as nucleic acid and that a small quantity of purines have been precipitated with the hexone bases.

Determination of Tyrosine and Tryptophane.—These were determined colorimetrically, tyrosine by the method of Folin and Looney[1] was present to the extent of 7.1%. By the method of May and Rose[2] we found tryptophane = 2.5%.

The Molisch test gave negative results.

SUMMARY.

Hydrolysis of the crude total protein of follicular fluid showed the presence of Arginine, 5.7%; histidine, trace; lysine, 11.0%; tryptophane, 2.5%; tyrosine, 7.1%. All of these values very closely approximate those of the albumin ovarian residue. The latter contains appreciably more sulphur (1.5%), only 1.1% being found in the protein of follicular fluid.

KALAMAZOO, MICHIGAN,
NOVEMBER 9, 1925.

CHEMICAL EXAMINATION OF OVARIAN RESIDUE.*[1]

I. THE PROTEIN FRACTION.

BY BRYANT FULLERTON[2] AND FREDERICK W. HEYL.

When, in general, any glandular material is extracted in the cold with water or dilute saline solutions, a precipitate is usually produced upon acidification (acetic acid). Preparations of this class have been classified by Samuely[3] in two groups (*a*) "cell globulins" and (*b*) tissue or cell nucleoproteins. Under (*a*) it is noted that from liver tissue of horse or cat, from kidney, spleen, lymph cells, testicles and thymus, such preparations have upon analysis shown from 0 to 1.3% phosphorus. Approximately the same substances are also listed under (*b*). Perhaps one should infer that if, on treating such extracts with methods suitable for the precipitation of globulins, a nonphosphorus compound results, we have a "cell globulin." For instance, from thyroid gland extracts, such a substance can be prepared.

Such substances, when containing phosphorus are best classified according to Jones[4] as α nucleoproteins, and they are probably variable colloidal mixtures or salt like combinations of cell globulins or albumins with nucleic acid.[5] In Osborne's work on wheat embryo, it was shown that such extracts contain both albumin (leucosin) and globulin associated with nucleic acid.

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[1] *J. Biol. Chem.*, 51, 421 (1922).

[2] *J. Biol. Chem.*, 54, 213 (1922).

* From the Chemical Research Laboratory of the Upjohn Company.

[1] Received for Publication December 1, 1925.

[2] This paper is based upon the thesis presented by Mr. Fullerton to the Faculty of Kalamazoo College, in partial fulfillment of the requirements for the degree of Master of Science.

[3] "Abderhalden's Hand-lexikon IV," p. 93 and 130.

[4] "The Nucleic Acids."

[5] "Pohl, Abder. Handbuch, V," 665 reports positive pentose reaction for liver nucleoproteins. See Fleugel, 1919.

In recent years interest in this fraction has lagged. Rather the nucleic acid has absorbed almost all the attention. It is generally held that such preparations do not represent true constituents of the cell, and Wells[1] has shown that any antigenic properties that they have are dependent upon the protein component. He goes further and states that these labile proteins are not specific for the cells in which they are found.

These proteins have not been hydrolyzed and the chemist cannot form an opinion on their specificity. As these unorganized proteins are readily soluble, in distinction to the major part of the cell contents, and since most of the metabolic changes may be assumed for that reason to revolve about them, some knowledge of their chemical make up is certainly of interest. Pohl has hydrolyzed liver nucleoprotein and compared the nitrogen distribution with the labile blood protein and believes they are not identical. That these proteins are labile and readily altered for metabolic purposes is shown by the fact that whilst in hunger the total protein of the liver increases, there is a marked destruction of the α nucleo protein fraction.

If after the tissue has been thus exhausted, a further extraction is made with dilute alkalies, further quantities of protein material are extracted, which may in turn be precipitated more or less completely with acetic acid. These also contain phosphorus, but it has been the custom not to include these among the so-called α nucleoproteins. In one of the more recent studies with this procedure McGregor[2] reported on the composition of the proteins of the central nervous system. When the extraction of this material is begun with water or dilute saline solutions, about 5% of protein was dissolved; subsequently 10% more was extracted with very dilute alkaline solutions. The phosphorus in the former proteins amounted to about 0.1% and in the latter 0.6%.

When the first aqueous or saline extracts are precipitated with acetic acid the protein is incompletely thrown down and has a variable composition depending upon the nature of the solution. The protein precipitated from alkaline solution is said to be uniform.

Reviewing the methods of preparation of substances listed under the title of α nucleoproteins we find that in some instances, these have been isolated by beginning at once to extract the gland with dilute alkalies. By way of illustration we may mention some examples of both processes.

