Fractionation and Characterization of Purothionin[1]

N. FISHER, D. G. REDMAN, and **G. A. H. ELTON,** Flour Milling and Baking Research Association, Chorleywood, Hertfordshire, England

ABSTRACT

Purothionin isolated from wheat flour by the method of Balls and Hale (Cereal Chem. 17: 243; 1940) has been fractionated on Sephadex G-75 to give two products with differing compositions and properties. The first fraction, A, gave weakly staining bands in the albumin region on starch-gel electrophoresis in aluminum lactate buffer at pH 3.3, but the second, B, gave a closely spaced doublet in these conditions. The high cystine and arginine contents first noted by Balls *et al.* (Cereal Chem. 19: 279; 1942) in purothionin have been found in the second fraction obtained in the present work, and a surprisingly low glutamic acid content has also been found, together with a high lysine content. This fraction had an average molecular weight of about 10,000 as determined by gel filtration in two different solvent systems in which proteins of known molecular weight (cytochrome-c, myoglobin, and soybean trypsin inhibitor) were used as standards. Purothionin B (and an ethanol-purified sample, C) were closely similar in amino acid composition and similar in molecular weight to the "fast-moving globulin doublet" (J. Sci. Food Agr. 13: 62; 1962) and had the same electrophoretic mobility at pH 3.3.

A low-molecular-weight protein, termed purothionin, was first isolated as its crystalline hydrochloride from a petroleum ether extract of wheat flour by Balls and Hale (1). The protein was believed to be derived from a lipoprotein containing lecithin, and had a most unusual composition (2), containing 20% arginine and 16% cystine, compared with the usual values for flour proteins of about 4% for arginine and 2% for cystine. The minimum molecular weight (MW) was estimated as 6,000 on the basis of its tyrosine content, and diffusion measurements gave a value of 10,200; hence a probable MW of about 12,000. However, it was not a single compound, and preparations from different sources varied in their composition. In subsequent work (3), bactericidal and fungicidal properties were demonstrated in purothionin, which was stated to be "the oxidized form of a powerful oxidation-reduction system," and it was also reported to be an activator of wheat proteinase. Surprisingly, no other studies followed these initial reports, and the present work describes some further attempts to purify and characterize this material. In the preliminary work purothionin gave electrophoretic bands corresponding in mobility to those given by the "fast-moving globulin doublet" (4), and a preparation of the latter fraction was therefore undertaken for comparative purposes.

MATERIALS AND ANALYTICAL METHODS

Materials

An untreated and unbleached (U.U.) soft flour ("Snowdown"), having the properties shown in the table below, was selected to correspond as closely as possible to the flour used by the original workers.

[1]Presented at the 52nd Annual Meeting, Los Angeles, Calif., April 1967. After completion of this work we were notified of a similar paper by C. C. Nimmo and his colleagues of Western Regional Research Laboratory, USDA, which was presented at the same meeting. (Editor's Note: See Nimmo *et al.*, page 28 in this issue.)

Properties of Snowdown U.U. Flour

Protein (N × 5.7), %	8.5	Resistance (R)	67
Moisture, %	14.0	Extensibility (E)	14.7
Color grade figure	1.4	10 E/R	2.2
Water absorption, %	49.6	Falling time (Hagberg)	136

Sephadex G-75 (40–120 μ): Product of Pharmacia Ltd., Uppsala, Sweden.

Reference proteins: myoglobin, cytochrome-c, and soybean trypsin inhibitor were purchased from Koch-Light Laboratories Ltd., Colnbrook, England.

Methods

Starch-gel electrophoresis was usually carried out according to the method of Elton and Ewart (4) in aluminum lactate buffer pH 3.3, $\mu = 0.05$ (which gave better resolution of albumins and globulins than the corresponding sodium lactate buffer), with a potential gradient of 5 V/cm. for 4 hr.

Gel filtration was carried out in water-jacketed columns at 25° ± 0.1°C. unless otherwise stated.

