# SCIENTIFIC SECTION

## CHEMICAL INVESTIGATIONS OF CORPUS LUTEUM. III. ON THE PRESENCE OF FREE AMINO ACIDS IN THE ACETONE EXTRACT.*

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The ovary is a tissue that represents at different periods in its cycle entirely different physiological structures and chemical compositions. These changes in structure and composition are correlated with its varying functions at different times. An important factor in regulating this changing function of the ovary is undoubtedly played by the enzymes of the gland. That is slight changes in the physical-chemical composition of the gland will set the conditions necessary for the optimum activity of certain enzymes. Coincident with the development of a maximum amount then of the protein constituents of the ovary, we should expect to find an excess of the protein synthesizing enzymes and as the proteins began to diminish in amount and the lipoid constituents became more prominent, this would also be accompanied by increase in the activity of the appropriate enzymes. Thus a complete study of the ovary, at different periods in its cycle, as far as its enzymatic activity is concerned, would give very valuable information concerning the mechanics of the physiology of the ovary.

As we have shown,[1] follicular fluid from the sow consists of 7.31 per cent total solids, 87 per cent of which is protein and only 1.6 per cent of which is total lipoids (phosphatides, fat, and cholesterol). With the development of the corpus luteum we find[2] that now the total protein of the dried material amounts to only 40 per cent. This change in the tissue is undoubtedly accompanied by an increase in the catabolic activity of the proteolytic enzymes and we should logically expect to find some of the protein hydrolytic products, such as peptides or amino acids, still left in the corpus luteum.

In a systematic examination of corpus luteum we should expect to find the largest amount of these protein split products in the water soluble extractive fraction. However, crystalline material separated from the acetone extract of corpus luteum, the alcohol insoluble part of which was shown to be a mixture of leucine, isoleucine and probably valine. Whether these amino acids existed preformed in the corpus luteum, or whether they were formed by subsequent autolytic action, we cannot definitely state. Our corpus luteum, however, consisted of material that was dried immediately after collection at 40° C. *in vacuo* and then ground up and extracted with acetone. This method of preparation allows very little opportunity for autolytic processes to occur to any extent.

Leucine has been reported to have been found in the pancreas, lymph, salivary, thyroid and thymus glands.[3] This work, though, is not conclusive as to whether leucine existed free in these glands or had been formed by autolytic processes. B. Demant claims[4] to have found it in the watery extract of the human fœtus.

* Received for publication July 24, 1925.

[1] F. W. Heyl, M. C. Hart, and W. B. Payne, J. Am. Pharm. Assoc., 14, 3, 210–215 (1925).

[2] B. Fullerton and F. W. Heyl, J. Am. Pharm. Assoc., 13, 3, 194–200 (1924).

[3] Radziejewski, *Zeitschr. f. Chem.*, 416 (1866).

[4] *Zeitschr. f. physiol. Chemie*, 3, 387–388 (1880).

Leucine is absent in the normal liver and blood.[1] In various pathological conditions though it is found in both the liver and urine.[2]

## EXPERIMENTAL.

In some previous work[3] reported from this laboratory on the crystalline material separating from the acetone extract of 600 Gm. of dried corpus luteum, we obtained 0.640 Gm. of material insoluble in absolute alcohol and decomposing at 260° C. This substance was entirely soluble in 2 cc. of water. This material was not of the nature of a soap, as the water solution on acidification with hydrochloric acid yielded no ether soluble fatty acids. Jaffe's picramic reaction for creatinine was negative. Only a trace of phosphorus was present.

Analysis. Subs., 0.0667; Cc. *N*/20 sodium hydroxide, 0.73. Phosphorus, 0.06.

This material was purified by dissolving in a little hot water, treating with animal charcoal, filtering and evaporating the filtrate to dryness. The residue was pulverized to a fine white powder that weighed 0.3788 Gm. and decomposed sharply at 261° C. on heating. It was analyzed for nitrogen by the micro-kjeldahl method.

Analysis. Subs., 0.201; Cc. *N*/50 ammonia, 7.26; Nitrogen, 10.12.

The nitrogen percentage and the physical properties of this substance suggested that it might be of the nature of an amino acid or a simple peptide. The biuret test was negative. The nitrogen by analysis for amino nitrogen by the Van Slyke micro-method indicated that all of the nitrogen was in the amino form, eliminating the possibility that this material was of a peptide nature.