Source.	Extraction fluid.	P %.	
Brain	NH_4Cl solution	0.56	Levene
Liver	Water	1.45	Halliburton
Suprarenal	2% ammonia	4.71	Jones & Whipple
Kidney	Salt Solution		
	Water	0.37	
Ox pancreas	Dilute saline	1.67	
Pig pancreas	Ammonia	5.05	Jones & Whipple
	Ammonium Acetate		Gamgee & Jones
Thymus	Water	5.23	
		0.97	
Spleen	Dilute $NaHCO_3$	1.2 to 1.8%	Levene & Mendel

[1] *J. Biol. Chem.*, 28, 11 (1917); *Z. Immunitäts.*, 19, 599 (1913).
[2] *J. Biol. Chem.*, 28, 403 (1917).

It appears to us that these variations confuse the study. With the exception of the confusing work on thymus extracts most of the interest in this work has been centered in the nucleic acids. Perhaps the nucleic acid is the same in every gland like that of thymus nucleic acid which contains 8.94% phosphorus.

However, these various preparations are without a doubt, not different salts of the same protein and the same nucleic acid. There is every reason to believe that various proteins are present, but with the exception of the above-mentioned work on thymus where an interesting histone was shown to be present, no extensive attention has been given to the protein fraction. It does indeed present difficulties which, since the work of Neuman and Kossel on nucleic acid has caused the interest to swing to the nucleic acid side, and with remarkably progressive results.

When glandular tissue is extracted with boiling solvents, coagulating the proteins and perhaps some protein nucleate, the filtrate contains organic phosphorus in non-coagulable form. This precipitates when acidified with acetic acid and these are the so-called β nucleoproteins of which, that studied by Hammarsten (1894) is the classic example. Similar nucleoproteins have been obtained as follows:

	Precipitated by.	% P.	
Mammary	Acetic acid	0.28%	Odenius
Spleen	Acetic acid	?	Levene
Liver	Acetic acid	3.06	Wohlgemuth
Ox pancreas	Acetic acid	4.98	

There is reason to believe that the phosphorus in these preparations is not derived from ordinary thymus nucleic acid, but from guanylic acid $H_2PO_4.C_5H_8O_3$ $C_5H_4N_5O$ (P = 7.67%) and that this is the source of pentose sugar reactions in animal extracts. The protein moiety is apparently a non-coagulable mucoid-like substance, perhaps somewhat similar in different tissues.

The materials extracted from glandular tissues with dilute alkalies have not, we believe, been classified as a group, but they are just as much amenable to such classification as the "glutelins" of the plant proteins. It will prove interesting to compare these with the mucoid substances. However, it is not impossible that this protein is the same as the soluble fraction, except that it is physically altered by the nucleic acid combination.

The residue left from the above-mentioned solvents may be termed the albuminoids of the tissue, the term "stromin" being frequently applied to the alkali soluble proteins (*e. g.*, "neurostromin").

We have applied these general principles to ovarian residue. We find that the proteins may be divided among the several above-mentioned groups as follows:

	Per cent.
Water soluble	6.8
Saline soluble (in addition)	4.3
Proteose[1]	1.2
Soluble in 0.1% alkali	10.3
Soluble in 1% (additional)	12,6
Albuminoids	22.0
Total isolated	57.2

[1] Calculated as N $\times$ 6¼ of the alcohol precipitable protein in the filtrates after coagulation.

The cold-water extract yielded without difficulty protein preparations which were practically phosphorus free. In terms of Abderhalden's nomenclature we would have here a "cell globulin." The material, however, instead of conforming to the solubility requirements of the globulin group is much more properly classified as an albumin. Most of it is precipitable at half saturation with ammonium sulphate, is not precipitated upon dialysis and can be isolated by heat coagulation.

Some protein remains in solution which has been half saturated with ammonium sulphate. It can be precipitated by increasing the acidity. It appears to be a further quantity of the same albumin contaminated perhaps by a non-protein moiety or a small amount of a nucleic acid (P = 0.2%). This protein is free from iron, in contrast to small amounts of globulin-like material which can be separated from the main albumin fraction.

It should be added that evidence was obtained of the presence of a non-coagulable mucoid in the filtrates from the albumin in the water extract. These preparations were not uniform in composition tending to lower carbon content.

The study of the saline extracts failed to reveal the presence of any conspicuous amount of a native globulin. In fact the presence of a native globulin is doubtful. A globulin-like artifact appears to form at the expense of the coagulable albumin.

