21 complexes of Tschugaeff mentioned above *give 135 different complexes which seem to be in harmony with this theory of biuret reaction.* This list is by no means complete.

Having thus shown what seems fairly conclusively, that the red color of these copper complexes is due to the presence of nitrogen groups alone, the next step is to find what the other details of these complexes are, and how the complexes are formed. Since experimental evidence on all points is not available, and on some points evidence is wholly lacking, our discussion on these questions, as well as our reply to recent criticism will be reserved for a subsequent paper.

V. Summary.

1. The absorption in the visible spectrum, of copper complexes of amino acids, peptides and proteins was studied quantitatively, in neutral and alkaline solutions.

2. From the nature and the amount of absorption, considerable support was found for the theory of biuret reaction proposed by Kober and Sugiura.

3. The biuret reactions with proteins seems to be nothing more than ring formations of the copper with the nitrogen groups.

HASTINGS-ON-HUDSON, N. Y.

[CONTRIBUTION FROM COLUMBIA UNIVERSITY AND THE HARRIMAN RESEARCH NO. 258.]

THE PHOSPHORIC ACID IN STARCH.

BY JOHN H. NORTHROP AND J. M. NELSON.

Received November 20, 1915.

Up to the present time no definite evidence has been brought forth as to whether the phosphoric acid present in starch is in chemical combination or not. Ford,[1] Fernbach,[2] Fernbach and Wolff,[3] Malfitano,[4] Malfitano and Moschkoff,[5] Fouard,[6] Thomas,[7] Grewzewska[8] and others, employing a variety of methods such as precipitation with alcohol, acetone, freezing or dialysis, etc., all were unable to completely free the starch from its accompanying phosphorus.

Samec,[9] seems to have shown quite conclusively that the starch granules are not homogeneous and that the phosphorus is associated with the exterior of the grains (amylopectin). He further states that the phosphoric acid and the amylopectin are chemically combined in the form of an amylophosphoric acid, since the only way to account for the decrease

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[1] *J. Soc. Chem. Ind.*, 23, 414 (1904).

[2] *Compt. rend.*, 138, 428 (1904); 155, 617 (1912).

[3] *Ibid.*, 140, 1403 (1905).

[4] *Ibid.*, 143, 400 (1906).

[5] *Ibid.*, 150, 711 (1910).

[6] *Ibid.*, 144, 501 (1907); 146, 285 (1908).

[7] *Biochem. Bull.*, 3, 407 (1914).

[8] *Compt. rend.*, 152, 785 (1911).

[9] *Kolloidchem. Beihefte*, 3, 123 (1911); 4, 133 (1912); 5, 141 (1913); 6, 23 (1914).

in viscosity and cataphoresis, and increase in solubility, osmotic pressure and activity, when starch solutions are heated or allowed to stand is by the hydrolysis, with the liberation of free phosphoric acid from a compound of this type. Samec measured what he considered to be the hydrolysis of the amylophosphoric acid, by the increase in the conductivity of the solution. Results obtained independently in this laboratory, however, show that little or no free phosphoric acid is liberated under these conditions and that, therefore, the change in properties of a starch solution under these conditions can not be attributed to the hydrolysis of such an amylophosphoric acid. It was found, on the contrary, that several hours' heating with 10% acid were necessary for the liberation of all the phosphoric as free phosphoric acid. This is in keeping with the behavior of other phosphoric acid esters studied by Fisher,[1] Anderson,[2] Plimmer,[3] and Levene and Jacobs.[4]

The results of the present investigation show:

First. The phosphorus is chemically combined in the starch grains and cannot be removed by extraction with dilute acid, either in the form of free phosphoric acid or in combination. This shows that its presence cannot be due to contamination with other material present in the potatoes from which the starch used in this investigation was obtained.

Second. A method has been found for the isolation, from partially hydrolyzed starch, of a compound of relatively high phosphorus content, of definite composition and containing a carbohydrate.

Third. This compouud differs from Samec's hypothetical amylophosphoric acid in that it is more stable towards hydrolysis.

