cirrhosis in the rats which is in contrast to the experience of other investigators (6, 7).

Alterations in iron metabolism also affect manganese metabolism. When iron absorption is increased in iron deficiency, so is manganese absorption increased, and decreased iron absorption in iron-loaded animals is associated with decreased manganese absorption. However, the body appears to compensate for the changes in manganese absorption by increased manganese excretion in the iron-deficient states and decreased manganese excretion in the iron-loaded state. Another interesting observation was the increased incorporation of ^{54}Mn into the porphyrin ring of hemoglobin in iron deficient red cells.

The mechanism of the interrelationship of the metabolism of iron and manganese and the biologic significance remain to be elucidated.

1. Borg, D. C. and Cotzias, G. C., Nature **182**, 1677 (1958).

2. Pollack, S., George, J. N., Reba, R. C., Kaufman, R. M., and Crosby, W. H., J. Clin. Invest. **44**, 9 (1965).

3. Brückman, G. and Zondek, S. G., J. Biol. Chem. **135**, 23 (1940).

4. Caraway, W. T., Clin. Chem. **9**, 188 (1963).

5. Labbe, R. F. and Nishida, G., Biochim. Biophys. Acta **26**, 2 (1957).

6. Findlay, G. M., Brit. J. Exptl. Pathol. **92**, 5 (1924).

7. Hurst, E. W. and Hurst, P. H., J. Pathol. Bacteriol. **31**, 303 (1928).

Received June 14, 1968. P.S.E.B.M., 1968, Vol. 129.

Dynamics of Luteinizing Hormone (LH) Secretion in the Cycling Ewe: A Radioimmunoassay Study* (33342)

IRVING I. GESCHWIND AND ROBERT DEWEY (Introduced by H. H. Cole)

Department of Animal Science, University of California, Davis, California 95616

A marked decrease in the concentration of pituitary LH occurs in the ewe between the fourth and thirty-fifth hours after the onset of estrus, as first shown by Santolucito *et al.* (1) using the ventral prostate bioassay method, and subsequently by Robertson and Hutchinson (2), with the ovarian ascorbic acid depletion (OAAD) assay. Robertson and Rakha (3) later refined these determinations, examining the shorter interval from 12 hr prior to estrus to 10 hr post-onset, and found that a significant decrease from the proestrus level occurred in the first 6 hr post-onset. A 48% decrease in content between the eighth and eighteenth hours post-onset was recently reported by Dierschke and Clegg (4).

It has been assumed that the observed depletions reflected release of the hormone into the circulation in order to induce ovulation. A more direct approach is the determination of circulating levels of LH, which has the additional advantage of allowing multiple sampling of the same animal since sacrifice of the animal is not required. This approach was attempted by Dierschke and Clegg (4), who assayed for LH in the cavernous sinus blood of cycling ewes by the OAAD procedure. They concluded that the serum data provided "only speculative evidence of hormone release". In the present paper we report determination of plasma LH levels in 4 cycling ewes through 2 complete cycles, using a radioimmunoassay technique which we had previously applied to the same samples of sera reported on by Dierschke and Clegg. At that time we found not only much lower values for LH than those reported by them, but also "a general lack of correlation in relative amounts as determined by the two methods" (4).

Materials and Methods. Four Targhee ewes, 30 months of age, all of whom had lambed the previous season, were the experimental subjects during one breeding season

* This investigation was supported in part by USPHS Research Grant HD 00394.

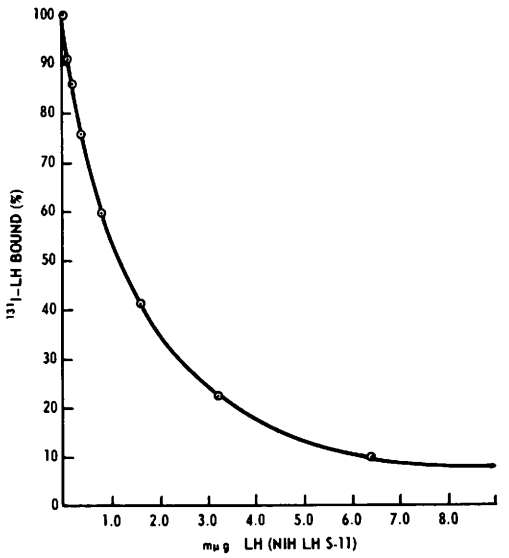

FIG. 1. A typical standard curve for the determination of ovine LH by radioimmunoassay. The amount of LH-^{131}I bound at zero added LH was set equal to 100%.

