48. *The Q-Enzyme of* Polytomella coeca.*

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For the synthesis of starch, *Polytomella coeca* utilises a phosphorylase and a Q-enzyme. The latter has been purified and shown to be very similar to potato Q-enzyme; it converts amylose into a polysaccharide which stains reddish-purple with iodine, there being little liberation of reducing sugar. The course of this conversion has been examined in detail. The new Q-enzyme and potato phosphorylase together show an autocatalytic liberation of inorganic phosphate when incubated with glucose-1 phosphate; the polysaccharide produced stains reddish-purple with iodine.

Polytomella coeca is a flagellated protozoa belonging to a group of organisms which are of particular interest inasmuch as they do not contain chlorophyll and yet are able to synthesise relatively large quantities of starch from simple carbon sources, but not from sugars (Lwoff, Ionesco, and Gutmann, *Biochim. Biophys. Acta*, 1950, **4**, 270). This organism has been extensively studied by Lwoff (*Ann. Inst. Pasteur*, 1941, **66**, 407) and Lwoff, Ionesco, and Gutmann (*loc. cit.*; *Compt. rend.*, 1949, **228**, 342), who have shown that it grows readily between pH 3·0 and 8·0 in a purely synthetic medium containing ammonium sulphate as the nitrogen source and acetic acid or ethyl alcohol as the carbon source; in addition, inorganic phosphate and traces of iron, magnesium, and thiamine are necessary. A culture medium such as this, in which radioactive sodium acetate is incorporated, furnishes a fruitful source of labelled starch (Bevington, Bourne, and Wilkinson, *Chem. Ind.*, 1950, 691).

In two recent papers Lwoff, Ionesco, and Gutmann (*locc. cit.*) reported the isolation from *Polytomella coeca* of an enzyme preparation containing phosphorylase and showed that the immediate precursor of the starch formed by the organism was glucose-1 phosphate. In the present work we have confirmed that a cell-free extract of the flagellate is capable of synthesising an iodine-staining starch-type polysaccharide from the Cori ester, but not, under the same conditions, from glucose, maltose, or sucrose. Thus it is very probable that *Polytomella coeca* synthesises chains of 1 : 4-α-linked glucose units by the agency of a phosphorylase, rather than of an amylosucrase (cf. Hehre and Hamilton, *J. Bact.*, 1948, **55**, 197; Hehre, Hamilton, and Carlson, *J. Biol. Chem.*, 1949, **177**, 267) or an amylomaltase (Monod and Torriani, *Compt. rend.*, 1948, **227**, 240; 1949, **228**, 718; Doudoroff *et al.*, *J. Biol. Chem.*, 1949, **179**, 921).

In a detailed examination of the composition of the starch formed by the intact cells of *Polytomella coeca*, three of us (*J.*, 1950, 2694) found that the major component, representing 84—87%, possessed the ramified amylopectin structure. Now, it is well known that phosphorylases isolated from plant and animal sources synthesise only amylose and that a second enzyme is necessary to establish the branch points found in amylopectin. This supplementary enzyme, which has been termed Q-enzyme, was first isolated by Haworth, Peat, and Bourne (*Nature*, 1944, **154**, 236) from potatoes and was obtained in crystalline form from that source by Gilbert and Patrick (*Nature*, 1950, **165**, 573). It is

* Presented, in brief, at the XIIth International Congress of Pure and Applied Chemistry, New York September 1951.

known to occur also in the broad bean and the wrinkled pea (Hobson, Whelan, and Peat, *J.*, 1950, 3566). Q-Enzyme appears to be closely related to the branching factor which is involved in the synthesis of glycogen (cf. Cori and Cori, *J. Biol. Chem.*, 1943, **151**, 57; Hehre, *ibid.*, 1949, **177**, 267). We now report the isolation of a Q-enzyme from *Polytomella coeca*.

