THE CHARACTERIZATION BY GEL ELECTROPHORESIS OF THE PROTEINS EXTRACTED BY DILUTE ALKALI FROM WHEAT FLOUR

By A. C. JENNINGS*

[*Manuscript received February* 2, 1968]

Summary

Procedures have been developed for characterizing, in a chemically unaltered form, the proteins usually extracted in dilute alkali from wheat flour or endosperm.

The electrophoretic zone patterns of the proteins migrating to the cathode in this fraction were qualitatively identical with those of a previously described high-speed supernatant fraction of wheat endosperm. In addition, there was a slow movement of proteins to the anode.

Intermolecular disulphide bonds do not appear to be present in flour or formed during the preparation of a dough.

It is concluded that most of the proteins previously considered to be of cytoplasmic origin are storage proteins. The following mechanism is proposed for the formation, in developing endosperm, of the protein fraction extracted by alkali. The proteins soluble in pyrophosphate buffer are neutralized by proteins, or other compounds, with a net negative charge at physiological pH values and which are synthesized in stoichiometric amounts. These complexes may then interact mainly through hydrophobic bonds to form aggregates insoluble in dilute aqueous buffers and dilute acids.

It is concluded that there are two types of storage proteins, synthesized by separate systems. One type is characterized, in gel electrophoresis, by relatively low mobilities, the other by relatively high mobilities.

I. INTRODUCTION

The proteins extracted from wheat flour by dilute salt solutions or by weak acids are readily resolved into zone patterns by gel electrophoresis but attempts to characterize, in a chemically unaltered state, those proteins normally soluble only in dilute alkali have been unsuccessful (Elton and Ewart 1960, 1962; Coulson and Sim 1961; Woychik, Boundy, and Dimler 1961; Graham 1963; Lee 1963).

This paper describes the resolution into zones of all the proteins of wheat flour by gel electrophoresis.

II. MATERIALS AND METHODS

Flour was prepared from grain of *Triticum vulgare* cv. Gabo and *T. durum* cv. Dural. A commercial sample of Queensland flour and a freeze-dried flour–water dough prepared from it were kindly supplied by Mr. M. V. Tracey, Wheat Research Unit, CSIRO, Sydney. Endosperm was dissected from immature grain of *T. vulgare* cv. Gabo and cv. Insignia and *T. durum* cv. Dural by the procedure of Jennings and Morton (1963*a*).

* Department of Agricultural Biochemistry and Soil Science, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, S.A. 5064.

Phenol and glacial acetic acid were redistilled before use. All other reagents were of A.R. quality. All chemical analyses were carried out by the procedures of Jennings and Morton (1963*a*, 1963*b*).

(*a*) *Preparation of Extracts*

(i) *Extract A*

Portions (1 g) of the freeze-dried dough and the flour samples were extracted with three portions (total volume of 10 ml) of phenol–acetic acid–water (1:1:1, w/v/v) containing 0·2M NaBr (Gallus and Jennings 1968). The suspensions were cooled in an ice–water bath then treated ultrasonically for 30 sec with a Branson Sonifier (model LS-75, Branson Instruments Inc., Stamford, Connecticut, U.S.A.). The three extracts were combined (extract A).

(ii) *Extracts B and C*

Portions of the freeze-dried dough and the flour samples were extracted with three portions (total volume 50 ml) of 0·05N acetic acid containing 2M urea, and treated ultrasonically as for extract A (extract B). Phenol–acetic acid (1:1, w/v) was added to the residues to give a ratio of phenol–acetic acid–water of about 1:1:1, w/v/v (Gallus and Jennings 1968). The residues were then extracted (as above) with 25 ml of the phenol–acetic acid–water solvent containing NaBr (extract C).

(iii) *Extracts D, E, and F*

The immature endosperm preparations were homogenized in a French pressure cell (French and Milner 1955) and extracted with n-butanol. The residues, the freeze-dried dough, and the flour samples were each extracted with one portion of n-butanol saturated with water, one portion of pyrophosphate buffer (extract D), and three portions of dilute acetic acid (extract E) by the procedure of Coates and Simmonds (1961). The residues were extracted with three portions of phenol–acetic acid–water (1:1:1, w/v/v) (extract F) instead of 0·1N NaOH, and then washed with ethanol and dried.

(*b*) *Fractionation of Developing Endosperm*

The endosperm of cv. Gabo (grown in 1959) was fractionated by the procedure of Graham, Morton, and Simmonds (1963).

