THE INSTITUTE OF BREWING RESEARCH SCHEME.

SECOND REPORT ON BARLEY PROTEINS.

COMPOSITION AND QUANTITATIVE ESTIMATION OF THE BARLEY PROTEINS.—II.

By L. R. Bishop.

The work on the quantitative estimation of the proteins of the barley grain which was described in the First Report (1) has been continued with the aim of checking and extending the methods described, in order that they could be applied to a study of the changes occurring during malting. The fractionation in more detail of the various substances extracted by salt solution was of particular interest since the protein breakdown products (very approximately equivalent to the "permanently soluble nitrogen") must necessarily be studied in following the changes during malting. In such a study it was also necessary to know if the methods can be successfully applied to malt as well as to barley. This would not be possible if the proteins of malt and barley were different.

Relation of the proteins of Barley to those of Malt.

Osborne and Campbell (2) in 1896 concluded, from the elementary composition of their preparations, that the proteins of malt were different from those of barley. They concluded that the hordein of barley gave rise in malt to a slightly different alcohol-soluble protein, which they named "bynin." Similarly they considered that the globulin of barley, which Osborne called edestin, was changed to "byn-edestin" in malt.

Luers (3) was unable to find significant differences between his Van Slyke analyses of hordein and "bynin" separated respectively from barley and from malt. The danger of contamination of the alcohol-soluble protein by salt-soluble nitrogenous compounds and carbohydrates is greater in malt than in barley and neither Osborne and Campbell nor Luers employed previous salt extraction to avoid this difficulty. Such contamination probably accounts for the 1 per cent. more carbon, and 1 per cent. less nitrogen in "bynin" (compared with hordein) on which Osborne and Campbell based the distinction.

Kraft (4) found the specific rotation and solubilities in alcohol, water and alkali of hordein and of "bynin" did not differ significantly. The same could be said of his determinations of the amount of glutamic acid in each.

Hence there is no doubt that hordein and "bynin" are very similar. It is still possible that hordein partly degraded by enzyme action during malting may still be alcohol-soluble and so would be extracted mixed with unaltered hordein and the mixture would constitute "bynin." The evidence is, however, against this. I found the ammonia percentage of the hot 70 per cent. alcohol extract of malt (previously repeatedly extracted by salt solution) to be almost the same as that for the hordein of barley.

Table I.

Ammonia per cent. of alcoholic extract		Values for pure protein.	
Barley	23·3	Hordein	23·3 Osborne
	23·2		23·00 Luers (3)
			23·38 Hoffman and Gortner
Malt	23·1	"Bynin"	23·55 Luers (3)

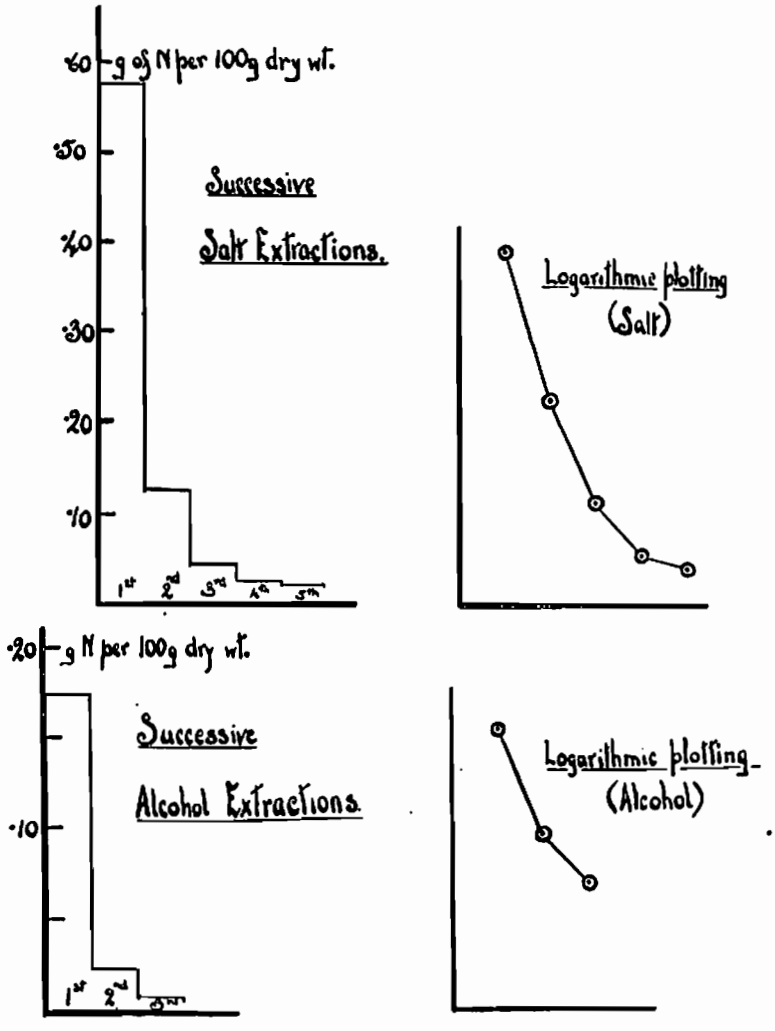
Hence it is proposed to discard the name "bynin" and to consider that both hordein and globulin persist throughout malting. Under these circumstances similar methods to those found satisfactory for barley should be applicable to the separation and estimation of the individual proteins of germinating barley or of malt.