Analysis. 0.0204 Gm. was made up to a volume of 5 cc. and 2 cc. aliquots were used for the determination of amino nitrogen.

Samples, 2.00 cc., 2.00 cc.; Ccs. nitrogen at 23° C. and 741 mm. pressure, 1.75, 1.75. Blank 0.12 cc.

Nitrogen in 2 cc. in amino form. .89 mg.

Nitrogen in 2 cc. from micro-kjeldahl. .83 mg.

This material was converted into the copper salt by boiling 0.2 Gm. in 200 cc. of water with an excess of freshly precipitated copper hydroxide. The hot solution was filtered and the deep blue solution concentrated under reduced pressure to a volume of 75 cc. A light blue copper salt separated on standing in the cold. This was filtered off, washed and dried to constant weight *in vacuo*. It weighed 0.0488 Gm. and was semi-crystalline under the microscope. The filtrate from this on being concentrated to 3 cc. yielded 0.0778 Gm. more of a more soluble, darker

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[1] F. Hoppe-Seyler, *Zeitschr. f. physiol. Chemie*, 3, 348 (1881).

[2] C. Neuberg and P. F. Richter, *Deutsche med. Wochenschr.*, 30, 499–501 (1904).
A. Englebert-Taylor, *Zeitschr. f. physiol. Chemie*, 34, 580–583 (1902).
T. S. Kirkbridge, *Centralbl. f. inn. Med.*, 18, 1057 (1897).
Städeler, *Jahresber d. Chem.*, 708 (1856).
E. Salkowski, *Jahresber. üb. d. Fortshritte d. Tier chemie*, 457 (1880).
Valentiner, *Jahresber. d. Chemie*, 675 (1854).
E. Salkowski, *Zeitschr. f. physiol. Chemie*, 13, 527 (1889).
B. Gmelin, *Zeitschr. f. physiol. Chemie*, 19, 23–24 (1894).

[3] Merrill C. Hart and Frederick W. Heyl, J. Am. Pharm. Assoc., 13, 1, 17–22 (1922).

blue copper salt. These two fractions were united, dried to constant weight at 115° C. (moisture 1.35 per cent) and analyzed for copper.

Analysis. Subs., 0.0756; CuO, 0.0188.
Calc., $(C_6H_{12}NO_2)_2Cu$; Cu, 19.64.
Found: Cu, 19.85.

These results above could be explained on the assumption that the above copper salt consisted of a mixture of the sparingly soluble leucine copper with the more soluble iso-leucine copper.

In some subsequent work carried out on the acetone extract of 4.073 kilos of dried corpus luteum we isolated again 2.798 Gm. of this material separating from the acetone extract and insoluble in absolute alcohol. This material was separated into a water soluble and a water insoluble fraction by treating it with two 50-cc. portions of water.

The water insoluble part of this fraction was washed with alcohol and dried to constant weight *in vacuo*. This was a slightly greyish solid that weighed 1.040 Gm. and had no melting or decomposition point up to 280° C. On analysis it showed the presence of 3.23 per cent ash, and 13.02 per cent nitrogen. It gave the biuret and Millons test. This material was evidently coagulated protein.

The water soluble part of this fraction formed a colloidal looking solution. This was treated with animal charcoal, filtered and evaporated to dryness. 1.3054 Gm. of a white solid was obtained. This decomposed quite sharply at 257–258° C. and was similar to the water soluble fraction previously studied.

This material was optically active. A filtered solution containing 1.2945 Gm. in 25 cc. showed $[\alpha]_D^{26} = -7.4$. Leucine shows an optical activity of $[\alpha]_D = -10.35$[1] and iso-leucine, $[\alpha]_D^{20} = -10.55$.[2]

The water solution after the determination of the specific rotation, was concentrated on the steam-bath to a small volume, diluted to 6 volumes with alcohol, cooled to 0° C. and the white insoluble precipitate filtered off. This was washed with ether and dried to constant wieght *in vacuo*. Weight 1.1760 Gm. On heating this decomposed at 278° C.