(*c*) *Gel Electrophoresis*

The apparatus used was as described by Graham (1963). The 0·017M aluminium lactate buffer (pH 3·2) used for the preparation of gels and in the electrode vessels was prepared by the procedure of Graham (1963).

A starch gel was prepared by mixing, in a 2-litre Buchner flask, 70 g of hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) and 168 g of urea (May and Baker Ltd., Dagenham, England) with 350 ml of the aluminium lactate buffer.* The mixture was heated in a boiling water-bath, with mixing, until a viscous homogeneous solution was obtained (Smithies 1955). This was boiled under vacuum for about 15–30 sec. After pouring, the gel was allowed to stand for 16 hr before use. The final composition of the gels was about 16·5% (w/v) starch and 6·4M urea in 0·014M aluminium lactate buffer (pH 3·2).

The samples were adsorbed on pieces of Whatman 3MM paper which were subsequently inserted into the gel. This was the only loading procedure which gave satisfactory gel patterns from the phenol–acetic acid–water extracts. However, the zone patterns obtained by this loading procedure were, generally, not as distinct as those obtained when aqueous extracts were loaded into slots in the gel (Graham 1963).

* The urea was not purified further before use since Marier and Rose (1964) have shown that cyanate in urea solutions is rapidly decomposed below pH 5·5. Cole and Mecham (1966) found only minor effects on zone patterns of wheat proteins separated in urea–starch gels in aluminium lactate buffer (pH 3·1) or, indeed, at pH 9. The identity of zone patterns from different extracts and not the relative arrangement of zones is the important feature of this work.

The electrophoretic separations were carried out with the gel in a horizontal position at the temperature of tap water (about 12°C) at voltage gradients of 6–10 V/cm for 4–6 hr. The two halves of the gel (Graham 1963) were stained in 0·125% (w/v) nigrosin (British Drug Houses, Ltd., Poole, England) in methanol–water–acetic acid (8:12:1 by vol.) (Hermans *et al.* 1960). This solvent composition caused less shrinkage of the gels and gave clearer backgrounds than that used by Graham (1963). The gels were not agitated during staining or washing in methanol–acetic acid–water (5:1:5 by vol.). The pieces of paper at the origin were stained with nigrosin (Gallus and Jennings 1968) to determine whether all the proteins had migrated from the origin.

III. Results

Extract A contained between 97·3% (for cv. Dural) and 98·4% (for the freeze-dried dough) of the total nitrogen. Extract B contained about 75% of the total nitrogen while about 4·7% (for cv. Dural)–3·2% of the total nitrogen was left in the residues remaining after the preparation of extract C.

The distribution of nitrogen between the individual extracts (D, E, and F) obtained in the modified extraction procedure of Graham, Morton, and Simmonds (1963) are shown in Table 1 for the freeze-dried dough and the flour samples, and

Table 1

PERCENTAGES OF THE TOTAL NITROGEN FOUND IN EACH EXTRACT AND THE RESIDUE AFTER FRACTIONATION OF A FREEZE-DRIED DOUGH AND FLOUR SAMPLES FROM VARIOUS SOURCES

The nitrogen contents of the extracts made with water-saturated n-butanol were not determined

Fraction*	Percentage of Total Nitrogen in Extract from:			
	Flour from cv. Gabo	Flour from cv. Dural	Queensland Flour	Freeze-dried Dough
Extract D	7·9	6·1	6·8	6·1
Extract E				
1	55·2	35·2	42·6	38·7
2	22·2	31·9	28·5	36·1
3	3·6	4·6	4·3	5·1
Total	81·0	71·8	75·4	79·9
Extract F				
1	10·2	15·5	10·5	9·9
2	2·5	4·0	3·2	3·4
3	0·6	0·4	0·7	0·7
Total	13·3	19·9	14·4	14·1
Residue	2·2	2·9	1·6	1·1
Recovery of N (%)	104·4	100·7	98·2	101·2

* For experimental details, see Section II(*a*).

Table 2 for the immature endosperm. The amounts of protein in extracts F were about the same or less than those in the alkali extracts made by Coates and Simmonds (1961), Bell and Simmonds (1963), and Graham, Morton, and Simmonds (1963).

The results of the fractionation of the developing endosperm of cv. Gabo (grown in 1959) are shown in Figures 1 and 2.