The variations in composition and alterations in solubility of the other proteins which, like the globulins, might be associated with the albumin are reminiscent of the serum-globulin fractionation studies which lead to Fibrinoglobulin, Euglobulin and pseudoglobulin and insoluble globulins.

Comparison of our analytical results by the Van Slyke process with those obtained by Lock and Thomas[1] on serum albumin and serum globulin show the protein of the gland to differ materially from the serum proteins, especially in the matter of histidine content.

	Total N.	Amide N.	Histidine N.	Arginine N.	Lysine N.
Serum albumin	100.0	5.18	5.71	9.11	12.88
Serum globulin	100.0	5.55–6.01	2.6–3.0	9.5–8.3	8.2–8.5
Ovarian albumin	100.0	7.8	0.2	11.6	14.69

Most of the phosphorus can be precipitated from a saline extract (made subsequent to a water extract) when acidified. The crude protein thus precipitated amounts to over 3 per cent of the glandular material and contains 1.39% phosphorus. We have not hydrolyzed this to prove the presence of nucleic acid.

A direct saline extract, upon dialysis, yields alteration products, one of which has globulin-like properties. From the salt-free solution the characteristic ovarian residue albumin was isolated and the presence of mucoid was again demonstrated.

EXPERIMENTAL.

Effect of Preliminary Extractions on Subsequent Protein Extraction of Ovarian Residue.—Ether extracted 8.53%. Cold absolute alcohol extracted 6.0% solids further, of which the non-lipoidal part amounts to approximately 4.0%. This extract contains N = 0.21% of the tissue. Cold 95% alcohol extracts 9.8% solids containing 0.42% N; while boiling 95% alcohol extracts 11.7% solids and 0.58% N.

[1] *Z. physik. Chem.*, 87, 78 (1913).

In order to find out what preliminary extractions might be permitted before the subsequent protein study, we have extracted separate samples amounting to 5.0 Gm. of the tissue as follows: (*a*) with ether; (*b*) ether followed by 95% alcohol; (*c*) with ether followed by absolute alcohol, 75% alcohol, 95% alcohol, absolute alcohol, and finally with ether. In each case the extracted residues which had been extracted with volatile solvents, were extracted three times with 10% salt solution and estimations were made of the α nucleo-protein, coagulable protein in the filtrate, and of non-protein nitrogen. Then the stromin proteins were extracted with 1.0% sodium hydroxide solution at 4° C. and lastly the albuminoids were weighed as residue. The results obtained may be tabulated as follows:

Sample.	α Nucleo-protein %.	Coagulable protein %.	Non-protein nitrogen %.	Non-protein N soluble in 90% alcohol.
A	3.6[1]	6.95	0.95	0.75
B	2.5	4.70	0.63	0.35
C	0.64	none	0.28	0.14

Sample.	Soluble in 1% alkali.	Residue %.	Filtrate from stromin % N.
A	20.5[2]	25.9	
B	24.8	23.1	1.07
C	22.43	35.3	

From these preliminary results it is at once evident that a prior alcohol (95%) extraction at once decreases the saline soluble proteins and they now appear in the alkali soluble protein extract. A considerable extraction in a preliminary way, as in C, causes the saline protein extract to be almost protein free and in this case the proteins are found in the residue.

NATURE OF THE PROTEINS.

A. The Water Extract.—The dilute water extract is sufficiently free from salts, so that very little protein is precipitable upon acidification, and furthermore it could not be coagulated. When sodium chloride was added to the aqueous extract a precipitate resulted upon acidification and again on heating. The liquid freed from protein material by both means still contained appreciable amounts of material giving the biuret test.

A quantity of ether extracted ovarian residue equivalent to 5 grams of glandular material was extracted with three successive portions of water and the volume made up to 250 cc. (p_H = 5.8). This extract was divided into three parts of 100, 100 and 50 cc. each.

To the 50 cc. aliquot, 5 Gm. NaCl was added, acetic acid equivalent to 0.1% and the solution was heated to coagulation: *total precipitable material* 6.78%.

The 100 cc. aliquots were treated with 10 Gm. salt and precipitated with 0.05% and 0.15% acetic acid. The precipitate at a concentration of 0.05% amounted to 0.66%; at 0.15% the precipitate amounted to 1.55% more, making a total of 2.2% of protein precipitable. Another determination at 0.25% acetic acid gave 2.4%.

From the filtrates from these fractions precipitable with acetic acid, heat coagulated 4.74% (4.38) further.