In the standard method for the preparation of the enzyme the bacterial cells were ruptured by grinding them with sand, and an aqueous extract of the debris was treated with lead acetate in a manner similar to that used by Barker, Bourne, and Peat (*J.*, 1949, 1705) in the case of the potato enzyme. The precipitated lead–protein complex was eluted with sodium hydrogen carbonate solution in a stream of carbon dioxide and the Q-enzyme was precipitated from the extract with neutral ammonium sulphate, at a concentration (25 g./100 c.c.) chosen to give maximal activity consistent with the removal of phosphorylase. No amylose-precipitating fatty acid was detected in any sample of Q-enzyme thus prepared. The activity of the enzyme was appreciably greater than that of an equal quantity of potato Q-enzyme at the same stage of purification. An alternative

FIG. 1. *Action of Q-enzyme on amylose.*

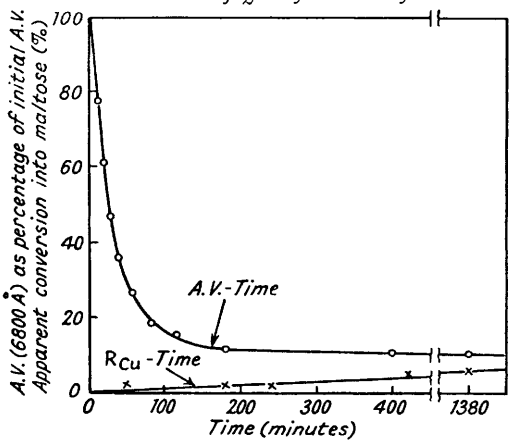
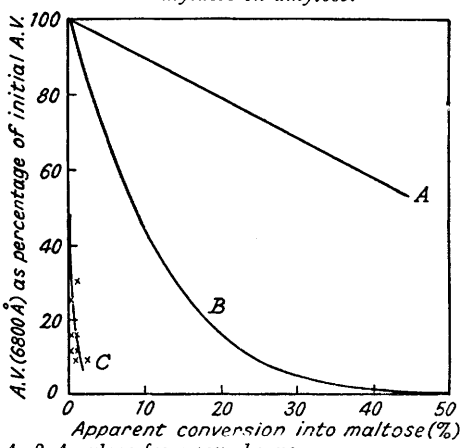


FIG. 2. *The action of Q-enzyme and amylases on amylose.*



A, β-*Amylase from soya beans.*
B, *Salivary* α-*amylase.*
C, *Composite curve of results with potato Q-enzyme and* Polytomella coeca *Q-enzyme.*

process, in which the lead–protein complex was eluted with citrate buffer (pH 7·0) instead of sodium hydrogen carbonate solution, was not adopted because, although it gave a slightly better yield of Q-enzyme activity, there was a greater contamination with both amylase and non-enzymic protein impurities. However, there is one respect in which the citrate elution process offers an advantage, namely, that the Q-enzyme sample finally obtained from such a process can be freeze-dried in 0·2M-citrate buffer (pH 7·0) with negligible loss in activity, whereas under similar drying conditions the product of the standard sodium hydrogen carbonate process loses almost the whole of its Q-enzyme activity. Thus when the Q-enzyme is to be stored over a prolonged period it is better that it should be kept as the less pure freeze-dried product of the citrate elution process and then purified further when required. It seems probable that the extraneous proteins eluted in the citrate method stabilise the Q-enzyme during drying (cf. Barker, Bourne, and Peat, *loc. cit.*; Barker, Bourne, Wilkinson, and Peat, *J.*, 1950, 84); the prevention of the development of acidity during freeze-drying is not in itself sufficient protection against deactivation of the enzyme.