This material was converted into the copper salt by heating it with 2 liters of water and an excess of freshly precipitated copper hydroxide. The solution was filtered hot and the deep blue filtrate was concentrated and held at various volumes from 450 to 2.00 Ccs., and the separated copper salts filtered off and dried. Each of these fractions in turn was exhaustively extracted with absolute methyl alcohol for the purpose of separating the more soluble copper salts of iso-leucine and valine. The more water insoluble copper salts were practically insoluble in hot methyl alcohol and the fractions that separated from the smaller volumes of water were almost entirely soluble in cold methyl alcohol.

The sparingly water soluble, methyl alcohol insoluble top fraction, corresponding to leucine copper in its solubilities, was crystallized twice from water. These were fairly well-formed, light blue, microscopic needles. Weight 0.1612 Gm. These crystals were dried to constant weight at 125° C. (Moisture 1.5 per cent) and analyzed for carbon, hydrogen, copper, and nitrogen.

[1] F. Ehrlich, *Biochem. Zeit.*, 1, 8–13 (1906).

[2] F. Ehrlich and A. Wendel, *Biochem. Zeit.*, 8, 394–437 (1908).
F. Ehrlich, *Ber. d. Deutsch. chem. Gesellschaft*, 37, 1809–1840 (1904).

Analysis. Subs., 0.1238; $H_2O$, 0.0848; $CO_2$, 0.1935; CuO, 0.0319.
Subs., 0.0292; cc. *N*/50 ammonia, 8.95.
Calc. for leucine copper $(C_6H_{12}NO_2)_2Cu$; C, 44.47; H, 7.47; N, 8.65; Cu, 19.63.
Calc. for valine copper $(C_5H_{10}NO_2)_2Cu$; C, 40.57; H, 6.83; N, 9.46; Cu, 21.49.
Found: C, 42.63; H, 7.66; N, 8.58; Cu, 20.57.

Calculating these results on a copper free basis, we have the following results:

Calc. for leucine, $C_6H_{13}NO_2$; C, 54.97; H, 9.99; N, 10.69.
Calc. for valine, $C_5H_{11}NO_2$; C, 51.24; H, 9.47; N, 11.96.
Found: C, 53.67; H, 9.64; N, 10.80.

The analysis and solubilities of this copper salt indicate that it is largely the copper salt of leucine contaminated with some valine copper salt.

The lower more water soluble copper salts separating from the smaller volumes of solution were progressively more soluble in methy alcohol. The lowest most soluble fraction of the water crystallization of the copper salts was dissolved twice in small volumes of cold methyl alcohol and filtered to remove traces of methyl alcohol insoluble material. The final residue obtained by evaporating off the methyl alcohol was crystallized twice from very small volumes of hot water, and the dark blue microcrystalline material filtered off and dried to constant weight *in vacuo*. Weight 0.1749 Gm. These crystals were then dried to constant weight at 120° C. (moisture, 2.23 per cent) and analyzed for carbon, hydrogen, copper. and nitrogen.

Analysis. Subs., 0.1276; $CO_2$, 0.2011; $H_2O$, 0.0846; CuO, 0.033.
Subs., 0.0332; cc. *N*/50 ammonia, 10.23.
Calc. for iso-leucine copper $(C_6H_{12}NO_2)_2Cu$; C, 44.47; H, 7.47; N, 8.65; Cu, 19.63.
Calc. for valine copper $(C_5H_{10}NO_2)_2Cu$; C, 40.57; H, 6.83; N, 9.46; Cu, 21.49.
Found: C, 42.97; H, 7.42; N, 8.63; Cu, 20.65.

Calculating these results on the copper free basis, we have:

Calc. for iso-leucine, $C_6H_{13}NO_2$; C, 54.97; H, 9.99; N, 10.69.
Calc. for valine, $C_5H_{11}NO_2$; C, 51.24; H, 9.47; N, 11.96.
Found: C, 54.16; H, 9.35; N, 10.91.

The analysis and solubities of this copper salt indicate that it is largely the copper salt of iso-leucine contaminated with some valine.

## SUMMARY.

In the systematic chemical examination of the acetone extract of corpus luteum crystalline material separates, the alcohol insoluble part of which is shown to be a mixture of the amino acids, leucine, iso-leucine, and probably valine. The presence of amino acids in corpus luteum is to be expected from theoretical considerations when we consider the change of follicular liquid from one in which the protein constituents predominate to the corpus luteum in which the protein part is greatly reduced.

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