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The potato starch hydrolyzate as a substrate of carrot tissue grown in vitro

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INTRODUCTION

A. Słabęcka-Szweykowska (1952) in investigations on the influence of different sugars on the formation of anthocyanin in vine tissue grown *in vitro* took maltose also into consideration. As she had difficulties in obtaining maltose she tried to replace it with the enzymatic hydrolyzate of potato starch. The result was astounding: the growth optimum of vine tissue on the starch hydrolyzate was about 300 per cent higher than with sucrose regarded hitherto as the best carbon source from synthetic media for vine tissue. Moreover, it is remarkable that the growth optimum was found when the concentration of sugar in the medium was 2—6 per cent, while in the case of the hydrolyzate the optimum corresponded to a 9 per cent. concentration. Gautheret (1956) described the starch hydrolyzate as "the most efficient source of carbon for vine tissue".

An explanation of this phenomenon could only be guessed at for the time being. Both Słabęcka-Szweykowska and Czosnowski (1952) expressed the same opinion that such stimulation of growth could only be due to the presence in the hydrolyzate of "some favourable plastic substances" or "the action of the products of hydrolytic breakdown of starch that have molecules larger than maltose..., to inherent phosphorus and or to some other elements".

The aim of this investigation was to search for the true explanation of the problem.

Starch consists of two essential fractions: amylose and amylopectin (Maquenne et Roux 1905a and b, Gruzewska 1908, 1911). In the course of hydrolysis amylose yields only maltose while amylopectin yields maltose and limit-dextrins (Maquenne et Roux 1905a and b, Myrbaeck 1938, Hodge, Montgomery and Hilbert 1948).

Besides glucose as a structural unit of starch the following non-sugar components are found in starch grains of different plants: Si, Cu, Mg, K, Na, Fe, N, P and lipids (Kerr 1950). In potato starch lipids are found in very small amounts (Schoch 1942) and all elements except P can be washed out with dilute HCl (Kerr 1950). This resistance of P to HCl washing was explained by Pasternak (1935) who was the first to define the chemical relationship of P and starch. Phosphorus in potato starch is linked as an ester to some glucose rings at the 6-th carbon. This ester linkage of P is typical only for amylopectin because amylose contains no phosphorus. (Baldwin 1930, Pasternak 1935, Myrbaeck 1938, Schoch 1942, Kerr 1950). In the course of enzymatic hydrolysis of starch all of its organic P remains in the limit-dextrins (Myrbaeck 1938, Myrbaeck and Neumueller 1950).

Amylases are not able to break down 1—6 linkages. A branch of amylopectin charged with the phosphoric acid radical is also an alien substrate for them (Hodge, Montgomery, Hilbert, 1948).

The enzymatic hydrolysis of starch never yields more maltose than 80 per cent of starch. What remains consists of limit-dextrins with unbroken 1—6 linkages, isomaltose (Myrbaeck and Neumueller 1950) and a small amount of monosaccharides. At their origin may be, 1) the presence of glucosidases in the diastase preparation and 2) the liberation of terminal glucose groups as reported for salivary amylase (Pazur 1953). Finally among the non-maltose products of hydrolysis are the phosporosugar compounds (Pasternak 1935).

MATERIAL AND METHODS

At the time this work was started the vine tissue used by Słabęcka-Szweykowska was no longer available, so it was decided to carry out the experiments on carrot tissue isolated by Gautheret and maintained in culture by regular transplantations since 1938. The carrot tissue has become adapted to self-formation of the auxin-type substances (Czosnowski 1952, Duhamet 1953a and b, Heller 1954).

Heller's (1954) mineral nutritive medium was used. When tissue was grown on a medium deprived of phosphorus $Na_2HPO_4H_2O$ was replaced by an equimolar amount of Na_2SO_4. 10 H_2O.

For control experiments 1 per cent glucose was used this concentration being the most effective (Lewicka 1950).

In all experiments the nutritive medium in batches of about 12 ml was poured into twelve culture tubes. The tubes with the medium were plugged with cotton and sterilized in an autoclave for 20 minutes at 1 atmosphere. Transplantations were made in sterile conditions.

The procedure followed for preparing starch hydrolyzate was the same as that developed by Słabęcka-Szweykowska.

A 5 per cent starch gel was prepared: 50 g of commercial potato starch were suspended in 100 ml of cold water distilled in a glass vessel to avoid inhibitory action of copper traces (Myrbaeck and Neumueller 1950) and stirred till the suspension seemed homogeneous. 850 ml of boiling bidistilled H_2O were poured onto the suspension. The gel was boiled and stirred for 5 minutes. It was then cooled to 50°C and diastase suspended in a few millilitres of water was added.