[1] Is phosphorus-free. By α nucleoprotein we mean precipitable with acetic acid.

[2] Dried at 110°; contains 0.58% P.

The filtrate from this coagulum contained nitrogen equivalent to 0.82% of the gland. This solution gave a red biuret test.

Unless salt is added as above, it was found that acidification of the extract yielded a precipitate amounting to only 1.4% and the filtrate cannot be coagulated upon heating. In another experiment only 0.44% precipitated upon acidification and coagulation, while 6.76% further was precipitated from the filtrate of the coagulation with 90% alcohol. This precipitation was practically complete at 70 vol. % alcohol.

It was found that concentrated aqueous extracts could not be filtered through Mandler filters, an appreciable part of the protein being held back and clogging the pores. Extracts prepared in this way gave very little precipitate with ammonium sulphate at 3/10 to 4/10 saturation.

PREPARATION OF PROTEIN FRACTIONS FROM AQUEOUS EXTRACT.

Two hundred Gm. fat free tissue (219 Gm. ovarian residue) was agitated with 3 liters of cold distilled thymol water for one hour, and allowed to stand in the ice chest for about one hour. The extract was removed by centrifugation; filtered through pleated filters, and through Mandler thimbles. The filtrate measured 2760 cc. It was half saturated with ammonium sulphate in substance and allowed to stand over night at 4°.

This fraction was easily removed by centrifugation, redissolved in water and the solution filtered through paper and reprecipitated at half saturation.

The precipitate was redissolved in water, transferred to a parchment bag and dialyzed against distilled water until free from the SO_4 ion. A considerable fraction (*A*) separated and was removed by centrifugation. The solution (*B*) from which it had precipitated was dialyzed for 24 hours more and nothing further precipitated.

The precipitate (*A*) was agitated with 200 cc. of 10% salt solution and allowed to stand in the ice chest for several hours. The insoluble part, (*C*), which is an altered protein, was centrifuged off, washed with brine, suspended in water, dialyzed free from chlorine ion, collected by centrifugation, washed with 50% alcohol, 95% alcohol, absolute alcohol, and finally with ether. The yield was 0.68 Gm. equivalent to 0.3%. This is the largest yield of this fraction we ever obtained.

The 10% saline filtrate from (*C*) was dialyzed free from salt. It yielded a precipitate of globulin (*D*) that weighed 0.42 Gm. This fraction is usually larger than this, frequently amounting to 0.85 gram or 0.4%.

Several analyses of this globulin were made:

Sample.	No.	Yield Gm.	Ash %.	C.	H.	N.	S.	P.
166	1	0.72	1.67[1]	52.8	7.3	..	1.1	0.08
166	1	..	..	52.7	7.1	..	..	..
162	a	0.77	1.54[1]	52.7	7.1	..	..	..
158 & 170	mixed[2]	..	1.17[1]	..	..	14.63	..	..
162	*a*	..	..	52.4	6.9	..	..	..

The chief part of the protein precipitated at half saturation has the solubilities of an albumin, and is found in solution (*B*) from which ammonium sulphate has

[1] Ash contained iron.

[2] Molisch test ++.

been removed by dialysis. It is easily removed by coagulation and represents 2.5% of the gland.

Several analyses of this material were made:

Sample.	No.	Yield Gm.	Ash %.	C.	H.	N.	S.	P.
152	1	5.4	0.85	52.0	6.95	..	..	..
152	1	..	..	52.05	7.05	..	..	..
152	1	..	..	52.3	7.0	..	..	..
152	1	..	..	51.95	6.9	..	..	..
162	B	4.66	..	..	..	15.26	1.5	absent
158[1]	B	4.1	0.73	..	..	15.32	1.58	absent

The filtrate from the coagulated albumin when poured into alcohol gave very uniform yields of a mucoid substance, amounting to 0.4%.

Several samples of this uncoagulable protein were analyzed: Molisch test +.

Sample.	No.	Yield.	Ash.	C.	H.	N.	S.	P.
164	..	0.88	1.84	51.1	7.1	..	..	..
..	..	..	..	50.7	7.0	..	..	..
160	1	0.91	3.10	..	..	14.3	1.47	absent
171	..	0.56	8.54	50.7	7.2	..	..	..

The half-saturated ammonium sulphate solution from which the above fractions separated was acidified with acetic acid, maximum precipitation occurring at $p_H = 3.2$. For the original liquid $p_H = 4.6$. This precipitate was suspended in water and dialyzed free from chlorides and sulphates, and there remained as an insoluble protein salt, *E*, 2.0 grams.