Conditions for the Extraction of the Proteins from Malt.

In order to decide whether any modifica-

DIAGRAM 1.

MALT FROM CHISELBOROUGH, 1926.



tions in the extraction methods described for barley in the First Report (1) were necessary for malt, separate determinations were made of the nitrogen extracted by the successive extraction of two different malts, first by 5 per cent. potassium sulphate solution and then by 70 per cent. alcohol. The quantities and conditions used were the same as those used in the barley extraction. These analyses indicated that the number of extractions used for barley was suitable also for malt. The results for one of the malts are given in Diagram 1, in which the vertical heights of the blocks represent the amounts of nitrogen dissolved in successive extractions. The logarithmic plotting departs from a straight line owing to proteolytic action and to slow outward diffusion of the proteins. The completeness of extraction was seen to be satisfactory, and it was assumed that the methods would apply equally well to samples from intermediate stages of malting.

If albumin or globulin are coagulated on the kiln the coagulated protein, being insoluble, would appear as glutelin in the estimations. The studies recorded in the Third Report indicate that no serious error

TABLE 2.

	Ammonia per cent. of fraction.		Amide nitrogen of purified protein.
	Barley	Malt	
Albumin	9·84, 8·64	—	9·40 per cent. (Luers and Landauer) 9·29 per cent. (Bishop)
Globulin	8·93	10·5	9·02 per cent. (Bishop)
Hordein	23·3, 23.2	23·1	23·3 per cent. (Osborne); 23·00 per cent. 23·3 (bynin) (Luers); 23·38 per cent. (Hoffman and Gortner)
Glutelin (Difference)	12·3	11.7	11·38 per cent. (Larmour) (5) 12·3 per cent. (Bishop)

arises in this way, and, taking this into consideration, the methods developed for barley would appear to be applicable to a study of malting.

Estimations of the amino-nitrogen showed that the slight degradation of proteins which takes place during the salt extractions occurs chiefly in the solid particles not in the solution. This is checked immediately the residue is mixed with the alcohol (under 2½ hours from the commencement of extraction) and the salt solution is fractionated as soon as possible. It is considered that under these conditions proteolytic activity is not a source of serious error, even in the green malts where proteolytic activity is relatively high.

Ammonia percentage of the proteins of Barley and Malt.

This useful index (equivalent to the amide nitrogen of the Van Slyke analysis) was used in the First Report to show the purity of the alcohol extract. Its use has now been extended to the other fractions, and the table above (Table 2) gives evidence for the assumption that the total nitrogen determined in each of the fractions comes almost entirely from that protein which it is supposed to represent.

