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The Action of a Hydrolytic Enzyme System from *Helianthus tuberosus* L. on Carbohydrates Present in the Tubers

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Green (1887) described an 'inulin-ferment' (later named inulase: Green, 1893) which was present in glycerol extracts made from sprouting tubers of the Jerusalem artichoke and which liberated from inulin reducing sugar and uncharacterized intermediate products. Wolff & Geslin (1918) made from chicory roots extracts in which reducing sugar was slowly liberated, and Colin (1919) reported a similar observation with extracts of artichoke tubers; in both cases the liberation of reducing sugar was correlated with a change of optical rotation in the negative direction. Pringsheim & Kohn (1924) attempted to prepare enzymes from dahlia tubers, but considered their efforts unsuccessful. Thaysen, Bakes & Green (1929) were led to suspect the presence of an inulase in artichoke-tuber extract, but could not confirm this experimentally. These various findings have led to the general conclusion that higher plants are not good sources of enzymes attacking inulin (Pringsheim & Leibowitz, 1929; cf. Lemoigne, 1942).

However, Vadas (1934) suggested the use of a short incubation of mashed tubers as a preliminary to ethanolic fermentation of the carbohydrates, and Asai (1938) noted that as much as 50% of the carbohydrate in the tubers was converted to reducing sugar when the crushed material was incubated for 20 hr. at 37° and pH 3.9.

Physiological studies of the artichoke plant (cf. Colin, 1919; Belval, 1946) indicate that enzyme systems exist concerned with the transformation of the tuber carbohydrates. The latter consist of a mixture of related oligo- and poly-saccharides as

well as inulin itself. In a previous paper (Bacon & Edelman, 1951) we have described the application of paper partition chromatography to these substances.

During that investigation we re-examined the possibility of preparing from the tubers extracts with enzymic activity towards inulin and related substances. The results presented here demonstrate the existence in tuber extracts of a hydrolytic enzyme system, which liberates fructose from inulin and related substances, but has relatively little action upon sucrose.

Part of this work was communicated to the Biochemical Society at Leeds on 23 September 1949 (Edelman & Bacon, 1949).

MATERIALS AND METHODS

Except where otherwise stated, the methods of analysis used were those described by Bacon & Edelman (1951), as also were the batches of artichoke tubers. Reducing substances (RS) are expressed in terms of hexose and by 'total RS' is meant RS after hydrolysis with 0.5% (w/v) oxalic acid for 30 min. at 100°. The sample of commercial inulin used was obtained from Messrs Thomas Kerfoot and Co., Vale of Bardsley, Ashton-under-Lyne, who kindly informed us that it was prepared from the root of the dandelion, *Taraxacum officinale*. All samples of inulin were purified by filtering the hot aqueous solution, freezing the filtrate, dehydrating the resulting precipitate with ethanol and acetone, washing it with light petroleum and drying it in a vacuum desiccator.

The lead acetate and sodium oxalate solutions used throughout were: 30% (w/v) $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$ and 3% (w/v) $\text{Na}_2\text{C}_2\text{O}_4$. All dialysis was carried out in Visking synthetic cellulose casing (John Crampton and Co. Ltd., Wythenshawe, Manchester).

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RESULTS

Characterization of enzymic activity

When tuber extracts made as described by Bacon & Edelman (1951) were incubated at 37–40°, their optical rotation was found to change in the negative direction, and the free RS to increase. There was no change in the RS liberated by mild acid hydrolysis (Table 1). The quantitative relations between change in RS and change in optical rotation were consistent with the liberation of fructose from laevorotatory oligo- or poly-saccharides.

Table 1. *Changes in reducing substances (RS) and optical rotation on incubation of tuber extract*

(Fresh extract, 17 November 1948, was incubated at 40°, and samples taken for deproteinization and measurement of free and total RS, and rotation (2 dm. tube).)

Time of incubation (hr.)	Optical rotation	RS (mg./ml.)	
		Free	Total
0	–2.73°	3.88	—
4.5	–3.20°	8.17	133
22	–4.75°	20.5	136

The same changes were observed when extracts were incubated in the presence of toluene or CHCl₃, and microscopical examination of incubated extracts, either by dark-ground illumination or after the use of Gram stain, showed that no significant growth of micro-organisms had occurred. The effect observed must therefore be attributed to constituents of the tubers; it was abolished when the extracts were boiled.