In all electrophoretic separations, provided the amounts of protein loaded onto the gels were not too large, the appearance of the origin slot surfaces and of the pieces of filter paper after staining with nigrosin indicated that there was very little, if any, protein left at the origin.

TABLE 2

PERCENTAGE OF TOTAL NITROGEN IN EXTRACTS AND THE RESIDUE AFTER FRACTIONATION OF IMMATURE ENDOSPERM

Nitrogen contents of the extracts made with n-butanol and water-saturated n-butanol were not determined. Values in parenthesis indicate the number of days after flowering of sampling the endosperm

Fraction*	Percentage of Total Nitrogen in Extracts of Endosperm			
	cv. Dural (9 days)	cv. Dural (29 days)	cv. Insignia (9 days)	cv. Gabo (18 days)
Extract D	27·5	4·2	17·5	20·0
Extract E				
1	11·7	48·0	3·6	14·9
2	4·1	4·0	2·7	4·1
3	5·6	14·6	1·1	4·0
Total	21·4	66·6	7·3	23·0
Extract F				
1	30·8	19·0	20·8	21·1
2	7·3	8·2	6·7	4·6
3	1·4	1·4	1·1	1·0
Total	39·5	28·6	28·6	26·6
Residue	1·5	3·5	0·5	1·3
Recovery of N (%)	89·9	102·9	53·8	70·9

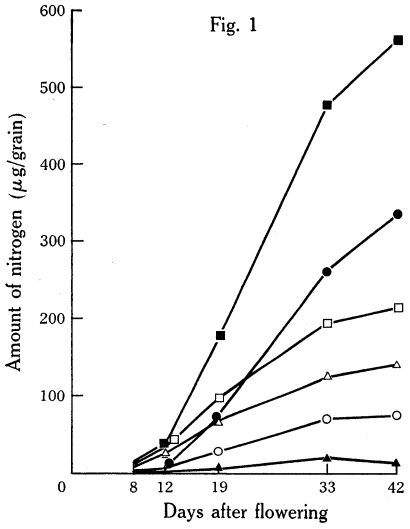
* For experimental details, see Section II(*a*).

To simplify the presentation of the results and their discussion, the zone patterns can be divided into three regions. Region 1 contains those fast-moving zones due to the proteins predominantly present in the 0·01M sodium pyrophosphate buffer (pH 7·4) extracts while region 2 encompasses the slow-moving zones due to the proteins found in greatest amount in the 0·05N acetic acid extracts (Graham 1963). Region 3 contains those zones due to proteins which migrate towards the anode.

The zone patterns obtained in this study differed slightly from corresponding patterns reported by Graham (1963) in that the slowest migrating zones in region 2 stained much more intensely. It was observed that the nigrosin did not penetrate into the gel in these zones as readily as in the faster zones but tended to form a precipitate on the surface of the gel which was readily dislodged with continuous agitation.

It was found that, in all the gels used in this study, there were no apparent differences between the zone patterns given by the freeze-dried dough and the parent flour sample.

The proteins in extract A gave zone patterns in regions 1 and 2 which were indistinguishable from those obtained by Graham (1963) from water extracts of flour of the same variety or from those of extract B in this study. In addition, some proteins present in extract A migrated very slowly towards the anode (in region 3) as a single narrow zone.



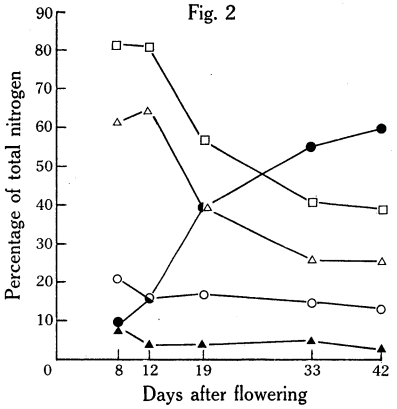


Fig. 1.—Amounts of protein nitrogen in the pyrophosphate (○), sodium hydroxide (△), combined pyrophosphate and sodium hydroxide (□), acetic acid (●), residue (▲), and total (■) fractions obtained from the endosperm of cv. Gabo during development (in 1959) by the extraction procedure of Graham, Morton, and Simmonds (1963). The horizontal axis shows the times of harvest after the mean date of flowering.

Fig. 2.—Percentage of the total nitrogen in the pyrophosphate (○), sodium hydroxide (△), combined pyrophosphate and sodium hydroxide (□), acetic acid (●), and residue (▲) fractions obtained from the endosperm of cv. Gabo during development (in 1959). Other details as for Figure 1.