Samples of Q-enzyme obtained from the flagellate by the standard method rapidly converted potato amylose into a polysaccharide which stained reddish-purple with iodine. The changes in the iodine-staining properties and reducing power of a typical digest of this type are shown in Fig. 1. It will be seen that the A.V. (6800 Å) of the solution decreased

rapidly, falling to 11% of its initial value in 3 hours; thereafter there was a further decrease of only 2% during the next 20 hours. Since the " apparent conversion into maltose," as measured by cuprimetric titration, was only 2 and 6% after 3 and 23 hours, respectively, there could have been very little, if any, contamination with either α- or β-amylase. When the time factor was eliminated by plotting A.V. (6800 Å) against " apparent conversion into maltose " the curve given in Fig. 2 was obtained; the points shown in this figure represent the results of several experiments in which different samples of Q-enzyme were employed. The striking similarity between this enzyme and potato Q-enzyme and their non-identity with either α- or β-amylase is clearly demonstrated. These results were consistent with the conversion of amylose into an amylopectin-type product by the Q-enzyme of *Polytomella coeca*, as also were the changes in the complete light-absorption curves of the polysaccharide–iodine solutions (Fig. 3).

The optimum pH for the amylose conversion is *ca.* 7·3, compared with 7·0 for potato Q-enzyme (Barker, Bourne, and Peat, *loc. cit.*) and 7·25—7·5 for the Q-enzyme of the broad bean (Hobson, Whelan, and Peat, *loc. cit.*). The optimum temperature is particularly sensitive to pH change and values ranging from 25° to 33° have been obtained; Barker, Bourne, and Peat (*loc. cit.*) recorded 21° ± 1° for potato Q-enzyme, and Hobson, Whelan, and Peat (*loc. cit.*) gave 20° for broad bean Q-enzyme.

FIG. 3. *Light-absorption curves obtained during the treatment of amylose with Q-enzyme.*

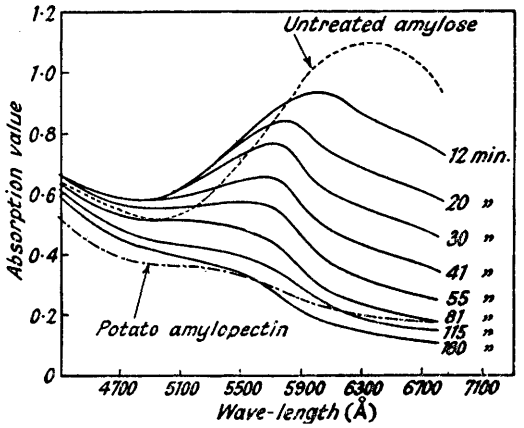
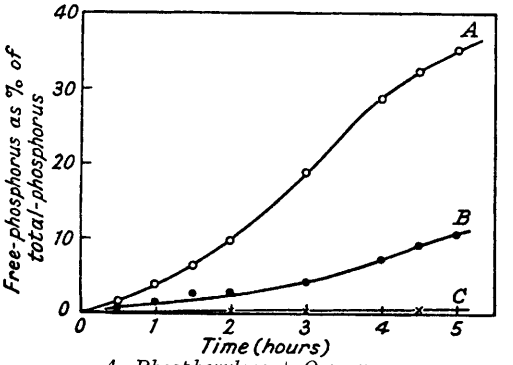


FIG. 4. *Autocatalysis in polysaccharide synthesis from glucose-1 phosphate.*



A, Phosphorylase + Q-enzyme.
B, Phosphorylase alone.
C, Q-Enzyme alone.

The Q-enzyme of *Polytomella coeca* had, *per se*, no action on glucose-1 phosphate, and therefore was free from phosphorylase. However, like potato Q-enzyme (Barker, Bourne, Wilkinson, and Peat, *loc. cit.*) and the " branching factor " of liver and heart (Cori and Cori, *loc. cit.*), it promoted the synthetic function of potato phosphorylase; this autocatalytic effect can be seen in Fig. 4. The joint action of the two enzymes resulted in the formation of a polysaccharide which stained reddish-purple with iodine. Although this effect is not specific for Q-enzyme, it does reveal the presence of an enzyme which increases the number of non-reducing chain-ends in the amylose produced by the phosphorylase action.