The activity was not affected by the presence of cyanide in concentrations up to 0.005M.

In order to distinguish between phosphorolytic and hydrolytic breakdown of carbohydrate, incubations were carried out with the addition of inorganic phosphate. A disappearance of inorganic phosphate under these conditions would suggest phosphorolysis; an increase in the rate of RS liberation without a change in inorganic phosphate might be interpreted as being due to the simultaneous action of a phosphorylase and a phosphatase. Phosphatase activity was present in the extracts, as was shown by the following experiment: 0.5 ml. of extract, diluted to 2.5 ml. with water, was incubated with 5 ml. 0.01M-disodium phenyl phosphate, and either (a) 5 ml. 0.4M-sodium acetate buffer (pH 5.0) plus 0.2 ml. water, (b) 5.2 ml. water, giving a pH value of 6.5, or

(c) 5.0 ml. water plus 0.2 ml. M-NaF, giving pH 6.5. The phenol liberated during 1 hr. incubation at 40° was estimated as described by King (1946), amounting to (a) 0.65 mg., (b) 0.056 mg., (c) 0.003 mg. Experiments to detect phosphorolytic breakdown of polysaccharide were therefore carried out in the presence of 0.02M-NaF, in order to reduce any effects of phosphatase to a minimum.

Several portions of extract were incubated at 26° for 40 hr. and samples taken at intervals for estimation of RS and inorganic phosphate (by the method of Fiske & Subbarow, 1925). No differences from controls without added fluoride or inorganic phosphate were observed at pH 6.4 with 0.01M added phosphate nor at pH 7.5 with 0.02M-phosphate (see Table 2); the extract already contained about 0.005M-inorganic phosphate. The increase in RS and inorganic phosphate during intermediate periods showed no differences from the overall change given in Table 2. At pH 6.9 with 0.17M added phosphate there was a decrease of 18% in RS liberation both with and without fluoride, but no corresponding decrease in inorganic phosphate; it is possible that the enzyme system was inhibited by the high concentration of phosphate.

The above results suggest that the activity of the tuber extracts is due to a hydrolytic, rather than a phosphorolytic, system (see also experiments with inulin, described below).

Measurement of activity

Since it was not known upon what substances the enzyme or enzymes concerned were acting, the possibility existing that inulin itself was not attacked, it was thought advisable to use as a substrate for measuring enzyme activity the whole mixture of soluble carbohydrates present in the tubers, and to define a unit of activity as the liberation of 1 mg. of RS (calculated as hexose) from such a mixture under conditions defined below.

Preparation of standard substrate. Washed tubers (350 g.) (bought and extracted 8 February 1949) were extracted in the usual way in the Waring blender. The extract obtained was used for the extraction of a further 350 g. Four such double extracts were made, yielding 2.17 l. This liquid was heated in a boiling-water bath for 10 min., 0.25 vol. M-sodium acetate buffer (pH 5.0) was added and the resultant liquid filtered after 10 min. Analyses gave values of 2 mg./ml. RS in the unhydrolysed filtrate, 101 mg./ml. RS after mild acid hydrolysis, and 80 mg./ml. total ketose. The liquid was clear and light yellow; when saturated with toluene it was stored in the refrigerator for several months without

Table 2. *Changes in reducing substances (RS) and inorganic phosphate during incubation of tuber extracts*

(An extract, made 14 November 1949, was incubated with and without 0.02M-sodium potassium phosphate buffer (pH 7.5), with and without 0.02M-NaF, the pH of all mixtures being adjusted to 7.5. After 50 hr. at 26° the reaction was stopped by heating the samples in a boiling-water bath.)

		mg./ml. incubation mixture			
		No added phosphate		0.02M added phosphate	
		With NaF	No NaF	With NaF	No NaF
RS:	0 hr.	1.6	1.8	1.6	1.7
	50 hr.	10.1	10.7	10.6	9.8
	Change	8.5	8.9	9.0	8.1
P of inorganic phosphate:	0 hr.	0.11	0.11	0.67	0.68
	50 hr.	0.18	0.19	0.72	0.72
	Change	0.07	0.08	0.05	0.04

detectable change in the appearance or analysis. This buffered extract is subsequently referred to as 'standard substrate'. The optical rotation and chromatographic analysis of the various 'spots' of this preparation are given by Bacon & Edelman (1951; Tables 1 and 4).