(1967–68). All were followed for 2 cycles and then bred at the onset of the third cycle, lambing subsequently. Blood from the jugular vein (10 ml) was collected in heparinized syringes, and the plasma was recovered by centrifugation. During much of the first cycle, and in the initial days of the second cycle, samples were obtained daily at 8:00 a.m., 4:00 p.m., and between 10:30 p.m. and midnight; during the period of gestation samples were taken infrequently. Onset of estrus was determined as the time when the ewe accepted mounting by a vasectomized ram. The exact time, however, was uncertain since observations were made only twice daily at 8:00 a.m. and 4:00 p.m.; the maximum error in the determination is therefore 16 hr. The arrows in Fig. 2 indicate when acceptance was first observed.

The same ewes were used for the half-life and distribution volume studies, approximately 3.5 months after lambing. Each ewe received an intrajugular injection of 0.9 or 1.0 ml of a 600 μg/ml solution of NIH-LH-S13, and blood samples (5 ml) were then taken at 15-min intervals, for 2 hr, for radioimmunoassay. Half-lives of the injected LH were calculated from the semilogarithmic plots of the data, and distribution volumes were obtained from 0 time extrapolation of plasma LH concentrations, and the body weights. The latter were determined after each ewe was sheared and deprived of water and food for 24 hr.

Plasma LH was determined by radioimmunossay, employing antisera obtained by a previously described procedure (5). LH-^{131}I and LH-^{125}I (the latter used in the half-life studies) were prepared by the method of Greenwood *et al.* (6), from a highly purified ovine LH-preparation, IV-28-BP [potency: approximately 2 times NIH-LH-S1; less than 0.05% contamination with FSH or TSH (7)] obtained from Dr. Harold Papkoff. NIH-LH-S11 (potency: 0.81 times NIH-LH-S1) was the standard for the endogenous hormone studies; the later half-life studies made use of NIH-LH-S13 (potency: 0.93 times NIH-LH-S1) as standard. The details of the radioimmunoassay are as follows:

Duplicate or triplicate 100-μl samples of standard or unknown were pipetted into 75 × 12-mm disposable plastic tubes, followed by 0.5 ml of a diluted rabbit antiserum capable of binding approximately 70% of the labeled ovine LH (whole antiserum heated at 56° for 30 min, centrifuged, and diluted approximately 1:100,000). The tubes were shaken, and to each one was added 0.5 ml of the labeled LH (0.25 mμg/ml of LH-^{131}I, ~ 300 mCi/mg, or 0.25 mμg/ml of LH-^{125}I, ~ 60 mCi/mg); the tubes were then refrigerat-

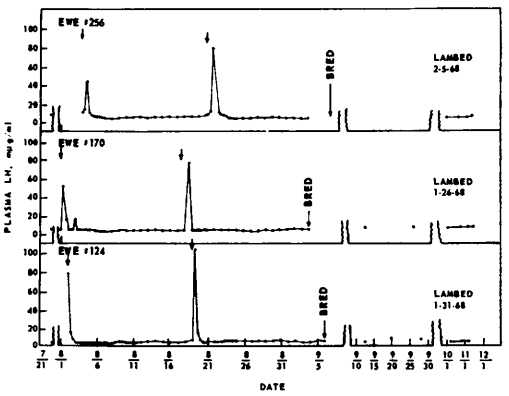

FIG. 2. Plasma levels of LH in three ewes during two complete cycles and the subsequent gestation periods.

ed for 3 days. Second antibody, 0.1 ml of a 1:10 dilution of goat antirabbit γ-globulin, was then added to each tube, followed by 0.1 ml of a 1:20 dilution of normal rabbit serum; the tubes were allowed to stand overnight at 4°, centrifuged at 3000*g* at 4° for 15 min, the supernatants were aspirated off, and the tubes containing precipitate were counted.