Fourth. The possibility that the compound is derived from proteins in the starch is shown to be very remote, in view of the small amount of nitrogen present in the original starch and of the fact that acetyl derivatives were obtained that contained phosphorus but no nitrogen.

Experimental.

Determination of Phosphorus.—The sample was decomposed by the Neumann method and the phosphorus precipitated as ammonium phosphomolybdate[5] and determined by titration with alkali,[6] formaldehyde being added to remove the ammonia.[7] The starch used in this investigation was potato flour and was found to contain 0.06% phosphorus by this method.

No phosphate could be extracted by dilute acid, while a known amount

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[1] *Ber.*, **47**, 3193 (1914).

[2] *J. Biol. Chem.*, **20**, 487 (1915).

[3] *Biochem. J.*, **7**, 43 (1913).

[4] *Ber.*, **41**, 1905 (1908).

[5] Hibbard, *J. Ind. Eng. Chem.*, **5**, 998 (1913).

[6] Falk and Sugiura, THIS JOURNAL, **37**, 1507 (1905).

[7] Bang, *Biochem. Z.*, **32**, 443 (1911).

of phosphate added to the starch before extraction could be completely recovered, showing that the phosphorus is not present as an adsorbed impurity.

Ten grams of starch were stirred twenty-four hours with 500 cc. of 2% hydrochloric acid, filtered, the filtrate evaporated to 150 cc. and the phosphorus precipitated with molybdate as usual. The amount of precipitate was too small to determine. The experiment was repeated with the addition of 0.0064 g. of phosphorus as secondary potassium phosphate to the solution before extraction and resulted in the recovery of 0.00638 g. of phosphorus.

Determination of Nitrogen.—Various figures are given in the literature for the percentage of nitrogen present in potato starch. König[1] gives 0.1%; Fernbach[2] found 0.018–0.038%; and Samec[3] reports a trace. Analysis of 5 g. of the starch used in this investigation gave negative results by the Kjeldahl method. The Dumas method, using a 1 g. sample also gave negative results. A slight coloration was obtained in the nitroprusside test, which was found to be less than that obtained from a mixture of crystallized egg albumin and cane sugar containing 0.1% nitrogen.

Isolation of a Compound of Higher Phosphorus Content.—Born and Nelson[4] have shown that the phosphorus bearing polysaccharide or invertase undergoes acetolysis in such a way as to increase the percentage of phosphorus. Acetylation of starch, however, led only to the isolation of products containing 0.06–0.13% phosphorus. Since no products containing a higher percentage of phosphorus could be isolated by this method, it was abandoned. The acetyl derivatives were free from nitrogen, so that it seems improbable that the phosphorus was of protein origin.

Acid Hydrolysis.—It was found, however, that starch could be partially hydrolyzed by acid without splitting off more than a small percentage of the total phosphorus and that compounds of relatively high phosphorus content could be isolated from this partially hydrolyzed starch solution.

Ten grams of starch were dissolved in 100 cc. of 10% hydrochloric acid and the solution heated on the water bath until no precipitate was obtained on adding a drop of the solution to alcohol. The solution was then filtered from the precipitate of retrograded starch which always formed and the amount of free phosphoric acid determined in the filtrate by precipitation first with magnesia mixture and then with molybdate. The experiment was then repeated with the addition of a known amount of a

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[1] *Nahrungs und Genussmittel,* 3rd ed., I, 626.

[2] *Loc. cit.*

[3] *Loc. cit.*

[4] THIS JOURNAL, 36, 393 (1913).

solution of secondary potassium phosphate to the solution before hydrolysis. The results obtained by difference in this way were the same as those obtained directly, showing that this phosphorus was not retained by adsorption. Only 3-5 % of the total phosphorus could be found in the solution as free phosphoric acid when the starch was hydrolyzed under these conditions. The results of four such determinations are given in Table I.

TABLE I.