Amino acid analysis: Samples were hydrolyzed for 22 hr. in constant-boiling hydrochloric acid (~ 6.1*N;* sample:acid ratio 1:2,000 w./v.) under nitrogen in screw-capped tubes at 105°C. (5). The acid was removed by rotary evaporation at room temperature and the hydrolysates were separated in a Technicon Auto-analyzer; Nor-leucine was used as an internal standard. Correction factors for destruction of threonine and serine and for incomplete hydrolysis of isoleucine and valine were obtained from hydrolyses carried out for various times. Reproducibility was ± 5%. Cystine analyses of proteins were occasionally checked by oxidation of the protein with performic acid before the hydrolysis, by a slight modification of the method of Hirs (6) in which the reagents were removed after oxidation by alternate freeze-drying and water treatments. Results agreed with those from unoxidized samples within 2%. Tryptophan was determined (5) by the method of Spies and Chambers (7,8); procedure N of the 1949 paper was used, and results were read from curve C, Fig. 4, of the 1948 paper.

Nitrogen was determined by a micro-Kjeldahl method; a selenium catalyst was used in the digestion and a Markham still for the distillation (9).

PROCEDURES

Flour Extraction

Flour was extracted in glass columns 3 or 9 in. in diameter, according to the scale of the extraction (3.6 kilos or 40.9 kilos of flour giving packing heights of approx. 120 and 150 cm. respectively). Column gaskets were of Neoprene sheathed in polytetrafluoroethylene.

The smaller-scale extraction and isolation procedures followed those of the original authors as closely as practicable (see flow chart, Fig. 1). Petroleum ether (boiling range 60°–80°C., B.P. grade; ~ 2.9 liters/kilo) was percolated down the column under pressure of nitrogen, and the yellow extract was concentrated in a rotary evaporator to approximately 95 ml. The concentrate was then stored at –2° to –3°C. for 26 days, and the resulting precipitate was centrifuged off at –2.5°C. (1,000 × *g*). The supernatant was evaporated to dryness, to obtain the "crude lipid" extract.