It was consistently observed that extract A appeared to contain relatively more of the proteins in region 1 than did extract B.

The proteins in extract C gave similar qualitative zone patterns in region 1 to those shown by extracts A and B, although quantitative differences were apparent. Distinct zones were not apparent in region 2, although there was some faint general overall staining. The zone pattern in region 3 was similar to that shown by extract A.

Extracts D and E gave typical zone patterns (Graham 1963) while extract F gave rather similar zone patterns to extract C, except that region 2 appeared to be almost free of proteins.

Separation of the proteins of extracts C, D, and F in gels of different buffer compositions did not show any significant differences between their zone patterns,

except in alkaline buffers, where much more of the proteins in extract F migrated towards the anode. However, the effect on electrophoretic mobility of phenolate ions adsorbed to proteins was not determined. It was also apparent that extract F contained more of the proteins with the highest electrophoretic mobilities than did extract D.

IV. Discussion

The anomalous effects noted in the staining of the proteins which migrate most slowly towards the cathode at pH 3 suggest that they are of relatively low molecular weight, thus allowing ready diffusion from the gel. This would account for the apparent absence, or presence in rather low amounts, of these proteins in gels which are agitated during staining (Graham 1963). Oh, Sanders, and Gehrke (1966) have also suggested that these proteins may have low molecular weights.

The results in Figures 1 and 2 support the conclusions of earlier authors (Woodman and Engledow 1924; McCalla 1938; Bilinski and McConnell 1958; Morton and Raison 1964) that synthesis of the proteins usually extracted by dilute acetic acid (or the gliadin fraction) commences later than, and proceeds independently of, that of the other endosperm proteins. [The data of Graham, Morton, and Simmonds (1963), when recalculated on the bases used in Figs. 1 and 2, shows the same general relationship.]

In addition, the present results (Figs. 1 and 2) are consistent with a precursor–product relationship between the proteins extracted by pyrophosphate buffer and dilute alkali. The qualitative similarities in the zone patterns shown by these protein fractions (extracts D and F) in immature and mature endosperm provide further evidence for this. Some of the results obtained by Graham and Morton (1963) and Graham, Morton, and Raison (1964), while studying the incorporation of amino acids labelled with radioactive isotopes into endosperm proteins, also appear to support this conclusion.

Graham, Morton, and Raison (1963) concluded that the incorporation of amino acids into their "high-speed supernatant" fraction (or proteins extracted by pyrophosphate buffer) preceded that into the small "protein bodies". These protein bodies had an amino acid composition rather similar to that of the proteins extracted by dilute alkali from the same preparation of endosperm (Jennings and Morton 1963*c*). Graham, Morton, and Raison (1963) and Morton, Raison, and Smeaton (1964) also found that their protein body preparations contained significant amounts of proteins which were soluble in pyrophosphate buffer.

Thus it is concluded that most of the proteins extracted by dilute alkali are identical with proteins extracted by pyrophosphate buffer.

It may be considered that the protein fraction extracted by dilute alkali is an artefact produced during the fractionation procedure. However, the differences in specific radioactivity between the protein fractions soluble in pyrophosphate buffer and dilute alkali found by Graham and Morton (1963) and Graham, Morton, and Raison (1964) makes this unlikely.

The chemical and cytological evidence (Jennings and Morton 1963*b*; Jennings, Morton, and Palk 1963) would support the suggestion that, in the earliest stages of development (Figs. 1 and 2), the proteins extracted by dilute alkali are derived from the rather large amounts of lipoprotein membrane material in the endosperm cells. However, the zone patterns of extract F from endosperm harvested 9 days after flowering and from the corresponding flour were qualitatively the same.

The observation that the protein fractions extracted by pyrophosphate buffer and by dilute alkali are qualitatively identical (with regard to the proteins with a net positive charge at pH 3) would account for the reported presence of salt-soluble proteins in washed gluten (Pence, Mecham, and Olcott 1956) and in gluten prepared and purified by more elaborate techniques (Gehrke, Oh, and Freeark 1964; Woychik, Huebner, and Dimler 1964; Elton and Ewart 1966). It also explains why, after repeated extractions with pyrophosphate buffer, proteins usually extracted by this solvent are detected in dilute acetic acid extracts (Graham and Morton 1963; Graham, Morton, and Raison 1963). This relationship also casts doubt on the assumption of Graham, Morton, and Raison (1964) that the proteins which migrated in gels in region 1 in their pyrophosphate extracts and high-speed supernatant fractions are cytoplasmic proteins. While cytoplasmic proteins undoubtedly occur in these fractions, it is likely that the proteins present in sufficient concentrations to give the observed zone patterns are storage proteins.

