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On the Presence of Nucleoproteins and Lipoproteins in the Salt-soluble Protein Fraction of Barley

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Considerable amounts of nucleic acids and protein-bound lipids are found in extracts obtained from ground barley grain with 1 M sodium chloride. About 5 % of the nitrogenous material precipitated from such extracts with cold trichloroacetic acid (TCA) consists of nucleic acids (both RNA and DNA), representing about a third of the total nucleic acids of barley. The lipids bound to salt-soluble proteins, represent only 2.5—5 % of the total barley lipids. Distilled water dissolves no nucleoprotein below pH 7 and only moderate amounts (only RNA) above this value. The solubility of the lipoproteins is also lower when extracted with distilled water. Calcium ion at a concentration of 0.01 moles/l completely inhibits the extraction of TCA-stable phosphorus-containing protein complexes at low ionic strengths.

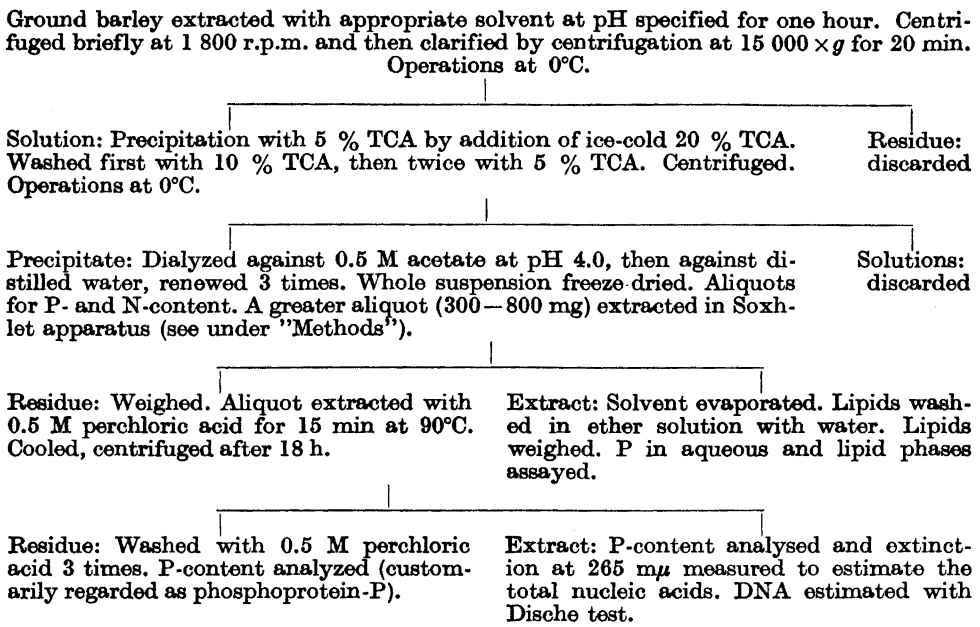
The methods used for nucleic acid and lipid assays, and for the determination of phosphorus distribution, and the origin and character of the nucleoproteins and lipoproteins are discussed.

Our knowledge of the nucleoproteins and lipoproteins of barley is very limited. Pool and Shooter [1] showed that small amounts of nucleic acid were present in a protein fraction. Similar observations have been made by Äyräpää [2], who also found lipids in the same fraction. A phosphorus-containing protein fraction supposed to be nucleoprotein has been found in brewery wort by Hopkins, Amphlett and Berridge [3]. The presence in brewery products of purine and pyrimidine derivatives, supposed to be degradation products of nucleic acids, has been demonstrated by several workers [4–6].

The corresponding protein complexes of wheat, which could be supposed to have their analogues in barley, are somewhat better worked out, but the investigations are almost exclusively limited to the nucleic acid complexes of wheat germ [7,8] (first recognized by Osborne [9]) and gluten-lipid complexes (*cf. e. g.* Olcott and Mecham [10]). A peculiar wheat lipoprotein has been isolated and described by Balls, Hale and Harris [11].

This paper gives no complete picture of the nucleoproteins and lipoproteins of barley grain. The aim of this investigation is to elucidate the extent to which such complexes occur in the much investigated so-called salt-soluble

Fig. 1. Outlines of the analytical procedure.



protein fraction of barley. Hence the extraction has been limited to the "natural" pH range (pH 5—8) mostly used in such investigations.

