ANALYSIS OF CORPUS LUTEUM.

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The extensive morphological and physiological studies which have been carried out upon this tissue, have not been accompanied by any extensive additions to our chemical information concerning it. It has however been attempted to show that certain crude extracts exert clinically some of the activities which have been described as the normal function of the gland. Among the various activities which have been ascribed to crude extracts are the following: (1) hyperemia of the uterus;[1] (2) stimulation of growth of genitalia;[2] (3) induced menstruation;[3] (4) an antagonistic body retarding excessive menstruation;[4] (5) temporary inhibition of ovulation[5] in hens; (6) induced hyperplasia of the mammary gland;[6] (7) stimulation of milk secretion.[7]

It is apparent that none of these recorded activities can satisfactorily serve in the laboratory as a guide for chemical work. Macht and Matsumoto[8] have shown that contractions of the vas deferens of the rat are excited in quantitative manner by corpus luteum extracts, but there is no reason to have any confidence in the relation of this finding to any natural activity which the extracts may possess.

As stated by Vincent:[9] "The whole subject of the psychological effects induced by extracts of ovary with and without corporo lutea, and both with and without interstitial cells, demands renewed careful investigation. In such investigation particular attention should be paid to the mode of preparation of the extracts and the chemical nature of the substances contained in them."

This is a common sense suggestion, and when any further physiological experiments are attempted, the chemical nature of the extract should certainly be as carefully ascertained as the biological results. It is well to appreciate that under improved conditions for the large scale preparation of desiccated fresh glandular material, that quite uniform preparations of the gland are being employed clinically at this time. As to the extracts used hypodermically, this may not be equally true.

The clinical use of these preparations at the present time is summed up as follows: they are used in the treatment of artificial and natural menopause, particularly the attacks of giddiness, trembling, palpitation, flushings, sweatings, and other nervous and vasomotor disturbances, and various skin disturbances occurring at this time. Headaches occurring at menstruation sometimes yield to organotherapy. Hirst[10] has reported on its use in vomiting of pregnancy.

It is our purpose to mention the clinical uses, in order to indicate the difficulty involved in correlating the chemical work with any clinical, or pharmacological results that might be obtained from the various constituents.

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[1] Hermann, *Verhand. deutsch. Gesell. f. Gynäk.*, II, 258, 1913.

[2,3,4] Seitz, Wintz and Fingerhut, *Münch. med. Wochens.*, 61, 1457, 1914.

[5] Pearl and Surface, *J. Biol. Chem.*, 19, 263, 1914.

[6] Lane-Claypon and Starling, *Proc. Roy. Soc.*, 77, 1906.

[7] Mackenzie, *Quart. Jour. Physiol.*, 4, 22, 1911.

[8] *Am. J. Physiol.*, 49, 149, 1919.

[9] "Endocrinology and Metabolism," II, 563, 1922.

[10] *Am. J. Obstetrics and Dis. of Women and Children*, 329, 1919.

Nevertheless the therapeutic use of this substance is growing and particularly interesting is the frequent clinical choice of the extract. Therefore a closer chemical investigation of the latter is all the more interesting and desirable, even if there can be no immediate correlation of results.

Considerable is already known concerning the chemistry of this tissue, with a general tendency to assign interest to the lipin fraction, and not to the protein part as in the thyroid, nor to the extractive part as in adrenals or posterior pituitary. Rather the interest appears to reside in the lipin fraction as with the anterior pituitary. This statement is made notwithstanding the toxic results reported by Wallis and Williams[1] on injection of extractive material. Thus Fränkel and Herrman[2] claim to have prepared an active phosphatide in which a relation of 5N:2P is stated to exist. Furthermore there has always existed an opinion that the various conflicting results, reported earlier in connection with ovarian treatment, were in part due to various stages of development of the corpus luteum, and that the increased quantity of phosphatides in this material in the pregnant cow was desirable as a therapeutic agent. The ovarian residue is deficient in this element.[3] However phosphatide P decreases as pregnancy develops.[4]

More recent patents[5] claim to separate the menses increasing from the menses controlling constituents. In the latter, the ovaries are extracted successively with alcohol, acetone, ether, chloroform, and hot alcohol; and using the alcoholic extracts as starting material. The former is said to be extracted from the corpus luteum. The gland is exhaustively extracted with alcohol and chloroform, adding an excess of the latter to the mixed extracts, shaking out the active principle with water and evaporating the aqueous liquor to dryness. The chloroformic-alcohol solution corresponds to the fraction used for the preparation of the menses increasing substance in the case of the ovarian extract. Roughly, this appears to be a separation of the lipin fraction from the water soluble extractives, and the assignment of antagonistic effects to these parts. It has been thought by some that the corpus luteum inhibits menstruation (since it develops during pregnancy) while the ovarian residue has been used for decreased menstruation (amenorrhœa).

A recent British patent[6] describes a product from corpus luteum which appears to be cholesterol or a cholesterol ester. This patent again mentions the phosphatides having 2:5 P:N ratio.

Serono and Palozzi[7] state that the lipoids in the ovary are similar to those of egg yolk. For the whole ovary the lipoids varied from 3.05–4.89%. The lipoids in fresh corpus luteum amounted to 6.93%. Desiccated tissue yielded 18.59% lipoids. Upon analysis the lipoid fraction gave: lecithin, 28.58%; cholesterol, 13.62%; fatty acids and saturated lipins, 54.81%. Fenger[8] reported 22% petroleum ether soluble material of which 43.8% is lecithin. Fenger's figure (2.12%

[1] *Lancet*, 784, 1922.
[2] Austrian Pat. 71,382, 1916.
[3] Rosenbloom, *J. Biol. Chem.*, 13, 511, 1912.
[4] Rosenbloom, *Ibid.*, 29, 141, 1917.
[5] U. S. P. 1,318,698-699, 1920, Seitz and Wintz.
[6] B. P. 113,311 (*Soc. pour. l'ind. chim. à. Bâle*), 1917. See U. S. P. 1,314,321.
[7] *Rass. clin. terap. sci. affin.*, 14, 293, 1915; *Ibid.*, 299.
[8] *J. Biol. Chem.*, 27, 305, 1917.

phosphatide for fresh tissue) is much higher than that of Corner[1] whose results give 0.7% phosphatide as the maximum in early pregnancy.

The pigment of corpus luteum has been isolated[2] by Escher and has been identified as carotin, $C_{40}H_{56}$. This substance which occurs to the extent of 0.0031 gram per kilo gram has been most completely described of any of the constituents.

In the present work we have found the purified phosphatides to amount to 9.4% of the glandular material. We have been particularly interested in convincing ourselves that there is no reason to believe that a specific phosphatide is present. It will appear probable from this analysis that the usual lecithin and kephalin mixtures are present. The usual methods for determining this mixture by ascertaining the nitrogen distribution after hydrolysis did not give very satisfactory results in the case of the hot alcoholic extractions. Nitrogen in excess of the atomic ratio required by the phosphorus was found even after purification. With the cold alcoholic extract the results are somewhat more satisfactory, but they are at best an approximation. The kephalin appears to predominate in these fractions. It is not improbable that the unknown sulphatides which are present in appreciable quantities render the quantitative method inapplicable in this case. It is interesting to observe that the excess of nitrogen found can be partly accounted for by adding one atom of N for each atom of S found. By calculation the phosphorus thus requires 0.095% nitrogen.

Alcoholic extract.	Found per cent. P.	S.	Chlorine N.	Amino N.	Sum.	Calculated N on basis of P.	S.	Total.
Hot	0.21	0.13	0.071	0.065	0.14	0.095	0.057	0.15
Cold	0.19	0.13	0.044	0.083	0.13	0.086	0.057	0.14

A few preliminary determinations have been made upon ovarian residue which had been very carefully separated. The lipins are practically absent.

The remaining results of this proximate analysis will be found at the conclusion.

EXPERIMENTAL.

Our sample represented carefully collected, hand dissected material, dried immediately after collection in high vacuum and at a temperature not exceeding 40°. It is representative of the material found on the pharmaceutical market, in that it has been taken from such a large number of cattle as to represent a good average sample.

The material contained 4.5% moisture, 5.4% ash, and duplicate nitrogen estimations gave N = 10.4, 10.2.

The analysis was begun by extracting with various volatile solvents. The extractions were made by exhaustive maceration, at room temperature.

Extract.	Per cent.	% Phosphorus.	% Nitrogen.
Acetone	16.15	0.14	0.16
Ligroin (28–46°)	19.6	0.35	0.27
Ether	20.1	0.26	0.22
Absolute alcohol[3] (boiling)	17.95	0.32	1.16
		0.35	1.26

[1] *J. Biol. Chem.*, 29, 143, 1917.

[2] *Zeits. f. physiol. chem.*, 83, 198, 1913.

[3] Made after the sample had been exhausted with ether.

In the first three extracts the observed atomic ratios representing P:N varied from 1:2.5 to 1:1.6 and as pointed out by MacLean,[1] it can be shown that a nitrogenous substance is present in these extracts, which is removed by his process of purification of phosphatides by emulsification.

Total Phosphatide Content.—When the ether extract is purified by emulsification, the purified phosphatides insoluble in water contain not more than 0.20% P. When now the subsequent alcohol extract is reëxtracted with anhydrous ether, the lipin fraction amounts to 0.22–0.25%. If the purification is carried out by emulsification, using dilute hydrochloric acid, 0.21% phosphorus is found, some passing into the extractive fraction along with more nitrogen. If we accept 0.42% as the value for total phosphatide phosphorus, the lecithin and kephalin fraction amount to 9.4% of the glandular material (factor = 24.7).

Analysis of Lipin Fractions.

(A) The Ether Extract.—This extract contains about 25% phosphatides (Lecithin = 11% and kephalin = 14%): cholesterol (partly as ester) 8.5%; fats, 65%, along with a small amount of extractives. Cerobrosides and sphingomyelin are absent.

The ether extract was first purified by emulsification. The purified lipins amounted to 19.05% of the original material.

Found: N = 0.13%; P = 0.20%; S = 0.025%. Ratio, P:N = 1:1.44.

This analysis was conducted as follows: Fifty grams were exhausted in the cold with anhydrous ether. The ether extract was made up to a volume of 500 cc. The exhausted residue was dried in a vacuum desiccator.

The ether extract was aliquoted into two units of 20 cc. each, one of 250 cc. and one of 210 cc.

Total solids: 20 cc. gave 0.4101 Gm. dried *in vacuo* (20.5%) and 0.4078 Gm. dried at 95° (20.39%).

The residue of total solids was redissolved in chloroform (20 cc.) and aliquots of 5 cc. (0.5 Gm. corpus luteum) taken for the determination of total phosphorus, and total cholesterol.

5 cc. gave 0.2005 Gm. and 0.2405 Gm. $PbMoO_4$ equivalent to 0.27 and 0.29% P, respectively.

5 cc. gave 0.0139 Gm. cholesterol digitonin from free cholesterol, and 0.0184 g. from the ester. This is equivalent to 0.7% and 0.92%, respectively.[2]

210 cc. of the original ether extract (21.0 Gm.) was concentrated and emulsified, by the method of Macarthur.[3] This process separates the ether extract into two parts: (1) the water soluble extractives; (2) the washed lipin fraction.

(1) **Analysis of Extractives.**—This solution contained ammonia N = 0.01% and total N = 0.065%.

(2) **The Lipins** were freed from chloroform and hydrolyzed by heating for 30 hours at 130° with 400 cc. *N*/5 sulphuric acid. A second hydrolysis was carried out on the lipin residue using 200 cc. acid for 10 hours. The lipin residue was washed and put aside.

The acid hydrolysate was made up to 200 cc. volume.

(*a*) 100 cc. cleared with baryta yielded 0.0949 Gm. choline platinum chloride.

(*b*) The platinum salt was dissolved in water and the solution made up to 3.3 cc. Of this, 1 cc. gave 0.14 cc. N_2 gas by Van Slyke = 0.07659 mg. N.

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[1] "Lecithin and Allied Substances," 1918.

[2] Mueller, *J. Biol. Chem.*, 21, 25, 1915.

[3] *Jour. Am. Chem. Soc.*, 41, 1230, 1919; *Bull. Hygienic Lab.*, 103, 72, 1916. When a duplicate analysis was made by the method of Brauns and MacLaughlin (*Jour. Am. Chem. Soc.*, 42, 2246) where the ether extract is washed with salt solution, we found upon direct determination of NH_2 in the hydrolysate, 0.091%, and a total of N of 0.144%.

(*c*) The filtrate from the choline platinum chloride was made to a volume of 10 cc. 2 cc. gave 1.76 cc. N_2 gas by Van Slyke (26.5° and 755 mm.). This is equivalent to 0.0463% amino nitrogen.

By calculation choline N = 0.036%; amino N = 0.048 (0.049) total = 0.084%.

A total N, made on 50 cc. (without baryta treatment) of the filtrate, which required 4.00 cc. *N*/10 acid, gave 0.106% N.

There was no galactose in this hydrolysate, and not more than a trace of sphingosine N was found in the lipin residue.

The total phosphorus in the purified extract amounts to 0.20%. Hence the calculated amount of nitrogen, if P:N is 1:1, would be 0.09%. Therefore it is evident from the above maximum and minimum results (0.084 to 0.106%) that the lipins are monoaminomonophosphatides. A small quantity of nitrogen in other forms is also present.

Lipin nitrogen × 100/1.8 = 5.9% lipins, which is in agreement with 4.95% found by using phosphorus as a basis of calculation.

It may be calculated that lecithin N/Kephalin N = 36/49; kephalin predominating somewhat.

(B) The Hot Alcoholic Extract of Ether Extracted Corpus Luteum.—A quantity of ether extracted corpus luteum equivalent to 15 Gm. of the original material was exhausted with boiling alcohol, which coagulated the proteins. The extract was made to a volume of 500 cc.

25 cc. contained 0.1346 Gm. = 17.95% solids.

25 cc. yielded 0.3525. Gm. $PbMoO_4$ = 0.32% P.

50 cc. required 12.42 cc. *N*/10 acid = 1.16% N.

15 cc. was concentrated to dryness and exhausted with ether. The ether yielded 0.145 Gm. $PbMoO_4$. Hence, ether soluble phosphorus = 0.22%.

400 cc. (12 Gm.) was emulsified by the process of MacArthur and divided into (1) lipin fraction; (2) extractives.

(1) **The Lipin Fraction.**—This was made up to a volume of 200 cc. with the customary chloroform + alcohol mixture.

20 cc. contains 0.0906 Gm. = 7.55% lipins.

10 cc. = 0.0058 Gm. $BaSO_4$ = 0.13% S.

10 cc. = 0.1818 Gm. $PbMoO_4$ = 0.21% P.

The lipins (equivalent to 10.8 Gm. corpus luteum) were hydrolyzed and there was obtained both the lipin residue, and the aqueous acid hydrolysate.

The acid hydrolysate (200 cc.) was analyzed as follows:

50 cc. required 3.47 cc. *N*/10 acid = 0.00486 Gm. N = 0.18%.

50 cc. contained no sugars.

100 cc. was analyzed for choline and aminoethyl alcohol. The choline platinum chloride weighed 0.0891 Gm. This was dissolved and made up to a volume of 6 cc. 2 cc. gave 0.15 cc. N_2 by Van Slyke.

The filtrate from the choline platinum chloride was made to a volume of 10 cc. Of this 2 cc. gave 1.22 cc. N_2 at 28° and 747 mm. and is equivalent to 0.0605% amino N.

By calculation we have choline N = 0.071% and amino N = 0.065%.

(2) **Extractives.**—This fraction resulting from the emulsification of the hot alcoholic extract, contained: ammonia N = 0.023% (0.028%); non-precipitable with lead subacetate, N = 0.93%; precipitable with phosphotungstic acid in filtrate from above, N = 0.21%. The lead precipitate contained N = 0.1%. Total N = 0.1%. Total N = 1.06%.

We interpret these results as follows: the extract consists of 42% lipins and 58% extractives. The extractive material contains approximately 10% of nitrogen. The lipins contain a mixture or a complex of phosphatides and sulphatides. The phosphatides are composed of lecithin and kephalin in about equal quantities. Sphingomyelin and cerebrosides are absent. Cholesterol was absent.

(C) Cold Alcoholic Extract of Ether Extracted Corpus Luteum.—We were interested to learn how complete the alcoholic extractive might be made in the cold, hoping to thus avoid the coagulation of the proteins. The extract is considerably less, only 12.26% in contrast to 17.95% obtained with hot alcohol. The lipin fraction weighed 5.9%, while 7.55% was obtained as described above. The phosphorus and sulphur in the lipin check closely with that extracted with hot alcohol, which indicates that considerable purer lipin has been obtained.

A quantity of ether extracted corpus luteum equivalent to 15 Gm. original glandular material was exhausted in the cold with absolute alcohol. The final volume of the extract was made up to 500 cc. A duplicate was run on a new sample.

25 cc. contained 0.092 Gm. = 12.26% solids (11.63%).

25 cc. yielded 0.3009 Gm. $PbMoO_4$ = 0.276% P (0.254%).

50 cc. required 7.44 cc. $N/10$ acid = 0.7% N (0.71%).

25 cc. evaporated to dryness in the presence of Na_2SO_4 and exhausted with ether. The ether yielded 0.2544 Gm. $PbMoO_4$. The ether soluble phosphorus = 0.23% (0.17%).

375 cc. (11.25 Gm. corpus luteum) was emulsified by the process of MacArthur and divided into two fractions (*a*) lipin fraction; (*b*) extractives.

(1) **The Lipin Fraction.**—This was made up to a volume of 200 cc. with chloroform and alcohol mixture.

20 cc. contains 0.0665 Gm. dried *in vacuo* = 5.9% solids (5.5%).

10 cc. gave 0.155 Gm. $PbMoO_4$ = 0.19% P (0.15%).

10 cc. 0.0054 Gm. $BaSO_4$ = 0.13% S (0.115%).

Cholesterol was not found in this extract.

The lipins (equivalent to 10.125 Gm. corpus luteum) were hydrolyzed and divided into two fractions, the lipin residue and the aqueous acid hydrolysate.

The acid hydrolysate (200 cc.) was treated as follows:

50 cc. required 2.05 cc. $N/10$ acid = 0.00287 Gm. N = 0.113% N (0.11%).

100 cc. was analyzed for choline and aminoethyl alcohol. There was found choline N = 0.044% (0.05) and amino N = 0.083%.

(2) **The Water-Soluble Extractives.**—This contained: total N = 0.54%, (0.56%); ammonia N = 0.028%; N, non-precipitable with lead subacetate = 0.48%. Phosphotungstic acid gave no precipitate.

Protein Extract of Corpus Luteum.

Ten grams of the glandular substances was extracted with ether. The residue weighed 7.718 Gm.

A quantity of ether extracted material equivalent to 2.2905 Gm. corpus luteum was extracted with 8.5% salt solution (thymol), filtered and the residue washed. The residue weighed 0.8045 Gm. (39.5%).[1]

Aliquots of the extract were taken for the determination of total soluble nitrogen.

A quantity of ether extracted corpus luteum was exhausted with boiling alcohol. The alcohol contained nitrogen equivalent to 1.24% of the original material, while the alcohol extracted residue contained N = 8.3%.

On an aliquot ($^1/_5$) of the saline extract to which a slight excess of $N/2$ acetic acid was added, the coagulable protein was estimated. Coagulation began at 28° and a first fraction separated up to 40°. A larger amount separated at 40–60°. Above this temperature only a trace separated.

An aliquot of the saline solution which had been completely coagulated was saturated with zinc sulphate in the presence of sulphuric acid, and a slight quantity

[1] When extracted with dilute alkali, there remained 0.1382 Gm. = 6.0% of supporting tissue of gland.

of proteose separated, amounting to 2.06% of the corpus luteum (0.33% N × 6¼).

The filtrate from the proteose contained 4.13% N.

An aliquot of the proteose free filtrate was precipitated with phosphotungstic acid. The precipitate contained nitrogen = 1.53%.

These results may be tabulated as follows:

DISTRIBUTION OF NITROGEN IN CORPUS LUTEUM.

Total N	10.2	
Alcohol Soluble N	1.2	
Alcohol Insoluble N	9.0	
Soluble in Saline N	4.8	
Coagulable N	0.7	Protein = { 5.0% / 4.4%
Proteose N	0.33	
Filtrate from Proteose N	4.1	
Phosphotungstic ppt. N	1.5	
Not precipitated (by difference)	2.6	
Extracted by 0.2% KOH[1]	3.2	

Composition of Ovarian Residue.—A sample of ovarian residue (moisture = 6.4; ash 3.5; nitrogen 11.8%) was exhausted with ether. The ether extract amounts to 8.53% of the material, and contains traces only of phosphorus. (P = 0.017%; S = 0.01%.)

A quantity of ether extract (equivalent to 40 Gm. residue) was emulsified and divided into (*a*) lipin fraction; (*b*) extractives.

The lipins were hydrolyzed in the usual manner but the hydrolysate contained only 0.0096% N.

The extractives contained in the ether solution, yielded: ammonia = 0.002%; lead precipitate = 0.003%; non-precipitable with lead = 0.012%. Total N found = 0.014%.

When ether extracted ovarian residue is extracted with alcohol the following results were obtained.

Extract.	Per cent.	% N
Cold Absolute Alcohol	6.0	0.21
Cold 95% Alcohol	9.8	0.42
Boiling 95% Alcohol	11.7	0.58

Summary (Proximate Analysis).

Corpus luteum as it occurs on the pharmaceutical market contains about 9.4% phosphatides along with appreciable quantities of sulphatides. These are monoamino-monophosphatides in which kephalin predominates somewhat over lecithin. Fat amounts to about 12.0% and cholesterol 1.6%. The material contains 10.2% nitrogen of which 1.2% is alcohol soluble. Of the remaining 9% of nitrogen 0.7% corresponds to coagulable protein, 0.3% to proteose and 3.2% more corresponds to alkali soluble protein. The total protein amounts to about 6.4% N = about 40% of the glandular material, although part of this is of course insoluble. The extractive material amounts to about 4.0% N, of which 1.2% is alcohol soluble.

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[1] Subsequent to alcohol and saline extractions.