Fig. 1. Effect of pH on the liberation of reducing substances (RS) and the changes in optical rotation in tuber extracts. Samples of extract (10 December 1948) were adjusted to the required pH value with N -HCl and changes in RS and optical rotation (2 dm.) were measured on incubation for 4 hr. at 37° . ●—●, RS; ○—○, optical rotation.

Effect of pH. The effect of pH on the change in RS and optical rotation during incubation is shown in Fig. 1. The pH of the extract was altered by adding N -HCl. During incubation there were small changes in pH, the greatest being from 5.98 to 6.14, but in other cases less than 0.1 unit. The maximum activity occurred at about pH 5, with only slight decrease at higher, and a more marked decrease at lower, pH values.

Progress curve of the reaction. During the early stages of the reaction liberation of RS was proportional to the time of incubation (Table 3).

Table 3. *Initial stages of liberation of reducing substances (RS) from standard substrate (tuber extract)*

(Enzyme preparation (made 29 March 1950, see Fig. 4) incubated as for activity measurement and samples withdrawn at intervals for estimation of total RS.)

Time of incubation (hr.)	In 6 ml. incubated mixture	
	RS (mg.)	Liberation of RS (mg.)
0	2.43	0
1	5.67	3.24
2	9.35	6.92
3	11.9	9.47

Effect of substrate concentration. An enzyme preparation free from carbohydrates was made from a tuber extract

(3 February 1949) by $(NH_4)_2SO_4$ precipitation at 60% saturation. Another portion of the extract was heated for 15 min. in a boiling-water bath, cooled, and diluted with 0.25 vol. M -sodium acetate buffer (pH 5.0); it contained 2.3 mg./ml. of free RS, 93.8 mg./ml. total RS, and 75 mg./ml. ketose. Dilutions with 0.2 M -acetate buffer were used as substrates for the action of the enzyme preparation. The results (Fig. 2) indicate that rates of reaction approaching the limiting velocity are reached with concentrations of about 75 mg. total RS/ml.

Fig. 2. Effect of carbohydrate concentration on the rate of liberation of reducing substances (RS) from tuber extracts. A quarter volume of enzyme preparation was added to boiled extract diluted as indicated (both preparations described in Text) and incubated for 3 hr. at 40° . The liberation of RS, expressed as mg./ml. is corrected for the RS content of the enzyme preparation, 0.21 mg./ml.

Effect of enzyme concentration. A preparation made by precipitation with $(NH_4)_2SO_4$ was added in varying amounts to standard substrate and incubated for 3 hr. at 37° . The amounts of RS liberated are shown in Table 4.

Unit of activity. Consequent upon the above experiments 1 unit of activity was defined as that which liberates 1 mg. RS (calculated as hexose on the basis of a fructose standard) from standard substrate diluted from 5 to 6 vol. (thus containing 84 mg. total RS/ml.) at pH 5.0 in 0.17 M -sodium acetate buffer in 180 min. at 40° .

Estimation of enzyme activity. For the estimation of enzyme activity 0.5 ml. of the enzyme solution was mixed with 2.5 ml. standard substrate, and two 1.0 ml. samples were transferred to test tubes. (In the case of solutions with low activity, i.e. less than 10 units/ml., 1.0 ml. was mixed with 2.0 ml., the remainder of the procedure being the same.) One of the tubes was incubated at 40° for 2 min. and then heated in a boiling-water bath for 3 min. to inactivate the enzymes. The other was incubated for between 32 and 182 min. according to the approximate enzyme activity and

then treated similarly. After cooling 0.1 ml. lead acetate solution was added to each tube and they were allowed to stand for 10 min. The solutions were filtered through a Whatman no. 1 paper (diameter 5.5 cm.) and the filter paper containing the precipitate was washed at least three times with several ml. of distilled water. To the combined filtrate and washings was added 0.5 ml. sodium oxalate solution, the precipitate of lead oxalate being filtered off and washed as before. The final filtrate was made up to known volume (50 or 100 ml.) for estimation of RS.