Fineness of Grinding.

Early studies showed the importance of this factor in the analysis of barley. In the earlier work the grinding was made as even as possible by the use of an adjustable large cone coffee mill. This was not altogether satisfactory, especially with barleys having a tough structure. Since then a mechanical mill (the "Wiley" mill) has been tested. This mill grinds, without heating the material, by the cutting action of knives. The material passes through a sieve out of the grinding region as soon as it has reached the necessary degree of fineness.

The following table illustrates the very large effect on the apparent salt-soluble nitrogen (and in consequence on the other fractions) of varying degrees of fineness of grinding. It also shows that even half millimetre fineness is not as good as the "coffee mill" grinding, which reduces most of the material to a finer state of division, but leaves some large particles. In consequence, in all the later work the material has been ground first as fine as possible in the "coffee" mill and then reground in the Wiley mill using the ½ mm. sieve to ensure evenness.

It will be seen that the hordein nitrogen is affected least and consequently is more reliable. Barley 166c was of fairly low

TABLE 3.—*Effect of Grinding on the Analyses.*

Barley.	Method of Grinding.	Salt-sol.	Hor-dein.	Glute-lin.
		%	%	%
166c (Archer N.I.A.B. 1924).	1 mm. Wiley ...	25·6	29·1	45·2
	½ mm. Wiley ...	31·2	28·7	40·0
	"Coffee Mill" ...	34·9	27·7	37·3
	"Coffee Mill" and ½ mm. Wiley ...	36·2	27·9	35·8
	Total nitrogen on dry barley 1.42%			
140 (Archer N.I.A.B. 1923).	1 mm. Wiley ...	23·6	34·2	42·3
	½ mm. Wiley ...	27·5	34·8	37·6
	"Coffee Mill" ...	30·8	36·3	32·8
	"Coffee Mill" and ½ mm Wiley	30·1	35·2	34·7
	Total nitrogen on dry barley 1·98%			

Note.—Sample numbers here and elsewhere in these Reports refer to barleys described in Sir John Russell's Reports on the Barley Research.

nitrogen content, and the slight increase of apparent hordein with coarse grinding may be explained as due to extraction of previously unextracted salt-soluble nitrogen. In barley 140 the nitrogen content was high, and apparently the larger amount of hordein was not efficiently extracted from the coarse particles, since slightly larger amounts are obtained from the finely ground samples.

Fractionation of the Salt-Soluble Compounds.

The salt extraction, it will be recalled, dissolves out from the barley the fully built up proteins, albumin and globulin, and also protein breakdown products, such as proteoses, peptones, amino-acids and other simple nitrogen compounds. In the work described in the First Report (1), only the albumin and non-protein nitrogen were estimated. Further work has now been carried out with the object of extending the methods of fractionation then adopted.

Albumin.

The difficulty encountered in the estimation of albumin referred to in the First Report (1) has been overcome by allowing the extract to stand with the acid buffer solution to remove that part of the globulin which is precipitated as the acid compound. The extract is then filtered, and used for the albumin estimation.