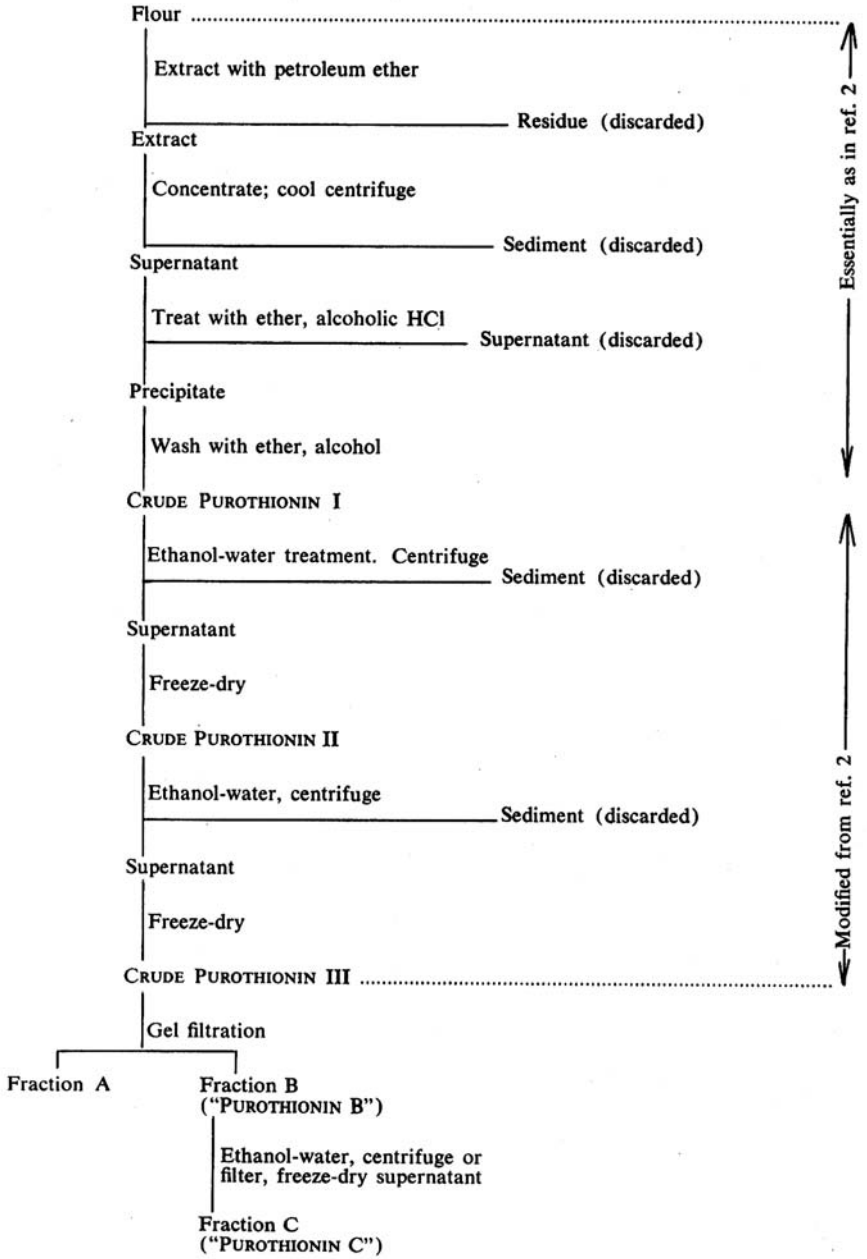

Fig. 1. Simplified flow-chart of extraction and purification of purothionin.

In the larger-scale extraction, concentration of the column effluent was carried out at reduced pressure and a distillation temperature not exceeding 45°C., but without use of a rotary evaporator. The concentrated extract (about 3 liters) was placed in a refrigerator at –20°C. Aliquots of the supernatant were then worked up as required.

Purothionin Hydrochloride

The "crude lipid" from either extraction procedure was dissolved in an equal volume of ether, and 3 volumes of a 1.3*N* solution of dry hydrogen chloride in dry ethanol was added. A precipitate formed almost immediately. The reaction mixture was held at 0°C. for 1 hr. and then centrifuged (1,000 × *g*, 30 min., –2°C.). The supernatant was decanted through a sintered-glass filter (porosity No. 2); the precipitate was washed repeatedly by decantation with absolute alcohol followed by anhydrous ether, and finally filtered and dried to a brown solid (I; 0.03% of flour weight). The yield did not vary significantly with the scale of extraction.

Purification

The crude product (I; 1.13 g.) was treated with 4 ml. water (2); it became sticky and most failed to dissolve. Alcohol (12 ml.) was added, followed by 4 ml. of 3:1 (v./v.) alcohol-water; much of the solid, though now less sticky, still failed to dissolve. The mixture was then centrifuged and the supernatant decanted. The residue was treated with 10 ml. water (much of it dissolved) followed by 30 ml. ethanol, and the mixture was centrifuged. This process was repeated twice more, and the extracts were combined and evaporated to dryness; this gave 740 mg. of product (II). This was suspended in a total of 12.5 ml. water and centrifuged (40 min., 1,000 × *g*). The liquid layer was removed (together with a minute amount of floating solid, which could not be separated), and 125 ml. absolute ethanol was added. No crystallization occurred after overnight refrigeration. The solution was therefore evaporated to dryness under reduced pressure and the product desiccated; this yielded 598 mg. brown vitreous solid (III; ~ 0.02% of flour weight). Starch-gel electrophoresis showed this material to be a mixture of components, the main constituents corresponding in mobility to the fast-moving pair of globulin bands previously described (4). Preparations of this globulin fraction were therefore carried out from Snowdown flour and also from a second U.U. flour (11.7% protein) as follows:

The flour (750 g.) was treated with 3 liters of a 1.0*M* solution of sodium chloride in 0.1*M* phosphate buffer, pH 7.0. The mixture was stirred for 2 hr. and then centrifuged (30 min., 1,200 × *g*). The supernatant was dialyzed at 12°C. against distilled water until free of chloride, and then for a further 24 hr. The dialysis residue was centrifuged at 1,000 × *g*, 12°C., and the sediment was dispersed in 100 ml. water and freeze-dried; this gave 3.8 g. of product. Starch-gel electrophoresis showed this to contain the desired "fast-moving globulin doublet" as a minor component (4). This preparation could be purified by means of gel filtration (Sephadex G-75; 78 × 2.4-cm. column), but better results were obtained with carboxymethyl cellulose (termed CMC; preswollen Whatman CM52) in a 0.32*M* solution of sodium chloride in 0.02*M* acetate buffer, pH 5.2, to form a column 30 cm. × 2.5 cm. Globu-