Trichloroacetic acid (TCA) at 0—+2°C has been chosen as the protein-precipitating agent, because cold TCA separates loosely bound phosphates [12–14], especially inositol phosphates [15], from the more stable posphorus-containing complexes (nucleoproteins, lipoproteins and phosphoproteins). It was also used to wash the tissues before lipid extraction [16]. TCA is generally regarded as the most selective protein-precipitating agent [17].

The TCA-precipitates from different extracts were freeze-dried and then subjected to an analytical procedure which may be regarded as a combination and modification of the methods of Schneider [12] and of Ogur and Rosen [18]. The outlines of the procedure are given in Fig. 1; the details are described under "Materials and Methods".

RESULTS

I. *Orientation experiments; comparison between barley and malt.* The amounts of phosphorus and nitrogen precipitated with 5 % TCA from different barley and malt extracts are shown in Figs. 2 and 3. (The experimental procedure was similar to the first two steps described in Fig. 1, with the exception that the extraction was performed at +25°C for 2 h.) The solubility properties of the phosphorus-containing protein complexes in barley and malt

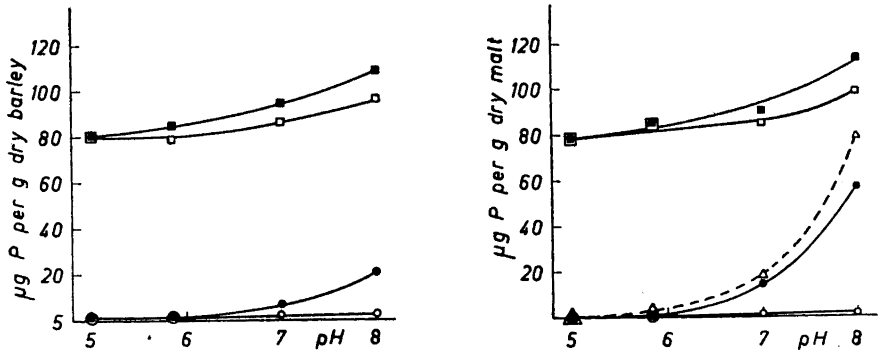


Fig. 2. The amounts of TCA-precipitated phosphorus in barley and malt extracts. Ordinate: μg precipitated P corresponding to 1 gram of dry barley or malt. Asbcissa: pH at extraction. Extraction solutions: -●—●- distilled water, -○—○- 0.01 M $CaCl_2$, -■—■- 1 M NaCl, -□—□- 1 M NaCl+0.01 M $CaCl_2$ and -△--△- 0.01 M EDTA.

are evidently very similar. Distilled water, however, in the alkaline range dissolves more protein-bound phosphorus, probably nucleic acid, from malt.

The nitrogen curves presented in Fig. 3, indicating the amounts of TCA-precipitable nitrogen extracted, suggest that a relatively phosphorus-rich fraction would be extracted with 1 M sodium chloride at pH 8 if the bulk of protein were first removed at pH 7 with distilled water. The activity of nucleases necessitates a rapid extraction and centrifugation procedure. A nucleic-acid rich protein fraction has been prepared in this way, but has not yet been further investigated.

The other conclusions drawn from these figures will be presented together with the results of phosphorus distribution analyses.

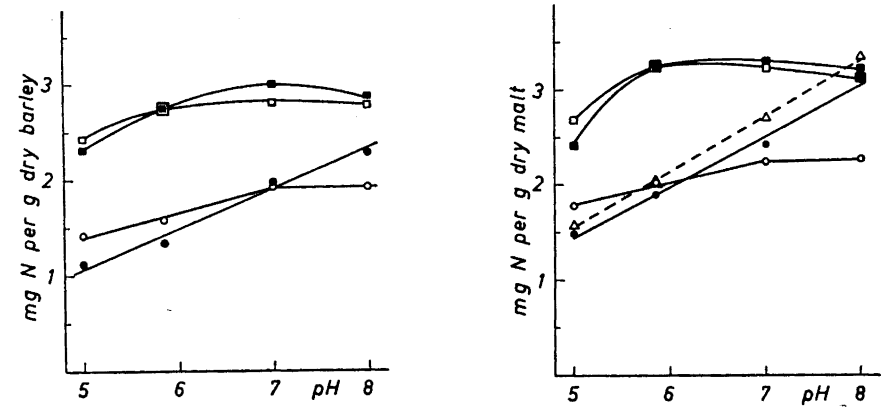


Fig. 3. The amounts of TCA-precipitated nitrogen in barley and malt extracts. Ordinate: mg precipitated N corresponding to 1 gram of dry barley or malt. Otherwise as in Fig. 2.