The power of diastase ("Diastase Merck"), used for saccharifying starch gel was such that during two hours at 55°C all the starch hydrolyzate was converted, the enzyme to substrate ratio being 1 : 250. Thus 200 mg of Merck's diastase were taken for 50 g of starch. The diastase was suspended in twice that amount of bidistilled water warmed to 50°C in a thermostat then mixed with the gel and stirred. To the liquified suspension a small amount of benzene was added as a preservative and the beaker covered with a Petri dish was placed in a thermostat at 50°C for 24 hours.

To obtain a high maltose content in the hydrolyzate the procedure described above was repeated 5 times, fresh bidistilled water being replaced by boiled hydrolyzate from the day before. Boiling inactivated diastase was added carlier. The hydrolyzate was filtered every time from a small amount of unhydrolyzed erythrodextrin flakes. After the fifth hydrolysis the whole was evaporated to about 1/3 of the original volume.

The amount of reducing sugars counted as maltose was determined by Bertrand's method.

The content of reducing sugars calculated as maltose in some hydrolyzetes was as follows:

Phosphate determinations of the blue phospho-molybdenum complex reduced by hydroquinone were made in a colorimeter.

The results were calculated as phosporus.

Organic phosphate was determined directly in 1 ml of the hydrolyzate by burning a sample of hydrolyzate, determing the total content of P and calculating the organic P content.

The hydrolyzate was dried in 105°C to a constant weight and the equivalent of 2 ml was treated with 10 ml of conc. HNO_3 and 1 ml of conc. H_2SO_4 and burned in a 250 ml Kjeldahl flask.

Determinations were made in a Leitz colorimeter with the Arrhenius method described in Lange's "Kolorimetrische Analyse".

The values obtained were:

1 ml of No 20 hydrolyzate (H 20) contained 320 μg of total phosphorus, 120 μg of inorganic P and 200 μg organic P.

EXPERIMENTS

At the time the experiments were carried out the carrot tissue did not grow well. Its growth was slow and uneven; quite frequently necrosis occurred in newly transplanted tissue fragments. Nevertheless, it has been possible to obtain definite results.

The weight of original fragments was 100—120 mg.

The aim of the first experiment was to define for what range of hydrolyzate concentrations the most prominent growth reaction of the carrot tissue would be obtained. It seemed probable that the sugar requirements of carrot tissue would be different then of *Vitis vinifera* tissue and consequently that carrot tissue would respond differently to starch hydrolyzate.

The growth optimum found in preliminary experiments corresponded to 2 per cent. It was thus necessary to investigate the effect of lower concentrations because concentrations used at first were 2, 4, 6, 8 per cent, whereas 2 per cent, gave the highest result. The tissue was then transplanted on a medium containing No 20 hydrolyzate in concentrations of 0.5, 1, 2, 4 and 8 per cent in two series: with and without phosphates in the mineral medium. The tissue grew for 52 days. Fig. 1 shows that at 0.5 and at 8 per cent there is no difference between the two series of experiments. It is, however, apparent that the osmotic pressure at the 8 per cent concentration is disadvantageous for the tissue (Szweykowska 1952).

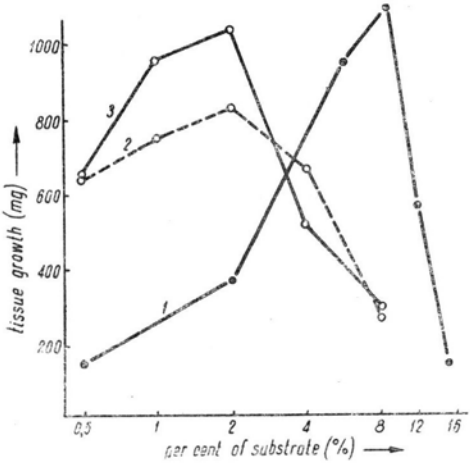

Fig. 1

1 — the effect of starch hydrolyzate on vine tissue in Słabęcka-Szweykowska's experiment (1952);
2 — starch hydrolyzate no 20 without standard P;
3 — starch hydrolyzate no 20 with standard P.

The control on 1 per cent glucose (optimum for carrot tissue) as compared with the experiment using No 20 hydrolyzate gave no clear difference between the series with and without P, but tissue growth on glucose was far weaker than on

the hydrolyzate. The lack of difference between the two glucose series was due to the fact that the transplantation was the first one to a medium without phosphate. The growth being weak, the production of new cells was not intense and the amount of phosphates saved from the earlier medium was probably sufficient to be distributed among the new cells.