lin residue (second U.U. flour), 1 g., was stirred in the buffer (15 ml.) for 24 hr., and then centrifuged (2,000 × *g*, 30 min.). The supernatant was applied to the column and eluted stepwise with successive 50-ml. portions of 0.02*M* acetate buffer made respectively 0.40, 0.50, 0.60, 0.70, and 0.80*M* in sodium chloride. Dialysis and freeze-drying of the eluates was followed by starch-gel electrophoretic analysis which showed that the 0.70*M* sodium chloride-eluted fraction contained the "fast-moving globulin doublet" (3.6 mg.), in this instance apparently free of other components (Fig. 2).

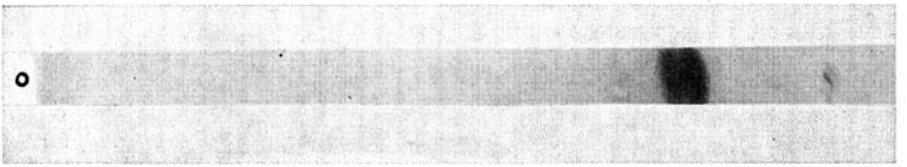

Fig. 2. Starch-gel electrophoretic pattern of a fraction obtained from carboxymethyl cellulose separation of wheat globulins.

Electrophoresis of mixed purothionin and globulin preparations gave a "doublet" of unchanged mobility.

Fractionation of Purothionin Hydrochloride

Sephadex G-75 equilibrated in 0.01*M* acetic acid was packed into a column 2 cm. in diameter to a height of 29 cm. (packing volume 91 cc.: void volume, determined with dextran blue, 10 cc.). The purothionin preparation (III; 74.1 mg.) was dissolved in 0.01*M* acetic acid (0.5 ml.) and loaded onto the column. Acetic acid, 0.01*M*, was passed through the column and 5-ml. fractions were collected at a flow rate of 1–2 ml./min. The absorbances of the fractions at 280 mμ are shown in Fig. 3, a and b, for preparations from small and large columns respectively (the latter preparation, however, corresponding to fraction II above: load 65.8 mg.).

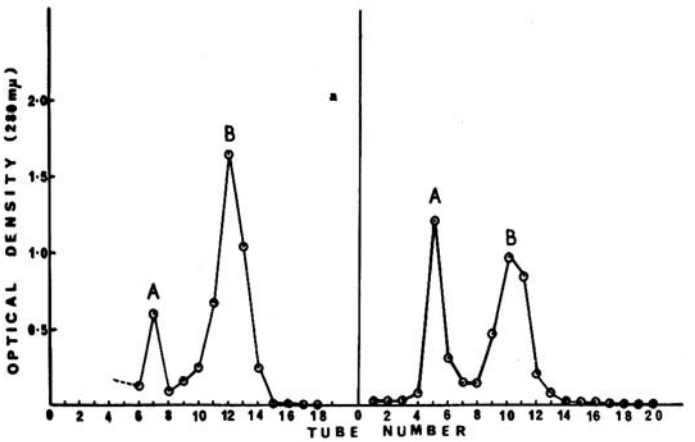

Fig. 3. Fractionation of crude purothionin hydrochloride preparations, III and II, on Sephadex G-75.

The contents of tubes 6 and 7 and of tubes 9–14 (Fig. 3, a) were respectively combined and freeze-dried, to give fractions A (6.6 mg.) and B (56.7 mg.). The products were obtained as white solids, fraction B showing birefringence in polarized light but no regular crystalline form. When fraction B was rerun on the same Sephadex G-75 column, a small forerun preceded a single large peak, with no subsequent fractions.

Starch-gel electrophoresis showed that fraction A gave a number of weakly staining bands in the albumin region, whereas fraction B gave a closely spaced doublet, shown in Fig. 4.

