261. *The Mechanism of Carbohydrase Action. Part II.* α-Amylolysis of Linear Substrates.*

By W. J. Whelan and P. J. P. Roberts.

The end-products of the action of purified salivary α-amylase on amylose and the maltodextrins derived from it have been examined. Only two end-products are found, namely, maltose and maltotriose. This examination supports the theory of α-amylase action first advanced by Meyer and Bernfeld, which states that in an amylose-type chain, α-amylase can hydrolyse any except the two terminal α-1 : 4-linkages. The ratios of maltose to maltotriose which are produced by α-amylolysis of these substrates are found to be in exact agreement with values obtained by calculation on the basis of Meyer's theory. It is also demonstrated that the susceptible linkages in a given substrate molecule are all hydrolysed by α-amylase at the same rate.

The present investigation of the action of a purified amylase on the separated components of starch had the purpose of gaining an insight into the mechanism of α-amylolysis by examination and characterisation of the end-products.

Human saliva was chosen as the source of the enzyme, and the purification method of

* Part I, preceding paper.

Meyer, Fischer, Staub, and Bernfeld (*Helv. Chim. Acta*, 1948, **31**, 2158) was used, the enzyme being purified to the stage before crystallisation. A preliminary account of the present work, which is concerned with the amylose component of starch, has already been given (Roberts and Whelan, *Biochem. J.*, 1951, **49**, lvi; 1952, **51**, xviii). Studies on amylopectin and glycogen have been reported elsewhere (Whelan and Roberts, *Nature*, 1952, **170**, 748). In Fig. 1 is demonstrated the action of salivary α-amylase (referred to subsequently as α-amylase) on potato amylose as measured by the development of reducing power. The reaction was of the first order and ceased abruptly at an apparent conversion into maltose of 90·8%. At this stage the enzyme was still active and no further reaction occurred when fresh enzyme was added. In large-scale experiments, considerable difficulty was experienced because of the precipitation of amylose when the enzyme was added. This effect was largely eliminated by the addition of sodium chloride to the digest.

After the attainment of the limiting conversion, a digest of amylose (3·023 g.) was heated to deactivate the enzyme, concentrated, and a portion of the products, equivalent to 2·708 g. of amylose, was fractionated on a charcoal–Celite column as described by Bailey, Roberts, and Whelan (preceding paper). The progress of the fractionation is recorded in Fig. 2. Perfusion of the column with water yielded no monosaccharide fraction although the method of determining the sugars, namely by measurement of optical rotation, was sufficiently sensitive to detect 10 mg. of glucose. Elution of the column with 7·5% ethanol

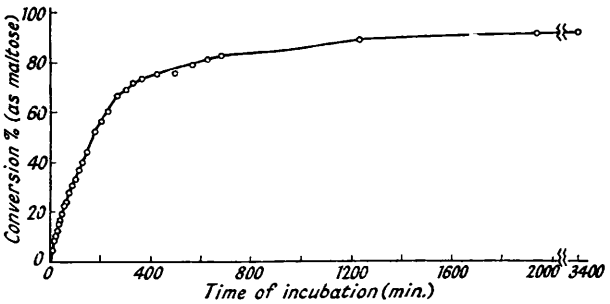


FIG. 1. *α-Amylolysis of potato amylose at* pH 7·0.

extracted maltose, identified by its β-octa-acetate, and 15% ethanol eluted maltotriose, identified as its β-undeca-acetate (Table 1) and by hydrolysis with β-amylase (Table 3). The products were also compared with authentic samples. These sugars were apparently the sole products since elution with higher concentrations of alcohol yielded no other substance (Fig. 2). The combined yield of maltose and maltotriose was equivalent

TABLE 1. *Properties of α-Limit Dextrins from Amylose.*

	Yield,			β-Acetate,*†			
Sugar	g.	10^{-3} mole	$[\alpha]_D$, in H_2O	m. p.	$[\alpha]_D$, in $CHCl_3$	R_F ‡ value	Reduction § equiv.
Maltose (anhydrous)	1·623	4·745	(*a*) 136°	159—160°	+63°	0·41	0·235
			(*b*) 136	159—160	+62·6	0·41	0·230
Maltotriose	0·999	1·983	(*a*) 160	134—136	+86	0·38	0·346
			(*b*) 160	135—137	+85	0·38	0·343

