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CHEMICAL EXAMINATION OF OVARIAN RESIDUE.[1]

II. Alcohol Insoluble, Water Soluble Nitrogenous Extractives.

BY FREDERICK W. HEYL AND BRYANT FULLERTON.

We have conducted the systematic examination of the extractives of ovarian residue, using the methods of Kutscher,[2] with the exception that preliminary percolations of the tissue for the purpose of exhausting the lipoids and alcohol soluble extractives have been carried out. The constituents of the alcoholic extract which may for example contain the betaines, and also the constituents of the filtrate of non-basic materials from the phosphotungstic acid precipitation will be reported upon later.

The phosphotungstates precipitated from the aqueous extract from 4.53 kilograms of the desiccated tissue yielded 27.8 Gms. of organic material containing 4.466 Gms. nitrogen. This material yields 1.11 Gms. creatinine = 0.413 Gm. creatinine N.

The purine nitrogen amounts to 0.508 Gm. But a small portion of this was accounted for by the adenine and xanthine isomer isolated. In fact the usual procedure for the analysis of the purines accounts for less than 0.4 Gm. of purine or perhaps 30% of the purine nitrogen.

The so-called Silver II fraction was not examined quantitatively, but nothing was definitely isolated.

The Silver III or arginine fraction contained 1.06 Gms. nitrogen; yielded 1.2 Gms. arginine equivalent to 0.386 Gm. nitrogen, so that 36% of the nitrogen of this fraction is accounted for.

The lysine fraction containing approximately 1.0 Gm. of nitrogen is almost entirely of unknown composition, 0.71 Gm. of lysine picrate containing but 0.05 Gm. of lysine nitrogen being isolated (0.275 Gm. lysine).

Since it has been customary in this kind of work to report the products isolated, without designating the quantitative considerations it will be noted that the

[1] From the Chemical Research Laboratory of The Upjohn Company. Received for publication April 17, 1926.

[2] "Handbuch Der Biochem. Arbeits. Abderhalden," II, p. 1044.

careful application of approved methods here indicates that they leave larger quantities of the basic constituents unaccounted for than is generally believed. In fact we have learned very little about this extract. It will perhaps prove interesting to hydrolyze either the original extract or the material precipitated by phosphotungstic acid and repeat these separations on the split products.

A summary of our findings will be found at the conclusion of the paper.

EXPERIMENTAL PART.

Preliminary Cold Absolute Alcoholic Extract.—Instead of extracting this tissue at once with water as is customary in the study of nitrogenous extractives, a preliminary extract of the fat free substance from 4.53 Kgs. of desiccated gland was made with absolute alcohol. This has the additional advantage of yielding the lipoids which, while they may have failed to dissolve in ether are now frequently found in the alcoholic percolate.

The alcoholic solution obtained by exhaustive percolation was concentrated in steps and three fractions of insoluble material (A) removed, washed with alcohol and with ether.

This material (A) was emulsified with water and readily separated into a water insoluble part (A, I lipoid) and a water soluble (extractive) part.

The water insoluble part (A, I lipoid) amounted to about 5.65 Gms. It will be reported with the lipoids.

The *water soluble part* of the extracts did not separate until extremely concentrated solutions were obtained, when sodium chloride was obtained. The *water soluble part* of the material which separated from the last alcoholic percolate separated in crusts upon concentrating the aqueous extract from the lipin part. The top fraction weighing about 0.4 Gm. decomposed at 285° and on analysis proved to be largely isoleucine.

Calc. for $C_6H_{13}NO_2$: N = 10.7. Found: N = 10.5%. NH_2 = 11.0%.

The total yield of isoleucine was 2.1 Gms.

The alcoholic filtrates from the less soluble part (A) and the alcoholic and ether washings of (A) were concentrated and poured into ether in which it proved to be very largely soluble. The *ether soluble part* was agitated repeatedly with water which extracted a small amount of material and the *lipoidal ether solution* was put aside.

The material which precipitated, when the alcoholic solution was poured into ether, was emulsified with water; to this mixture ether was added, and after agitation the ether extract was added to the main lipoidal solution. The aqueous layer was also shaken with chloroform, which was returned (after removal of the chloroform) to the lipoid fraction. The united lipoid fraction (B) will be reported upon by Dr. M. C. Hart.