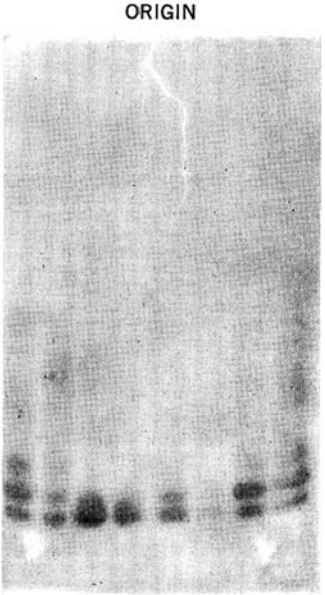

Fig. 4. Starch-gel electrophoretic comparison of purothionin and the "fast-moving globulin doublet." The four patterns at center are of purothionin, the outer four of globulin (Sephadex preparation, less pure than that shown in Fig. 2).

Treatment of fraction B (26.2 mg.) with water (0.2 ml.) gave a clear amber solution, which became turbid on addition of ethyl alcohol (0.6 ml.). When this had cooled to 0°C. for 48 hr., a very small amount of white solid was deposited. This was separated off by centrifugation, and the supernatant was cooled to –20°C. overnight. No crystallization occurred, and the solution was therefore freeze-dried to give a white solid (C; 18.4 mg.). Electrophoresis gave the same doublet as shown in Fig. 4.

Molecular Weight

Determinations were carried out on fraction B by gel filtration in the presence of standard crystalline proteins. The latter comprised horse-heart cytochrome-c, myoglobin, and soybean trypsin inhibitor, with MW's of 12,400, 17,800, and 21,500 respectively.

A Sephadex G-75 column, 38 cm. × 2 cm. diam. (volume 119 ml., void volume 22.5 ml.), was calibrated with the cytochrome and myoglobin

samples (the trypsin inhibitor being insoluble in the eluting solvent). With 0.01*M* acetic acid as eluant, fractions of 2.25 ml. were collected at a flow rate of 1.5 ml./min., with a column load of cytochrome-c, 0.5 mg., purothionin fraction B, 2 mg., myoglobin, 0.4 mg., and dextran blue solution, 1 drop, in 0.5 ml. solvent. A plot of "tube number" (i.e. elution volume) against log MW of the standards, assuming the linear relationship found by Andrews (10) to hold, gave a MW for purothionin of 9,550.

A Sephadex G-75 column was then prepared with a 0.1*M* solution of KCl in 0.05*M* *tris* buffer, pH 7.5. Column diameter was 2.3 cm. and packing height 95 cm.; this gave a bed volume of 395 ml. The column was loaded with cytochrome-c, 0.5 mg.; myoglobin, 0.4 mg.; trypsin inhibitor, 0.6 mg.; purothionin fraction B, 3 mg.; and dextran blue, 1 drop, dissolved in minimal quantity of buffer (about 1 ml.). Fractions, 2.25-ml., were collected at a flow rate of 1.5 ml./min. The linear relation of elution volume with log MW was confirmed, and gave a MW for purothionin of 10,500. The ultraviolet spectrum of purothionin showed maxima at 206 and 275 mμ. The infrared spectrum is given in Fig. 5.

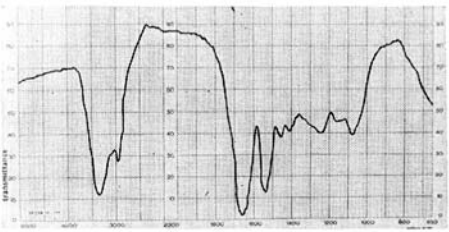

Fig. 5. Infrared spectrum of purothionin (KBr disk).

Purothionin

The crude hydrochloride (III; 38.4 mg.) was treated with 0.7 ml. 0.01*N* ammonium hydroxide and loaded onto a column (35 cm. × 2 cm. diam.) of Sephadex G-75 in 0.01*N* ammonium hydroxide. Elution with this solvent gave an elution diagram similar to that of the crude hydrochloride (Fig. 3, a), with two peaks, the first of which yielded 8.4 mg. of product and the second 13.7 mg. The products were subjected to starch-gel electrophoresis and found to correspond to fractions A and B of Fig. 3. The purothionin "free base" was a white solid which was still very soluble in water, in contrast to the globulin preparation which precipitated from aqueous salt solution on dialysis against water.