In conclusion, an enzyme similar to potato Q-enzyme has been isolated from *Polytomella coeca*; this enzyme catalyses the conversion of amylose into a polysaccharide which stains reddish-purple with iodine and may well be of the amylopectin-type. The amylose conversion has now been carried out on a relatively large scale and the polysaccharide product has been isolated; as will be shown in the following paper, rigorous chemical examinations have revealed that it is a member of the amylopectin–glycogen class.

EXPERIMENTAL

Definitions of Starch–Iodine Colours.—Two expressions, absorption value (A.V.) and blue value (B.V.), are used in this paper to describe the intensities of the iodine stains of polysaccharides; they were defined by Bourne, Haworth, Macey, and Peat (*J.*, 1948, 924). Since the B.V. is determined under standard conditions (polysaccharide, 1 mg./100 c.c.; iodine,

2 mg./100 c.c.; potassium iodide, 20 mg./100 c.c.) and at λ 6800 Å, it is characteristic of the polysaccharide under examination. On the other hand, the A.V. is a convenient term for an absorption measurement made under any given set of conditions, for example, during the course of a reaction in which the polysaccharide concentration may be changing.

Storage and Culture of Polytomella coeca (cf. Lwoff, Ionesco, and Gutmann, *loc. cit.*).—(a) *In a peptone medium.* For maintaining the culture over long periods a 0·5% peptone broth, adjusted with sodium hydroxide to pH 6·0, was employed. The medium was sterilised for 30 minutes at 15 lb. pressure. When it was cool, it was inoculated with a pure culture of *Polytomella coeca* and kept in the dark at room temperature. When a mass culture was required, ethyl alcohol (1 c.c. per 250 c.c. of culture) was added and in 2—3 days vigorous growth occurred.

(b) *In a synthetic medium.* The synthetic medium consisted of ammonium sulphate (4·0 g.), magnesium sulphate (0·4 g.), sodium acetate (4·0 g.), potassium dihydrogen phosphate (2·0 g.), ethyl alcohol (12·0 c.c.), and thiamine hydrochloride (0·04 mg.) in distilled water (4 l.). It was adjusted to pH 7·0 with sodium hydroxide and sterilised for 40 minutes at 15 lb. pressure. When it was cool, 0·4% ferric ammonium citrate solution (10 c.c.), which had been sterilised separately, was added.

In order to obtain a mass culture, a portion (250 c.c.) of the synthetic medium was inoculated with a portion (2 c.c.) of a young mass peptone culture, and incubated at 25°, whilst being continuously aerated with a fine stream of air, which had been previously freed from carbon dioxide. After 2—3 days, a portion (25 c.c.) of the vigorous culture was introduced into a large volume (4 l.) of the synthetic medium, which was then incubated for 3—5 days, as before.

Standard Method for the Isolation of Q-Enzyme.—A mass culture was grown in a synthetic medium (4 l.), as described above. The flagellates were collected in the centrifuge and ground with washed white sand until microscopical examination revealed that almost complete disruption of the cells had been effected. The ground mass was extracted with three portions (15 c.c. each) of 0·02M-citrate buffer (pH 7·0), and the volume of the combined extracts was adjusted to 50 c.c. Lead acetate solution (pH 7·25; 10 c.c.), prepared as described by Barker, Bourne, and Peat (*loc. cit.*), was added and the precipitated lead–protein complex was collected in the centrifuge. The precipitate was stirred with 0·2N-sodium hydrogen carbonate (50 c.c.) for 5 minutes, and a stream of carbon dioxide was then passed through the suspension for $2\frac{1}{2}$ minutes. The insoluble residue was removed by the centrifuge and discarded. To the supernatant liquid, ammonium sulphate solution (pH 7·0; 50 g./100 c.c. of solution) was added, the final concentration of this salt being 25 g./100 c.c. The small amount of proteinaceous material thus precipitated was collected in the centrifuge and re-dissolved in 0·2M-citrate buffer (pH 7·0; 30 c.c.).