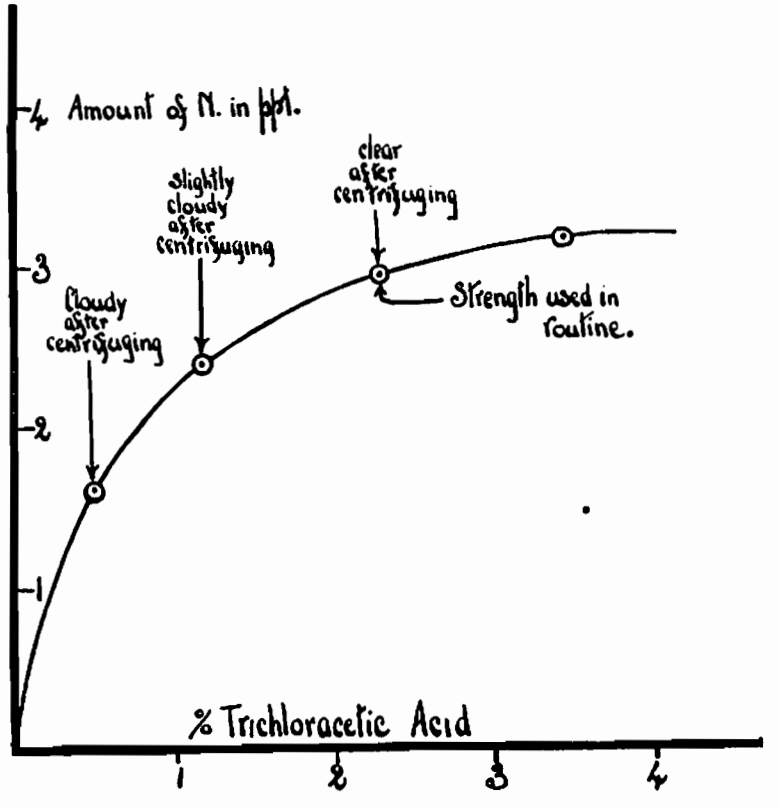
Total Proteins.

Brief reference was made in the First Report (1) to the use of trichloracetic acid for the estimation of total proteins (*i.e.*, albumin+globulin). Further investigation of the method has shown it to be satisfactory as applied to barley and malt.

Luck (6) gives a list of papers which show that trichloracetic acid (at 2-4 per cent. concentration) is a specific precipitant for

DIAGRAM II.

NITROGEN PRECIPITATED FROM SALT EXTRACT BY VARIOUS CONCENTRATIONS OF TRICHLORACETIC ACID.



fully built-up proteins, and is quantitative for albumins and globulins. My experiments showed that the amount of nitrogen precipitated from barley extracts is affected by the concentration of the precipitant. See Diagram II. (p. 319). The strength chosen (2·27 per cent.) is probably about the best, since the slow increase with higher concentrations is attributed to precipitation of the higher proteoses.

The free amino nitrogen of this precipitate is considered to be a measure of the amount of fully built up protein present. In several proteins Wilson (7) found this to range from 2-5 per cent. of the total nitrogen. Van Slyke demonstrated that it corresponds to the amount of the nitrogen in the form of the ϵ group of lysine. Calculating from my Van Slyke analyses of albumin and globulin (which are preliminary), and assuming equal amounts of each, the value on these assumptions should be 4·52 per cent. Wilson (7) showed that hydrolysis occurred in the determination of the free amino-nitrogen by means of the Van Slyke method, so that it is not entirely satisfactory. The result of my determinations of the free amino nitrogen of about 5 per cent. (duplicates 5·01 per cent. and 4·85 per cent. with 15 minutes' shaking at 17° C.) indicates that the major part of the trichloracetic precipitate consists of fully built-up protein. It is interesting to note that the use of the ratio of free amino to total nitrogen as a measure of the complexity of protein degradation products was introduced by Horace Brown (8) and that the Van Slyke method is a modification of his method.

Proteoses, etc.

No method for the estimation of proteoses and other intermediate protein breakdown products was described in the First Report (1).

In the experiments carried out this year an estimation of the amount of nitrogen in this form has been obtained by a difference method. It represents the difference between the amount of nitrogen left in solution by trichloracetic acid, and that left in solution by the copper hydroxide precipitation. Such a difference method is unsatisfactory, and in one barley a small negative result was obtained. The figures obtained, however, probably have a relative value. The method used by Luck (6) appears to be preferable. He adds kaolin to the solution after trichloracetic acid precipitation (of liver and muscle extracts), and states, with references and experiments to support it, that kaolin adsorbs proteoses and peptones, but does not adsorb amino-acids. It is difficult to test critically this specificity with barley extracts, but a determination by the Van Slyke method of the free amino-nitrogen of the compounds adsorbed under these conditions indicates that the claim is substantially correct. The amino nitrogen was 10·7 per cent. of the total nitrogen (after 15 minutes' shaking at 17° C.). Wilson (7) found 8 per cent. of the nitrogen of proteoses as free amino nitrogen and 27 per cent. with peptones. It is proposed to use the kaolin adsorption method in future, and discard the copper precipitation. Comparative estimations of the proteoses by the difference method and by kaolin adsorption showed that the latter gave higher results with a corresponding reduction of the "non-protein" or amino-acid nitrogen.