Table 1. The amounts of TCA-precipitates obtained from barley extracts and their nitrogen and phosphorus content.

Solvent and pH at extraction	Yield mg dry matter per g dry barley	% N in dry matter	% P in dry matter
Distilled water, pH 5.85	8.04	14.4	0.025
» » pH 7.0	9.48	14.5	0.040
» » pH 8.0	13.1	14.0	0.175
1 M NaCl, pH 5.85	22.1	13.7	0.440
» » pH 7.0	21.0	14.4	0.445
» » pH 8.0	20.4	14.3	0.545

II. *The distribution of phosphorus.* The amounts of freeze-dried TCA-precipitates obtained from barley extracts and their nitrogen and phosphorus contents are shown in Table 1. The distribution of phosphorus in these precipitates is shown in Table 2. The results presented in Table 2 and in Figs. 2 and 3 may be condensed to the following conclusions:

1) The nucleic acids are extracted by 1 M sodium chloride in considerable amounts at all the pH values investigated. The amounts extracted at pH 8 are only slightly higher than those obtained at pH 5.85. Distilled water dissolves moderate amounts of nucleic acids at pH values above 7 but not at lower values.

2) The lipoprotein phosphorus is found in considerable amounts only in sodium chloride extracts at pH 8, but small amounts are present in all the extracts, somewhat more in sodium chloride extracts than in distilled water extracts.

3) The other phosphorus-containing fractions are small. The phosphorus left in the residue after extraction with perchloric acid, customarily designated as phosphoprotein phosphorus, and another unknown phosphorus fraction, extracted with neutral alcohol-ether mixture but found in the aqueous phase after the washing of the lipids, are both present in greater amounts in sodium

Table 2. The distribution of phosphorus in TCA-precipitates from barley extracts.

Solvent at extraction	Lipid-P µg/g barley	Nucleic acid-P µg/g barley	Unknown P		Control	
			P in rest substance "Phosphoprotein-P"	P extracted with alcohol-ether but water-soluble	P by direct assay	P by addition
Dist. water pH 5.85	0.7	0.6	0.3	0.2	2.0	1.8
» » pH 7.0	1.1	1.9	0.3	0.8	3.8	4.1
» » pH 8.0	2.6	16.1	0.5	1.0	22.9	20.2
1 M NaCl pH 5.85	3.7	86.4	2.8	2.9	97.0	95.8
» pH 7.0	3.3	83.6	1.5	2.2	93.0	90.6
» pH 8.0	19.3	97.0	1.8	2.4	111.5	120.5

chloride extracts. Their amounts are not much influenced by the pH value at extraction.

4) The influence of calcium ion is marked in distilled water extracts: at a concentration of 0.01 M it inhibits the extraction of practically all the protein-bound phosphorus. In 1 M sodium chloride extracts, the presence of similar amounts of calcium has only a slight inhibiting effect above pH 7 (as seen in Figs. 1 and 2).

III. *Nucleic acids.* The amounts of nucleic acids calculated on the basis of phosphorus analyses compared with the amounts calculated from the ultraviolet absorption data are shown in Table 3. The agreement (except when only traces of phosphorus are present) is generally good. Only in the sodium chloride extract obtained at pH 5.85 is there appreciably more phosphorus. This excess of phosphorus seemed to be acid-stable (it was not hydrolysed to inorganic phosphate by 1 M hydrochloric acid at 100°C in 10 min). Since, in the course of attempts to prepare nucleic acid from whole barley, phytic acid was found in some combination with nucleic acid, the presence of phytic acid was especially tested for, but with negative results.

Table 3. The amounts of nucleic acids in TCA-precipitates from barley extracts. (Phosphorus and U.V. extinction values compared).

Solvent at extraction	Nucleic acid by P-det. µg/g barley	Nucleic acid by extinction µg/g barley	DNA by Dische reaction µg/g barley	% nucleic acid N of the total N of TCA-ppt. (by extinction)
Dist. water pH 5.85	6(?)	0	0	—
» » pH 7.0	20(?)	11	0	0.15
» » pH 8.0	169	165	0	1.9
1 M NaCl, pH 5.85	890	748	274	4.0
» » pH 7.0	870	835	300	4.4
» » pH 8.0	1000	978	344	5.4

The nucleic acids are calculated to constitute 4.0—5.5 % of the TCA-precipitable nitrogen in the sodium chloride extracts.