Standard Digest of Amylose and Q-Enzyme.—The standard digest was composed of amylose solution (7·5 c.c.; containing 14 mg. of amylose), maltose solution (1·5 c.c.; containing 3·0 mg. of maltose), 0·2M-citrate buffer (pH 7·0; 2·5 c.c.), and Q-enzyme solution (2·5 c.c.), making a total volume of 14 c.c. The amylose solution was prepared by dissolving the polysaccharide in sodium hydroxide and neutralising the solution with sulphuric acid, as described by Barker, Bourne, and Peat (*loc. cit.*). The digest was incubated at 25°; at intervals, aliquot portions (1 c.c.; equivalent to 1 mg. of amylose) were stained with iodine (2 mg.) and potassium iodide (20 mg.) in a final volume of 100 c.c. and the absorption values were measured. Other portions (5 c.c.) were subjected to the Shaffer–Hartmann determination of reducing sugar (*J. Biol. Chem.*, 1921, **45**, 377). This reagent is insensitive to small amounts of sugar (<0·2 mg. of glucose and <0·4 mg. of maltose) and it was for this reason that maltose was introduced into the digest. A control digest, containing water (7·5 c.c.) in place of the amylose solution, was simultaneously incubated at 25°, and its reducing power was measured at intervals. The difference between the reducing values of the two digests was a measure of the reducing sugar liberated by the action of the enzyme on amylose and is expressed as " apparent conversion into maltose."

Polysaccharide Synthesis by a Cell-free Extract.—The enzyme solution employed was that obtained when the disrupted flagellates were extracted with 0·02M-citrate buffer, as described in the standard method. Portions (2 c.c.) of the enzyme solution were incubated at 18° with 0·125M-solutions of various sugars in 0·2M-citrate buffer (pH 6·0, 8·0 c.c.). The sugars used were glucose, glucose-1 phosphate, maltose, and sucrose. After 36 hours, the digest containing the Cori ester gave a deep reddish-purple stain with iodine, whereas the other three digests did not give a stain.

Development of Optimum Conditions for the Isolation of Q-Enzyme.—(a) *Fractionation with ammonium sulphate.* The crude enzyme, extracted from a 4-l. mass culture grown in a synthetic

medium, was treated with lead acetate and the precipitate was eluted with sodium hydrogen carbonate solution, as described in the standard method. The eluate was treated with neutral ammonium sulphate solution (50 g./100 c.c.), giving successive concentrations of this salt of 18, 24, 30, and 37·5 g./100 c.c. Each precipitate was collected in the centrifuge and redissolved in 0·2M-citrate buffer (pH 7·0; 15 c.c.). The four enzyme solutions thus obtained were examined for Q-enzyme activity in standard digests containing potato amylose (B.V. 0·95), and for phosphorylase activity by Green and Stumpf's method (*J. Biol. Chem.*, 1942, **142**, 355). The results are recorded in the following table.

$(NH_4)_2SO_4$ concn. (g./100 c.c.)	Phosphorylase activity	Test for Q-enzyme: Fall (%) in A.V. (6800 Å) of amylose : 45 min.	6 h.	Apparent conversion into maltose (%) in 6 h
0—18	Nil	74·7	90·6	Nil
18—24	Nil	83·1	90·6	1·9
24—30	Slight	81·5	91·5	Nil
30—37·5	Nil	1·0	26·4	Nil

In order to obtain a good recovery of Q-enzyme with little or no contamination by phosphorylase, the fraction precipitated in the 0—25 g./100 c.c. range of ammonium sulphate concentrations was retained in the standard method.