The united aqueous solution of alcoholic extractives will be reported upon later.

Examination of Alcohol Insoluble Residue for Water Soluble Bases.[1]—The ovar-

[1] A portion of the residue which had been percolated with cold alcohol was boiled several times with absolute alcohol and the solution was cautiously concentrated to observe whether or not any cerebrosides could be crystallized, but none were found. The material was in fact entirely water soluble; was treated with normal lead acetate solution, filtered from a slight precipitate and the phosphotungstates prepared in the usual manner. The filtrate from this phosphotungstic precipitate was added to the corresponding main filtrates, and the alcohol soluble bases proved so slight in amount that they were added to the main base solution.

ian residue (extracted as described above) was macerated with 25 liters of water, and heated to 80° for thirty minutes. After cooling, the supernatant extract was removed, and the residue again heated at 80° for thirty minutes with 30 liters further. This extract was removed by filtering through linen bags and a third extract was made with 25 liters further in the same manner. By trial the correct amount of acetic acid for complete coagulation was determined and the extracts were coagulated separately, and the last one concentrated to a volume of 1 liter. Finally the other extracts and washings of the coagulum were concentrated, the final volume being 4 liters, and a small amount of normal lead acetate was added as a preservative.

This extract solidified to a solid gel upon standing. It was diluted to about 10 gallons.

In order to see if anything could be directly crystallized from this extract, a portion of it amounting to approximately one-fifth of the solution was removed, treated with an excess of tannic acid, baryta, sulphuric acid, lead oxide as in the method of Kutscher.[1] The final solutions failed to crystallize either directly or after treatment with hydrogen sulphide.[2]

The entire fraction was now treated in this manner and the final solution was acidified with sulphuric acid and precipitated with an excess of phosphotungstic acid in the usual manner.

Filtrate from Bases.—The filtrate from the base fraction was freed from the excess of phosphotungstic and sulphuric acids with barium hydroxide and then CO_2 and concentrated to a small volume, and the barium was removed quantitatively with sulphuric acid. This will be reported on later.

The precipitated bases were removed by filtration and decomposed by the method of Wechsler.[3]

The concentrated solution was set aside in a desiccator to observe if anything could be crystallized directly, but nothing separated upon prolonged standing. The volume was made up to 350 and a sample of 3.5 cc. was taken out for analytical purposes.

Analysis of Phosphotungstic Acid Precipitable Fraction.—The 3.5 cc. (1% of entire solution) was made up to 50 cc.

5 cc. contained 0.0603 Gm. total solids = 60.3 Gms.

5 cc. gave 0.0325 Gm. ash = 32.5 Gms. ash, leaving only 27.8 Gms. organic material.

2 cc. required by Kjeldahl, 6.38 cc. $N/50$ acid equivalent to 0.0017864 Gm. N or 4.466 Gms. in the entire solution. The organic material contains therefore 16% N.

Creatinine (total) in Base Fraction.—1 cc. + 1.8 cc. 25% H_2SO_4 + 6 cc. water was heated for several hours on the steam-bath and made up to 10 cc.[4] Of this 9 cc. was neutralized and read against a standard creatinine solution containing 0.2 mg. Colorimetrical readings showed 0.000222 Gm. creatinine, or 1.11 Gms. creatinine in the entire solution.

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[1] See "Abderhalden's Handbuch Biochem. Arbeits.," II, p. 1044.

[2] A considerable crystallization of potassium sulphate was obtained. Slight crystallizations, one of which consisted of balls of needles giving murexide test was obtained.

[3] The distillates from the decomposed phosphotungstates yielded 0.5 Gm. ammonia. 5 cc. of solution of the chlorides required 16.85 cc. $N/10$ alkali with methyl red; and 17.05 cc. more after the addition of formaldehyde (Weber and Wilson, *J. Biol. Chem.*, 35, 385 (1918).

[4] Janney and Blatherwick, *J. Biol. Chem.*, 21, 579 (1915); Falk, Baumann and McGuire, *Ibid.*, 37, 528 (1919).