RESULTS AND DISCUSSION

Amino Acid Composition of Purothionin and "Fast-Moving Globulin Doublet"

The amino acid compositions of the purothionin hydrochloride preparations were compared with those of the purified globulin fraction described earlier, with the results shown in Table I.

The original method of preparation of purothionin is excessively time-consuming and could be substantially accelerated by removing the sterols which precipitate rapidly in the cold and immediately proceeding to the preparation of the crude hydrochloride. This would necessitate repeated aqueous extraction of the crude first product, which may in any case

TABLE I

AMINO ACID COMPOSITION[a] OF PUROTHIONIN[b] AND WHEAT GLOBULIN PREPARATIONS

AMINO ACID	PUROTHIONIN FRACTIONS[c] A	B	C	GLOBULIN[d]
Aspartic[e]	9.15	6.83	6.85	6.40
Threonine	6.13	5.19	5.23	4.50
Serine	7.07	8.28	8.49	8.88
Glutamic[e]	11.84	7.11	6.37	6.07
Proline	4.56	3.98	4.27	3.26
Glycine	5.11	4.54	4.59	4.99
Alanine	5.96	3.62	3.65	4.00
Valine	5.74	2.92	2.63	2.15
Cystine/2	5.45	12.19	12.65	12.60
Methionine	2.43	0.71	0.65	0.43
Isoleucine	4.39	2.09	1.90	1.43
Leucine	8.32	10.81	9.54	9.39
Tyrosine	3.03	2.44	2.91	2.45
Phenylalanine	4.34	3.60	3.73	3.33
Lysine	7.33	11.60	12.20	12.56
Histidine	1.94	0.61	0.50	2.24
Arginine	7.19	13.49	13.84	15.33

[a] g. Anhydroamino acid per 100 g. recovered anhydroamino acid.

[b] After correction for known acid hydrolysis losses, recovery of amino acids completely accounted for the total nitrogen of the sample.

[c] A tryptophan value (refs. 7,8) of 0.70 g./100 g. protein was obtained for purothionin fraction C.

[d] CMC preparation.

[e] Including asparagine and glutamine, respectively.

become necessary (as in the present study) even after the cold storage for 3 weeks specified in the original method.

It seems highly unlikely that the conditions used by Balls *et al.* (2) for preparation of the crude hydrochloride (1*N* HCl in anhydrous ethanol-ether at 0°C.), resulting in immediate reaction, could really constitute a hydrolysis unless the linkage were exceedingly labile. Decomposition of an electrostatically bound complex seems more likely, and we have found that successive extractions of the petroleum ether extract of flour ("crude lipid") with 1 and 15% NaCl solutions gave products which with 1% NaCl showed both ester and amide absorptions in the infrared, but with 15% showed only amide absorption. Yields were exceedingly low, however, and further work is required on this aspect. The use of aqueous HCl to extract the "crude lipid" solution is also being investigated.

We have not found it possible to purify the crude hydrochloride adequately by crystallization from aqueous ethanol alone. Gel filtration has, however, given excellent separations, and the gel-purified products could be further slightly purified by ethanol treatment, indicating that despite some overlapping, these processes are essentially complementary. Repetition of either process failed to change the electrophoretic behavior of the product, although the ethanol treatment did cause some change in amino acid composition (compare glutamic acid, cystine, leucine, tyrosine, and lysine contents of preparations B and C in Table I). Later work with much longer Sephadex G-75 columns has partially resolved the bands of Fig. 3.

Any "molecular weight" determined for purothionin at this stage must

be a mean value based on the presence of at least two components (the bands of the "doublet" obtained on electrophoresis). The mean MW of fraction B of 10,000 ± 5%, obtained with the use of two gel-filtration systems in the present work, agrees well with the value of 10,200 obtained by a diffusion method by the previous authors, but is lower than their final value of 12,000 assumed on the basis of "minimum MW" determination, a method which is liable to very large errors when applied to a mixture of components. Fraction A of the crude purothionin is also a mixture of components with MW's about 45,000 and above.