* From 2·708 g. of amylose.
† See Wolfrom *et al.*, *J. Amer. Chem. Soc.*, 1949, **71**, 2874.
‡ In pyridine–fusel oil–water (1 : 1 : 1).
§ Expressed as mg. of sugar/ml. of 0·005N-thiosulphate, the Somogyi copper reagent being used (*J. Biol. Chem.*, 1945, **160**, 61).
(*a*) Values for authentic specimens; (*b*) experimental values.

to 92·4% of the amylose hydrolysed. Elution of maltose and higher saccharides from a charcoal column is not, however, quantitative (see later), but the losses of maltose and maltotriose are almost the same. When the two sugars are mixed in the proportion found in the above experiment, the mixture has a reducing power equivalent to 88·9% of apparent maltose. The reducing power observed at the end of the reaction was 90·0% of maltose.

The fact that the only detectable products of α-amylolysis of potato amylose are maltose and maltotriose may be held to conflict with the demonstration of the presence of β-linkages in potato amylose by Peat, Thomas, and Whelan (*J.*, 1952, 722). The presence of these

linkages is made manifest by the incompleteness (*ca.* 70%) of the β-amylolysis of amylose but inasmuch as one such linkage per amylose chain would suffice to account for the fact that β-amylolysis is incomplete and as the molecule of potato amylose contains about 1000 glucose residues (Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488) the yield of, for example, a disaccharide containing the β-linkage need not be greater than 0·2% of the weight of amylose used.

The problem now arises of interpreting the action of α-amylase in terms of its products. Whilst this work was in progress, Meyer and Gonon (*Helv. Chim. Acta*, 1951, **34**, 294) reported a study of the action of two α-amylases on amylose in which it was shown, by differential fermentation with yeast, that glucose, maltose, and unfermentable material were produced. The last-named substance (presumed to be maltotriose) was slowly hydrolysed by the α-amylases, yielding glucose and maltose; the reaction was not, however, carried to completion. Glucose and maltose were therefore stated to be the ultimate end-products. These results were deemed to support the hypothesis advanced by Meyer and Bernfeld (*Helv. Chim. Acta*, 1941, **24**, 359E) to the effect that α-amylase acts in a random fashion on all the α-1 : 4-links in an amylose-type chain except only such as are terminal linkages. If this is the course of events then amylose must of necessity give rise to maltose and maltotriose as end-products since both sugars contain only terminal linkages. Meyer