Purines.—35 cc. was analyzed by the method of Falk, Baumann and McGuire[1] and the final copper precipitate was suspended in 400 cc. boiling water and decomposed with H_2S. After the addition of a few drops of 2 *N* acetic acid, the CuS was filtered off and washed with boiling water. The combined filtrate was concentrated on a hot plate to a volume of 50 cc. Of this; 20 cc. required by Kjeldahl 5.08 cc. *N*/50 acid equivalent to 1.4224 mgs. nitrogen or 0.508 Gm. purine nitrogen in the entire solution (11.4% of the nitrogen here is purine N).

Uric Acid.—Another aliquot of the solution, examined by the method of Folin,[2] showed the presence of 0.096 Gm. uric acid in the entire solution.

Separation of the Bases.—The main solution (99%) was acidified slightly with nitric acid and precipitated with 25% silver nitrate solution. The purine silver nitrate compounds were centrifuged. This precipitate was washed twice with water containing a few drops of dilute HNO_3 and a few drops of silver nitrate solution.

The Purine Fraction, which was voluminous, was shaken with about 200 cc. of 10% ammonia and the ammoniacal fluid was removed by the centrifuge. This was repeated twice again, and the insoluble silver compound was finally digested with 500 cc. of warm 10% ammonia. The ammoniacal fluids contained small quantities of soluble silver compounds, which when decomposed with H_2S amounted to 0.15 Gm. amorphous material which contained no guanosine.

The main silver purine residue after the above treatment with ammonia, was freed from ammonia, suspended in water and decomposed with H_2S. The filtrate from silver sulphide was concentrated to a small volume, but nothing crystallized. The material was treated with an excess of 2 *N* hydrochloric acid and concentrated. It proved to be largely soluble; the insoluble material contained no uric acid.[3]

The solution of the hydrochlorides was now examined by the methods of Krüger and Salomon[4] and Krüger and Schittenhelm.[5] The excess of hydrochloric acid was removed by cautious evaporation and it was hydrolyzed with an excess of water at moderate temperature. The insoluble part was slight and yielded neither xanthine nor uric acid.

The soluble part (1.6 Gms.) was made ammoniacal and a heavy precipitate separated, most of which dissolved when shaken with fresh volumes of 1% ammonia. The slight amount of insoluble material gave a doubtfully positive test for guanine with nitric acid. It gave a positive diazo test, and a precipitate with sodium picrate.

The combined ammoniacal filtrates were evaporated to dryness to remove ammonia, redissolved in water and enough hydrochloric acid to make the solution acid to methyl orange. The addition of sodium picrate yielded 0.6 Gm. of very crude adenine picrate equivalent to 0.22 Gm. of adenine or 0.005% of the tissue.

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[1] *J. Biol. Chem.*, 37, 529 (1919).

[2] "Physiological Chem.," Mathews, p. 1019.

[3] The insoluble amorphous material was soluble in dilute sodium hydroxide and precipitated with acetic acid. This was repeated thrice. N = 36.02%; C = 39.42%; H = 2.51%. Calc. for $C_5H_4N_4O_2$: C = 39.46%; H = 2.65%; N = 36.85%. Evaporation with nitric acid yielded little or no yellow substance, and the subsequent addition of alkali caused no alteration in color. Diazo test positive but very slight. While the analytical data agrees closely with xanthine it may be confidently stated not to be this purine.

[4] *Z. physiol. Chem.*, 26, 350 (1898).

[5] *Ibid.*, 35, 153 (1902).

A fraction melting at 270–282° was analyzed.

0.0403 Gm. picrate gave 0.0594 Gm. nitron picrate.
Calc. for $C_5H_5N_5$: $C_6H_3O_7N_3$: $C_6H_3O_7N_3$ = 62.9%.
Found: $C_6H_3O_7N_3$ = 62.4%.

The filtrate from adenine picrate was acidified with nitric acid, picric acid removed with ether, and the solution was reprecipitated with ammoniacal silver nitrate. The silver compound was decomposed with H_2S and on evaporation a small amount of material was obtained. This was redissolved in water and converted into the picrate which in turn was converted into the nitrate,[1] but hypoxanthine could not be identified.