The following section gives details of the procedure which has been followed in all the analyses discussed in the Third Report, together with a modification to be used in the future. They include the methods adopted in the First Report (1) with additions and slight modifications in procedure. It is considered that the fractionations adopted are sufficient to give sound estimates of the amounts of nitrogen in different states of complexity.

Details of the Methods used for the Quantitative Estimations of the Proteins of Barley and Malt.

Thousand corn weight is determined in triplicate on 20 grm. samples.

The dry grain (10 per cent. moisture or less) is finely ground in a "coffee" mill, and re-ground in a "Wiley" mill, using the $\frac{1}{2}$ mm. sieve.

Moisture and total nitrogen determinations are made as described in the First Report (p. 109).

Salt Extraction.

Exactly 10 grms. of material is stirred with 70 ccs. of 5 per cent. potassium sulphate solution (p_H 5-6) in a 100 cc. centrifuge tube. This is shaken for 15 minutes, and then centrifuged for 5 minutes at 2,500 revs. per min. The supernatant liquid is filtered through cotton wool into a 500 cc. flask containing a little toluene. The extraction of the residue is repeated in the same way to give five extractions in all. The resulting

extract is made up to 500 cc. and 100 cc. taken for determination of total nitrogen.

Albumin Nitrogen—140 cc. of the extract is buffered to p_H 4·6 by adding 10 cc. of acetate-acetic buffer (equal volumes of normal solutions of acetic acid and of sodium acetate). The solution is allowed to stand for 1 hour, and is then filtered through a No. 41 Whatman filter paper into a 200 cc. boiling tube. This tube has previously been carefully cleaned with chromic acid. The tube is placed for 20 mins. in a sloping position half immersed in a bath maintained at 82° C. Under these conditions, a steady circulation is maintained, and large even coagula are formed which do not adhere to the tube. The precipitate is filtered off through a No. 41 Whatman filter paper, and washed with hot water. The nitrogen content of the precipitate is then determined, and the necessary blank for the filter paper, etc., deducted.

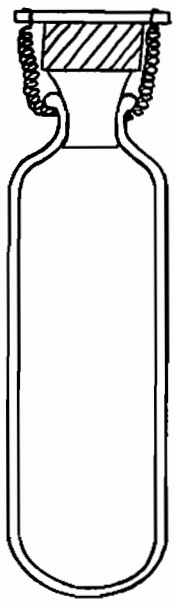
Total Protein.—10 cc. of 25 per cent. trichloracetic acid solution are added to 100 ccs. of extract in a 150 cc. centrifuge flask. This is allowed to stand 1 hour, and is then centrifuged. The nitrogen is determined in the precipitate (total protein) and in the solution. The sum of these amounts should be equal to the total salt-soluble nitrogen.

Globulin Nitrogen.—An estimate can be obtained of the globulin nitrogen by subtracting the albumin nitrogen from the total protein nitrogen.

"Non-protein" Nitrogen.—This was determined as described in the First Report (p.110).

Proteose and Peptone Nitrogen.—An estimate of the nitrogen in this form has previously been obtained by subtracting the "non-protein" nitrogen from that left in solution by trichloracetic acid.

DIAGRAM III.



In future it is proposed to dispense with the copper precipitation previously employed for determining the non-protein nitrogen, and proceed as follows:

The trichloracetic acid precipitation is carried out in duplicate, the nitrogen in the precipitate giving, of course, total protein. The clear solution, after centrifuging, is poured into another 150 cc. centrifuge flask, and 2 grms. of acid-washed kaolin added, well shaken and centrifuged off. The duplicate fractions are combined for the nitrogen determinations. The nitrogen adsorbed on the kaolin represents proteose and peptone nitrogen (a blank determination of the nitrogen in the kaolin must be made). The nitrogen in solution represents the nitrogen in the form of simple compounds, such as amino-acids (grouped under the term "non-protein" nitrogen).