Results. Radioimmunoassay procedure. A standard radioimmunoassay curve is shown in Fig. 1, and indicates a useful range from 2 to 30 mμg/ml; for samples containing greater concentrations, dilutions must be made. Since the curve was obtained using a diluent (2.1 g of $NaHCO_3$, 9.0 g of NaCl, 50 g of bovine serum albumin fraction V powder, and 0.1 g of Merthiolate diluted to 1 liter), it was imperative to demonstrate that a similar curve could be obtained by adding ovine LH to sheep plasma. To 100 μl of sheep plasma containing 0.4 mμg of radioimmunoassayable LH were added 0.64, 1.28, or 3.2 mμg of LH; radioimmunoassay values indicated recoveries of 97% of added LH. In order to check specificity, 20 mμg of a highly purified ovine FSH, G-IV-5 (obtained from Dr. Harold Papkoff; potency: approximately 45 times NIH-FSH-S1, and less than 0.003 times NIH-LH-S1), and of a highly purified TSH, CM-17-II (obtained from Dr. John Pierce; potency: approximately 20 TSH U/mg, and approximately 0.08 times NIH-LH-S1, determined in our laboratory by the OAAD assay) in 100 μl of diluent were run in the assay. The assay of the FSH indicated less than 1% LH contamination or cross-reaction (less than 2 mμg/ml); the assay of the TSH, however, indicated 17% LH contamination or cross-reaction (34 mμg/ml).

Plasma levels of LH. In the six cycles investigated in three of the ewes, peak LH values were always observed within a short period after the onset of estrus (Fig. 2). Although samples were taken every 8 hr from each animal, it is obvious that there is some variability in the time after onset of estrus when the peak value was observed. Furthermore, the peak was quite sharp, markedly elevated levels being found at only a single time interval in the immediate post-onset period. This level was as high as 30 times the baseline level, and returned to baseline rather rapidly, the baseline level being 2–3 mμg/ml. No other significant elevation was observed during the rest of the cycle or during that part of the pregnancy period followed. The fourth ewe, no. 072, showed no elevation in plasma LH levels in either cycle although she displayed behavioral estrus; this animal was bred and subsequently lambed normally.

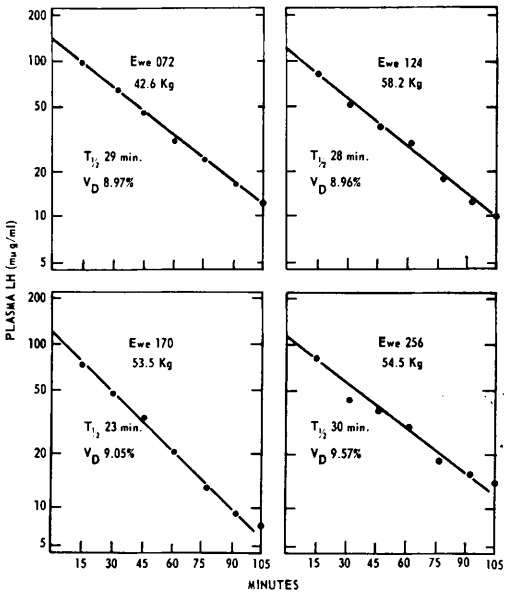

FIG. 3. Half-lives and distribution volumes (% of body wt.) of intravenously injected NIH-LH-S13 in four ewes. The distribution volumes are probably too low by about 10% (see text).

Half-life and distribution volume of ovine LH. The ranges of half-lives (23–30 min) and distribution volumes (8.96–9.57% of body wt.) were found to be quite narrow in the 4 ewes (Fig. 3); the average half-life was 28 min and the average distribution volume was 9.14%.