Histidine Silver Fractions.—The filtrate from the silver purines was treated with an excess of silver nitrate and barium hydroxide solution added. After the addition of 10 cc. the solution was still acid to litmus and gave further precipitate with ammoniacal silver nitrate and with baryta. The precipitate H, 1 was removed.

H, 1.—This slight silver precipitate was decomposed in the usual manner and the solution of the bases in dilute sulphuric acid was precipitated with mercuric sulphate solution which yielded a small amount of insoluble mercury salt. The mercury salt decomposed in the usual manner gave a solution containing approximately 7 mgs. of histidine when estimated colorimetrically.[2] No satisfactory preparation of picrolonate could be isolated.

The filtrate from the insoluble mercury salt, when freed from mercury and SO_4 contained approximately 2 mgs. histidine but nothing was isolated.

Baryta was further added to the main solution (40 cc.) the reaction being alkaline to litmus, but precipitates still resulted with ammoniacal silver nitrate or baryta. The precipitate H, 2 was removed.

Baryta was further added (44 cc.). The p_H was about 5.4 but precipitates still resulted with ammoniacal silver and baryta, but were becoming very slight. Precipitate = H, 3.

Baryta was added (40 cc.) and the p_H now = 6.0 and the other histidine tests are negative or very slight. The precipitate is H, 4.

The solution was now saturated with barium hydroxide in substance. The precipitate is H, 5.

The Histidine Fractions, H, 2 and H, 3, were jointly examined exactly as in H, 1. A slight insoluble mercury salt was obtained which upon decomposition yielded a solution containing 42 mgs. of histidine nitrogen equivalent to 157 mgs. histidine. To this solution picrolonic acid was added and 0.12 Gm. of a crude picrolonate separated. Washed with 95% alcohol and recrystallized from water it melted at 205–211° and could not be further purified. This material was impure histidine picrolonate.

The filtrate from the insoluble mercury compound contained 158 mgs. of nitrogen. Freed from $HgSO_4$ quantitatively the concentrated solution was treated with an excess of silver nitrate and the trace of histidine silver removed by the cautious addition of baryta. Arginine could be isolated in small amount (0.1 Gm.) as picrolonate (m. p. 220–228°) from the silver salt precipitated from alkaline solution. The histidine silver fraction showed the presence of 53.8 mgs. of histidine, but nothing could be isolated.

Histidine Fraction, H, 4.—While this fraction continued to show light precipitates with a drop of baryta and ammoniacal silver nitrate, suggestive of the incomplete precipitation of histidine, the solution was nevertheless quite alkaline and might prove to be the arginine fraction;

[1] A. Hunter, *J. Biol. Chem.*, 18, 111 (1914).
[2] Koessler and Hanke. *Ibid.*, 39, 504 (1919).

in short we had difficulty in finding the end-points of this precipitation, and for this reason took out H, 2 and H, 3 in hope of getting a purer preparation of histidine in it.

This precipitate was decomposed in the usual manner, and the mercuric sulphate precipitation carried out. In the histidine mercury precipitate was found 17.7 mgs. histidine nitrogen, but no characteristic picrolonate could be prepared. The sum of the various histidine determinations reported give a maximum value of 0.3 Gm. histidine equivalent to 0.007% of the tissue.

The filtrate from the mercuric sulphate precipitation contained 93.8 mgs. of nitrogen, still yielding 18 mgs. of histidine but nothing except a trace of arginine picrolonate was isolated.

Arginine Fraction, H, 5.—This silver fraction precipitated at high alkalinity, which might be expected to contain arginine, carnosine, methyl guanidine, agmantine, but which should be free from histidine, was decomposed with H_2S in the presence of diluted H_2SO_4. The filtrate from the HgS contained 1.06 Gms. nitrogen.

Carnosine Color Test.[1]—Compared by the diazo color with standard histidine solutions, there was found, histidine = 0.15 Gm. which is equivalent to 0.22 Gm. carnosine.