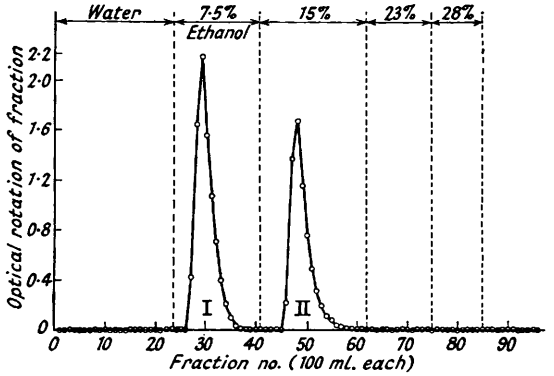


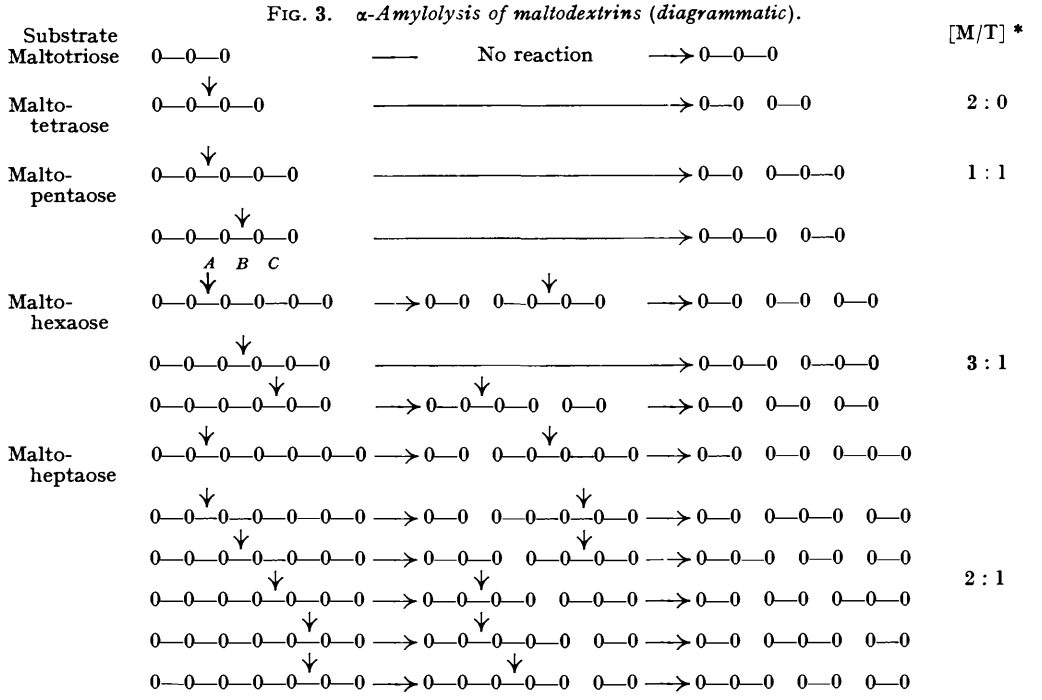
FIG. 2. *Charcoal fractionation of amylose α-limit dextrins.*
I, Maltose. II, Maltotriose.

and Gonon's results would appear to be the same as those reported by us except that the crystalline enzymes used by the Swiss authors seem to effect the further hydrolysis of maltotriose to maltose and glucose. This observation is obviously at variance with the proposed theory of α-amylase action if the crystalline α-amylases are to be regarded as enzymically pure. Our amorphous preparation of salivary amylase has been repeatedly tested for action on maltotriose but no evidence of hydrolysis has been observed (Table 3). Descloux, quoted by Meyer and Gonon (*loc. cit.*), has been able, by a mathematical treatment of α-amylase action, to calculate the maltose : glucose ratio at the end of the reaction for amylose-type chains of any length. On the same mathematical basis we have calculated the relative amounts of maltose to maltotriose which result when the latter is not further hydrolysed. In the case of amylose the calculation leads to a molar ratio of maltose : maltotriose of 2·35 : 1. The molar ratio found by experiment was 2·39 : 1 (Table 1), in excellent agreement with the calculated value.

Further evidence in support of Meyer's conception of α-amylase action comes from an examination of the yields of maltose and maltotriose produced by α-amylolysis of the maltodextrins, the linear dextrins of low molecular weight isolated from acid-hydrolysed amylose as described in the preceding paper. Since the calculated ratios of maltose : maltotriose for the dextrins differ widely, it is possible to subject the above hypothesis to a rigorous test. These ratios may be calculated by the method quoted above or obtained by inspection. The inspection method is illustrated in Fig. 3 for the series of dextrins from maltotriose to maltoheptaose and consists in the consideration of all possible ways in which the dextrin may be hydrolysed and summation of the amounts of maltose and maltotriose which would result.

In studying the α-amylolysis of the maltodextrins about 30 mg. of each dextrin were used. The dextrin was hydrolysed to completion by the enzyme and a portion of the digest was fractionated on a small charcoal–Celite column. The recoveries of maltose and

FIG. 3. *α-Amylolysis of maltodextrins (diagrammatic).*



* [M/T] = molar ratio [maltose : maltotriose].

maltotriose from an artificial mixture (28 mg.) were 94·4 and 95·2%, respectively. Maltotriose, as mentioned earlier, was not attacked by the α-amylase, maltotetraose gave only maltose, while malto-pentaose, -hexaose, and -heptaose were each degraded to mixtures of maltose and maltotriose. The corrected yields of maltose and maltotriose, expressed as molar ratios, are shown in Table 2, and are compared with the calculated ratios. The observed reducing powers of the digests after α-amylolysis are also compared with predicted values for the particular mixture of maltose and maltotriose. In every case the results confirm the validity of the hypothesis.

TABLE 2. *α-Amylolysis of maltodextrins; maltose and maltotriose ratios.*

	Calc. values		Exptl. values	
Maltodextrin	% Conversion (as maltose)	[M/T] *	% Conversion (as maltose)	[M/T] *
Maltotriose	0·0	—	0·0	—
Maltotetraose	100·0	2·00 : 0	99·4	2·00 : 0
Maltopentaose	80·0	1·00 : 1	79·4	1·00 : 1
Maltohexaose	88·8	3·00 : 1	86·1	2·98 : 1
Maltoheptaose	85·7	2·00 : 1	84·6	2·00 : 1

* [M/T] = molar ratio [maltose : maltotriose].