(b) *Elution of the lead–protein complex.* The ruptured cells from a mass culture, grown in a synthetic medium (4 l.), were extracted with 0·02M-citrate buffer (pH 7·0; 50 c.c.), and the extract was divided into two equal portions, each of which was treated with lead acetate, as described in the standard method. One of the lead–protein complexes was extracted with 0·2N-sodium hydrogen carbonate (25 c.c.) in a stream of carbon dioxide (see the standard method), and the other with 0·2M-citrate buffer (pH 7·0; 25 c.c.). Each of the two extracts was treated with neutral ammonium sulphate solution (final concentration, 25 g./100 c.c.) and the proteinaceous precipitate was dissolved in 0·2M-citrate buffer (pH 7·0; 15 c.c.). The two enzyme solutions thus obtained were incorporated in standard digests containing potato amylose (B.V. 0·95); the changes in A.V. (6800 Å) and in reducing power are shown in the following table. It was observed that ammonium sulphate precipitated considerably more material from the citrate extract than it did from the sodium hydrogen carbonate extract.

Extraction by :	Test for Q-enzyme: Fall (%) in A.V. (6800 Å) in : 30 min.	2 h.	Apparent conversion into maltose (%) in 23 h.
0·2N-$NaHCO_3$	67·0	83·9	4·7
0·2M-Citrate buffer	82·8	89·4	8·4

(c) *Freeze-drying of Q-enzyme solutions.* The preceding experiment was repeated and extended. A sample (10 c.c.) of each of the final 0·2M-citrate solutions of Q-enzyme was freeze-dried and redissolved in water, the volume of the solution being adjusted to 10 c.c. The effect of the drying process on enzymic activity can be seen in the next table; these observations were confirmed in several other experiments.

Extraction by :	Freeze-drying	pH of final enzyme solution	Test for Q-enzyme: Fall (%) in A.V. (6800 Å) in : 40 min.	4·5 h.	Apparent conversion into maltose (%) in 21 h.
0·2N-$NaHCO_3$	Omitted	7·39	65·5	87·2	1·5
	Included	6·95	14·4	19·2	1·3
0·2M-Citrate buffer	Omitted	7·40	77·8	87·2	4·3
	Included	6·79	73·4	87·6	4·3

A More Detailed Examination of the Action of Q-Enzyme on Amylose.—A solution of Q-enzyme, prepared by the standard method, was incorporated in a standard digest, which was prepared on thrice the usual scale; the polysaccharide employed was potato amylose (B.V. 0·95). The digest was incubated at 25° and the course of the enzyme action was followed by measuring the changes in the reducing power and iodine-staining properties, as in the standard digest. The complete absorption spectra of the polysaccharide–iodine stains in the visible region were measured. The results are shown graphically in Figs. 1 and 3. In a second experiment, in which a different enzyme preparation was employed, similar results were obtained.

Optimum Temperature for the Action of Q-Enzyme on Amylose.—A sample of Q-enzyme, prepared by the standard method, was incorporated in six standard digests, the polysaccharide being potato amylose (B.V. 0·95). The digests were incubated at temperatures ranging from 10° to 35°, and, after 32 and 75 minutes, respectively, withdrawals (1·0 c.c.) were made for the

determination of A.V. (6800 Å). The results are shown in the following table; when plotted, they reveal the optimum temperature to be 25—27°. That the enzyme employed in this experiment was substantially free from amylases was shown by the fact that, at 25° and pH 7·0, it effected an 84% decrease in A.V. (6800 Å) while the " apparent conversion into maltose " was only 1·3%.

Temp. of incubation		10°	15°	20°	23°	30°	35°
Fall (%) in A.V. (6800 Å) in	32 min.	40·2	51·0	58·0	67·0	59·0	34·0
	75 min.	75·5	79·7	81·0	83·0	78·0	44·8

In a duplicate experiment, 0·2M-citrate buffer (pH 6·8) was employed, both in the digest and as a solvent for the enzyme. When plotted, the result given in the following table show that, under these conditions, the optimum temperature was 32—34°.

