hormonal activity have almost uniformly neglected the protein fraction, in spite of the fact that a large amount of cholesterol is found closely associated with the latter.[3] However, a variable amount of estrogen has been found associated with

[1] Pincus, G., and Werthessen, N., personal communication.

[2] Astwood, E. B., *Endocrinology*, 1938, **23**, 25.

[3] Tronsegaard, N., and Koudahl, B., *Z. Physiol. Chem.*, 1926, **153**, 111.

TABLE III.
Dialysis of Estrogen in Blood.

| Sample | Volume cc | Duration of dialysis in hrs. | Estrogen as μg α-Estradiol per 100 cc |
|---|---|---|---|
| Cow serum | 160 | 48 | 0.25 |
| Cow* | | | |
| Whole blood† | 200 | 46 | 0.20 |
| Plasma† | 100 | 46 | 0.40 |
| Laked cells† | 100 | 46 | 0.35 |
| Pregnant cow (6 mo.) | | | |
| Whole blood† | 50 | 36 | 0.34 |
| Plasma† | 100 | 26 | 0.10 |
| Laked cells† | 100 | 26 | 0.20 |
| Pregnant human | | | |
| Whole blood† (3 mo.) | 100 | 72 | 0.22 |
| Whole blood† (5½ mo.) | 100 | 72 | 0.55 |

* Gonadotropin treated.
† Preserved with potassium oxalate 0.2%.

the protein in mare serum[4] and in human pregnancy serum.[5] This bound estrogen could be liberated by prolonged strong acid hydrolysis, but not by refluxing 4 hours with alcohol, nor by ultrafiltration or tryptic digestion.

Our data suggest that an equilibrium exists in blood between estrogen and protein. Thus, two-thirds of the total estrogen is normally closely associated with the protein. The remainder is apparently present in an esterified form in the aqueous phase. The hydrophilic nature of this esterified estrogen may be involved in the formation of the protein complex. It is improbable that the latter represents merely simple adsorption, since the relative amount of estrogen found associated with the protein by different extraction procedures is quite constant (Table I).

The estrogen-protein complex is of such a nature that dialysis results in its progressive dissociation. It is probable that this is accomplished by preserving the ratio between dissociated and protein-bound estrogen inside the collodion bag. As dialysis proceeds, gradually removing the protein-free estrogen, further dissociation of the estrogen-protein complex is permitted. This postulated equilibrium, then, seems to obey the law of mass action, enabling dialysis to proceed toward completion, depending upon the concentration gradient of estrogen across the membrane.

The above results bear on the fundamental problem of steroid transport and activity *in vivo,* and suggest that estrogen bound to protein is potentially available for physiological action by dissociation at the cell membrane.

*Summary.* The total estrogen content of blood and sera obtained from normal, pregnant and gonadotrophin-injected animals and women was uniformly low. Values approximating 0.5 μg of alpha-estradiol equivalents per 100 cc of blood were found.

Two-thirds of the total estrogen was closely associated with the protein fraction. The remaining one-third existed mainly in a hydrophilic, non-protein conjugated form.

Blood estrogens, as well as pure solutions of the sodium salts of estrone, alpha- and beta-estradiol, and estriol, were shown to dialyze quantitatively past a collodion membrane.

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[4] Haussler, E. P., *Festschrift E. C. Barrell,* 1936, 327 (Basel).

[5] Rakoff, A. E., Paschkis, K. E., and Cantarow, A., *Am. J. Obstet. and Gynec.,* 1943, **46**, 856.