It should be noted that the assumption is implicit in all these calculations that in a given substrate each link which may be attacked by α-amylase is hydrolysed at the same rate. The demonstration, by the above method, of the mode of action of α-amylase provides at the same time evidence which justifies this assumption. Our argument may be illustrated by the case of maltohexaose. α-Amylase can hydrolyse the three linkages, marked *A*, *B*, and *C* in Fig. 3. If *B*, being the most remote from the chain ends, is more easily hydrolysed than *A* or *C*, as is postulated by Myrbäck and Sillen (*Svensk Kem. Tid.*, 1944, **56**, 60), then the ratio maltose : maltotriose should be less than 3 : 1, since hydrolysis

of link *B* gives rise only to maltotriose, which is not futher hydrolysable. These experiments shed no light on the question as to whether the rate of hydrolysis of a linkage is the same in substrates of different molecular size and this question is being examined by other methods. Myrbäck and Magnusson (*Arkiv Kemi, Mineral., Geol.*, 1945, **20**, *A*, No. 14) claim that the end linkages (which are resistant to α-amylase) are more readily split by acid than are the internal links, all of which are presumed to be hydrolysed at the same rate.

Experimental

Preparation of Salivary α-Amylase.—The method of Meyer *et al.* (*Helv. Chim. Acta*, 1948, **31**, 2158) was used with the following variation. Human saliva (750 ml.) was purified as described to the stage of the first precipitation with ammonium sulphate at pH 8·0. The precipitate was then dissolved in water (40 ml.) and 0·2M-citrate buffer (pH 7·0; 20 ml.) and freeze-dried. The resulting preparation was a white, slightly hygroscopic powder, stable for an indefinite period when stored in the refrigerator.

Preparation of Potato Amylose.—The method given in *J.*, 1951, 801, was used.

Preparation of Maltodextrins.—The method recorded in the preceding paper was used.

Small-scale Digestion of Amylose.—Potato amylose (B.V., 1·41; 115·2 mg.) was dissolved in hot 0·1N-sodium hydroxide, the solution was cooled and neutralised with 1N-sulphuric acid, and 0·2M-citrate buffer (pH 7·0; 10 ml.) was added. α-Amylase solution (1 mg.; 1 ml.) and water to a total of 100 ml. were added, and the digest was incubated at 35°. An immediate partial precipitation of the amylose occurred. At intervals portions (5 ml. each) of the digest were removed for the measurement of reducing power as maltose, the Somogyi copper reagent (*J. Biol. Chem.*, 1945, **160**, 61) being used. A control digest from which amylose was absent was used to determine the reducing power of the other digest components. After 24 hours a portion of the digest was removed and centrifuged, and the supernatant solution (10 ml.) was hydrolysed with acid (Pirt and Whelan, *J. Sci. Food Agric.*, 1951, **2**, 224) in order to determine the polysaccharide concentration; 22·2% of the amylose had been precipitated. After allowance for precipitation the measured reducing powers after 20, 24, and 44 hours corresponded to conversions of amylose into maltose of 91·0, 90·0, and 91·0%, respectively.

