for optimum precipitation depending on the relative amounts of the drug and the protein. Repeated precipitation by acid followed by solution at pH 7.5 does not reduce the suramin content of the precipitated complexes.

3. Considerable amounts of suramin become firmly attached to proteins such as the serum proteins and crystalline egg albumin at pH 7.5, but much more is bound by the protein in acid solutions, particularly at pH's below 6.

4. The absence of suramin from the red cells of animals injected with the drug is due to the inability of the drug to pass through the cell membrane. Suramin readily combines with the proteins of haemolysed red cells.

5. Suramin injected intravenously as a complex with rabbit serum proteins, or with egg albumin, is not retained longer in the blood than is the injected free drug.

6. The blocking of the free amino groups of proteins by the action of di-2-chloroethylsulphone does not reduce the capacity of the proteins to combine with suramin.

7. Suramin does not react with the thiol groups of cysteine or proteins.

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The Phosphorus Compounds of Wheat Starch

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In a previous paper, Lampitt, Fuller & Goldenberg (1941) showed that the insoluble portion separating out when wheat starch retrogrades has a much higher phosphorus content than the soluble fraction, and the presence of a phosphorus-rich fraction in wheat starch was postulated.

In the extensive literature on the phosphorus compounds of starches (summarized with many references by Lampitt *et al.* 1941; Lampitt, Fuller & Goldenberg, 1948), the consensus of opinion appears to be that, whereas in potato starch the phosphorus is present as an amylo-phosphoric ester,

in wheat starch it is combined as phosphatide loosely bound to carbohydrate, possibly by polar adsorption. This phosphatide, however, has apparently not been isolated and characterized, its presence being presumed by several authors on not very conclusive evidence. That wheat flour contains a phosphatide was shown by Winterstein & Hiestand (1907) who demonstrated in the gluten residue obtained on washing out starch from a dough the presence of a substance, associated with reducing sugar, which contained choline and gave β -glycerophosphoric acid on acid hydrolysis. Nottbohm & Mayer (1934)

found that most of the choline present in wheat flour remains in the starch fraction washed out of a dough; the starch contained no free choline, but 90 % of the choline present was isolated in a form soluble in benzene after the starch had been ground with pumice powder and water. (It is to be noted that the phosphatide content calculated from the choline values accounted for only 35 % of the total phosphorus present in the starch.) By acid hydrolysis of wheat starch, Posternak (1935) obtained only glycerophosphoric acid and no glucose-6-phosphate. Schoch (1942) showed that most of the fatty matter and most of the phosphorus in wheat starch were soluble in 85 % methanol or 80 % dioxan, the soluble fraction having the high phosphorus content of 2.25 %. Lehrmann (1930), by extraction of the solution resulting from the acid hydrolysis of de-fatted wheat starch, obtained 1 % of fatty matter consisting of fatty acids, but the aqueous solution contained no nitrogen and no glycerol. Lehrmann, who made no attempt to determine how this fatty acid was combined in the starch, later (Lehrmann, 1945) confirmed Schoch's (1942) results. He agreed with the conclusion of the latter, on the meagre evidence adduced, that the major proportion of the phosphorus in wheat was present in the form of loosely bound phosphatide, a small proportion of the phosphorus being combined directly with carbohydrate.

From the extensive literature, it appears that phosphorus is combined in the wheat berry in the following forms: (a) inorganic phosphate (probably potassium dihydrogen phosphate), (b) phytin (chiefly in bran and germ), (c) phosphatide, (d) nucleic acid (especially in germ), (e) associated with the starch, probably as a starch phosphoric ester, (f) possibly also hydrolysis products of these. It is possible that any or all of these types of phosphorus may be present in wheat starch, even if only as contaminants due to incomplete separation of the starch from the other flour constituents. It is also possible that decomposition products of these phosphorus compounds may be produced during isolation of a fraction.

Preliminary work showed that starch yielded very little soluble phosphorus and nitrogen when extracted by the normal method for phosphatides using ethanol and ether. The starch was therefore completely peptized with chloral hydrate as a preliminary step, the method of Meyer, Brentano & Bernfeld (1940) being chosen as it was claimed that degradation and hydrolysis of the starch molecule are thus avoided. The peptization was followed by precipitation with acetone, both soluble and insoluble fractions being examined.