Discussion. The general virtues of radioimmunoassays are considered to be economy, sensitivity, and selectivity; the present assay possesses the first two of these virtues, and is selective in that no cross-reaction with FSH is evident. We have confirmed this by also using high concentrations of NIH-FSH-S3, alone or added to known quantities of LH, dissolved in either sheep plasma or diluent. The cross-reaction of TSH in this assay, how-

ever, is more troublesome. The particular sample of TSH employed was found by us to contain the equivalent of 0.08 mg of NIH-LH-S1 per mg preparation by the OAAD assay, and 0.17 mg of S1 per mg preparation by the radioimmunoassay. Obviously only about half can be accounted for by bioassayable LH contamination. The remaining cross-reactivity could result from inactivated LH in the TSH preparation, from common antigenic determinants in LH and TSH, or from the use of an ovine LH standard to determine concentrations of bovine hormones. We are not certain what the exact cause is, but favor the concept of common antigenic determinants, previously suggested by other workers [e.g., (8)]. Baron *et al.* (9) have recently described a radioimmunoassay for sheep LH which also showed no cross-reaction with FSH, but did with TSH. Nevertheless, since the degree of cross-reaction of the TSH itself is relatively small (less than 10%), we believe that it cannot account for the results obtained with ewe plasma.

At all times, except for a short period soon after the onset of estrus, the ewe has a low circulating LH level of 2–3 mμg/ml. The level in rams is even less, since in these animals we have never been able to detect circulating LH (i.e., levels less than 2 mμg/ml), but in wethers average levels of about 8 mμg/ml have been determined. In ewes, the large increase in plasma LH shortly after the onset of estrus is expected on the basis of the pituitary data: from the onset of estrus to 6–16 hr post-onset, when the minimum pituitary content of LH is found (3, 4), about 70% of the total pituitary LH, or approximately 1500 μg equivalents of NIH-LH-S1, disappears (3, 4). The finding that almost all the increase was restricted to a single sample, with little evidence of it either 8 hr earlier or later, would indicate that the hormone is released as a surge whose duration is relatively short. It was precisely because of this finding that the studies on half-life and distribution volume were undertaken. The half-life values calculated were not unexpected, whereas the distribution volumes were, since Gay and Bogdanove (10) had reported distribution volumes of less than 2% of the body weight for ovine LH in the *rat*. With a distribution volume of about 9%, which is probably a minimum value since the body weights are probably overestimated by about 10% (H. H. Cole and W. N. Garrett, personal communications) as a result of the fill remaining after the 24-hr fast, and with a half-life of approximately 30 min, a peak value of about 80 mμg/ml (cf. Fig. 2) would be obtained if almost all the LH were steadily released over a 3-hr period. Increasing plasma LH values would be observed for the 3 hr of the surge, followed by a decline to baseline in the next 2.5 hr. Thus, if readings were made every 8 hr, only a single very high value would be observed, *if the timing for taking the samples were correct.* Since almost the entire elevation would last less than 6 hr, it would also be possible to miss the peak entirely, and this apparently occurred on two occasions in ewe 072.

Additional calculations indicate that if a baseline plasma LH concentration of 2–3 mμg/ml is to be maintained, a secretion rate of about 400 μg/day is required. The surge is then superimposed upon this basal level of release. Finally, a plot of the data of Robertson and Hutchinson (2) reveals a linear reaccumulation of LH in the pituitary from the thirty-sixth hour post-onset to day 15 of the cycle. In order to permit the storage of the amount of LH required for the ensuing cycle, approximately 100 μg must be synthesized daily specifically for storage purposes. If this is added to the synthetic rate required to maintain the baseline secretion rate referred to above, a total LH synthetic rate of approximately 500 μg/day of NIH-LH-S1 equivalents is calculated for the cycling ewe.