Grams starch.	10.	10.	10.	10.
Grams P found	0.00023	0.00024	0.00662	0.00664
Grams P added	...	...	0.0064	0.0064
P from starch	0.00023	0.00024	0.00022	0.00024
Per cent. P hydrolyzed	3.83	4.00	3.66	4.00

These results render it improbable that the increase in conductivity noticed by Samec, when starch solutions were heated or allowed to stand, was due to the liberation of free phosphoric acid.

Long-continued hydrolysis with acid resulted in the liberation of all the phosphorus as free phosphoric acid. Thus it will be noticed that the starch compound differs from phytin in this respect, since Anderson and others have shown that phytin is completely hydrolyzed only by heating with strong acid under pressure.[1]

Ten grams of starch were dissolved in 100 cc. of 10% hydrochloric acid and heated on the water bath with a reflux condenser for fourteen hours. Found, by precipitation as magnesia ammonium phosphate and then as molybdate, 0.0059 g. phosphorus, equivalent to 98.3%.

A compound containing the combined phosphorus could be precipitated from the solution by neutralizing the acid with barium hydroxide and adding two volumes of alcohol. This precipitate, after removal of the barium with sulfuric acid and subsequent precipitation with alcohol, contained 3-5% phosphorus and was later shown to be an organic phosphoric acid. In order that the material could be obtained in sufficient quantities to work with, it was necessary to determine the conditions for its isolation so that it could be carried out on a large scale.

Conditions for Hydrolysis.—As it was necessary to precipitate with alcohol, attempts were made to hydrolyze the starch in as concentrated a solution as possible. A 25% starch solution was found to be the maximum concentration that could be used, since a higher concentration gave too thick a paste. Hydrolysis with strong acid was found to be the best, since dilute acid favors the formation of retrograded starch, a substance that is exceedingly troublesome to filter.

When working with small quantities, it was found possible to evaporate the solution after hydrolysis and subsequent neutralization with ammonia, but when large amounts were used a caramel-like substance was

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[1] Anderson, *J. Biol. Chem.*, **20**, 487 (1915); Plimmer, *Biochem. J.*, **7**, 43 (1913).

formed that was insoluble in alcohol.[1] The formation of this insoluble substance was obviated by allowing hydrolysis and evaporation to take place together. The following conditions were found to be the most satisfactory for this purpose:

Ten kilograms of starch were suspended in forty liters of water and two liters of concentrated hydrochloric acid added. The mixture was stirred and steam under fifty pounds' pressure led in until the stiff paste which first formed had completely liquefied. The solution was then transferred to large evaporating dishes and heated on the water bath at 65° until no precipitate was formed on the addition of a few drops of the solution to alcohol. It was then neutralized by solid barium hydroxide and the solution filtered from the precipitate of retrograded starch, barium phosphate, and barium carbonate, through large fluted papers. The solution, usually twenty to twenty-five liters in volume, was precipitated with two volumes 85% alcohol in large precipitation jars and allowed to settle overnight. As much of the supernatant liquid as possible was then siphoned off and the remainder filtered with suction. About 60 g. of a white hygroscopic substance were obtained in this way from each ten kilograms of starch.

Sixty kilograms of starch were treated by this method, yielding about 300 g. of the barium precipitate. The product was suspended in water and decomposed by a slight excess of sulfuric acid, the solution filtered and the residue extracted three times with water and filtered with suction. The original filtrate and washings were combined and the excess acid removed by long stirring with lead carbonate; the lead carbonate and sulfate filtered off, and the solution made slightly alkaline with ammonia and saturated with hydrogen sulfide to remove the lead. The filtrate from the lead sulfide was evaporated under 25 mm. pressure to a thin syrup and precipitated by the addition of eight liters of glacial acetic acid. By pouring the acid carefully down the side of the precipitation jar and then stirring slowly, a granular precipitate was obtained that could be easily filtered. The precipitate was washed with absolute alcohol and dried in a vacuum desiccator, first over sulfuric acid and then over potassium hydroxide. Yield, 110 g., marked "Acetic acid precipitate." The acetic acid filtrate after evaporation under diminished pressure and subsequent precipitation with alcohol yielded 75 g. of a slightly