EXPERIMENTAL

The starch used. This was prepared in the usual way by washing out from a dough made from a strong wheat flour

of 70 % extraction. The starch was allowed to sediment out from the wash water, washed by decantation several times with water and finally filtered on a Büchner funnel. The starch was dried in a current of hot air at a temperature below the gelling temperature, the lumps of dried starch pulverized and passed through a 48-mesh silk sieve. Microscopical examination showed that very few swollen and burst grains were present. Two samples of starch used contained approximately 8 % moisture, and (in the dry solids) 0.08 % P and 0.11 % N (N/P, 3.0/1), and 0.07 % P and 0.08 % N (N/P, 2.6/1), respectively. Starch prepared by the method used in this investigation invariably contains free phosphate. In the present case, 14 % of the total P in the starch was extractable by shaking for 1 min. with water at room temperature, and this fraction was almost entirely inorganic P. This 'adventitious P' has been borne in mind during this study; it is considered that its presence does not affect the main conclusions reached.

Methods of analysis. Nitrogen was determined by the micro-Kjeldahl procedure using Cu as catalyst; phosphorus by the Kuttner & Lichtenstein (1932) method after destruction of organic matter with H_2SO_4 and H_2O_2 (except where otherwise mentioned N and P values are calculated as percentages of the respective total N and P contents of the original starch and N:P ratios are calculated in terms of gram atoms); inorganic P by colorimetric estimation after precipitation with magnesia mixture.

Choline was determined where there was sufficient material, using the colorimetric reineckate method of Engel (1942). Two modifications in this method were found to be desirable: (1) hydrolysis with baryta for 3 hr. instead of 2 hr., (2) washing the reineckate precipitate with 3×2 ml. portions of ice-cold water according to the method of Ramsay & Stewart (1941); it was established that under these conditions the solubility of the precipitate was only 6 mg./100 ml. so that the loss on washing was negligible. When the acetone solution of the precipitate was unsuitable for colorimetric measurement of the colour (e.g. if the colour was too weak or the solution slightly turbid), the amount of choline present was calculated from the N content (micro-Kjeldahl) as described by Ramsay & Stewart (1941); it was found that the colorimetric and N methods gave identical results.

Glycerol was determined by the periodate procedure of Ramsay & Stewart (1941). To obtain satisfactory results with this method, it was found essential to proceed under rigidly controlled conditions and to observe the following precautions: (a) not more than 6 ml. of the baryta hydrolysate of the phosphatide should be taken when the volumes of reagents specified by Ramsay & Stewart are used; (b) the time of oxidation should be exactly 5 min.; (c) distillation of the formaldehyde produced should continue until the solution is almost dry before water is added prior to the second distillation; (d) the liquid must remain slightly alkaline during distillation to avoid evolution of formic acid (also formed on oxidation) which interferes with the subsequent colorimetric determination of the formaldehyde.

Peptization in chloral hydrate

The general procedure was as follows: 9 g. portions of starch were each dissolved by heating to 80° with 300 ml. 33 % (w/v) aqueous chloral hydrate solution (previously brought to pH 7.0 by addition of a little NaHCO_3) and the whole maintained at 80° for 30 min. Microscopical examina-

tion showed that the starch grains were then completely dispersed. The starch was recovered as a fine precipitate by blowing the hot solution through a fine jet into 600 ml. of dry acetone which was vigorously stirred. The solution was decanted off and the precipitate washed by decantation with 2×200 ml. portions of dry acetone, finally filtered on a Büchner funnel and dried at $95-100^\circ$ in a steam oven. The weight of precipitate was practically equal to that of the original starch taken.

The acetone solution and washings (referred to later as 'acetone filtrate') were combined and concentrated to small bulk (50 ml.) under reduced pressure at a temperature below 60° . The concentrate, which contained chloral hydrate, was treated with 50 ml. water and again concentrated as before, whereby a small part of the chloral was removed. Further treatment to remove chloral hydrate by distillation with water was not carried out owing to the possibility of hydrolysis or other decomposition of P compounds.

In three such experiments the acetone filtrate contained on the average 60 % of the original phosphorus and 45 % of the nitrogen of the starch, and the N/P ratio was materially lower than that of the starch, indicating that fractionation of the P compounds had been effected.