Summary. Three out of four cycling ewes followed for two full cycles showed a marked increase in radioimmunoassayable circulating LH levels shortly after the onset of estrus; although samples were taken every 8 hr, only one sample showed the increase in each cycle. The same four ewes were used to determine the half-life and distribution volume of exogenous ovine LH; an average half-life of 28 min and an uncorrected distribution volume of 9.14% of body weight were found. The results have been interpreted as indica-

ting that almost all the LH was released from the pituitary in a surge lasting not more than 3 hr, and that the normal cycling ewe pituitary synthesizes approximately 0.5 mg of NIH-LH-S1 equivalent per day.

We wish to thank Dr. Harold Papkoff for the gift of the highly purified ovine LH and FSH preparations employed in these studies, and Dr. John Pierce for the purified TSH sample.

1. Santolucito, J. A., Clegg, M. T., and Cole, H. H., Endocrinology **66**, 273 (1960).

2. Robertson, H. A. and Hutchinson, J. S. M., J. Endocrinol. **24**, 143 (1962).

3. Robertson, H. A. and Rakha, A. M., J. Endocrinol. **35**, 177 (1966).

4. Dierschke, D. J. and Clegg, M. T., J. Reprod. Fertility **15**, 321 (1968).

5. Samli, M. H. and Geschwind, I. I., Endocrinology **81**, 835 (1967).

6. Greenwood, F. C., Hunter, W. M., and Glover, J. S., Biochem. J. **89**, 114 (1963).

7. Papkoff, H., Gospodarowicz, D., Candiotti, A., and Li, C. H., Arch. Biochem. Biophys. **111**, 431 (1965).

8. Odell, W. D., Wilber, J. F., and Paul, W. E., J. Clin. Endocrinol. Metab. **25**, 1179 (1965).

9. Baron, G., Terterin, C., and Jutisz, M., C. R. Acad. Sci., **265**, 2058 (1967).

10. Gay, V. L. and Bogdanove, E. M., Endocrinology **82**, 359 (1968).

Received June 18, 1968. P.S.E.B.M., 1968, Vol. 129.

Histological Effects of Procedural and Environmental Factors on Isolated Rat Heart Preparations* (33343)

JOHN W. BROWN,[1] DENNIS CRISTIAN,[2] AND RAYMOND R. PARADISE

Department of Pharmacology, Indiana University School of Medicine, Indianapolis, Indiana 46202

Studies of isolated tissue preparations constitute the basis of much of our present knowledge concerning biochemistry and physiology. A controlled environment and a thorough understanding of the variables involved in isolated tissue experimentation is of primary importance.

There were three objectives of this study: (i) to determine the effects of certain environmental conditions on the histological appearance of isolated rat heart preparations; (ii) to determine if significant differences exist between incubated and perfused preparations under identical conditions of treatment-duration and temperature; and (iii) to determine the best type of preparation for further isolated tissue studies.

The effects of duration of treatment, temperature of the environment, and the presence of various drugs on the morphology of the isolated rat right ventricular strip, isolated atria, and perfused whole heart were evaluated. Attention was focused on a possible explanation for the significant differences. All histological observations were made with a light microscope.

Isolated, incubated, and perfused hearts have been extensively employed in experimental procedures. A survey of the literature disclosed that few studies have been done on the histology of isolated preparations. Tanz (1) reported that 0.5 μg/ml of 9-alpha-fluorohydrocortisone greatly reduced the histological signs of cardiac degeneration which occurred in the isolated cat papillary muscle incubated at 38° for 6 hr. Tanz (2) subsequently reported that chlortetracycline at 20 μg/ml prevented loss of cross striations and increased the height of contraction in isolated cat papillary muscle over a 6-hr period of

* This study was supported in part by U. S. Public Health Service Research Grant HE-07718 and in part with facilities provided by the Heart Research Center Grant H-6308 from the National Heart Institute, U. S. Public Health Service.

[1] Sophomore medical student, 1968 K. K. Chen Pharmacology Awardee, Indiana University School of Medicine.

[2] Dow Chemical Loan Scholarship Awardee, 1967-1968 NDEA Graduate Fellow, Pharmacology Predoctoral Candidate.