Tissue grown on hydrolyzate had a decidedly healthy appearance: necrotic symptoms were few, whereas controls on glucose had numerous brown patches.

Since the chief product of starch hydrolysis was maltose (up to 80 per cent) the tissue was transplanted on media with 0,5, 1, 2, and 4 per cent of maltose in two series: with and without P.

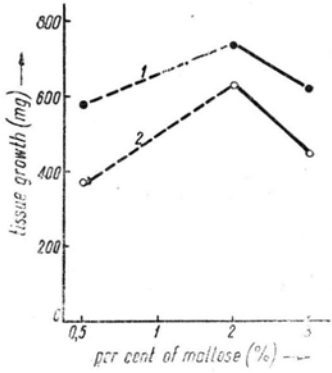

Fig. 2
1. maltose + standard P;
2. maltose — standard P.

Owing to infection the culture on 1 per cent maltose medium perished (at that time experiments with molds very dangerous for in vitro culture were being carried out at the Institute). However, it seems very unlikely that carrot tissue would grow better on 1 per cent than 2 per cent maltose because maltose is a disaccharide. It is clear from the diagrams that lack of P inhibited tissue growth. Even the highest growth rate on maltose was less than on hydrolyzate but higher than on glucose. It is

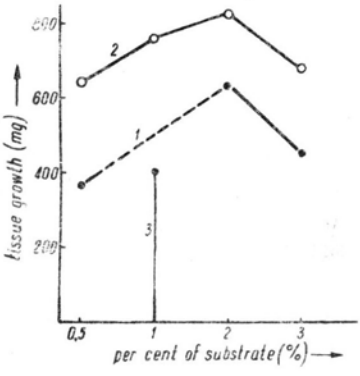

Fig. 3
1 — maltose + P;
2 — hydrolyzate no 20 + P;
3 — glucose + P.

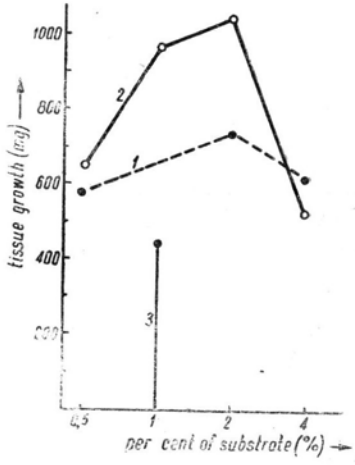

Fig. 4
1 — maltose — P;
2 — hydrolyzate no 20 — P;
3 — glucose P.

to be noted that on 4 per cent maltose plus P (normal mineral medium) growth was better than for the corresponding concentration of the hydrolyzate plus standard P.

Here are the results:

Table 1

Medium \ Concentration	Tissue weight in mg (mean values and standard errors)				
	0,5%	1%	2%	4%	8%
glucose + P	—	450 + 54	—	—	—
glucose — P	—	410 + 35	—	—	—
maltose + P	580 + 59	—	740 + 56	620 + 39	—
maltose — P	370 + 20	—	630 + 59	450 + 38	—
H 20 + P	650 + 43	960 + 50	1040 + 96	520 + 29	300 + 52
H 20 — P	640 + 46	760 + 56	830 + 60	680 + 61	260 + 23

DISCUSSION

The above results lead to the following conclusions and enable one to judge how far they are justified. The opinion of Czosnowski (1952) and Słabecka-Szweykowska (1952) on the favourable influence of starch hydrolyzate was confirmed.

It is clear from the presented diagrams that carrot tissue grows far better on starch hydrolyzate than on maltose. Maltose amounts to 70—80 per cent of hydrolyzate substances and being a disaccharide produces the best growth of the tissue when its per cent (by weight) concentration is twice that of such a mono-sugar as glucose. A molecule of maltose exerts in solution the same osmotic pressure as a molecule of glucose but maltose contains a double amount of carbon substrate. For this reason the effort in absorbing sugars through the cell wall is in both cases the same but in the case of maltose it is twice as effective. The lesser effect of glucose is shown in figs. 3 and 4. In this aspect it is not probable that in the case of maltose the one per cent combination which was omitted in the experiments would give better results than the 2 per cent concentration.