Fractionation of the phosphorus compounds in the acetone filtrate

Five fractionations were carried through on the acetone filtrates obtained from 18 g. portions of the wheat starch using the procedure shown in Fig. 1.

The process was carried out in a quantitative manner and samples of the various fractions were taken for determinations of N, P and choline.

The results of one such fractionation are shown in Table 1. The separation of Fractions II and III is dependent on the relative solubilities of the material in ether and in the aqueous phase, the distribution being influenced by the amount of chloral hydrate remaining after concentration of the acetone filtrate. As a consequence, in certain experiments the bulk of the P and N was found in Fraction III, and the ratio N/P was found to be approximately 1/1 in this fraction, as for Fraction IIa in Table 1.

From these results, namely, (a) the solubility of P and N in ether or chloroform (Fractions IIa or III respectively), (b) a N/P ratio in this fraction approximately 1/1 and (c) that the N was present almost exclusively as choline, it was tentatively concluded that the acetone filtrate contained a phosphatide of the lecithin type.

In most of these experiments the ether-soluble fraction (presumably phosphatide) was insoluble in light petroleum. It is well known that the solubility of phosphatides is considerably modified by the action of light and oxygen during isolation and purification. This may have occurred in the present instance in spite of the precautions taken to effect concentration below 60° in an inert atmosphere as described by Kirk, Page & Van Slyke (1934).

Fig. 1. Fractionation of P compounds in acetone filtrate.

* All concentrations were effected *in vacuo* at temperatures below 60° , in some cases with N_2 instead of an air leak.

† As a considerable amount of emulsion formed during the first ether extraction, the mixture was allowed to stand overnight before separation. No difficulty was then experienced in subsequent extractions.

‡ This was to remove any non-phosphatide N (Kirk, 1938).

Table 1. *Fractionation of acetone-soluble phosphorus compounds*

	Original starch	Starch ppt.	Fractions from acetone filtrate					
			I	IIa	IIb	III	IV	V
Percentage of total P	100	37	0.6	52	2.7	1.0	Trace	Trace
Percentage of total N	100	54	1.5	24	3.8	2.1	1.2	1.7
Ratio N/P	2.6/1	3.7/1	6/1	1.15/1	2.7/1	5.6/1	—	—
Percentage of N in fraction combined as choline	—	—	—	95	—	93	—	nil

Isolation of phosphatide

The acetone filtrate prepared from a total of 112 g. of wheat starch was fractionated into the ether-soluble portion and the aqueous residue, as described in Fig. 1. These two portions were further fractionated by the method outlined in Fig. 2, when six fractions were obtained. These were dissolved in suitable solvents and samples taken for analysis. From the results shown in Table 2, it was concluded that Fraction *D* consists largely of phosphatide, as the N present was mostly in the form of choline, and the ratio of choline N/P was close to 1/1. Further, a positive test for glycerol was obtained when the method of Ramsay & Stewart (1941) was applied to this fraction.

It will be noted that small amounts of choline were present in the other fractions, but in the largest fraction (*A*) most

of the N was not present as choline. The fraction which appears to be mostly phosphatide (*D*) was again soluble in CHCl_3 but insoluble in light petroleum.

Purification of phosphatide

A further quantity of fraction *D* was isolated by the procedure of Fig. 2 from 86 g. of wheat starch. The material was purified by solution in CHCl_3 and precipitated with excess of acetone, repeated three times. The product (50 mg.) was a white waxy solid. (Found: P, 3.85; N, 1.76; choline, 15.64; glycerol, 11.8. Calc. for oleopalmitophosphatide of mol. wt. 777; P, 4.0; N, 1.8; choline, 15.6; glycerol, 11.8 %.)

These results indicate that a phosphatide is definitely present in wheat starch, having a composition similar to that of lecithin.

Fig. 2. Further fractionation of acetone filtrate. All acetone filtrates combined to form Fraction *F*. All acetone precipitations made at 0–5°.