Table 4. *Relation between enzyme concentration and rate of liberation of RS*

(Different amounts of an enzyme preparation, prepared 21 February 1949 by a single precipitation between 25 and 55% saturation $(\text{NH}_4)_2\text{SO}_4$ (activity: 33 units/ml.) were incubated with 5 ml. of standard substrate for 3 hr. at 37°. Water added to make vol. 6 ml.)

Vol. enzyme preparation in 6 ml. incubation mixture (ml.)	RS liberated (mg./ml.)	RS liberated/ml. enzyme preparation (mg./ml.)
0.0	— 0.40	—
0.2	0.95	41
0.4	1.79	33
0.6	2.81	32
0.8	3.71	31
1.0	5.17	34
2.0*	9.14	29

* 4 ml. substrate used.

In preliminary experiments on activity estimation, it was assumed that addition of lead acetate to the solutions used in these estimations was sufficient to inactivate the enzyme. It was found, however (Bacon & Edelman, 1951), that the optical rotation of tuber extracts deproteinized by adding 0.1 vol. lead acetate solution changed slowly in the negative direction, no such change occurring in extracts boiled before deproteinization. Unboiled deproteinized extracts were perfectly clear and almost colourless, but gave a slight haziness with 5% (w/v) aqueous trichloroacetic acid. The failure of lead to remove the enzyme system is illustrated by the following experiment.

To four samples *A*, *B*, *C* and *D* of an enzyme preparation were added 0.1 vol. of 30, 22.5, 15 and 7.5% lead acetate solution respectively. The solutions were centrifuged, and the enzyme activities of the supernatant fluids were estimated: these were 18.0 (control), 7.8 (*A*), 9.6 (*B*), 10.8 (*C*) and 11.4 (*D*) units/ml. After this observation all solutions in which enzyme inactivation was required were immersed in boiling water for at least 3 min.

Example of estimation of activity. Standard substrate (2.5 ml.) incubated with 0.5 ml. enzyme concentrate. RS in 1.0 ml. samples incubated for 2 and 62 min. were 3.50 and 5.65 mg. respectively. Increase during 60 min.: 2.15 mg./ml. The activity is obtained by multiplying this value by the time factor 3, and by the enzyme dilution factor 6, giving 39 units/ml. enzyme solution.

Preparation of enzyme concentrates

In the first stages of attempts to prepare enzyme concentrates the estimations of activity were not exactly

comparable, because the original extract contained large amounts of carbohydrate.

The concentration of substrate was thus higher than in the estimations at later stages when the contaminating carbohydrate had been largely or completely eliminated. As the activity of the initial extracts was low, the concentration of standard substrate was decreased by the necessity of adding a larger proportion of enzyme solution in order to give a measurable increase of RS; this may have counterbalanced the first effect to some extent, but in any case the alteration in substrate concentration was not large enough to introduce any serious error.

The concentration of activity by fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ is illustrated by the following preparation (7 April 1949). Five batches of 400 g. scrubbed tubers were disintegrated in the Waring blender. Water was added to the first batch only, sufficient liquid from this being used to extract the next batch and so on. During each extraction 5 or 10 ml. 0.2M-KCN were added to inhibit phenol oxidases. The mash obtained after each blending was squeezed through gauze, and the extracts combined to give a total of 1.28 l. A sample was removed for estimation of enzyme activity (5.5 units/ml.) (stage 1).

To 1.27 l. (total units 6970) were added 183 g. powdered $(\text{NH}_4)_2\text{SO}_4$ (A.R.) with stirring (0.25 saturation), N-NaOH being added dropwise to keep the solution approximately neutral (green to bromothymol blue). The liquid was filtered on four large Büchner funnels using thick pads of 'Hyflo Supercel' (Johns Manville Co. Ltd., Artillery Row, London, S.W. 1), on Whatman no. 1 filter paper; filter aid was also added to the solution. Yield: 1.29 l. clear filtrate having activity, 4.6 units/ml. (estimated on dialysed sample, corrected for volume increase during dialysis) (stage 2).