Temp. of incubation		19°	25°	30°	35°	39·5°
Fall (%) in A.V. (6800 Å) in	17 min.	41·5	52·1	57·8	58·9	42·8
	27 min.	65·4	69·7	74·2	72·3	53·2

Temperature optima of 28° and 32—33° were found on using 0·02M-citrate buffers of pH 7·2 and 6·8, respectively.

Optimum pH for the Action of Q-Enzyme on Amylose.—Q-Enzyme, prepared by the standard method, was incorporated in seven digests of the standard type, except that 0·2M-citrate buffer solutions of different pH values were employed; these buffers differed only in the amounts of sodium hydroxide which they contained. The digests were incubated at 25°, withdrawals (1·0 c.c.) being made after 45 and 90 minutes for the determination of A.V. (6800 Å). The results are shown in the next table; the pH values recorded are those of the complete digests. Below pH 5·0 protein was precipitated from the digests and no fall in A.V. (6800 Å) could be detected. The Q-enzyme sample employed effected, at pH 7·0 and 25°, an 89% fall in A.V. (6800 Å) with only 2·1% " apparent conversion into maltose."

pH value of digest		4·11	5·24	6·29	6·86	7·30	7·55	7·62
Fall (%) in A.V. (6800 Å) in	45 min.	0·0	13·3	52·1	69·6	74·6	72·4	72·4
	90 min.	0·0	29·8	75·5	83·0	85·0	83·5	83·5

Autocatalysis by Q-Enzyme during Polysaccharide Synthesis by Phosphorylase.—(a) *Isolation of phosphorylase.* The source of phosphorylase was a sample of fraction P2, which had been isolated several months earlier from potatoes and freeze-dried in 0·2M-citrate buffer (pH 6·0), as described by Barker, Bourne, Wilkinson, and Peat (*loc. cit.*). The enzyme was further purified by two fractional precipitations with neutral ammonium sulphate, the fraction which was precipitated between salt concentrations of 18 and 35 g./100 c.c. being retained at each stage. The product was dissolved in 0·2M-citrate buffer (pH 6·0; 20 c.c. per 100 c.c. of potato juice employed) and freeze-dried. When required, the free-flowing powder was dissolved in water (20 c.c. per 100 c.c. of potato juice employed).

(b) *Preparation of dipotassium glucose-1 phosphate.* The Cori ester was prepared from potato starch by phosphorolysis, as recommended by Sumner and Somers (*Arch. Biochem.*, 1944, **4**, 11).

(c) *Compositions of digests.* Three digests, having the compositions shown below, were incubated at 25°. The Q-enzyme solution used had been prepared by the standard method. At intervals, aliquot portions (2·0 c.c.) were withdrawn and inactivated with 6% trichloroacetic acid (2·85 c.c.). The precipitates were collected in the centrifuge, and the inorganic phosphate in each supernatant liquid was estimated by Allen's colorimetric method (*Biochem. J.*, 1940, **34**, 858), corrections being applied for traces of inorganic phosphate in the enzyme samples. The results are shown graphically in Fig. 4. Similar results were obtained in a second series of digests. In addition, it was shown that heat-inactivated Q-enzyme did not increase the rate of polysaccharide synthesis by phosphorylase.

Solution	Volume (c.c.) in digest: A	B	C
0·1M-Dipotassium glucose-1 phosphate	9·00	9·00	9·00
Phosphorylase	6·75	6·75	—
Q-Enzyme	6·75	—	6·75
0·2M-Citrate buffer (pH 7·0)	9·00	15·75	15·75

The authors express their gratitude to Dr. A. Lwoff of the Pasteur Institute for kindly supplying the culture of *Polytomella coeca* used in this work, and to Courtaulds' Scientific and Educational Trust and the Department of Scientific and Industrial Research for financial assistance to two of them (I. A. W. and A. B.).

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[*Received, July 27th*, 1951.]