Table 2. Analysis of fractions obtained by procedure in Fig. 2

Fraction ...	A	B	C	D	E	F
Wt. of fraction (g.)	3.96	1.83	0.011	1.118	0.79	The weight of solid matter in this fraction could not be accurately determined owing to the presence of MgCl_2 : the fraction contained 2.3 mg. P and 4.7 mg. N of which 0.9 mg. was combined as choline; N/P, 4.5/1; choline N/P, 0.9/1
P (%)	0.158	0.433	Trace	3.13	0.132	
N (%)	0.235	0.450	Trace	1.74	0.118	
Choline N (%)	0.031	0.354	—	1.68	0.105	
Ratio N/P	3.0/1	2.4/1	—	1.2/1	2.0/1	
Ratio choline N/P	0.43/1	1.8/1	—	1.2/1	4.5/1	

Table 3. Phosphorus and nitrogen compounds in acetone filtrate

	Acetone precipitate	Acetone filtrate		Total in acetone filtrate (calc. as % of weight of original starch)
		Ether-soluble	Aqueous residue	
P as % of total P in starch	45	15.8	40*	P, 0.0370
N as % of total N in starch	70	13.5	21	N, 0.0240
Choline N as % of total choline N in starch	—	9.8	16.9	Choline N 0.0186
Ratio N/P	3.9/1	1.8/1	1.3/1	N/P, 1.5/1
Ratio choline N/P	—	1.3/1	1.05/1	Choline N/P 1.1/1

* In another similar fractionation, 5% of the P of the original starch was combined as inorganic phosphate in this aqueous residue.

*The proportion of phosphatide
in wheat starch*

The P compounds in the acetone filtrate from 18 g. of wheat starch were separated merely into the ether-soluble and aqueous residue fractions in Fig. 2, and determinations of N, P and choline were made on these fractions (see Table 3). It will be noted that the ratio of total choline N to total P in the acetone filtrate is 1.1/1, from which it appears reasonable to assume that the bulk of the choline was initially present as phosphatide. From the total choline figure for the acetone filtrate, the calculated phosphatide content is 1.0 %, and from the total P content of this filtrate 0.9 %. These values, which are obviously maximum values, are comparable with the value of 0.86 % calculated from the total P contents of Fractions IIa and III in Table 1.

*Examination of the acetone precipitate (starch residue)
from the chloral hydrate peptization process*

The starch residue obtained by removal of the solvent from the acetone precipitate and drying in the steam oven was soluble in 0.5N-HCl, but was precipitated on addition of an equal volume of ethanol. The ethanolic filtrate contained 10–15 % of the P of the original starch (the acid-soluble P), while approx. 30 % remained associated with the precipitate. Similar results were obtained respectively for the extract and the insoluble residue obtained on refluxing the acetone precipitate with 85 % methanol for 12 hr. Part of the acid-soluble P was combined as inorganic phosphate and part as organic phosphate (glycerophosphate or hexose phosphate), but there is a possibility that some may have been combined as nucleotide or phytin or even as phosphatide liberated by the acid from a complex with carbohydrate. A larger quantity of the acetone precipitate was therefore extracted with 0.5 N-HCl and the extract fractionated.

The acid-soluble phosphorus fraction. The dried acetone-precipitated material was moistened with a little alcohol (to avoid the formation of lumps later), 20 times the weight of 0.5 N-HCl added and the mixture shaken for 1 hr. at 15–20°. An equal volume of ethanol was added, and portions of the solution obtained on filtering off the precipitated starch were treated with (a) FeCl₃ after neutralization and (b) excess of Ba(OH)₂, as in the scheme of LePage & Umbreit (1943). The Fe precipitate was materially reduced by solution in acid and reprecipitation by neutralization, so that the presence of phytin was considered to be very doubtful. The Ba precipitate contained a little inorganic P, and an organic P compound containing glycerol but no combined sugar. In all the soluble and insoluble fractions, qualitative tests for guanine, adenine and choline before and after acid hydrolysis were negative, indicating the absence of nucleotide and phosphatide. The acid-soluble P is therefore largely glycerophosphate with some inorganic P.

DISCUSSION

The data presented afford proof that phosphatide is present in wheat starch, and therefore the statements in the literature to this effect, based on inconclusive evidence, have been substantiated. The phosphorus present (approx. 0.07 %) has been divided into three main fractions: (1) approx. 50–60 % of the total phosphorus remains in solution when a chloral hydrate dispersion of the starch is precipitated with excess acetone. This soluble phosphorus is largely present as phosphatide with a little inorganic phosphate. (2) 10 % of the total phosphorus is extractable from the acetone precipitate (starch residue) by 0.5N-hydrochloric acid, as inorganic phosphate and glycerophosphate. (3) Approx. 30 % of the total phosphorus remains associated with the starch residue. The nature of the phosphorus compound in this fraction is being investigated.