To 1.28 l. (total units 5890) were added 246 g. $(\text{NH}_4)_2\text{SO}_4$ (0.55 saturation) and the filtration was carried out on a single large Büchner funnel using filter aid as above. The filter cake was sucked as dry as possible without passage of air, turned out on to a sheet of filter paper sprinkled with Hyflo Supercel, and pressed between filter papers until it crumbled readily. It was ground in a mortar with 30 ml. water and centrifuged. The precipitate was washed three times by centrifugation with about 20 ml. water, and the initial supernatant fluid and the washings were combined to give 90 ml. This liquid was dialysed for 1.5 hr. against running tap water at 8°. The volume after dialysis was 144 ml. The slight precipitate which had formed was removed by centrifugation and rejected. Activity of supernatant, 26.3 units/ml. (stage 3).

To 91 ml. (total units 2400) were added 30.3 ml. neutralized saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and to 116 ml. supernatant after centrifugation were added a further 77.5 ml. saturated $(\text{NH}_4)_2\text{SO}_4$ solution. On centrifuging a loosely packed precipitate formed, to which were added 2 ml. of water to give 13 ml. of enzyme concentrate. Activity, 114 units/ml. (corrected after dialysis). Total units 1480 (stage 4).

The final concentrate was a viscous suspension in approximately half-saturated $(\text{NH}_4)_2\text{SO}_4$ solution, and was stored in the refrigerator for several weeks with only slight loss in activity. Small portions were dialysed overnight before use, the dialysed solution being an opaque mobile liquid. No further attempt was made to purify the enzyme system.

Unless an enzyme preparation with high activity was required experiments were carried out using preparations

corresponding to stage 3. Activities of between 30 and 40 units/ml. could be obtained at this stage by centrifuging the solution at fairly high speed (about 3000 g) instead of filtering it, and taking up the precipitate in a minimum of water.

Nature and specificity of enzymic action

The action of enzyme concentrates was tested on a number of substances, including, in addition to the tuber carbohydrates, sucrose, inulin, and two other fructose-containing polysaccharides.

Action on the soluble carbohydrates of the tuber. Paper partition chromatograms of samples taken during the incubation of tuber extracts showed that free fructose and traces of glucose were formed. It appeared, therefore, that the appearance of free RS was probably caused by the hydrolysis of non-reducing higher saccharide to reducing monosaccharide, and it became of interest to study the final products of this action.

When the enzyme concentrate described above was incubated at 40° with standard substrate so diluted as to give a concentration of 10 mg. combined RS/ml. detectable appearance of RS ceased before the theoretical value for total acid hydrolysis (Fig. 3). The amount not appearing in this experiment was 33.5% of the combined RS. After 50 hr. standard substrate was added to the solution to bring the concentration of combined RS approximately to the initial value. Free RS was estimated immediately, and after incubation for a further 2 hr. That the enzyme was still active was shown by the appearance of a further 1.43 mg. RS/ml. during this period, although the activity was now less than at the beginning of incubation, when 4.2 mg. RS/ml. had been liberated in an equal time. Addition of 16 mg. fructose/ml. at 50 hr. had no significant effect on the RS already present, the values being 24.2 mg. RS/ml. immediately after the addition, and 24.5 mg. RS/ml. 2 hr. later. It seemed probable, therefore, that the cessation of hydrolysis was due to part of the combined RS not being attacked by the enzyme, and not to an equilibrium with the fructose liberated.

The nature of this unattacked material was investigated chromatographically. Qualitative chromatography indicated the disappearance of all spots with R_F values lower than that of sucrose. A large amount of fructose, but little or no glucose, had appeared and there was an apparent increase of 'spot 1'. These findings were borne out by quantitative analysis of chromatograms from a similar experiment

(Table 5). It had been shown (Bacon & Edelman, 1950b, 1951) that the spot 1 initially present in tuber extracts is mainly, if not all, sucrose. That the material causing the

Fig. 3. Course of liberation of reducing substances (RS) from artichoke extract. Standard substrate, diluted with 0.2M-sodium acetate buffer (pH 5.0) to give 12 mg. combined RS/ml. (12 ml.), was incubated at 37° with 2.5 ml. of enzyme preparation (7 April 1949, described in text). RS, before (●) and after (▲) hydrolysis, and ketose (○), were estimated on 1.0 ml. samples.

increase in this spot during incubation with the enzyme system may also be sucrose was indicated by the following:

(a) The theoretical reducing sugar values calculated from the fructose content for this spot (assuming it to be sucrose) were 458 and 480 $\mu\text{g.}/20\ \mu\text{l.}$ in the 600 and 1980 min. samples respectively. The values given after extraction from the paper and hydrolysis with invertase were 440 and 438 $\mu\text{g.}/20\ \mu\text{l.}$ (4 ml. samples of the extracts were incubated for 90 min. at 40° with 1 ml. of a 1-25 dilution of British Drug Houses Ltd. 'Invertase Concentrate', which had been

Table 5. *Effect of hydrolytic enzyme preparation on the components of tuber extract*

(0.5 ml. standard substrate was incubated at 40° with 0.5 ml. of an enzyme preparation, prepared 16 January 1950 by a single precipitation between 25 and 52% saturation $(\text{NH}_4)_2\text{SO}_4$, activity 36 units/ml., dialysed before use. The ketose contents of the individual components were followed as previously described (Bacon & Edelman, 1951).)

Spot	Time of incubation (min.)						
	0	60	200	360	600	1440	1980
	$\mu\text{g. ketose}/20\ \mu\text{l. incubation mixture}$						
<i>F</i>	—	97	240	384	500	563	590
1	91	101	140	177	229	254	240
2	87	87	92	77	51	29	27
3	97	95	74	47	92		
4	87	70	40	24			
5	84	66	40	24			
6 to <i>n</i>	392	307	220	124			
Total	838	823	846	857	872	846	857

dialysed overnight against running tap water; no attempt was made to remove the enzyme before estimation of RS. A paper 'blank' was treated similarly; it amounted to about 25% of the RS measured.) These values, assuming complete breakdown by invertase, give ratios of fructose: glucose of 1:1.02 and 1:1.13.

(b) Only traces of free glucose appeared on the chromatogram even at 1980 min. incubation, and all the glucose in the extract could be accounted for by the material in spot 1, if that was assumed to be sucrose (as in the following calculations). Total fructose in the 1980 min. sample was 857 $\mu\text{g.}/20\mu\text{l.}$, therefore total RS should be $857 + 240 = 1097\mu\text{g.}/20\mu\text{l.}$, making the glucose as percentage of combined RS = $(240/1097) \times 100 = 21.8$. Similarly, the glucose in the 1440 min. samples would constitute 22.7% of the combined RS. The determined value for this extract was 21%.

(c) The enzyme preparations had very little action on sucrose (see below).

Action on sucrose. Enzyme concentrates were only very weakly active against sucrose under the conditions of temperature and pH normally used. Thus an enzyme preparation acting on standard substrate with activity 99 units/ml., had an activity of only 4.7 units/ml. against a sucrose solution with the same concentration (w/v) of combined RS. As the tuber extract material probably has a mean molecular weight of the order of 1000 at least, the molar concentration of sucrose was considerably greater than that of the standard substrate.

Action on fractions of standard substrate. To investigate the action of enzyme preparations on the components of the standard substrate with higher R_F , five fractions (A to E) were separated from standard substrate by paper partition chromatography in phenol followed by Soxhlet extraction from the paper. (For further details of these fractions see Bacon & Edelman, 1951.) Fraction B consisted of a mixture of spots 2 and 3 and contained 38.7% glucose, 61.3% fructose. Fraction D had R_F close to zero and probably contained more than two components. It contained 10.3% glucose, 98.7% fructose. Fraction B, on incubation with an enzyme concentrate of activity about 100 units/ml., showed progressive disappearance of spots 2 and 3 with time and a corresponding appearance and increase of spots in the same positions as spot 1 and fructose. At 180 and 360 min. incubation there was little or no carbohydrate material visible with R_F less than that of spot 1. At 1080 min. spot 1 itself had largely disappeared. The fructose spot increased throughout the incubation. Glucose was not shown by the spraying reagent used (phloroglucinol). Fraction D showed spots 1-3 and a higher streak on incubation with the enzyme, and an even greater quantity of fructose than fraction B. The weakness of spots 1-3 could be ascribed to the small amount of combined glucose in this fraction, as these three spots contain a relatively high glucose: fructose ratio, spot 1 probably being sucrose. These intermediate spots eventually disappeared except for spot 1, which was present even at 1080 min.

The R_F values of the substances detected in these experiments cannot be taken as proof of their identity with the spots of the original tuber extract, and the possibility is not excluded that some at least of the material in these positions may have been composed entirely of fructose residues.