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[1] Six hundred grams of this substance were obtained in this way from ten kilograms of starch. It contained 0.24% phosphorus and yielded acetyl derivatives containing 0.06–1.59% phosphorus. They could not be sufficiently purified to warrant further investigation. Acid hydrolysis led to the isolation of a substance containing 3.9% phosphorus and similar in properties to those obtained direct from starch. These results suggest the possibility of the formation of phosphorus-bearing polysaccharides from simpler compounds under these conditions.

yellowish powder that contained 3.4% phosphorus. This substance has not been investigated farther.

Acetic Acid Precipitate.—The crude substance when dry was obtained as a dull white, very hygroscopic powder. It was soluble in water, from which solution it could be precipitated by the addition of alcohol, acetone or acetic acid. Upon analysis it gave 4.9% phosphorus, none of which was present as inorganic phosphates. Qualitative analysis of the ash showed the presence of small quantities of magnesia, sulfates, chlorides and silica. It contained no nitrogen and did not reduce Fehling's solution.

Acyl Derivatives.—Owing to the insolubility of the substance in organic solvents, purification was attempted by means of acylation, in the hope that in this way products of definite chemical constitution could be isolated. Acetylation, however, led to the formation of uncrystallizable syrups. This difficulty was avoided by benzoylating the compound by means of the Schotten-Baumann reaction, but the products were colloidal and could not be crystallized, so that purification could only be accomplished by precipitation from chloroform or acetone solution by means of ligroin, ether, water, etc. Two amorphous substances were obtained in this way that were fairly constant in phosphorus content.

Compound "A".....	1.02, 1.04, 0.99, 1.08, 1.03% phosphorus.
Compound "B".....	1.48, 1.42, 1.40, 1.41% phosphorus.

Since the products could not be crystallized and gave varying values for phosphorus after more extended treatment it is difficult to say whether or not they were chemical individuals.

Lead Salt.—Purification was then attempted by means of the lead salt, which it was found could be precipitated from alcohol solutions by means of lead acetate. The compound obtained in this way contained constant amounts of lead and phosphorus when prepared under different conditions and appeared to be a definite chemical compound. It was free from inorganic phosphates and from metals other than lead. The results of the analyses of five such preparations with the methods used for their isolation are as follows:

Preparation No.	1.	2.	3.	4.	5.
Per cent. lead........................	30.46	30.30	30.53	30.33	30.38
Per cent. phosphorus..................	3.86	3.80	3.78	3.99	3.90

Method of Preparation.

(1) One gram of the substance was dissolved in 100 cc. of water and precipitated by the addition of 400 cc. of alcohol. The precipitate was then extracted several times with hot 80% alcohol and the combined filtrates precipitated by the addition of lead acetate. A white flocculent precipitate was obtained which was filtered off, dissolved in dilute acetic acid and precipitated with alcohol.

(2) Five grams of the substance were dissolved in 100 cc. of water and a 10% solution of lead nitrate added until no further precipitate was obtained. One hundred cc. of alcohol were then added and the solution allowed to stand in the ice box overnight. The solution was filtered and the filtrate precipitated by the addition of a saturated solution of lead acetate in 80% alcohol. The resulting precipitate was filtered off and reprecipitated from water solution by means of alcohol.

(3), (4) Twenty grams of the substance were dissolved in 200 cc. of water, neutralized with lead carbonate and 9 g. of lead nitrate added. The solution was allowed to stand in the ice box or overnight, filtered and the filtrate precipitated by the addition of 400 cc of alcohol.

(5) Five grams of the substance were dissolved in 50 cc. of water and 10% silver nitrate solution added until no further precipitate was obtained. The silver chloride was filtered off and the excess silver removed by hydrogen sulfide. The hydrogen sulfide was removed by boiling and the solution neutralized with lead carbonate and treated as described under (3).