The electrophoretic behavior of purothionin, the recovery of all the nitrogen of the sample as amino acids after hydrolysis, and the ultraviolet and infrared spectra (with amide I, II, and III bands) leave little doubt that it is a protein, a point much discussed in the original work.

The complete amino acid composition of purothionin has not previously been reported. Individual values were given for cystine, arginine, tyrosine, and histidine, and while these differ in detail from the values reported here, there is general agreement with the exceptionally high arginine and cystine values in these results. We have additionally shown the presence of very high lysine values and very low glutamic acid and proline values, compared with those of most wheat proteins.

The remarkable similarity in the amino acid compositions of purothionin preparations B and C to the composition of the "globulin pair" raises the question whether or not these materials are identical. Elution volumes on Sephadex G-75 have been found to coincide, indicating similar MW's. While differences in threonine, proline, isoleucine, and particularly histidine suggest nonidentity, all the remainder of the values, including the unusually high cystine, arginine, and lysine values and the low glutamic acid contents, agree within the limits of experimental error or are very closely similar. The identical electrophoretic mobilities observed at pH 3.3 must to some extent be offset by the results of a large number of electrophoretic experiments in various alkaline conditions in which the mobilities were found to differ. Starch-gel electrophoresis in these conditions gave poorly reproducible results, but in all cases, purothionin had a different mobility from that of the globulin. The solubilities of purothionin and the globulins appear to differ, the purothionin being soluble in water whereas the globulin requires the presence of salt to ensure solution. While, therefore, purothionin and the fast-moving globulins are evidently closely related, their identity is not unequivocally established.

The question of the lipoprotein precursor of purothionin requires much further work. We have found that the petrol extract contains dispersed material which can be separated by centrifugation, giving products which show amide and ester absorptions in the infrared. Purothionin can, however, be prepared from the clear residual oil. (We have eliminated the slight possibility that the water present in the flour and saturating the petroleum ether passed through the column might be responsible for the extraction of the purothionin.) Work on the structure of purothionin and its precursors is in progress.

Acknowledgments

The valuable advice of J. A. D. Ewart, the skilled technical assistance of P. Maple and D. Smith, and the photographic work of C. H. Groves are gratefully acknowledged.

Literature Cited

1. Balls, A. K., and Hale, W. S. A sulphur-bearing constituent of the petroleum ether extract of wheat flour (preliminary report). Cereal Chem. 17: 243–245 (1940).
2. Balls, A. K., Hale, W. S., and Harris, T. H. A crystalline protein obtained from a lipoprotein of wheat flour. Cereal Chem. 19: 279–288 (1942).
3. Stuart, L. S., and Harris, T. H. Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. Cereal Chem. 19: 288–300 (1942).
4. Elton, G. A. H., and Ewart, J. A. D. Starch-gel electrophoresis of cereal proteins. J. Sci. Food Agr. 13: 62–72 (1962).
5. Ewart, J. A. D. Amino acid analyses of glutenins and gliadins. J. Sci. Food Agr. 18: 111–116 (1967).
6. Hirs, C. H. W. The oxidation of ribonuclease with performic acid. J. Biol. Chem. 219: 611–621 (1956).
7. Spies, J. R., and Chambers, D. C. Chemical determination of tryptophan. Study of color-forming reactions of *p*-dimethylaminobenzaldehyde and sodium nitrite in sulfuric acid solution. Anal. Chem. 20: 30–39 (1948).
8. Spies, J. R., and Chambers, D. C. Chemical determination of tryptophan in proteins. Anal. Chem. 21: 1249–1266 (1949).
9. Jones, A. S. Determination of nitrogen by Kjeldahl's method. *In* Comprehensive analytical chemistry, ed. by C. L. Wilson and D. W. Wilson; vol. 1B, pp. 495–509. Elsevier: Amsterdam (1960).
10. Andrews, P. Estimation of the molecular weights of proteins by gel-filtration. Biochem. J. 91(2): 222–232 (1964).

[Received May 10, 1967. Accepted August 25, 1967]