Since the amounts of protein extracted into each fraction were similar (Table 1) and the zone patterns shown by similar fractions of the freeze-dried dough and the parent flour were identical, it seems unlikely that irreversible aggregation of proteins occurred during the preparation of the dough. This is in agreement with the conclusions of Stanley, Jennings, and Nicholas (1968), who found that the weight average molecular weights of the proteins in extract F were less than 30,000. Thus it seems unlikely that the formation of intermolecular disulphide bonds can account for the incorporation of the proteins soluble in pyrophosphate buffer into the fraction normally extracted by dilute alkali.

The presence of proteins with a net negative charge at pH 3 in extracts A, C, and F and the ease of dispersal of the proteins in extract F in phenol–acetic acid–water suggest a mechanism to account for this incorporation.

It is postulated that the proteins soluble in pyrophosphate buffer are neutralized by proteins, or other material, with a net negative charge at the pH of the cell contents and which are produced in stoichiometric amounts. The neutralized proteins then may interact mainly through hydrophobic bonds to form stable, insoluble aggregates. It is suggested that hydrophobic rather than hydrogen bonds are involved because of their greater stability in aqueous solvents (Nemethy and Scheraga 1962; Scheraga 1963). Also, these aggregates do not dissolve in urea solutions (see above and Daftary and Pomeranz 1966) but dissolve incompletely in the presence of a detergent and urea (Meredith and Wren 1966) and completely in phenol–acetic acid–water.

It is perhaps pertinent that Jennings and Morton (1963*b*) found, in the developing endosperm, the phosphorus:nitrogen ratio in the protein fraction isolated by the procedure of Martin and Morton (1956) remained relatively constant from about

18 days after flowering, indicating the presence of phosphorylated groups in one or more of the proteins. Phospholipids may also be synthesized at an appropriate rate (Jennings and Morton 1963*b*).

No experimental evidence is available at present to account for the apparent differences in the ratios of proteins in extracts D and F.

It is concluded that there are two distinct types of storage proteins present in the wheat grain. One type is characterized by their solubility in dilute acetic acid, their low mobilities in gels (at pH 3), their high content of glutamine and proline, and their low content of basic amino acids (Jennings and Morton 1963*c*). The other type is characterized by their high mobilities in gels (at pH 3), a lower content of glutamine and proline, a higher content of basic amino acids (Jennings and Morton 1963*c*), their solubility in pyrophosphate buffer, and their aggregation into insoluble complexes, probably after interaction with proteins or other compounds with net negative charges produced in stoichiometric amounts.

The protein bodies (Jennings, Morton, and Palk 1963) undoubtedly represent sites of accumulation of storage protein in the endosperm. Bulk preparations of these have been shown to contain proteins soluble in pyrophosphate buffer, dilute acetic acid, and dilute alkali (Morton, Raison, and Smeaton 1964). Purified preparations of small protein bodies, with an amino acid composition rather similar to the protein fraction extracted with dilute alkali (Jennings and Morton 1963*c*), have been shown to contain proteins soluble in pyrophosphate buffer and dilute acetic acid (Graham, Morton, and Raison 1963). Since the two types of storage proteins are synthesized independently by separate systems, it is probable that individual proteoplasts (Morton and Raison 1963) contain only one of these two systems.

V. Acknowledgments

Milling of the flour samples was carried out by the Bread Research Institute of Australia, Sydney. The author wishes to thank Professor D. J. D. Nicholas for his interest in this work. Grateful acknowledgment is made of financial support from the Wheat Industry Research Fund.