All preparations were dried for analysis at 60° *in vacuo* over sulfuric acid. Attempts to determine the percentage of carbon and hydrogen were unsatisfactory, owing to the difficulty experienced in burning the substance.

Free Acid.—The free acid was prepared from the lead salt by removing the lead with hydrogen sulfide and precipitating the resultant solution with alcohol, after evaporation of the solution under diminished pressure. The substance contained a constant amount of phosphorus as shown by the following analyses:

Preparation No.[1]	1.	2.	3.	4.	5.	6.
Per cent. phosphorus	5.30	5.30	5.56	5.24	5.27	5.33
Carbon[1]	35.45 and 35.20%					
Hydrogen	5.84 and 5.93%					

The substance as obtained by the above method was a perfectly white amorphous powder. It was extremely hygroscopic, contained no ash other than phosphoric acid and was free from nitrogen. It reduced Fehling's solution and on hydrolysis with acid yielded a reducing sugar equivalent to 65% glucose, and gave a heavy precipitate of glucosazone. The substance was optically active. $[\alpha]_D^{22}$ 108° 30′.

Hydrolysis and Identification of the Sugar.—One gram of the material was dissolved in 100 cc. of 10% hydrochloric acid and heated on the water bath for four hours. A large excess of sodium acetate and 2 g. of phenyl hydrazone were then added and the solution heated for two hours longer. A heavy precipitate formed which was recrystallized from methyl alcohol and water containing a small amount of pyridine, and identified as

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[1] It was necessary to remove the ash and grind it with potassium dichromate and repeat the combustion in order to burn the substance completely.

glucosazone by means of the melting point and optical rotation. M. p. 204° (uncorr.).

Two-tenths of a gram dissolved in 4 g. of pyridine and 6 g. of absolute alcohol gave a negative rotation[1] in a 25 mm. tube of 27′.

Quantitative Estimation of the Sugar.—0.3384 g. of the substance was dissolved in 100 cc. of 10% hydrochloric acid and heated on the water bath for eight hours. The solution was then made up to 100 cc. and the sugar determined in two aliquots of 25 cc. each by Defren's method.[2]

Gram copper oxide	0.1417	0.1416
Glucose equivalent	0.0630	0.0629
Per cent. glucose	64.86	64.81

Probable Constitution of the Compound.

The simplest formula that will conform to the above analyses is $C_{17}H_{31}O_{16}(H_2PO_3)$ corresponding to the calculated percentages; carbon, 35.66; hydrogen, 5.77; phosphorus, 5.42. According to the formula the lead salt contains a higher percentage of lead than should be present (26.73%), were the metal combined only with the phosphoric acid. The percentage of phosphorus in the lead salt calculated on a lead free basis agrees, however, with that found in the free acid. This shows that the excess of lead is not present as an impurity in the form of some other lead salt, since in that case the phosphorus value would have to be less. The excess of lead might be due to the fact that the salt was formed in a strong alcoholic solution, a condition which we know favors the formation of lead salts of the carbohydrates, and that therefore some of the lead might be combined in this way.

The amount of reducing sugar present corresponds to the ratio of one molecule of maltose to one of phosphoric acid (found, 61.70; calc., 59.79). The remainder of the molecule, 26.04%, aside from the phosphoric acid corresponds to the empirical formula $C_5H_9O_5$. No evidence for the presence of pentose could be obtained. It was mentioned above that difficulty was encountered in completely burning the sample during combustion owing to the presence of the phosphoric acid. It therefore seems possible that the remainder of the molecule is also a hexose. In view of the fact that it does not reduce Fehling's solution, it might be inosite, or, if another molecule of hexose, be still combined with the phosphoric acid in such a way as to destroy its reducing power. Eight grams of the crude material were examined for inosite, but none could be found, although 60% of the inosite were recovered from phytin treated in a similar way. It is impossible, therefore, to make any definite statement as to the nature of the remainder of the molecule.

NEW YORK, N. Y.

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[1] Neuberg, *Ber.*, **32**, 3384 (1899).

[2] Sherman, "Organic Analysis," **1912**.