Analysis of protein salt *E* resulted as follows:

Sample.	Yield.	Ash.	C.	H.	N.	S.	P.
..	..	..	52.0	7.1	..	..	..
171 P	2.05	0.3	51.9	6.85	14.76	1.75	0.22
167 P[2]	2.85	0.3	..	..	14.64	1.83	0.27

The aqueous fluid which was separated from the above protein salt yielded upon coagulation (in the presence of salt) small yields (approximately 0.1%) of a coagulated protein.

The total protein separated from the water extract of 219 Gm. gland varied from 10.3 Gm. to 8.2 Gm. Since the extract obtained measured about 90% of the three liters used, it may be concluded that the yield amounted to about 4.5 to 5%.

B. The Saline Extract.—When fat-free ovarian residue is directly extracted with 10% saline solution a protein extract of the following composition resulted:

Protein precipitated by acetic acid	3.60%
Protein coagulable in filtrate	6.95
Total nitrogen in filtrate	0.95
Alcohol soluble nitrogen (90% alcohol)	0.75

It is apparent that salt solutions extract a somewhat larger quantity of protein, than does water, indicating the presence of several per cent of globulin.[3] In

[1] Molisch test ++.

[2] Molisch test +++.

[3] A saline extract subsequent to a preliminary water extract yielded 2.6% coagulable protein to 4.3%.

order to isolate this as pure as possible the albumin fraction was removed with an aqueous extract, after which the residue was extracted with 10% salt solution (thymol).

PREPARATION OF PROTEINS FROM SALINE EXTRACT. AFTER PRELIMINARY WATER EXTRACT.

(*a*) Two hundred Gm. fat-free ovarian residue was exhausted with 3 liters of distilled water, and the extracted residue after washing with further quantities of water was extracted with 3 liters of 10% salt solution. The mixture stood in the ice chest over night. The extract was removed by centrifugation and filtered through a diatomaceous filter. The filtrate measured 2870 cc. (p_H = 6.).

To this extract 30 cc. 2 N hydrochloric acid was added and after standing in the ice chest over night a precipitate (205:1) weighing after purification 6.4 Gm. separated. This preparation was purified by suspension in water and dialysis against distilled water until free from Cl^-.

The filtrate (p_H = 3.) was further acidified by addition of 20 cc. 2 N, hydrochloric acid (p_H = 2.). The precipitate weighed 0.45 Gm. (205:2).

The filtrate from the above preparations was heated to coagulation when 0.53 Gm. further resulted (205:3).

The total protein in this extract therefore amounts to 7.38 Gm. equivalent to 3.4% of the original glandular material.

The analyses of these protein preparations gave the following results:

205:1; Moisture, 7.58%; Ash, 1.52%; On ash- and moisture-free basis, S = 0.89%; P = 1.39%.

205:3; Moisture, 8.2%. On moisture-free basis, S = 1.6% and P = 0.06%.

(*b*) A further quantity saline extract was prepared exactly as above, 3 liters of filtered extract being obtained. This extract was 1/4 saturated with ammonium sulphate and after standing over night in the ice chest the precipitate was centrifuged out, suspended in water and dialyzed free from salts and collected in the usual manner. It weighed 2.1 Gm. and was readily soluble in dilute salt solution.

Analysis 210:1; Moisture 12.2%; Ash, not determined.
On moisture-free basis, S = 1.43%; P = 0.34%.

The filtrate from the less soluble globulin was now half saturated with ammonium sulphate. By the same procedure as above mentioned it was found that the protein precipitated at 0.25 to 0.50 saturation weighed 1.28 Gm. To this weight should be added, however, 0.5 Gm. which was obtained by heating the dialyzed fluid in which the precipitate was originally suspended.

Analysis, 210:2; Moisture, 14.7%; Ash, not determined.
On moisture-free basis, S = 1.42%; P = 0.56%.

The filtrate from the material precipitated at half saturation yielded on coagulation 2.2 Gm. further, but this was not examined.

It therefore appears that the preliminary removal of water-soluble proteins, through the removal of phosphorus-free proteins causes the subsequent saline extract to yield rather highly phosphorized products which are unsuitable for analysis by hydrolysis. There is some evidence also that a considerable part of

the protein found in the saline extract may in fact be the same albumin found in the water extract.

Assuming the phosphorus to be present as nucleic acid (8.94% P) the material, 205:1, is probably a combination of nucleic acid (15%) and protein (85%). The corrected sulphur content for the protein moiety is therefore 1.07%.