In the shortened form of the estimation the total nitrogen of the salt extract is determined in duplicate on 200 ccs. samples and fractionations are not made.

TABLE 4.

ACCURACY OF KJELDAHL ESTIMATIONS OF FRACTIONS.

Sample No.	cc. of standard acid (approx. N/10) required in the estimation of								
		Salt Extract. Total nitrogen per 100 cc.							
	Total Nitrogen.	Direct	From Copper pptn.	From trichlor-acetic acid pptn.	Copper ppt. (ex 140 cc.)	Copper soln. (ex 140 cc.)	Trichlor-acetic acid ppt. (ex 100 cc.)	Trichlor-acetic acid soln. (ex. 100 cc.)	Alcohol Extract (100 cc.)
1	19·44 19·39	5·63	5·66	5·75	5·25	2·68	2·77	2·98	12·55 12·56
2	19·78 19·69	6·92	6·92	6·88	5·95	3·73	3·20	3·68	11·93 11·81
3	18·85 18·89	8·55	8·67	8·68	6·91	5·23	3·47	5·21	9·58 —

The sample numbers refer to the first three samples in malting expt. II.

Hordein Nitrogen.

The alcoholic extraction is carried out as described in the First Report (1), but a more convenient type of centrifuge tube and fittings is used. The arrangements can be seen in Diagram III. (p. 321) The modified form of tube is stronger than that previously described, and springs are used to hold the rubber stopper in position instead of the screw in the older form.

ACCURACY OF THE METHODS.

The individual values obtained in each analysis are all checked (except the albumin value) either directly by duplicates or indirectly. Examples showing the order of agreement found are given in Table 4 (p.321.)

The accuracy which is attained in any single set of estimations has been checked by repeating the extractions from the beginning on fresh samples. Table 5 gives some of these duplicate results from which an idea of the probable error of any determination can be gained.

TABLE 5.
ACCURACY FROM REPLICATES OF EXTRACTIONS.

	Nitrogen per 1,000 corns in the form of								
Malting Expt. III.	Total Nitrogen.	Salt soluble.	Hordein	Glutelin	Albumin	Non-protein	Total protein.	Globulin	Proteose
Sample 1.									
a	—	·279	·258	·270	·101	·132	·170	·070	—·023
b	·807	·272	·264	·264	·097	·131	·178	·081	—·026
Average	—	·276	·261	·267	·099	·132	·174	·076	—·025
Sample 2.									
a	—	·275	·259	·261	·085	·093	·173	·088	·012
b	·795	·268	·265	·263	·093	·089	·154	·060	·021
Average	—	·272	·262	·262	·089	·091	·164	·074	·017
Sample 3.									
a	—	.294	·247	·242	—	·107	·172	073*	·012
b	·783	.286	·251	·237	·099	·121	·170	·071	·004
Average	—	.290	·249	·240	—	·114	·171	·072	·008

* Using albumin value from (b).

SUMMARY.

The conditions and accuracy of the methods, described in the First Report, for the estimation of the separate proteins of barley, have been checked and slightly modified, and the methods have been amplified with the object of making a study of malting.

It is concluded that Osborne and Campbell's "bynin" in malt can be regarded as almost, if not quite, the same as the hordein of barley, and the distinction has been dropped. It is considered better to drop also the term "bynedestin" and refer to barley globulin throughout.

The quantities and conditions employed for the extraction of the proteins of barley have been found satisfactory also when applied to malt.

The more detailed fractionation of the salt-soluble constituents has been examined experimentally and standardised conditions of procedure are described.

Determinations of the ammonia nitrogen percentage of the various fractions give evidence of the purity of the protein estimated. Results are given showing that checks and duplicates agree closely.

The great importance of fineness and evenness of grinding has been demonstrated, and the best method found is described.

I am very grateful to Dr. A. C. Chibnall for his supervision during the course of this work.

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