VI. References

Bell, P. M., and Simmonds, D. H. (1963).—*Cereal Chem.* **40**, 121.
Bilinski, E., and McConnell, W. B. (1958).—*Cereal Chem.* **35**, 66.
Coates, J. H., and Simmonds, D. H. (1961).—*Cereal Chem.* **38**, 256.
Cole, E. G., and Mecham, D. K. (1966).—*Analyt. Biochem.* **14**, 215.
Coulson, C. B., and Sim, A. K. (1961).—*Biochem. J.* **80**, 46P.
Daftary, R. D., and Pomeranz, Y. (1966).—*J. Sci. Fd Agric.* **17**, 72.
Elton, G. A. H., and Ewart, J. A. D. (1960).—*Nature, Lond.* **187**, 600.
Elton, G. A. H., and Ewart, J. A. D. (1962).—*J. Sci. Fd Agric.* **13**, 62.
Elton, G. A. H., and Ewart, J. A. D. (1966).—*J. Sci. Fd Agric.* **17**, 34.
French, C. S., and Milner, H. W. (1955).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. I. p. 64. (Academic Press, Inc.: New York.)
Gallus, H. P. C., and Jennings, A. C. (1968).—*Aust. J. biol. Sci.* **21**, 1077.
Gehrke, C. W., Oh, Y. H., and Freeark, C. W. (1964).—*Analyt. Biochem.* **7**, 439.
Graham, J. S. D. (1963).—*Aust. J. biol. Sci.* **16**, 342.
Graham, J. S. D., and Morton, R. K. (1963).—*Aust. J. biol. Sci.* **16**, 357.

GRAHAM, J. S. D., MORTON, R. K., and RAISON, J. K. (1963).—*Aust. J. biol. Sci.* **16**, 375.
GRAHAM, J. S. D., MORTON, R. K., and RAISON, J. K. (1964).—*Aust. J. biol. Sci.* **17**, 102.
GRAHAM, J. S. D., MORTON, R. K., and SIMMONDS, D. H. (1963).—*Aust. J. biol. Sci.* **16**, 350.
HERMANS, P. E., MCGUCKIN, W. F., MCKENZIE, B. F., and BAYRD, E. D. (1960).—*Proc. Mayo Clin.* **35**, 792.
JENNINGS, A. C., and MORTON, R. K. (1963*a*).—*Aust. J. biol. Sci.* **16**, 318.
JENNINGS, A. C., and MORTON, R. K. (1963*b*).—*Aust. J. biol. Sci.* **16**, 332.
JENNINGS, A. C., and MORTON, R. K. (1963*c*).—*Aust. J. biol. Sci.* **16**, 384.
JENNINGS, A. C., MORTON, R. K., and PALK, B. A. (1963).—*Aust. J. biol. Sci.* **16**, 366.
LEE, J. W. (1963).—*Biochim. biophys. Acta* **69**, 159.
MARIER, J. R., and ROSE, D. (1964).—*Analyt. Biochem.* **7**, 304.
MARTIN, E. M., and MORTON, R. K. (1956).—*Biochem. J.* **64**, 221.
MCCALLA, A. G. (1938).—*Can. J. Res.* C 16, 263.
MEREDITH, C. B., and WREN, J. J. (1966).—*Cereal Chem.* **43**, 169.
MORTON, R. K., and RAISON, J. K. (1963).—*Nature, Lond.* **200**, 429.
MORTON, R. K., and RAISON, J. K. (1964).—*Biochem. J.* **91**, 528.
MORTON, R. K., RAISON, J. K., and SMEATON, J. R. (1964).—*Biochem. J.* **91**, 539.
NEMETHY, G., and SCHERAGA, H. A. (1962).—*J. phys. Chem.* **66**, 1773.
OH, Y. H., SANDERS, B. E., and GEHRKE, C. W. (1966).—*Can. J. Biochem.* **44**, 917.
PENCE, J. W., MECHAM, D. K., and OLCOTT, H. S. (1956).—*J. agric. Fd Chem.* **4**, 712.
SCHERAGA, H. A. (1963).—In "The Proteins". (Ed. H. Neurath.) Vol. II. p. 1. 2nd Ed. (Academic Press, Inc.: New York.)
SMITHIES, O. (1955).—*Biochem. J.* **61**, 629.
STANLEY, P. E., JENNINGS, A. C., and NICHOLAS, D. J. D. (1968).—*Phytochemistry* **7**, 1109.
WOODMAN, H. E., and ENGLEDOW, F. L. (1924).—*J. agric. Sci., Camb.* **14**, 563.
WOYCHIK, J. H., BOUNDY, J. A., and DIMLER, R. J. (1961).—*Archs Biochem. Biophys.* **94**, 477.
WOYCHIK, J. H., HEUBNER, F. R., and DIMLER, R. J. (1964).—*Archs Biochem. Biophys.* **105**, 151.