The protein part of this fraction consists, however, of more than one protein. At 1/4 saturation the material 210:1 consists of 96% protein so that the corrected sulphur content for the protein part of this precipitate is 1.49%. The material precipitated at half saturation, 210:2, on the same basis contains 94% protein with 1.51% S. These two estimations of sulphur indicate that the material precipitated at this particular acidity with ammonium sulphate up to half saturation might consist of the same albumin found in the water extract and which, as shown above, contains 1.5% sulphur.

This identity is also indicated by the fact that when it was purified by dialysis a considerable portion of the suspension dissolved in the water as dialysis proceeded and it could again be separated by coagulation.

C. Direct Saline Extract.—Two hundred grams fat-free tissue (219 Gm. ovarian residue) was shaken with 3 liters of cold 10% sodium chloride solution and allowed to stand in the ice chest over night. The extract was centrifuged and filtered through Mandler filters. The filtrate measured 2850 cc. A test portion (5%) was removed. It showed $p_H = 5.4$. When heated, two coagulation points could be detected. It showed a very slight turbidity at 70° and a dense curdling at 74–75°. After filtering this off, the filtrate remained clear up to 83°, was quite turbid at 88° but for flocculation it was heated at 90° to 95°. If the original extract ($p_H = 5.4$) is slightly acidified with acetic acid, precipitation results. If a solution containing 0.1% acetic acid is centrifuged clear and heated it grows turbid at 55° and flocks at 60°. After filtering this off a second and last coagulum can be produced at 74° to 75°.

The saline extract (2700 cc.) was now dialyzed against distilled water and a globulin fraction precipitated. This was removed, rinsed with water and shaken with 10% salt solution (100, 50, 50 cc.) and the saline extract removed from insoluble material by means of the centrifuge. The *insoluble part* or altered globulin weighed 1.12 Gm.

Analysis, 212; Moisture, 5.32%. On moisture-free basis: S = 1.16%; P = 0.23%.

The clear saline solution from which this altered globulin had separated was dialyzed and 1.27 Gm. of *globulin* resulted. We might add that prolonged dialysis failed to render this precipitation complete, and that 0.22 Gm. was precipitated by the addition of acetic acid to the filtrate from the globulin.

Analysis: 216; Moisture, 7.00%. On moisture- and ash-free basis: P = 0.038%. C = 50.9, 50.15; H = 7.2, 6.95. It therefore is not identical in this respect with the same fraction prepared from the direct water extract.

In order to check up the results previously obtained on the aqueous protein extracts, the original aqueous liquid from which approximately 2.6 Gm. had been removed upon dialysis was divided into two equal parts. The first portion was heated on the steam-bath but failed to coagulate until salt and acetic acid were added. The coagulum removed in the usual manner weighed 4.56 Gm., which

would correspond to 9.6 Gm. from 219 Gm. of tissue; a yield entirely disproportionate to the coagulated albumin found in the aqueous extract and exceeding the weight of all the water-soluble proteins previously found.[1] The product was, however, entirely unsatisfactory, being very dark and had evidently occluded much foreign material.

This filtrate yielded 0.54 Gm. mucoid (S = 1.25%, P = 0.28%).

The other half of the solution was therefore half saturated with ammonium sulphate in substance and allowed to stand in the ice chest. The precipitate was redissolved in water (200 cc.) and reprecipitated with ammonium sulphate.

The reprecipitated protein was redissolved and the solution filtered to remove a small amount of insoluble material. This solution was dialyzed, but nothing separated. The coagulation of this liquid yielded 1.0 gram of coagulated albumin.

The filtrate from the coagulated albumin yielded a small amount (0.07 Gm.) of mucoid.

The albumin 212; 214, on moisture-free basis, S = 1.1; P = 0.015%.

The half-saturated filtrate from which the above had separated gave a maximum precipitation at p_H = 3, and at this point (HCl) precipitation was effected. We suspended the precipitate in water and dialyzed it free from sulphates and in this process a considerable part of it dissolved. The entire fraction was therefore coagulated. Weight 1.5 Gm.

The weights obtained by the ammonium sulphate procedure are too low and mucoid fraction is conspicuously absent. The work was repeated. Two hundred and fifty Gm. fat-free ovarian residue, extracted with 3800 cc. saline solution (10%) yielded an extract which when dialyzed gave a globulin precipitate. This precipitate was treated with 10% saline solution. The insoluble part or *altered globulin* weighed 0.75 Gm.