The solution was concentrated to 100 cc. acidified with H_2SO_4 (5%) and precipitated with mercuric sulphate solution in the presence of alcohol. A small amount of mercury salt separated which was filtered off, washed and decomposed as usual. The sulphate ion was completely removed with $Ba(OH)_2$ and upon evaporation finally in a desiccator a slight amount of syrup resulted, which could be crystallized neither from water nor alcohol. The addition of picrolonic acid gave a slight precipitate which could not be purified.

The filtrate from the mercury salt precipitate was freed from Hg and SO_4, concentrated and treated with somewhat less than the calculated quantity of picrolonic acid. The total yield of arginine picrolonate was 3.03 Gms. equivalent to 1.2 Gms. of the base. The picrolonate decomposed at 228–229°.

Calc. for $C_{10}H_8N_4O_5.C_6H_{14}N_4O_2$: C = 43.8%; H = 5.05%.
Found: C = 43.7%; H = 4.75%.

The filtrate from arginine picrolonate was acidified with normal sulphuric acid and exhaustively extracted with ether. The aqueous solution was treated with a slight excess of barium hydroxide, filtered and the filtrate concentrated at 45° to a volume of 50 cc. This solution contains 0.403 Gm. of nitrogen of unknown source. A quantitative examination for creatinine showed but 0.09 Gm. The solution was treated with picric acid, concentrated to 10 cc. About 0.2 Gm. of an amorphous product separated which was filtered off and then there separated on standing a crystalline crop of prismatic blocks melting, after washing with ether, alcohol and water at 230°. It was recrystallized from water and the melting point was unchanged.

0.0625 Gm. gave 0.1135 nitron picrate.
Calc. for $C_4H_7ON_3{:}KC_{12}H_5O_{14}N_6$: picric acid = 75.2%.
Found: picric acid = 76.8%.

This substance is potassium creatinine picrate and Jaffe's test was positive.

The filtrate was acidified with hydrochloric acid and the picric acid removed with ether. It was concentrated to a small volume and treated with H_2PtCl_6 (0.4 Gm.) but the platinichloride was exceedingly soluble in water. Upon the addi-

[1] Koessler and Hanke, *J. Biol. Chem.*, 39, 504 (1919).

tion of alcohol it separated as an oil which did not crystallize. The residue was dissolved in water, and the platinum precipitated as sulphide which was filtered off and the solution concentrated to a syrup. No crystalline gold salt could be prepared.

The Lysine Fraction, i. e., the baryta saturated filtrate from the arginine fraction was slightly acidified with H_2SO_4 and this reagent was added as long as any more $BaSO_4$ precipitated but the acidity was restrained by the addition of sodium hydroxide. The solution was saturated with H_2S, filtered and slightly concentrated; the lysine fraction was precipitated with phosphotungstic acid. The filtrate amounted to 2 liters.[1]

The precipitated phosphotungstates were decomposed by the method of Wechsler and the solution of the bases concentrated to a syrup in a desiccator. The material is somewhat crystalline, but alcoholic solutions could not be brought to yield a crystalline crop.

An unsuccessful effort was made at this point to separate spermine phosphate.[2] After the removal of the reagents the solution was concentrated to a small volume. It contained 0.9 gram nitrogen.

It was fractionally precipitated with alcoholic picric acid solution and a considerable quantity of potassium picrate separated. This was recrystallized and the solution of the bases was freed from picric acid after the addition of an excess of hydrochloric acid. The solution of the hydrochlorides was taken to a syrup which was finally separated into 3 parts, (*a*) hydrochlorides soluble in absolute alcohol, (*b*) insoluble in absolute, soluble in 95%, (*c*) insoluble in 95% but water soluble.

(*a*) Nothing crystalline was present. It was precipitated with alcoholic $HgCl_2$ yielding a slight indefinite precipitate. Converted into chloroplatinate without obtaining crystals. The filtrate from the mercury salt contained N = 0.45 Gm.; amino N = 0.227 Gm. Amino N after 10 hours hydrolysis = 0.269 Gm. α-Amino N (5 min.) is therefore increased from 50.4% to 64.4%. Solution taken up with water, rendered alkaline with baryta, and the alkaline mercury precipitate yielded 0.1 Gm. lysine picrate. The filtrate from the alkaline mercury precipitate was freed from reagents, reprecipitated with phosphotungstic acid and here 0.17 Gm. lysine picrate resulted.