Action on inulin. The rate of liberation of reducing sugar from standard substrate by the action of an enzyme concentrate was greater than that from an equivalent concentra-

tion of inulin (w/v) under the same conditions of pH and temperature. The molecular weight of inulin is, however, probably considerably greater than that of the mean molecular weight of the carbohydrates in standard substrate, so that its molar concentration would be correspondingly lower. However, no constant relationship was found between activity against standard substrate and activity against inulin. This variability, which was not fully investigated, may have been due to variation in the degree of aggregation of inulin in the incubated mixture; on prolonged incubation inulin was often seen to precipitate.

Fig. 4. Liberation of reducing substances (RS) from dandelion inulin, grass levan and irisins 'B'. Approximately 4% (w/v) solutions of the polysaccharides in 0.2M-sodium acetate buffer (pH 5.0) were incubated at 40° with equal volume (7 ml.) of an enzyme preparation, made 29 March 1950 by a single precipitation between 20 and 50% saturation $(\text{NH}_4)_2\text{SO}_4$ (activity: 58 units/ml.). The solutions lost water by evaporation during the long incubation, so the RS is plotted as percentage of total ketose.

The action on dandelion inulin at pH 5 (see also Fig. 4), when investigated by qualitative chromatography, was found to produce a large fructose spot, a trace of glucose, a spot identical in R_F (butanol-acetic acid) with sucrose, and a much weaker spot apparently in the same position as spot 2 of artichoke extracts. The spot in the sucrose position became progressively stronger with increasing time of incubation. Its colour with the benzidine-trichloroacetic acid reagent was at first the typical yellow given by fructose, but became darker brown and thus more suggestive of the presence of combined glucose. These observations were corroborated by two-dimensional chromatography using an invertase spray between developments (see Bacon & Edelman, 1951). At first the substances in the positions of spots 1 and 2 showed no glucose, but after longer incubation significant amounts of glucose were detected in the spot 1 position, suggesting that sucrose is produced from inulin and may be an integral part of its molecule. Control incubations of buffered enzyme, alone and with a mixture of fructose (10% w/v) and glucose

(0.3% w/v), gave no formation of a spot in the sucrose position.

Inorganic phosphate, at a concentration of 0.02M (as KH_2PO_4), had no effect upon the liberation of RS from inulin in 0.17M-sodium acetate buffer (pH 5.0) during 24 hr. at 40°. There was no change in inorganic phosphate during the first 10 hr. of incubation.

No inorganic phosphate was detected at any stage during the control incubation, in contrast to the control experiment with fresh extract in which inorganic phosphate was present initially, and increased significantly during incubation (Table 2).

Action on irisin and grass levan. The liberation of RS by the action of enzyme concentrate on irisin 'B' (kindly supplied by Dr D. J. Bell) and a levan prepared from a dried sample of Italian rye-grass is shown in Fig. 4. The breakdown of inulin under the same conditions is also shown. Control incubations of the three polysaccharides in the absence of the enzyme showed no significant appearance of RS during 24 hr.

DISCUSSION

Although the action of inulases from moulds has been studied fairly extensively, there have been few reports of investigations of higher plants since the original observations of Green (1887). Pringsheim & Kohn (1924) referred to attempts to extract inulase from inulin-bearing plants but gave no experimental details. Neither Wolff & Geslin (1918) nor Asai (1938) examined the action of their extracts on inulin alone.

The preparations described here were fully as active on a volume basis as the most active preparations from *Aspergillus niger* cited by Pringsheim & Kohn (1924), and the activity of fresh extracts of tubers, made in the Waring blender, was measurable over relatively short periods of incubation (2-3 hr.). There would thus seem to be no practical objection to using the tissues of higher plants as sources of inulase; there is also the advantage of working with tissues which normally contain and presumably metabolize the polysaccharide.

The purest yeast-invertase preparations so far studied (Adams, Richtmyer & Hudson, 1943) showed some hydrolytic activity towards inulin, although it is not certain (cf. Pigman, 1944) whether this is to be ascribed to an unspecific action of invertase itself, or to a contaminating enzyme. The mould preparations studied by Pringsheim & Kohn (1924) had appreciable invertase activity which was diminished to a lesser extent than the inulase activity by adsorption on calcium phosphate. The preparations described here were never altogether free from action upon sucrose, but the rate of liberation of fructose and glucose from this substance was never more than 20% of the rate of fructose production from inulin at similar concentrations (w/w). Pringsheim & Kohn (1924) found three times as much invertase as inulase activity in their preparation. It may be significant that no sub-

stances other than sucrose, glucose, and fructose were seen on chromatograms when the artichoke preparations acted upon sucrose, in contrast with observations on yeast invertase (Blanchard & Albon, 1950; Bacon & Edelman, 1950a).