The soluble globulin separated from its saline solution upon dialysis. It weighed 0.9 Gm. and the salt-free filtrate when acidified with acetic acid yielded 0.3 Gm. further.

The dialyzed and porcelain-filtered solution from which these globulins had separated, when coagulated directly again in the presence of salt and acetic acid, gave an unsatisfactory black preparation of coagulated albumin weighing 11.5 Gm. The filtrate from this material after dialysis was concentrated to a small volume and some insoluble material filtered off and the concentrated solution was poured into alcohol and a typical mucoid fraction weighing 2.15 Gm. It should be pointed out that in the immediately preceding experiment practically no mucoid had been obtained, the first coagulum undoubtedly having carried it down.

(*c*) The experiment was repeated a third time. From 250 Gm. of fat-free ovarian residue, we isolated 0.97 Gm. insoluble altered protein and 2.0 Gm. of *globulin*.

Analysis of globulin 220:1; C = 49.9, 49.35, 49.5; H = 6.8, 6.8, 6.8.

The dialyzed solution from which the globulin fraction had separated was now half saturated with ammonium sulphate. The precipitate was redissolved in

[1] A typical experiment on the aqueous extract would yield, after dialysis, approximately 5.4 Gm. of coagulated albumin, 0.8 Gm. mucoid, 2.0 Gm. of protein salt precipitated with acid, 0.2 Gm. of coagulum in the filtrate from the protein salt; a total of 8.4 Gm.

750 cc. water and again precipitated at half saturation. The precipitate was dissolved in water and the solution filtered through porcelain and dialyzed until free from sulphates. The slight turbidity always observed at this step amounted in this case to a definite precipitation, which was removed by vigorous centrifugation. This material represents an alteration product (of globulin-like nature) which has resulted from the main protein which has always shown albumin-like properties. This precipitate was entirely soluble in salt solution and could be almost entirely reprecipitated upon dialysis. The material thus isolated weighed 0.65 Gm. and we will compare it critically with the globulin fraction. It is to be noted that the globulin yield (2.0 Gm. obtained) is already higher than usually obtained. In this particular experiment the saline extract stood over Sunday in the ice chest instead of being worked up within 12 hours.

The dialyzed solution from which the globulin-like artifact had been removed was coagulated and the albumin fraction amounted to 4.5 Gm.

This material agreed with the albumin previously isolated from the aqueous extract.

Analysis, 221:1. On moisture- and ash-free basis; C = 52.25; H = 6.9.

The filtrate from this coagulum was concentrated to 200 cc. and salt added (20 Gm.) and upon acidification with acetic acid a small amount (0.13 Gm.) further separated. The solution was freed from salt by dialysis, evaporated to a small volume and poured into alcohol. Weight 0.45 Gm.

Analysis, 222:3; Ash = 7.71%. On ash- and moisture-free basis; C = 47.8; H = 6.95. Molisch Test ++.

The solution from which the proteins had been removed by half saturation with ammonium sulphate was now completely saturated and a heavy precipitate removed by means of the centrifuge. It was redissolved in water and upon dialyzing this solution a slight amount of a globulin-like alteration product again separated. Most of the protein dissolved, however, and it was recovered by coagulation. Weight 1.55 Gm.

Analysis, 221:2. On moisture- and ash-free basis; C = 51.1; H = 7.0.

The filtrate from 221:2 was concentrated on the steam-bath. To a test portion was added some sodium chloride and acetic acid, but no coagulation occurred. The main portion was precipitated with alcohol yielding 3.26 Gm. of material which has not been investigated.

D. Alkaline Extractions.—Residues from the glandular material which had been extracted with ether, water, and 10% salt solution, were shaken with water and several aliquots equivalent to 20 Gm. of the ether extracted gland (21.9 Gm. gland). To these suspensions enough 10% NaOH solution was added to make the concentration of alkali 0.1, 0.2, 0.3, 0.5 and 1.0 per cent respectively. The volume of extracting material is 500 cc. in each case. The extractions were repeated three times. The extracts were acidified with acetic acid, separated, dried and weighed in the usual manner. The yields were as follows: 0.1% = 10.3%;[1] 0.2% = 16.3%; 0.3% = 14.9%; 0.5% = 22.5% and 1.0% = 22.9%.

[1] Moisture = 10%; Ash- and Moisture-free basis, S = 1.13%; P = 0.63%.

It is therefore apparent that when this residue, consisting no doubt largely of connective tissue, is extracted with 1.0% sodium hydroxide, that approximately half of the extract consists of mucoid (?) material readily soluble in 0.1% alkali, while the remainder consists perhaps of protein which is liberated by the action of the stronger alkali, perhaps from chondroitin acid combination.