SUMMARY

1. Peptization of wheat starch in chloral hydrate solution followed by precipitation with acetone yielded an acetone solution containing 50–60 % of the phosphorus in the original starch. From this solution a fraction was separated, soluble in ether and chloroform, in which the N/P ratio was nearly 1/1 and 95 % of the N was present as choline. Part of the 'adventitious P' present in the starch was also found in this fraction.
2. By precipitation of the chloroform-soluble material, 50 mg. of a waxy substance was isolated from 86 g. of starch, having a composition agreeing with that of lecithin. The presence of a phosphatide was therefore confirmed.
3. The phosphatide content of wheat starch was approximately 1.0 % calculated from the choline content of the acetone solution mentioned above.
4. From the carbohydrate material precipitated by acetone, approximately 10 % of the phosphorus in the original starch was extracted by 0.5N-hydrochloric acid, 30 % remaining associated with the carbohydrate.
5. The acid-soluble phosphorus was combined as inorganic phosphate (the residual 'adventitious P') and glycerophosphate.
6. Hexosephosphate and nucleotide were not present, while there was no conclusive evidence for phytic acid.

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Studies in Vitamin A

9. THE ROLE OF THE THYROID IN CAROTENE AND VITAMIN A METABOLISM

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Apart from the action of the tocopherols in preventing the oxidation of carotenoids in the intestinal lumen, not much is known of other factors which may control the absorption and metabolism of carotene and vitamin A. Attention has, however, been focused on the thyroid gland in this connexion, especially in regard to the conversion of carotene into vitamin A. Kunde (1926) noted the appearance of vitamin A deficiency in thyroidectomized rabbits fed carotene and a little later von Fellenberg & Grüter (1932) and Fasold & Heidemann (1933) claimed that carotene appeared in the milk of thyroidectomized goats. Abelin (1933) at the same time noted that in guinea pigs the administration of thyroxine adversely affected alike the metabolism of carotene and vitamin A. The clinical aspects of the subject have been reviewed by Drill (1943).

After Abelin's (1933) work there was a long interval before the effect of the thyroid on carotene metabolism was again investigated. Drill & Truant (1947), using the remission of xerophthalmia as criterion of vitamin A production, failed to demonstrate vitamin A formation from carotene in thyroidectomized animals. An objection to this work is that the carotene was administered parenterally and there are serious doubts whether injected carotene is utilized to any great extent (see, e.g., Sexton, Mehl & Deuel, 1946). Canadell & Valdecasas's (1947) experience appears to confirm Drill & Truant's (1947) work, but Remington, Harris & Smith (1942) state that eye symptoms in thyroidectomized rats are cured by the oral administration of carotene.

Di Bella (1940*a, b*) also found carotene effective in such animals but with 'reduced efficiency'.

Johnson & Baumann (1947), using the storage of vitamin A in the liver as the criterion of carotene conversion, found that the same dose of carotene produced less liver vitamin A in thiourea-treated animals than in controls and that, perhaps surprisingly, control rats stored less than did rats dosed with desiccated thyroid. Administration of thyroxine and thiourea together produced normal liver storage; this indicated that the action exerted by thiourea was antithyroid and that it did not reduce liver storage of vitamin A by virtue of another (unknown) pharmacological action. Kelley & Day (1948) have confirmed the findings of Johnson & Baumann (1947).

Wiese, Mehl & Deuel (1948) emphasize the importance of allowing for the growth-inhibiting power of thiouracil when the effect of the drug on carotene metabolism is assessed by means of biological assays involving the measuring of weight increments. They proved that growth inhibition was due to thiouracil *per se*, and not to its action on carotene metabolism, by demonstrating that a normal response could be elicited in thiouracil-treated animals when desiccated thyroid, but not vitamin A, was added to the diet. Wiese *et al.* (1948) overcame this difficulty with regard to the bioassay by evaluating the dose of carotene required to produce half the maximum growth obtained in the control and experimental groups. Using this technique they found that β -carotene was equally effective in both groups.