In order to estimate how much of the total nucleic acid of barley is extracted by 1 M sodium chloride solution, an attempt to estimate the nucleic acid content of barley is made by the cupric precipitation method, as described by Vendrely [19] (but on a larger scale, see under "Methods"). By assuming that the nucleic acids contain 10.7 % purine nitrogen and by neglecting the presence of free nucleotides, the nucleic acid content is calculated to be 0.27 % of the barley dry matter. Thus 1 M sodium chloride dissolves about 35—40 % of barley nucleic acid under the conditions described above.

The differentiation of RNA and DNA by hydrolytic methods [13,18] has not been found successful (*cf.* the experiences of Martin and Morton [20]). Thus the presence of DNA in the perchloric acid extracts containing total nucleic acid

was tested by the method of Dische [21], which is often considered to give too high values (*cf.* Markham [22]). The color given by perchloric acid-extracted nucleic acids, derived from sodium chloride extracts was the same as that of standard DNA (maximum at 595 mμ), whereas the nucleic acids derived from distilled water extracts gave no color. Moreover, in preliminary experiments starting from sodium chloride extracts we have obtained appreciable amounts of fibrous DNA, which is not digested by ribonuclease and shows an intense Feulgen reaction. Although the amounts given in Table 3 may be approximate, the presence of considerable amounts of DNA in sodium chloride extracts thus seems well established.

IV. *Lipids.* The amounts of lipids obtained from the TCA-precipitates and the phosphorus contents of these lipids are shown in Table 4.

Table 4. The lipids in TCA-precipitates from barley extracts and their phosphorus content.

Solvent at barley extraction	Lipid extracted with alcohol-ether		Lipid extracted with acidified alcohol-ether		Lipid as % of total barley lipid	Lipid as % of TCA-precipitated dry matter
	μg per g barley	% P in lipid	μg per g barley	% P in lipid *		
Dist. water pH 5.85	110	0.09	30	1.9	0.4	1.8
» » pH 7.0	330	0.16	30	1.9	1.1	3.6
» » pH 8.0	820	0.21	30	3.0	2.6	6.5
1 M NaCl pH 5.85	920	0.15	110	1.2	3.1	4.7
» » pH 7.0	820	0.12	100	2.4	2.8	4.4
» » pH 8.0	1460	1.25	50	2.8	4.6	7.4

* See under »Methods» how these values are estimated.

The amounts of lipids extracted with the HCl-containing alcohol-ether mixture is in most cases of the same order of magnitude as the error in the lipid analysis. But since the phosphorus content of this fraction is relatively important and since the removal of this fraction is necessary for the subsequent estimation of nucleic acids (*cf.* Ogur and Rosen [18]), this step cannot be omitted.

The nature of these lipids has not yet been investigated. Since their phosphorus content is generally low, the majority probably consist of various neutral components. (Abundance of neutral fat seems to be characteristic of plant lipoproteins [20,23]).

It has hitherto been possible to demonstrate the presence of steroids only in the lipids from the sodium chloride extract at pH 8 (by the method of Pijoan and Walter [24]; they amount to about 5 % of the protein-bound lipids).

DISCUSSION

1) *Accuracy of results.* The values presented above must be regarded as tentative only. In the first place, the extraction cannot be made exhaustively, as the enzymic degradation of nucleic acids, which is found to be appreciable

even in ice-cold solutions, necessitates a rapid extraction and clarification procedure. The long washing in TCA-solutions may also remove some of the nucleic acids.

As the clarification of the extract is another critical point, the barley cannot be ground too finely. Pool and Shooter [1] have shown that extracts from fine meal are difficult to clarify by centrifugation. Vendrely, Palmade and Vendrely [25], have found that the grinding of microorganisms in a ball mill renders the extraction of DNA more difficult. Filtration through filter pads (*e. g.* Seitz K 10) removes an appreciable fraction of the soluble high molecular nitrogen components, as was also pointed out by Pool and Shooter [1]. Hence we have chosen a grinding giving 70 % fine meal (as recommended by E.B.C.-Analytica [26]), extraction at 0°C for one hour and the clarification of extracts by centrifugation at 15 000 × *g*, as described under "Methods". Under other conditions, different values may be found.