Large-scale Digestion of Amylose.—In a similar experiment with 3 g. of amylose in a 1-l. digest, an almost complete precipitation of the amylose occurred on the addition of enzyme. Since a chance observation had indicated that the extent of precipitation was lessened in the presence of sodium chloride the effect of various salts in preventing precipitation was investigated. Amylose (100 mg.; air-dried) was heated in boiling water for 10 minutes and undissolved material was removed. The amylose concentration was determined by acid hydrolysis to glucose (as above). The clear supernatant solution was incorporated in digests of the following composition: amylose solution (7 ml.), 0·2M-citrate buffer (pH 7·0; 1 ml.), enzyme solution (1 ml.), and 1M-salt solution (1 ml.), each digest being contained in a 15-ml. centrifuge tube. The following salts were used: sodium chloride, sodium sulphate, disodium hydrogen phosphate, magnesium chloride, magnesium sulphate, and (0·667M) aluminium sulphate. Two additonal digests were prepared; in one, water replaced salt solution (digest *A*) and in the other water replaced enzyme and salt solutions (digest *B*). All digests were incubated at 35° and after 7 hours each digest was centrifuged and an aliquot (8 ml.) removed. Acid hydrolysis, as above, was used to determine the concentration of polysaccharide remaining in solution. The following results refer to the percentage of the amylose which had precipitated from solution in each case: digest *A*, 9·5%; digest *B*, 0%; NaCl, 1·3%; Na_2SO_4, 10·0%; Na_2HPO_4, 5·3%; $MgCl_2$, 36%; $MgSO_4$, 39%; $Al_2(SO_4)_3$, 19%. Sodium chloride was thus shown to be effective in preventing precipitation. A further difficulty encountered was that this salt was ineffective when the amylose had been dried *in vacuo* at 60°. Air-dried amylose containing about 20—30% of volatile matter was therefore used. This is the concentration found in amylose prepared as in *J.*, 1951, 801. A digest containing air-dried amylose (443·8 mg. $\equiv$ 294·7 mg. dry wt.), α-amylase (2 mg.), 0·2M-citrate buffer (pH 7·0; 12 ml.), 1N-sodium chloride (12 ml.), and water to 100 ml. was incubated at 35°. Hydrochloric acid, instead of sulphuric acid, was employed to neutralise the alkali used in dissolving the amylose. By using the methods given above, the apparent degree of conversion of the amylose into maltose and the extent of precipitation were determined. The results of the experiment, in which the precipitation (6·4%) has been allowed for, are given in Fig. 1.

A similar experiment on a ten-fold scale was carried out. The digest (1 l.) contained amylose (B.V. 1·47; 4·5533 g. wet wt., 3·023 g. dry wt.), 0·2M-citrate buffer (pH 7·0; 100 ml.), 1N-

sodium chloride (120 ml.), and freeze-dried α-amylase (20·8 mg.). After incubation for 24 hours at 35° the polysaccharide concentration was found by acid hydrolysis to be equivalent to 2·827 g. of amylose/l. Consequently, 0·196 g. or 6·5% of the amylose had been precipitated on addition of the enzyme. At this stage the measured reducing power of the digest corresponded to an apparent conversion into maltose of 87·4%. After 32 hours the conversion was 90·0% and the same value was found after 69 hours, a qualitative test then showing the enzyme to be still active. The digest was then heated in a boiling-water bath for 5 minutes in order to deactivate the enzyme. After cooling, 958 ml. of the digest (≡ 2·708 g. of amylose) were transferred to a distillation flask and concentrated to dryness under diminished pressure.

Fractionation of α-Dextrins.—The methods and apparatus used in charcoal chromatography are described in Part I (preceding paper); a column (3 × 45 cm.) of charcoal–Celite 535, prepared and treated as previously described, was used. The amylose α-dextrins were dissolved in a small volume of water and transferred to the column which was then irrigated successively with water, 7·5%, 15%, 23%, and 28% aqueous ethanol, as indicated in Fig. 2. Fractions (100 ml. each) were collected and filtered through a Seitz filter, and the optical rotation measured in a 4-dm. tube. For the recovery of the disaccharide, fractions 26—38 were combined and taken to dryness under diminished pressure. Similarly, the trisaccharide was obtained from combined fractions 45—58. The solids were each dissolved in water, and the solutions transferred to 500-ml. flasks and diluted to the mark. The concentration of each sugar was determined by acid hydrolysis, the optical rotations were measured, and the specific rotations calculated (Table 1). The reducing power of each sugar was compared with that of a corresponding authentic specimen. The mobility of each sugar on paper in the solvent system fusel oil–pyridine–water was similarly compared (Table 1). The sugar solutions remaining were then taken to dryness under diminished pressure.