The chromatographic studies of the action of tuber preparations show that fructose is the first product of their action on inulin; the appearance of fructose-containing substances in the di- and tri-saccharide positions fairly early in the hydrolysis may be due to a transfructosidation reaction (Edelman & Bacon, 1950) between the fructose formed and the inulin not yet broken down. It would thus seem that the enzyme preparation has the properties of a fructofuranosidase and that it is most active towards the 2-1'-linked terminal residue of inulin and related compounds. The crude preparations employed in the present investigation possessed a limited activity towards grass levan and irisin 'B', two other polysaccharides consisting chiefly of fructose. The irisin molecule is highly branched (Bell & Palmer, 1949), having both 2-1' and 2-6' linkages, though its exact structure cannot be decided upon. The slow rate at which it is attacked by the artichoke preparation might be taken as suggesting that its 'backbone' is linked in the inulin manner, while the side groups have the levan linkage, the action of the enzyme thus being limited by the speed with which the side groups could be removed to expose the more vulnerable 2-1' linkage.

A preliminary study by Miss M. Holden, at Rothamsted Experimental Station, of the action of certain fungal enzyme preparations (cf. Holden, 1950) on inulin, grass levan and irisin showed similar results, in that the degree of hydrolysis of irisin was in each case similar to that of the levan (Holden, 1949). The preparations with most inulase activity were most active against the other two polysaccharides, but in every case the rate of liberation of reducing sugar from inulin was considerably greater. A bacterial levan gave results similar to those with the grass levan.

The slow but significant hydrolysis of sucrose by our preparations might be ascribed to the action of a not wholly specific fructofuranosidase, the converse of the inulase activity of invertase preparations. However, clarification of this and other problems concerning specificity and the more important problem of the relation between hydrolysis and transfructosidation in artichoke-tuber preparations must await the results of a systematic attempt to purify the enzyme or enzymes concerned.

From the metabolic point of view the present results do not represent any advance on the original discovery of Green (1887), which conformed to the general observation that reserve carbohydrates may often be hydrolysed by extracts of the tissues which

contain them. It is notable, though, that the artichoke preparations described here had very little hydrolytic action upon sucrose, which was present in concentrations up to 1.5% (w/w fresh tuber) in all the tubers examined.

SUMMARY

1. Aqueous extracts of tubers of the Jerusalem artichoke (*Helianthus tuberosus*) show, on incubation, an increase in reducing substances and a decrease in optical rotation.

2. The enzyme or enzymes responsible have been freed from the tuber carbohydrates and concentrated twenty-fold by fractional precipitation with ammonium sulphate.

3. By the use of paper partition chromatography such preparations have been shown to liberate fructose from various substrates, including sucrose, inulin, a levan, and irisin 'B'; glucose was detected among the hydrolysis products of inulin.

4. The liberation of reducing sugars from the tuber carbohydrates and from inulin virtually ceased before complete hydrolysis had taken place. The residual combined sugar was in the form of a disaccharide, probably sucrose.

5. From these observations the main hydrolytic activity of tuber preparations has been characterized as that of a fructofuranosidase, with a moderate degree of specificity for such residues linked to position 1 of another fructose residue.

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The Metabolism of Pyruvate by *Lactobacillus plantarum*

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When pantothenate is added to suspensions of *Lactobacillus plantarum* deficient in that factor the yield of acetylcholine is much increased (Stephenson & Rowatt, 1947). Hills (1943) showed that the rate of oxidation of pyruvate by pantothenate-deficient suspensions of *Proteus morganii* is increased by the addition of pantothenate. It was thought on the

basis of these observations that pantothenate might be required for the metabolism of pyruvate in *Lb. plantarum*, and an investigation of the latter was planned. Pyruvate was found to be broken down to acetoin and carbon dioxide without the participation of pantothenate. This finding and various other aspects of pyruvate metabolism in this organism are described in the present paper.

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