As seen in Table 2, the accuracy of the values obtained in the phosphorus distribution analyses is scarcely greater than ±10 %. The lipid extraction and washing procedures may be somewhat incomplete. Further, the evaluation of nucleic acids is approximate owing to the use of standards obtained from different organisms. It must be emphasized that the method described in this paper is limited to TCA-precipitates from barley extracts. It is not applicable to whole barley, which contains both UV-absorbing and phosphorus-containing disturbing components.

2) *The origin of complexes.* The solubility properties of the nucleoproteins and lipoproteins of barley are similar to those of such complexes in general. For instance, from animal mitochondria the nucleoproteins may be extracted with 1 M sodium chloride at neutrality and the lipoproteins then with a more alkaline solution (for complete extraction a pH value of about 11 is needed) [27]. To what extent the nucleoprotein and lipoprotein complexes of saline barley extracts are derived from cytoplasmic particles remains to be shown. Some observations suggest that a part of the RNA may be derived from particles centrifuged down at 15 000 × *g* (probably plastids and mitochondria present in all the parts of the grain [28]). As the extraction of the nucleoproteins and lipoproteins is enhanced by the same factors, the lipoproteins may partially originate from similar particles. The submicroscopic particles (microsomes), if present, are probably left in solution after centrifugation at 15 000 × *g*, and may thus contribute to the nucleic acid and lipid content of the TCA-precipitates. (A centrifugation experiment at 150 000 × *g* has yielded slightly opaque pellets from sodium chloride extracts, previously clarified at 15 000 × *g*).

The DNA found in sodium chloride extracts may also partly originate from cytoplasmic particles, for Martin and Morton [23] have found appreciable quantities of DNA in cytoplasmic particles from wheat rootlets. On the other hand, when we tried to prepare nucleic acid from saline extracts of wheat flour (= endosperm fraction), a preparation consisting mainly of DNA was obtained. As RNA always preponderates in cytoplasmic particles, the presence of nuclear constituents in the saline extracts cannot be excluded.

3) *On the general character of nucleoproteins and lipoproteins.* The fractionation methods hitherto used in the study of barley proteins cannot be expected to give much information about native nucleoprotein and lipoprotein com-

plexes. During the slow fractionation procedures, an appreciable degradation by different enzymes is probable, since plant ribonucleases [29] and some lipases [30] are stable and active in a wide variety of conditions. Such procedures as salt fractionation may result in a dissociation of nucleoproteins, and in the subsequent dialysis the nucleic acid may then combine with new protein components [7,31,32].

In some preliminary fractionation experiments, we have certainly found lipids and nucleic acids in the globulin fraction and in the insoluble dialysis precipitate but never in the albumins. But just as phytic acid, on combining with proteins, may change their solubility properties [33,34], so nucleic acids and lipids may behave in a similar manner. For the reasons mentioned above, these experiments warrant no definite conclusions.

The presence of artificial lipoprotein complexes in barley extracts is possible, as evidenced with respect to wheat gluten by Olcott and Mecham [10]. But as all organisms contain lipoproteins, at least a part of those found in barley extracts may be expected to be natural.

The observations made in the course of this work suggest that greater attention paid to the structure of the grain, along the lines introduced by Säverborn, Danielsson and Svedberg [35], Danielsson [36] and Hess [37], might be fruitful in continued investigations on cereal proteins in general. In examination of lipoproteins and nucleoproteins, the separation of different cell fractions by differential centrifugation would be especially useful.

MATERIALS AND METHODS

Barley, Variety Herta, grown in 1955 in South Sweden, was used. It was milled immediately before the experiments with an Electrolux mill, adjusted to give about 70—75 % "fine meal" (passing through a Pfungstadt No. 3 sieve). N 1.69 %, P 0.37 % and total lipid 3.27 % (dry basis). Moisture 10.1 %.

Malt, Herta, grown in 1953 in South Sweden, was used. Milled as barley, about 90 % fine meal was obtained. N 1.57 % and P 0.42 % (dry basis). Moisture 6.8 %.

Nucleic acid standards. As RNA-standard, yeast nucleic acid from the Nutritional Biochemical Co. was used. It was deproteinized according to the method of Sevag, Lackmann and Smolens [38], dialyzed for one day and reprecipitated with 2 vols. ethanol. P 8.0 %, N 14.0 %, after heating with 0.5 M perchloric acid for 15 min at 90°C the extinction measured in 0.1 M HCl at 260 mμ was 10 800 per mole phosphorus.