E. Hydrolysis of the Ovarian Residue Albumin.—It is perfectly obvious from the foregoing that the chief soluble protein in ovarian residue consists of water-soluble protein, also soluble in saline solution, not precipitated by dialysis and precipitated at half saturation with ammonium sulphate. It can be prepared phosphorus free and has a sulphur content of 1.5%. (C = 51.9, H = 7.0.)

The material was examined by the Van Slyke process: 3.0181 Gm. (162B, containing 5.46% moisture and 0.16% ash) of ash- and moisture-free protein was hydrolyzed with 20% hydrochloric acid and hydrolysis was found to be complete after 24 hours. (Amino N = 13.37, 13.38%.)

Total N = 15.28, 15.37, 15.23, 15.15; average = 15.26%.

The results may be tabulated as follows:

	Per cent of protein.	Per cent of N.
Total N	15.26	100.00
Amide N	1.19	7.80
Humin N	0.22	1.44
Cystine N	0.11	0.70
Arginine N	1.77	11.60
Histidine N	0.03	0.20
Lysine N	2.27	14.69
Mono Amino N	9.24	60.55
Non Amino N	0.47	3.08
		99.43

This protein contains 8.5% tyrosine[1] and 2.1 to 2.27 of tryptophane.[2]

F. Hydrolysis of the Acid Precipitated Protein from Ovarian Residue.—In the foregoing discussion of the preparation of proteins it will bave been observed that after the above-described albumin has been precipitated at half saturation with ammonium sulphate, there remains in the original extract a considerable amount of protein. This can be precipitated by the addition of acid. According to the current methods described by Abderhalden, half saturation with ammonium sulphate is a routine procedure for separating albumins from globulins. It is customary to designate the material more readily precipitated as a globuliin, while complete saturation is required to precipitate the albumin. In this case, however, the material precipitated at half saturation has many albumin-like properties.

Now the material precipitated by acidification of the half-saturated solution shows a composition almost identical with the albumin. (See analysis of 171 P.) However, when precipitated as a salt it does not dissolve when dialyzed against distilled water.

In order to establish whether or not this preparation differed from the albumin, it also was hydrolyzed.

The material was examined by the Van Slyke process. 3.2687 grams (171 P + 173 P containing 6.08% moisture and 0.82% ash) of ash- and moisture-free

[1] Folin and Looney, *J. Biol. Chem.*, 51, 421 (1922).

[2] May and Rose, *Ibid.*, 54, 213 (1922).

protein was hydrolyzed for 25 hours with 20% hydrochloric acid. (Amino N = 12.8%.)

	Per cent of protein.	Per cent of N.
Total N	14.76	100.00
Amide N	1.35	9.14
Humin N	0.38	2.55
Cystine N[1]	0.10	0.69
Arginine N[1]	1.70	11.52
Histidine N[1]	0.00	0.00
Lysine N[1]	2.30	15.58
Mono Amino N	8.43	57.11
Non Amino N	0.55 (0.50)	3.41.
		100.00

	Albumin.	Acid precipitated protein.
Arginine N	1.77	1.70
Total N	4.15	4.10
Amino N	2.80	2.94

These proteins are remarkably alike in their content of hexone bases. Recalculating these agreeing results they are found to have the following composition:

	Per cent of protein.
Arginine	5.5
Histidine	0.10 or less
Lysine	11.8

There is, however, a distinct difference in the mono amino acid composition of the filtrate as will be observed by noting 9.24% found in the case of the albumin, whereas the acid precipitated protein yields only 8.43% and we have rigorously determined this by comparative analysis.

By colorimetric methods we found for this protein; tyrosine = 7.1% and tryptophane = 1.88 to 2.07%.

Since little confidence can be placed in the identity of the other fractions such as mucoid, they have not been hydrolyzed.

SUMMARY.

The water- and saline-soluble proteins of ovarian residue, that is of the gland from which corpus luteum has been removed, amounting to 10 to 11% of the desiccated tissue consists largely of an albumin for which C = 52.0; H = 7.0; N = 15.3; S = 1.5%.

The structural formula may be approximated as follows: $Arginine_3$, $Lysine_7$, $Tyrosine_4$, $Tryptophane_1$, $Cystine_2$, Mono amino $acids_{56}$. M Wt. = 8500.

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NOVEMBER 10, 1925.

[1] The base fraction gave results agreeing with those found for the albumin. In the base fraction we have: