Condensation of Diketohydrindene and Succinic Anhydride.—A similar product was obtained by condensing 1.5 g. of diketohydrindene and one g. of succinic anhydride in presence of 30 cc. of conc. sulfuric acid, and warming the mixture for one hour at 50°. On pouring into water a greenish mass was obtained, which was recrystallized from nitrobenzene. The substance separates very slowly, requiring sometimes the addition of alcohol. It does not melt even at 316° but sublimes on heating, and is insoluble in alcohol, acetic acid, acetone and benzene. Its solution in aniline is greenish in color, and the substance obtained by pouring the solution into dil. hydrochloric acid did not contain any nitrogen.

Subs., 0.1245: CO_2, 0.3776; H_2O, 0.0391.
Calc. for $C_{21}H_{10}O_2.H_2O$: C, 83.1; H, 3.3. Found: C, 82.7; H, 3.5.

Our thanks are due to Principal Südmersen for the kind interest he has taken in the work.

ASSAM, INDIA.

[CONTRIBUTION FROM THE STANFORD MEDICAL SCHOOL.]

A METHOD OF TISSUE ANALYSIS: APPLIED TO THE POSTERIOR AND ANTERIOR LOBES OF CATTLE PITUITARIES.

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The cattle pituitaries used in these experiments, were obtained from a local packer. They had been collected over a period of several days and kept frozen until ready for analysis. After freeing the pituitary from bone, connective tissue, fat, etc., it was separated into anterior and posterior lobes. Exactly 10 g. of skin-free, ground material from each lobe was weighed in conical flasks and treated as described under "Method."

Method of Analysis.

The method of analysis used is based on the separation into proteins, lipins and extractives that has already been proven satisfactory.[1] The original method has been simplified, extended to new determinations, and been made available for much smaller amounts of material. The amounts of tissue usually used (10 g.) are probably the most satisfactory, but most of the determinations described can be carried out on 2 g., though with somewhat less accuracy. It will be noticed that the new methods for small quantities are applied in some cases. In others it will be seen that the usual quantitative methods are made available and accurate by decreasing the volume of solutions used to about $^1/_{10}$, avoid-

[1] W. Koch, "Methods for the Quantitative Chemical Analysis of Animal Tissue," THIS JOURNAL, 31, 1329 (1909); M. L. Koch and C. Voegtlin, "Chemical Changes in the Central Nervous System in Pellagra," *Hygienic Lab. Bull.*, 103, 51 (1916).

ing transference of material whenever possible, often by use of centrifuge tubes, and the titration of the material in these smaller volumes with 0.01 *N* instead of 0.1 *N* solutions. It is believed that in this way the usual accuracy is maintained, though only small amounts are actually estimated. Obviously greater care must be used in gradation when smaller apparatus is used. Only redistilled organic solvents were employed.

In the alcohol method used in this investigation, 10 g. of tissue was placed in a 100 cc. conical flask and redistilled alcohol added until the concentration of the alcohol in the solution was about 80%. The corked flask was then allowed to stand in a dark place for several weeks, although the time could be shortened when necessary by repeated heatings. Then on each of several successive days the mixture was brought just to the boiling point 3 or 4 times. Finally the hot alcohol was decanted through a weighed, paper-lined, perforated crucible into a weighed 75 cc. beaker. More alcohol was added to the residue in the flask, the mixture heated and decanted as before. This was repeated 4 times. The residue was then allowed to stand overnight in ether, warmed several times and decanted, after which it was transferred to a glass mortar, thoroughly ground, and again placed in a flask. The alcohol and ether extractives in the meantime were concentrated on a steam bath.

The residue in the flask was then digested 3 times with about 20 cc. of hot water, and the water extracts decanted through the same weighed crucible into a separate beaker. After concentrating to about 5 cc., enough alcohol was added to make the alcohol concentration 80%. It was heated to boiling until the protein was coagulated, then poured through the crucible, again heated to boiling and poured through crucible into the *first weighed* beaker. The residue was then heated and transferred to the crucible. 95% alcohol was heated in separate beaker and used to wash out the flask, used repeatedly for this purpose, and finally collected in the weighed beaker. By proper manipulation with a glass rod, all of the residue was now in the weighed crucible.

This crucible was suspended in a continuous extractor and extracted with 95% alcohol for 30 hours. The alcohol extract was then transferred to the weighed beaker residue. The residue in the crucible was then extracted 10 hours with chloroform, the extract transferred to the weighed beaker and evaporated carefully to dryness, and dried to constant weight in a vacuum desiccator. This gives the weight of the lipins plus the extractives. The protein residue in the crucible was then heated to constant weight in a 105° oven and kept in vacuum desiccator. The total solids equal the sum of these two weights.

About 40 cc. water was added to the beaker containing the extractives and lipins. After considerable stirring and some time, a homogeneous emul-

sion was obtained. This was transferred to a 50 cc. centrifuge tube and $^1/_2$ cc. of hydrochloric acid added with stirring. As soon as the lipins were precipitated, the material was centrifuged and then decanted carefully into a 100 cc. measuring flask (lipin flask) containing about 5 cc. of chloroform. This flask was thoroughly shaken for several minutes, allowed to settle and the watery part added to a second centrifuge tube, centrifuged, and the clear, almost colorless solution of extractives filtered into a 100 cc. measuring flask. The residue in the first centrifuge tube was washed with about 30 cc. of water containing $^1/_4$ cc. of hydrochloric acid. The centrifuged solution was placed in the 100 cc. lipin flask and shaken with the chloroform, treated just as the original solution, and finally added to the 100 cc. extractive flask. The residue in the tube was washed once more with 30 cc. of water containing $^1/_4$ cc. of hydrochloric acid and treated as before.

The material in the extractive flask was made up to 100 cc. Aliquots of this solution were used later for the various determinations.

The residue in the centrifuge tube was dissolved in a mixture of absolute alcohol and chloroform (approximately 10 to 1), and added to the lipin flask. All the apparatus used for the precipitation of the lipins was washed thoroughly with alcohol-chloroform mixture and the latter transferred to the lipin flask and diluted to the mark with absolute alcohol. If the proper amounts of alcohol, chloroform and water (not much of the last two) are present, a clear solution is obtained.

Extractives.

By evaporating 30 cc. (or another aliquot part) of the extractives to dryness in weighed crucible or dish and bringing to constant weight in a vacuum desiccator over calcium chloride, the weight of extractives was obtained. Subtracting the weight of extractives from that of lipins and extractives gave the total weight of the lipins. This same organic and inorganic residue was carefully ignited to constant weight. The ash is not a measure of the inorganic salts alone, and probably the original ions are considerably rearranged; yet it is a helpful determination. This ash was dissolved in warm dil. nitric acid, made to 10 or 25 cc. and part used for the determination of one or more of the following: sodium, potassium, calcium, magnesium, phosphorus, iron (chlorine, sulfur).

Calcium was determined by placing an aliquot part ($^1/_2$) in a centrifuge tube, making slightly alkaline to phenolsulfonphthalein with ammonia, boiling till neutral, then while hot adding slowly one cc. of a solution of 1.25% oxalic acid in 0.25 *N* hydrochloric acid. The mixture was allowed to cool and 0.5 cc. of 20% sodium acetate solution added. After standing overnight this was centrifuged, washed twice by centrifuging with 5 cc. portions of cold water and 2 cc. of 5% sulfuric acid added.

It was then heated and titrated with 0.01 N potassium permanganate solution to a faint pink color.[1]

Magnesium was determined in the filtrate from the calcium determination. This was incinerated with a small amount of sulfuric acid, then transferred with water to a centrifuge tube and precipitated as magnesium ammonium phosphate. After washing with dilute ammonium hydroxide it was dissolved in a few drops of nitric acid. Thereafter the procedure was as in the phosphorus determination given below.

Total nitrogen in the extractives was determined in a 2 cc. portion by combustion with 1 cc. sulfuric acid (and small amount of cupric sulfate) in a good-sized, hard test-tube. It was diluted to 10 cc. in same tube, made alkaline, and the ammonia aerated into 5 cc. of 0.01 N hydrochloric acid.

For **ammonia nitrogen** 10 or 20 cc. of the extractives, after being made *slightly* alkaline with sodium hydroxide, was aerated into 0.01 N hydrochloric acid.

Urea Nitrogen.—The alkaline solution from the ammonia nitrogen determination was then carefully neutralized to litmus and urease added. The mixture was then incubated one hour, made alkaline, and the ammonia aerated into 10 cc. of 0.01 N hydrochloric acid.

Amino Nitrogen.—Five cc. of extractives was evaporated to 2 cc. and the amino nitrogen determined in small amino apparatus.[2]

For **Creatinin** 5 cc. of the extractives was saturated with picric acid and made alkaline with $^1/_2$ cc. of 10% sodium hydroxide as usually stated, and made up to 10 cc. Whether the resulting color is due to creatinin only is doubtful. After 20 minutes it was compared in a colorimeter with a standard made in the same way.

Creatin and Creatinin.—Five cc. of extractives was saturated with picric acid, diluted to 50 cc., boiled for 2 hours down to 5 cc., made up to the required volume and alkalinity as for creatinin, and compared with standard in colorimeter.

Uric acid[3] was determined if any was present by taking a large aliquot part of extractives and evaporating to 5 cc. 2 cc. of silver magnesia mixture was added and the mixture centrifuged. The residue was dissolved in 2 drops of 5% potassium cyanide solution, stirred, and 0.5 cc. of water, 0.5 cc. of phosphotungstic acid reagent, and 2 cc. of 20% sodium carbonate solution added. After making up to 10 cc. it was compared in a colorimeter with standard uric acid solution treated in same way.

[1] J. O. Halverson and O. Bergeim, "Determination of Small Amounts of Calcium Particularly in Blood," *J. Biol. Chem.*, **32**, 159 (1917).

[2] D. D. Van Slyke, "The Micromethod for Gasometric Determination of Aliphatic Amino Nitrogen," *Ibid.*, **23**, 407 (1915).

[3] O. Folin and W. Denis, "On the Colorimetric Determination of Uric Acid in Urine," *Ibid.*, **14**, 95 (1913).

Total extractive sulfur was determined by evaporating 5 (or 10) cc. in a nickel crucible with not more than 0.5 cc. of a 20% solution of potassium hydroxide and sodium nitrate (4–1). (It is also safe to use the magnesia fusion method. See lipin sulfur.) The amount of alkali salts must be kept low, for they interfere with precipitation of benzidine sulfate. When dry, it was heated on electric plate or over an alcohol lamp and finally with sulfur-free or asbestos protected burner if necessary, dissolved in water, transferred to a 25 cc. beaker, made acid with hydrochloric acid, evaporated to dryness, taken up with hydrochloric acid, again evaporated to dryness. The treatment with hydrochloric acid was repeated, and the solution taken up in 2 cc. of water and transferred to a centrifuge tube. Ten cc. of benzidine solution was added and the mixture allowed to stand, after which it was centrifuged and washed 3 times with saturated benzidine sulfate solution. Finally the benzidine sulfate was titrated with 0.01 *N* sodium hydroxide solution.

For **Sulfur as sulfates,** 20 cc. was neutralized, evaporated slowly to about 2 cc., transferred to a centrifuge tube, and made slightly acid with hydrochloric acid. It was then heated in a water-bath and 0.01 *N* barium chloride solution added drop by drop. The end-point was moderately sharp, when a drop did not produce a cloudiness in the clear centrifuged liquid after some time standing. A 0.01 *N* sulfuric acid solution was used to titrate back if necessary. This determination is somewhat tedious, but is the most satisfactory one for small amounts of sulfates.

Neutral sulfur is the difference between total extractive sulfur and sulfate sulfur.

Extractive sugar (+ other copper reducing substances) was estimated by neutralizing 2 cc. of extractive solution in a test-tube. To this was added one cc. of mixed Fehling's solution. This was heated in boiling water for exactly 5 minutes, then placed in ice water for several minutes. Five cc. of 40% sulfuric acid and 5 cc. of 100% potassium iodide were slowly run in and the cold solution titrated immediately with 0.02 *N* thiosulfate solution to faint salmon pink color, with starch as indicator. It is usually best to run one or two tubes on the copper solution and one or two on known sugar solutions of the concentration expected.

Total extractive phosphorus was estimated in 2 cc. after destroying the organic material by heating with 0.5 cc. of sulfuric acid and 3 cc. of conc. nitric in a test-tube. If the sulfuric acid residue was colorless, it was transferred with water to a 15 cc. centrifuge tube, neutralized with ammonium hydroxide, and made slightly acid with nitric acid. To this, the volume of which should be about 5 cc., was added 2 cc. of 50% ammonium nitrate solution, and the mixture was heated to 70° in water, 3 cc. of ammonium molybdate solution added, and allowed to stand at 65°

for 20 minutes. By a judicious use of a rubber tipped rod and a fine spray from a wash-bottle (with occasionally a drop of alcohol) the precipitate was centrifuged satisfactorily. After decantation, it was twice washed with one per cent. ammonium nitrate solution and once with water by centrifuging. An excess of 0.1 *N* sodium hydroxide solution was added with a carefully graduated one cc. pipet (using the same one in the same way for the 0.01 *N* acid value), one drop of phenolphthalein solution added and the solution titrated immediately with 0.01 *N* hydrochloric acid.

For **Phosphates,** ammonium nitrate and ammonium molybdate solutions were added to 2 cc. of extractives in a centrifuge tube at 65°, the mixture centrifuged, washed and titrated as above.

Neutral phosphorus is the difference between total extractive phosphorus and phosphate phosphorus.

Lipins.

An aliquot part of the lipin solution (30 cc.) was evaporated to dryness in a 200 cc. round-bottomed flask and 25 cc. of water and one cc. of concentrated hydrochloric acid added. The contents were refluxed for 30 hours, after which the solution was decanted and filtered into measuring cylinder or flask. The fatty acid residue was again heated with 15 cc. of water and 0.5 cc. of hydrochloric acid for 10 hours, and filtered into same cylinder or flask. After washing the residue a few times with acidulated water, the combined solutions were made up to 40 or 50 cc.

The lipin residue was dissolved in ether or chloroform and evaporated to constant weight in weighed beaker, then dissolved in chloroform and made to 10 cc.

Lipin Residue Nitrogen and Phosphorus.—Two cc. was evaporated and digested with 2 cc. of sulfuric acid and a drop of cupric sulfate until colorless. Water was added, the solution made accurately to 10 cc., and 2 cc. used for the phosphorus determination (see extractive phosphorus above) and 8 cc. used for nitrogen (see extractive nitrogen above).

Lipin Residue Sulfur.—Another 2 cc. was evaporated in a nickel or porcelain crucible with one g. of calcined, washed, light magnesia containing about 0.01 g. of sodium carbonate. When dry it was ignited slowly over alcohol lamp. Fumes should not appear. The heat was gradually increased until the mass was colorless. It was then extracted for $^1/_2$ hour with 10 cc. of water on a water-bath, and filtered. The residue was then extracted 3 times more with 5 to 10 cc. portions of water. To the combined filtrates 0.5 cc. of conc. hydrochloric acid and 0.5 cc. of bromine water, were added, and evaporation carried to dryness. A few cc. of hydrochloric acid was then added, and again the residue was evaporated to dryness. This was repeated and the mass taken up in 3 cc. of water, transferred to a centrifuge tube with 10 cc. of benzidine hydrochloride

solution, and after 15 minutes, centrifuged, washed 3 times with water saturated with benzidine sulfate, and 5 cc. of water and 1 drop of phenolphthalein added and the solution titrated hot with 0.01 *N* potassium hydroxide solution.

Amino Nitrogen.—Five cc. of the lipin hydrolysis filtrate was neutralized and evaporated to 2 cc. and the amino nitrogen determined in an amino apparatus.

The total filtrate nitrogen, in another 5 cc. portion, was determined by the usual combustion with one cc. of sulfuric acid and a drop of cupric sulfate solution, and aeration into 10 cc. of 0.01 *N* hydrochloric acid. Phosphorus can be determined in aliquot part following digestion, if desired.

Volatile Amine Nitrogen.—Ten cc. of the filtrate was made slightly alkaline in a test-tube and the ammonia aerated into 5 cc. of 0.01 *N* hydrochloric acid, and the nitrogen calculated as ammonia.

Cholin Nitrogen.—The contents of this test-tube was neutralized and washed into a small flask, evaporated to dryness, and covered with absolute alcohol. The residue was pulverized and allowed to stand till the extraction was complete. It was then filtered and washed several times with hot absolute alcohol; an excess of alcohol solution of chloroplatinic acid was added and the mixture allowed to stand overnight, then centrifuged, and washed with alcohol. The residue was dissolved in water, transferred to a test-tube and the nitrogen estimated in the usual way. The results were expressed as choline nitrogen, although there may be other forms present.

Iodine Absorbed.—Five cc. of the filtrate was evaporated to dryness in a 25 cc. flask, and 2 cc. of chloroform or glacial acetic acid added. Exactly 1 cc. mercury chloride and iodine (Hübl's) solution carefully mixed into this, the mixture shaken and allowed to stand overnight in an ice-box. One cc. of reagent was treated in exactly the same way as a blank. Each was then titrated with 0.01 *N* thiosulfate solution. From the data obtained the iodine absorbed by the lipin hydrolysis filtrate was calculated.

Sulfur.—After evaporating 5 cc. of the filtrate to dryness it was digested with 0.5 cc. potassium hydroxide-sodium nitrate fusion solution (or one can use the magnesia fusion, as described under Lipin Residue Sulfur). The organic matter was destroyed and the sulfur estimated as benzidine sulfate as described under extractive sulfur.

Sugar was estimated in 2 cc. of the filtrate as described under extractives; or, after neutralization, by adding 2 cc. of saturated picric acid solution, 2 cc. of 20% sodium carbonate solution, and heating for one hour in boiling water. A known sugar solution of about the same strength was treated in the same way, and the two compared in a colorimeter.

20 cc. of the original lipin solution was evaporated to dryness in a nickel crucible, 5 cc. of potassium hydroxide-sodium nitrate solution added, and the mixture evaporated to dryness on a water bath. It was then taken up in water and again evaporated to dryness, after which it was placed on an electric stove or over an alcohol lamp and heated until white. It was sometimes necessary to take up in water, evaporate and heat a second time. Finally it was taken up in water and made up accurately to 10 cc. or any other convenient volume. 0.5 or one cc. of the above 10 cc. was made acid with nitric acid, and *phosphorus* determined as previously described. This was a check on the acid fusion described below.

Total Sulfur.—Two cc. of this solution was placed in an evaporating dish, an excess of hydrochloric acid added and evaporated to dryness. The residue was dissolved in hydrochloric acid and evaporated to dryness. After repeating this treatment twice, the residue was transferred to a centrifuge tube with a small amount of water and sulfur determined by benzidine method described above.

As a check on this method, another 2 cc. portion was treated with hydrochloric acid, as described, then 5 cc. of water was added, and the solution brought to boiling. 2 cc. of barium chromate solution (4 g. pure barium chromate in one liter of *N* hydrochloric acid) was added and the solution boiled 2–5 minutes, a piece of litmus paper was introduced, then calcium carbonate in excess, the mixture shaken, allowed to stand a few minutes, filtered and washed until the volume of filtrate was 10 cc. To the filtrate was added 0.5 cc. of hydrochloric acid and 0.2 g. of potassium iodide. Titrate with 0.01 *N* thiosulfate solution. It was necessary to check the reagents and it was found best to run a known amount of 0.01 *N* sulfuric acid as a control experiment. All conditions must be kept uniform. Whenever the amount of salts is appreciable, this method is better than the benzidine one (but the results are uncertain in the extractive fraction, for there may be present substances other than sulfates that bring about a liberation of iodine).

Calcium and Magnesium.—Using a 5 cc. portion calcium was estimated as described under extractives. The filtrate from calcium was used for magnesium if present in appreciable quantities.

Cholesterol[1] was estimated in the following way: A small amount of calcium carbonate and anhydrous sodium sulfate was added to a one cc. portion of the lipin solution and the mixture evaporated to dryness in a warm but not hot place. When dry, the residue was extracted several times with 2 cc. portions of warm chloroform. These were filtered into a 10 cc. graduate, and made to 10 cc. Two cc. of acetic anhydride and 0.2 cc. of sulfuric acid was added. The mixture was then shaken and

[1] W. R. Bloor, "The Determination of Cholesterol in Blood," *J. Biol. Chem.*, **29**, 437 (1917).

allowed to stand $^1/_2$ hour. One or two mg. of cholesterol, or the amount expected, in chloroform, was treated in same way at the same time. The color of standard was compared with the unknown in a colorimeter.

The **iodine number** was found for 2 cc. of the lipin solution by evaporation to dryness, dissolving in 3 cc. chloroform, adding one cc. mercuric chloride iodine solution, allowing to stand in the cold overnight, and titrating with 0.01 *N* thiosulfate solution.

Lipin Nitrogen.—The organic matter in the residue from 2 cc. of lipin solution was destroyed with one cc. of sulfuric acid and one drop of cupric sulfate solution in large test-tube. The *lipin nitrogen* was determined in the whole sample, after making it alkaline, by aerating the ammonia into 0.01 *N* hydrochloric acid, or the solution was made up to 10 cc., and 8 cc. used for nitrogen and 2 cc. for phosphorus as described above.

Lipin phosphorus was best determined by digesting one cc. of lipin solution with 0.3 cc. of sulfuric acid and enough conc. nitric acid to make the solution colorless when evaporated until white fumes appeared. When the solution was boiled very slowly so that the nitric acid was not rapidly removed, the organic matter was more quickly destroyed. The residue was taken up in 3 cc. of water, most of the acid neutralized with ammonium hydroxide and the phosphorus determined as described above.

Proteins.

About 0.20 g. of protein material was exactly weighed out in a nickel crucible. Because of the hygroscopic nature of proteins this had to be done very quickly, or better, the weight taken was determined by difference in weighing bottle weights. Water and 5 cc. of the potassium hydroxide and sodium nitrate fusion mixture were added and fusion was completed as described above. The mixture was finally made to 10 cc.

Phosphorus in one cc. was determined as described above.

2 cc. was used for **sulfur** determination by the benzidine method as given under lipin sulfur.

In another 2 cc. sulfur was determined by the chromate method described under lipin sulfur.

Calcium was determined in 5 cc. as described for calcium in extractives.

Chlorine was estimated in one cc. by making it slightly acid with nitric acid in a centrifuge tube adding one cc. of 0.1 *N* silver nitrate solution, centrifuging and titrating with 0.02 *N* potassium thiocyanate solution, using ferric ammonium sulfate as an indicator. If desired, the solution in tube can be decanted into flask, the residue washed twice, and the contents of the flask titrated for excess silver.

Nitrogen.—Using about 25 mg. of protein in a test-tube, organic matter was destroyed with sulfuric acid and cupric sulfate as usual. The residue

was made alkaline and the ammonia aerated into 0.1 *N* hydrochloric acid, or made up to 10 cc., and 6 cc. used for phosphorus determination and 4 cc. for nitrogen.

0.25 g. of protein was hydrolyzed in a 300 cc. round-bottomed flask with 20 cc. of 10% sulfuric acid for 25 hours[1]. The completely hydrolyzed solution was made up to 40 cc. and the *total protein nitrogen* determined in two 1 cc. portions in usual way.

The remainder of this solution was filtered through a dry filter into a 50 cc. centrifuge tube, and the nitrogen determined in two more 1 cc. portions. The difference between the two sets was the *insoluble humin nitrogen.* The measuring cylinder (and filter paper) was washed. $^{36}/_{40}$ of the original sample was then in the centrifuge tube. 3 g. of phosphotungstic acid in saturated solution was added, the solution stirred, and let stand overnight. It was then centrifuged, decanted into a beaker, washed twice by centrifuging with 25 cc. portions of 2.5% phosphotungstic acid 5% sulfuric acid solution.

The phosphotungstic precipitate was washed into a large test-tube with about 15 cc. of water, made slightly but distinctly alkaline and the ammonia aerated into 5 cc. of 0.1 *N* hydrochloric acid. This is the *ammonia nitrogen.*

Barium chloride solution was added in slight excess and the mixture filtered into a 50 cc. measuring flask, washed and made up to volume.

Total nitrogen in 2 cc. and *amino nitrogen* in another 2 cc. were determined, allowing the gas to generate for 30 minutes for complete formation of nitrogen from lysine.

To 20 cc. in small flask was added 5 g. of sodium hydroxide, and the flask was placed in series as for an ammonia determination. It was then heated for 6 hours just to boiling while passing in a very slow stream of air, and thus aerated into 5 cc. 0.1 *N* hydrochloric acid. During the last hour it was boiled and aerated more rapidly. The result was calculated as *arginin nitrogen.*

The phosphotungstic filtrate was treated with a slight excess of finely ground barium hydroxide, filtered and washed thoroughly. This solution should always be tested for ammonia by aeration, because some phosphotungstic acids do not precipitate all of it. The slightly alkaline solution was made slightly acid with sulfuric acid and evaporated to about 25 cc. filtered and washed into a 50 cc. centrifuge tube. 0.3 *N* barium hydroxide solution and 0.3 *N* sulfuric acid were alternately added until approximately no precipitate formed on adding a drop of either. After centrifuging, a drop of either reagent very soon formed a precipitate. The

[1] D. D. Van Slyke, "Improvement in the Method for Analysis of Proteins by Determination of the Chemical Groups Characteristic of the Different Amino Acids," *J. Biol. Chem.*, **22**, 281 (1915).

removal of the barium and sulfate ions was continued alternately, adding and testing with 0.1 *N* barium hydroxide solution and 0.1 *N* sulfuric acid. When this point was reached, one or two drops of either reagent produced a distinct cloudiness in a few minutes. Some patience was required to get this point correctly. The solution was then filtered into a 50 cc. measuring flask, washed, made up to volume, a few drops of toluene added, and the whole shaken.

A 2 cc. portion of this filtrate was used for *amino nitrogen* determination.

The *total nitrogen* in this fraction was determined by means of a sulfuric acid combustion, as described above.

A 5 cc. portion was used for *amino acid nitrogen* by the copper method.

Another 5 cc. was used for the formol titration.

Though not definitely proven to be correct, a few data indicate that the aspartic and glutamic acids can be estimated by titrating 10 cc. to a faint pink with phenolphthalein, running a blank with an alanin solution of similar concentration to that of amino acids present. Or one can precipitate them as calcium salts and estimate the nitrogen. It should be possible to estimate tyrosin in this fraction with the phosphotungstic molybdate reagent by comparison with a tyrosin standard.

The soluble humin **nitrogen** was calculated by adding the total nitrogen found in the phosphotungstic precipitate and filtrate, including the ammonia + insoluble humin nitrogen, and subtracting this from the total nitrogen before precipitation of the basic nitrogen. Obviously errors in previous determination collect in this estimation.

General Discussion.

There are several questions of physiological importance upon which an analysis of the pituitary may shed light. The glandular anterior lobe has its origin in the alimentary canal, while the nervous posterior lobe originates from the same structure as the nervous system. It is natural, then, to ask to what extent have the differences due to independent origin been preserved; and how far have these original differences been changed by juxtaposition and coöperation in function? It might be expected that the posterior lobe would be more nearly like the nervous system than like the ailmentary canal in amounts of the various chemical substances present and that the anterior lobe would resemble more closely the alimentary canal.

It is necessary to add that because of the early union of the two portions, it is probable that the material from which they were formed had not yet assumed many chemical differences. In this case the peculiarities in the chemical nature of the two lobes would be of a common development. Even then, however, there must have been some potential or actual differences at the time of union that would persist in making the

anterior lobe more like the alimentary canal tissue and the posterior like nervous tissue.

It is pertinent to ask, too, what the data show in regard to the origin or presence of the active principles of the pituitary. There should be indications of such active compounds if present in appreciable quantities. Also, what tissues or what kinds of tissues do the lobes of the hypophysis most nearly resemble? There is some evidence, for example, that the anterior lobe is similar in function to the thyroid; therefore it may be similar in composition. There should also be comparison with other glands, especially those of internal secretion.

Discussion of Results.

Water and Solids.—Judging from the fact that the posterior lobe has 2.4% less solids than the anterior, it is physiologically the more active and younger.[1] The water content of the hypophysis is about half way between that of white and gray matter of the brain. It is about 8% higher than that for either muscle or liver and very much higher than for the supporting tissues. It resembles other secretory glands most closely in water content as well as in many other respects.

Proteins.—The fresh anterior lobe consists of 4% more proteins than the posterior lobe. Both lobes contain considerably more than do white or gray matter of the brain, more than muscle, but about the same as liver. The spleen and thyroid contain amounts of protein similar to the hypophysis.

If it is permissible to judge of the amount of nucleoprotein in the hypophysis by the phosphorus present in the protein fraction, it would seem that the percentage of total protein found as nucleoprotein is about the same in the two lobes. It is safe to say that there is about 3 times as much nucleoprotein in the pituitary as in any of the divisions of the nervous system, many times more than in muscle, but probably less than in liver and spleen.

In comparing the distribution of the protein nitrogen in the two lobes, the most apparent fact is the similarity between them. Humin, ammonia, basic, and the monoamino forms are about equally distributed in the two lobes. However, the larger amount of histidin and the smaller amount of cystin in the anterior lobe are probably significant differences. Though the proteins of the lobes of the pituitary have not been isolated and investigated separately, the data above would indicate that the proteins of the two lobes are qualitatively very similar.

Lipins.—It will be noticed that the total lipins are 0.84% higher in the posterior lobe than in the anterior, reversing the protein percentages in the two lobes. Though the white matter of the nervous system has 5

[1] F. Fenger, "Composition and Physiological Activity of the Pituitary Body," *J. Biol. Chem.*, 25, 417 (1916).

TABLE I.

Constituents in Percentages of Fresh Tissue and of Total Solids.

	Anterior lobe.		Posterior lobe.	
	Fresh tissue.	Total solids.	Fresh tissue.	Total solids.
Water	77.23		79.68	
Solids	22.77		20.32	
Proteins	17.66	77.53	13.46	66.22
Protein phosphorus	0.116	0.509	0.097	0.477
Protein sulfur	0.178	0.782	0.205	1.01
Protein calcium	0.017	0.074	0.032	0.157
Protein nitrogen	2.390	10.49	1.68	8.27
Protein humin nitrogen	0.487	2.15	0.256	1.26
Protein ammonia nitrogen	0.117	0.51	0.073	0.359
Protein basic nitrogen	0.653	2.87	0.513	2.52
Protein arginin nitrogen	0.373	1.64	0.321	1.59
Protein histidin nitrogen	0.104	0.462	0.027	0.132
Protein lysin nitrogen	0.148	0.651	0.075	0.367
Protein cystin nitrogen	0.078	0.34	0.090	0.443
Protein monoamino nitrogen	1.133	4.97	0.841	4.14
Protein non-amino nitrogen	0.285	1.25	0.190	0.935
Protein α-amino nitrogen	0.848	3.73	0.651	3.20
Lipins	3.16	13.87	4.00	19.68
Lipin phosphorus	0.095	0.418	0.12	0.59
Lipin sulfur	0.0012	0.0053	0.0016	0.0079
Cholesterol	0.375	1.65	0.430	2.12
Iodine absorbed	1.94	8.52	2.63	12.94
Lipin residue	2.02	8.87	2.58	12.69
Lipin nitrogen	0.054	0.237	0.077	0.379
Lipin residue nitrogen	0.011	0.048	0.013	0.064
Lipin filtrate nitrogen	0.043	0.189	0.064	0.315
Lipin amino nitrogen	0.025	0.110	0.037	0.181
Lipin choline nitrogen	0.016	0.070	0.021	0.103
Lipin sugar	0.13	0.57	0.15	0.74
Extractives	1.95	8.56	2.87	14.12
Organic extractives	1.56	6.85	2.44	12.00
Inorganic extractives	0.39	1.71	0.43	2.12
Extractive, sulfur	0.003	0.013	0.005	0.025
Extractive, calcium	0.002	0.009	0.008	0.039
Extractive, phosphorus	0.056	0.246	0.068	0.335
Extractive (phosphate) phosphorus	0.036	0.158	0.028	0.138
Extractive neutral phosphorus	0.020	0.088	0.040	0.197
Extractive sugar	0.342	1.50	0.225	1.107
Extractive nitrogen	0.085	0.374	0.168	0.827
Extractive amino nitrogen	0.053	0.233	0.093	0.458
Extractive ammonia nitrogen	0.003	0.013	0.002	0.010
Extractive urea nitrogen	0.007	0.031	0.004	0.020
Extractive creatin + creatinin nitrogen	0.012	0.053	0.009	0.044

TABLE II.

Percentage of Lipins in Fresh Tissue.

	Anterior lobe.	Posterior lobe.
Total lipins	3.16	4.00
Cholesterol	0.375	0.430
Phosphatids	2.45	3.09
Cephalin	1.47	2.16
Lecithin	0.84	1.11
Cerebrosides	0.596 (sugar) 0.55 (res. nitrogen)	0.688 (sugar) 0.65 (res. nitrogen)
Sulfatids	0.06	0.08

TABLE III.

Distribution of Nitrogen, Phosphorus and Sulfur.

	Anterior lobe.			Posterior lobe.		
	% total nitrogen.	% total phosphorus.	% total sulfur.	% total nitrogen.	% total phosphorus.	% total sulfur.
Protein	94.5	43.4	97.7	87.3	34.0	96.9
Lipin	2.13	35.6	0.7	4.0	42.1	0.8
Extractive	3.36	21.0	1.6	8.7	23.9	2.4

times as much lipins as the posterior lobe (20% as against 4%), the gray matter has about the same percentage amount as the pituitary. The percentage of total solids present as lipins is similar to, but less than that in the nervous system, before medullation has occurred. There is considerable similarity between fetal brain and adult pituitary in the percentage of total solids present as lipins and as proteins. Muscle has about $^1/_4$ as much lipins, most of the glandular tissues have less.[1] The liver, however, contains nearly as much; the thyroid somewhat more.

About the same portion of the total lipins in the two lobes is in the form of *cholesterol*. Both lobes contain less cholesterol than any part of the brain, less even than in very young fetal brains, but contain more cholesterol than do other organs.

The **phosphatids** are calculated from the lipin phosphorus on the assumption that the phosphatids contain 3.88% of phosphorus. This group is about equally distributed between the two lobes. The phosphatids are probably present in amounts about equal to those in gray matter of brain, somewhat greater than in liver, much greater than in muscle, and similar to that in thyroid and spleen.

In the table the lipin amino nitrogen is calculated to *theoretical cephalin*, assuming that it has 1.7% amino nitrogen. However, it is probable that there are small amounts of other lipins that contain amino nitrogen. Cephalin is thus the principal lipin in both lobes of the pituitary, as it is in all divisions of the brain.

Theoretical lecithin is calculated from the choline nitrogen, assuming thar 1.9% (all) its nitrogen is in this form. There is about $^3/_5$ as much

[1] F. Fenger, "Phosphatids in the Ductless Glands," *J. Biol. Chem.*, 27, 303 (1916).

lecithin as cephalin in each lobe. These proportions are similar to those in the brain.

Some nitrogen in filtrate is not amino or choline nitrogen. This may be one of the constituents of tethelin, or possibly of some phosphatids. This may be concerned in the formation of the active principle of the posterior lobe.

The *cerebrosides* can be calculated from the residue nitrogen (cerebrosides = 2.00% N). By this estimation the posterior lobe has 0.65% and the anterior lobe 0.55% cerebrosides. It is probably more accurate to calculate from the sugar formed on hydrolysis assuming that the cerebrosides contain 21.8% sugar. In this way the cerebrosides equal 0.69% in the posterior lobe and 0.60% in the anterior. This is remarkably similar to the amounts found in gray matter, about $^1/_{10}$ that of white matter, but much greater than in most tissues. Pus cells have about the same quantity as the pituitary. The glands of internal secretion may contain large amounts of cerebrosides; the data are meager.

The amount of *sulfatids* is very small, much smaller than in the fetal nervous system. In this respect it is similar to the non-nervous parts of the organism.

Extractives.—If the amount of extractives is a measure of the activity of a tissue, both lobes are among the more active tissues. The posterior lobe has considerably more extractives than the anterior. The posterior has about as much as young nervous tissue, and more than adult nervous tissue. The anterior lobe has approximately the same amount as the muscles, though the amounts of the various forms of extractives differ greatly.

The amount of *inorganic material* in the two lobes differs but slightly, while the amount of *organic material* is almost 1% higher in the posterior. One would expect the secreting portion to be higher in all extractives unless some special material of organic nature is being elaborated. In both lobes the amount of the inorganic extractives is considerably less than in the brain or muscles and most other organs.

There is more *sulfur* and more *calcium* in the posterior lobe. The *phosphorus* as *phosphate* is higher in the anterior lobe, but the *neutral phosphorus* is considerably greater in the posterior. The amount of sugar or other reducing substances in the anterior lobe is greater. The amount of nitrogen is about twice as high in the posterior lobe. The amount of nitrogen in the form of *urea*, *ammonia*, *creatin* and *creatinin* is slightly greater in the anterior lobe.

Probably the most significant data on extractives is the comparatively large amount of nitrogen in the posterior lobe as *amino nitrogen* and *undetermined nitrogen*. These are the two forms that are probably present in the pressor principle of the hypophysis.

It is evident from Table III that the distribution of nitrogen, phosphorus and sulfur is very much the same in the two lobes.

Conclusions and Summary.

1. A method of tissue analysis using small quantities is described. Two to 10 g. of tissue is used for complete analysis. The compounds are separated into 3 groups, proteins, lipins, and extractives. Many determinations are made on aliquot parts of each of these groups.

2. The anterior lobe has 2.4% more solids, 4% more protein, 0.9% less lipins, and 0.9% less extractives than the posterior lobe.

3. The distribution of the various elements and compounds in the protein fraction indicates a close similarity in the nature of the proteins of the two lobes.

4. Except for the small amount of sulfatids in the hypophysis the distribution and amounts of the various lipins are similar to those of gray matter of the cortex. Because of lack of quantitative data it cannot be stated whether the particular pituitary lipins do or do not closely resemble those of the other glands of internal secretion (thyroid in particular).

5. The extractives decidedly predominate in the posterior lobes. In attempting to see in what substances this predominance consists, non-phosphate phosphorus, amino nitrogen and undetermined nitrogen are found to be conspicuous. The two latter may be present in the pressor compound.

6. The chemical composition of the hypophysis resembles the gray matter of the brain or young nervous tissue in most respects. There are many points of resemblance to the thyroid and spleen, quite a few to the liver, but practically none to the connective tissues, muscle, or white matter of the brain. There is no part of this data that would indicate that the pituitary could not have a vicarious relationship to the thyroid.

7. In those respects in which the two lobes differ, the posterior is more like gray brain substance than the anterior. Both lobes resemble each other more closely than either resembles any other tissue. It cannot be stated that the pituitary is more like unmedullated nervous tissue than it is like undifferentiated pus cells. It can be assumed as an hypothesis that the partly differentiated embryological material, after union of the two parts, develops together, resulting in a similarity of composition.

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