(*b*) Alkaline mercury precipitation gave a lysine fraction yielding 0.25 gram lysine picrate.

(*c*) Alkaline mercury precipitate yielded a small amount of lysine picrate.

Calc. for $C_6H_{14}N_2O_2.C_6H_3N_3O_7$: picric acid = 61.05%; amino N = 7.47%.
Found: 61.3, 61.2, 61.3 and 60.8. Amino N = 7.34, 7.20, 7.32, 7.62.

SUMMARY.

The basic fraction obtained from desiccated, alcohol and ether exhausted ovarian residue amounts to 0.51% by weight. Basic nitrogen amounts to 0.098%

[1] On account of the dilution here, a portion of this filtrate was freed from phosphotungstic and sulphuric acids with baryta; from the excess of Ba with CO_2 and concentrated to small volume, from which the phosphotungstates were precipitated. Only 0.12 Gm. N is lost in the original phosphotungstic precipitation.

[2] Dudley, Rosenheim and Rosenheim, *Biochem. J.*, 18, 78 (1925).

which includes 0.009% creatinine nitrogen, so that the base fraction may be stated to contain 0.09% basic nitrogen. About 11% of this falls into the purine group from which we isolated adenine as picrate and a substance not xanthine which agreed upon analysis with the formula $C_5H_4N_4O_2$. Separation by means of silver and baryta gives a "histidine" fraction from which nothing but a very doubtful trace of histidine picrolonate could be obtained. It contains considerable material giving the diazo color test. In the arginine fraction amounting to about 24% of the basic nitrogen a considerable quantity of arginine was isolated as picrolonate. Aside from a trace of creatinine picrate also found in this fraction more than half of the material is unaccounted for. In the lysine fraction a very small part of the nitrogen is accounted for by the isolation of lysine picrate equivalent to 0.05 Gm. lysine nitrogen from a solution containing about 1.0 Gm. of nitrogen.

Isoleucine was incidentally identified.

KALAMAZOO, MICH.

STUDY ON THE ANTI-DIABETIC PROPERTIES OF TECOMA MOLLIS.*,[1] PRELIMINARY REPORT.

BY G. G. COLIN.

For many years we have been deeply interested in the researches carried out by several investigators who have devoted much time to the scientific study of pancreatic hormones of animal origin.

More recently it has been found that active metabolizing vegetable tissue contains an insulin-like substance which lowers blood sugar.[2,4,6,7] Dr. J. B. Collip[1] experimented with old dry leaves of *Diascia spicata* with negative results.

We have experimented with dry leaves of *Tecoma mollis*[15] (commonly known as "Tronadora" in this country) and obtained a decrease of blood sugar in the diabetic patient. This herb has been and is used considerably by diabetics on account of its beneficial effect. Many of them claim to have been cured by it. This material is carried by all druggists as dry leaves and as a fluidextract, and it is hard to find a doctor who has not prescribed it or at least heard of it.

Dr. Manuel Barreiro of Dr. Gabriel Malda's Clinic has had experience with the administration of this drug and informed us that in many cases very remarkable results are obtained by the use of a simple infusion of Tronadora.

There are, of course, a hundred varieties of herbs claimed to have various curative properties, but in the majority of cases the claimed therapeutic value is mostly imaginary. Bearing this in mind we thought it would be interesting to determine as far as possible the actual therapeutic value of this drug.

Upon questioning some diabetics who have used this drug we found that in some of them sugar in the urine had decreased and finally disappeared in from

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* Central Chemical Laboratory, Mexico.

[1] The funds for this investigation were granted by N. G. Colin to whom the authors express appreciation. Dr. Alvaro Sosa Granados of the Hospital d' Jesus, enthusiastically coöperated by giving valuable suggestions and taking charge of the patients under examination.

EDITOR'S NOTE: The author will welcome coöperation of an institution, equipped for the investigation of the physiological action of this material.