The DNA standard was prepared from calf thymus according to the procedure of Mirsky and Pollister [39]. It was digested with crystalline pancreatic ribonuclease (Sigma Chemical Co.) and then deproteinized (as RNA above); dialyzed and reprecipitated with 1 vol. ethanol. P 8.53 %, N 14.1 %; the extinction measured as above was at 268 mμ (maximum) 10 100 per mole phosphorus.

The calculations of nucleic acid amounts are based on the assumption that the purine: pyrimidine ratio is unity, that RNA contains 9.6 % P and DNA 9.9 % P. Thus the DNA-RNA mixture present in sodium chloride extracts is considered to have a molar extinction of 10 600 at 265 mμ and a phosphorus content of 9.7 %.

Extraction of barley. The extraction was made on an ice bath or at room temperature. The flour was added as a fine stream to the stirred solution, in order to avoid the formation of artificial lipid complexes on moistening the flour by doughing (*cf.* Olcott and Mecham[10]). The pH value was repeatedly checked and adjusted by addition of 2 M sodium hydroxide or hydrochloric acid through a capillary to the vortex of the stirred solution.

Centrifugation. The extracts were first centrifuged at 1 800 r.p.m. and the turbid solution then clarified by centrifugation at high speed for 20 min at 15 000 × *g*. The clear or slightly opalescent middle layer was collected by pipette, contamination with the lipid-

containing top layer being avoided. Extraction and clarification of a sufficient quantity of extract generally required 3 h.

Precipitation with TCA and dialysis. This was performed as described in Fig. 1. The precipitate was washed ready for dialysis 10—12 h after the beginning of the extraction. Practically no nitrogen and little phosphorus (less than 10 %) was lost during the dialysis, which required 42 h.

Extraction of lipids. Generally 300—800 mg of the freeze-dried TCA-precipitate was extracted in a semimicro Soxhlet apparatus, first with 80 ml of ethanol-ether (3:1) for 48 h, then with 60 ml of absolute ethanol to which had been added 0.8 ml of concentrated hydrochloric acid per 100 ml (about 0.1 M). After 24 h 20 ml of ether was added to the solvent and extraction continued for a further 24 h. The two extracts were dealt with separately. They were evaporated on a water bath, the last 10 ml *in vacuo* without heating. The residues were moistened with 0.6 ml of absolute ethanol, after which 20 ml of ether and 20 ml of distilled water were added and the mixtures shaken in separatory funnels. The separated ether phases were washed twice with water and the original aqueous phases (probably containing proteinaceous material, carbohydrates and polyphenols) shaken again twice with 15 ml of ether, which was likewise washed with water. The ether phases were transferred to 50 ml volumetric flasks with 2 g of anhydrous sodium sulphate. The flasks were filled to the volume. After drying overnight aliquots of 40—45 ml were removed and evaporated and the residues transferred with ether to small weighed capsules through a little funnel with a minute filter paper disk in the stem. The ether was finally evaporated in a desiccator by cautious suction, with ether in the vacuum trap. The capsules with their contained lipid droplets were finally weighed after 24 h drying *in vacuo*.

It may be mentioned that we have tried many different lipid extraction methods, including enzymatic proteolysis and acid pressure hydrolysis, extraction with chloroform and *n*-butanol (Morton [40]). The hydrolytic methods seem not to offer any advantage: they are tedious and much evidently non-lipid material which is soluble in alcohol-ether is obtained. The chloroform extracts gave a dark and solid residue. Extraction with *n*-butanol seemed in some cases suitable, but in other cases gave definitely less lipids; it is evidently useful but has not yet been systematically investigated.

The method described above is not ideal. Phospholipids are hydrolyzed during the acid extraction, which is proved by treating known amounts of soybean lecithin in the same manner. The lipids obtained contain some other material (they often give a positive Molisch reaction). Actually, it cannot be said if this material is an integral part of the lipid or not (*cf.* Lovern [41], p. 42).

From experiments with soybean lecithin it appears that the lipid phosphorus extracted with acid alcohol and ether is to be found in the aqueous phase after the washing of lipids. The phosphorus content of the "firmly bound" lipids listed in Table 4 is only a rough approximation. Owing to the various possible sources of error, the phosphorus percentage is calculated without regard to the lost unknown lipid fragments.