Acetylation of the α-Limit Dextrins.—The maltose (290 mg.) was heated at 120—130° with anhydrous sodium acetate (240 mg.) and acetic anhydride (3·2 ml.) until the solid had dissolved. The cooled solution was poured into ice–water, and the precipitated acetate (487 mg., 87%) was crystallised from ethanol (see Table 1). The maltotriose (389·9 mg.) was similarly treated but the solid obtained could not be satisfactorily crystallised, showing a tendency to revert to a syrup on exposure to air. Its optical rotation (+88·5°) was somewhat higher than that reported for maltotriose β-undeca-acetate, namely +86° (Wolfrom, Georges, Thompson, and Miller, *J. Amer. Chem. Soc.*, 1949, **71**, 2875). These workers had isolated the acetate by chromatogaphy on Magnesol–Celite, and we resorted to this method of purification. Maltotriose (106·3 mg.) was acetylated and the resulting solid (148·3 mg., 79%) was dissolved in benzene (5 ml.) and placed on a column (25 × 3 cm.) of Magnesol–Celite (5 : 1). The column was eluted under suction with benzene–ethanol (100 : 1, vol./vol.) and 100-ml. fractions were collected, the optical rotation of each being determined. Fractions 7—11 were combined and evaporated to dryness under reduced pressure; the resulting solid (69·4 mg.) was crystallised without difficulty from ethanol and its properties determined (see Table 1).

α-Amylolysis of Maltodextrins.—In a control experiment maltose monohydrate (17·58 mg.) and maltotriose (10·44 mg.) were mixed and fractionated on charcoal–Celite (24 × 1·5 cm.), following the procedure outlined in the preceding paper. The optical rotation of each fraction (20 ml.) was measured in a 2 dm. tube. After elution with water (200 ml.), maltose was removed with 7·5% ethanol (140 ml.), and maltotriose with 15% ethanol (140 ml.). The sum of the rotations due to maltose was 0·430° and for maltotriose was 0·318°. These values corresponded to recoveries of 94·4% and 95·2%, respectively. In the experiments to be described the yields of maltose and maltotriose were corrected for this measured loss of sugar. The method has the advantage of avoiding any error due to the effect of alcohol on the optical rotation of the sugar. In a typical digestion of a maltodextrin, maltohexaose (32·1 mg.) was treated with α-amylase (1 mg.) at 35° in a 25-ml. digest containing 0·2M-citrate buffer (pH 7·0; 3 ml.). Portions (1 ml. each) were removed at intervals for determination of reducing power. After 37 hours the degree of apparent conversion into maltose was 85·4% and after 61 hours was 86·1%. At this stage a portion of the digest (13·58 ml.) was removed and fractionated on charcoal–Celite as described above. The corrected sum of the rotations due to maltose was 0·333°, and for maltotriose, 0·194°. These values correspond to molar quantities of $3·58 \times 10^{-5}$ and $1·20 \times 10^{-5}$, respectively, and a molar ratio of 2·98 : 1 (see Table 2). The yield of maltose (anhydrous) was also determined by measurement of reducing power and found to be 11·47 mg.; the actual yield as determined by optical rotation was 11·54 mg.

Action of α- and β-Amylase on Maltotriose.—Maltotriose (25 mg.), α-amylase (2 mg.), and 0·2M-citrate buffer (pH 7·0; 3 ml.) were mixed, diluted to 25 ml., and incubated at 35°. At

intervals, portions (2 ml.) were removed and the reducing power measured by using the Somogyi reagent (*loc. cit.*). In Table 3 these reducing powers are expressed in terms of 0·005N-sodium thiosulphate solution. Similar digests were prepared containing crystalline sweet-potato β-amylase (*ca.* 1000 units; see *J.*, 1945, 882) or purified soya-bean β-amylase (*ca.* 1000 units; *J.*, 1952, 714) instead of α-amylase and were incubated at pH 4·8 and 35°. In these cases a slow but complete conversion of maltotriose into equimolar amounts of maltose and glucose took place (see Table 3).

TABLE 3. *Action of α- and β-amylase on maltotriose.*

Enzyme	Period of incubation (hours)					
	0	22·5	72·8	120	167·5	
Salivary α-amylase	5·46	5·33	5·29	5·36	5·28	ml. of 0·005N-thiosulphate
Sweet-potato β-amylase	0	47·6	87·7	98·9	101	% conversion
Soya-bean β-amylase ...	0	39·9	79·0	96·8	101	% conversion

The advice and encouragement given by Professor Stanley Peat, F.R.S., are gratefully acknowledged. The Department of Scientific and Industrial Research is also thanked for the award of a maintenance grant (to P. J. P. R.).

UNIVERSITY COLLEGE OF NORTH WALES, BANGOR. [*Received, September 24th*, 1952.]