However, in the case of hydrolyzate the influence of some other factors is superimposed on the influence of maltose. These can be only limit-dextrins or compounds charged with the phosphate radical (phosphotetrasaccharides or similar dextrins with P). From earlier considerations it follows that the action of mono-sugars is negligible and can by no means be responsible for the highly positive effect of hydrolyzate. The amount of dextrins introduced with hydrolyzate could range from 20 to 30 per cent of the original weight of starch. Since the amount of the hy-

drolyzate was calculated in respect to maltose the amount of dextrins the reducing value of which amounts to about 0,1 per cent of the reducing value of maltose (Hodge, Montgomery and Hilbert 1948) was not made apparent in the maltose value. The real by weight content of sugars in the medium was greater than the content calculated in respect to maltose only. This is the reason why the superposition of the effect of "some profitable plastic substances" was so distinct.

The two parallel series of experiments, one on a standard mineral medium, the other on a medium deprived of phosphates gave different results. The behaviour of phosphorus differs because of the two forms in which it occurs in the hydrolyzate:

1) free inorganic phosphate ions independent of dextrins and
2) organic phosphorus linked to dextrins.

The following table explains the problem quantitatively:

Table 2

H 20	0,5%	1%	2%	4%	8%
ml per 100 ml of medium	1,7	3,4	6,9	13,8	27,6
P of standard medium per 100 ml	2,5 mg	2,5 mg	2,5 mg	2,5 mg	2,5 mg
Inorganic P introduced with hydrolyzate per 100 ml	0,2 mg	0,4 mg	0,8 mg	1,6 mg	3,2 mg
Organic P introduced with hydrolyzate per 100 ml	0,35 mg	0,7 mg	1,4 mg	2,8 mg	5,6 mg
Sum of per cents of inorganic P of standard medium and organic P of hydrolyzate	108%	116%	132%	164%	228%
Content of organic P in medium lacking standard P	8%	16%	32%	64%	128%
Sum of P in medium lacking standard P	22%	44%	88%	176%	352%
Sum of P contents in standard medium	122%	144%	188%	276%	452%

It is apparent from the table that starch hydrolyzate contains considerable amounts of inorganic and organic P. At higher concentrations inorganic P in the hydrolyzate replaces the missing amount of standard P. This is shown by the fact that at 4 per cent when inorganic P of the hydrolyzate amounts up to 64 per cent of the standard medium the tissue grew better when standard P was excluded. Higher P concentrations inhibit growth not only because sugars are in amounts exceeding the optimum but also because the P concentration becomes harmful (H e l l e r 1954). For this reason tissues grow better on 4 per cent maltose than on 4 per cent hydrolyzate. However, the best result is obtained with a medium containing hydrolyzate and standard mineral P. It is to be concluded, therefore, that the hydrolyzate contains inherent P in amounts too small to replace the missing P of the standard mineral medium at the concentration of 2 per cent of the hydrolyzate. The advantageous influence of the hydrolyzate and especially of its more complex fractions is interesting in view of the fact that a plant during active growth produces more α-amylase than β-amylase (K n e e n 1950). For this reason K n e e n called α-amylase "amylase of activity", and β-amylase "amylase of dormancy".

Since besides maltose α-amylase gives limit-dextrins while decomposing amylopectin we observe here the correlation between active growth and utilization of dextrins by more intense working protoplasm. At rest the essential work is done by β-amylase which produces maltose at a slower rate. Metabolism is then by far less vigorous and the small amounts of released maltose are sufficient. Starch hydrolyzate is undoubtedly a more natural substrate than pure glucose or maltose. It is thus to be supposed that the products of starch breakdown do not necessarily have maltose or glucose as intermediate stages. Starch is rapidly decomposed by α-amylase to large or small dextrins which may become (H a s s i d 1951) substrates for the action of 1,6-glucosidases. In this manner the large molecules of the two starch fractions are rapidly made accessible for mass action of phosphorylases detaching one by one the glucose rings. Thus, the phosphorylases which produce phosphate esters of glucose shorten the way of amylopectin transformation from a static form to the intense metabolism of growing tissue.

This explanation is also confirmed by F. L i p m a n's (1941) well known supposition that phosphorolysis of glycogen or starch followed by glycolysis yields 4 high-energy phosphate bonds while utilization of glucose yields only two.

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SUMMARY

The growth stimulating effect of potato starch hydrolyzate reported for vine tissue by Słabęcka-Szweykowska was investigated and carrot tissue. It was found that the stimulation was due to maltose or polyglucose products of starch amylolysis.

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STRESZCZENIE

Celem niniejszej pracy było wyjaśnienie korzystnego wpływu hydrolizatu skrobi na wzrost in vitro tkanki winorośli, co stwierdziła Słabęcka-Szweykowska.

Okazało się, że pobudzenie wzrostu tkanki marchwi jest wynikiem działania maltozy i wielo-glukozowych produktów amylolitycznego rozkładu skrobi.

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