Extraction of nucleic acids. As already mentioned, we did not obtain any reproducible results with the differential hydrolytic extraction method of Ogur and Rosen [18]. The amounts of RNA extracted with cold perchloric acid were generally low. Thus the total nucleic acids were extracted with hot perchloric acid. Heating at +70°C for 20 min produced extracts which were difficult to clarify and which showed an appreciable extinction at 300—330 mμ. When the heating temperature was raised to +90°C and the extracts cooled overnight at 4°C, the turbidity was easy to centrifuge off and the absorption curve between 220 and 330 mμ was close to those of the nucleic acids, as shown in Fig. 4. The heating time seemed not to be critical; 10 and 30 min gave practically identical results.

Purine nitrogen determination. The method of Vendrely [19] was adopted, but on a larger scale. Barley samples of 2 g were hydrolyzed in 20 ml of 1 M hydrochloric acid by gently boiling for 6 h with reflux. After centrifugation, the brown solutions were neutralized (with bromocresol purple as indicator), acidified with acetic acid to about pH 5, heated to 100°C and centrifuged again. A sample of the solution corresponding to a known amount of original extract was then taken for the copper precipitation, performed according to the original method but with sixfold amounts of reagents (3 drops was taken to be 0.1 ml). The nitrogen was determined in the second precipitate by the usual Kjeldahl method (modification of Blom and Schwartz [42], see below).

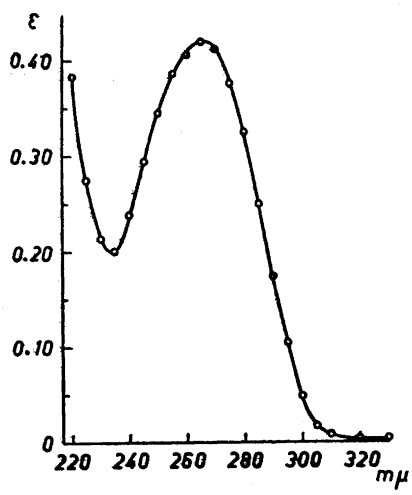


Fig. 4. The ultraviolet absorption spectrum of hot perchloric acid extract containing nucleic acids. (Delipidated TCA-precipitate from sodium chloride extract, obtained at pH 8).

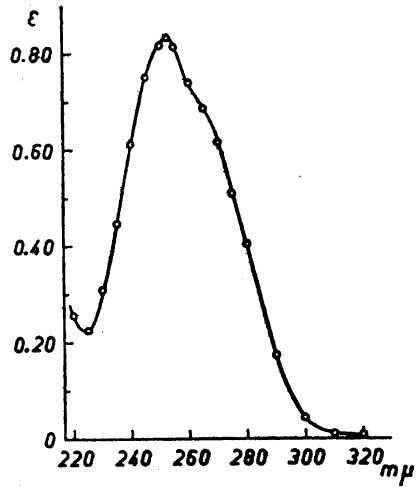


Fig. 5. The ultraviolet absorption spectrum of the decomposed copper-purine complex from barley hydrolysate, measured in 0.1 M hydrochloric acid.

Since the values given by the cupric precipitation method may be too high, they were checked for some samples by measuring the ultraviolet absorption of the decomposed cupric precipitate as follows: The second cupric precipitate was decomposed by 1 % sodium sulphide (exactly as the first precipitate according to the original method). The cleared supernatant was diluted with 0.1 M hydrochloric acid and the ultraviolet absorption measured. Fig. 5 shows the absorption curve of such a solution, corresponding well to the curve of an adenine-guanine mixture. If the molar extinction coefficient of an equimolar adenine-guanine mixture is considered to be 12 000 (at 253 mμ) [22], virtually equal values were found by nitrogen determination and by extinction measurement.

Assay of nitrogen. The semimicro modification of the Kjeldahl method suggested by Blom and Schwartz [42] was used. Distillation was performed in Markham's apparatus.

Assay of phosphorus. The Fiske-SubbaRow method as modified by King [43] was used. Especially when the presence of phytic acid was suspected, a droplet of 0.4 % ammonium molybdate was added to the digestion tube to ensure rapid hydrolysis of phytic acid [44].

Phytic acid analysis. The method of Leva and Rappoport [45] was adopted, since preliminary experiments showed that relatively large amounts of mucleic acid do not interfere.

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