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# Secretion of Amylase by Rumex Virus Tumors In Vitro

L. G. Nickell
*Chas. Pfizer and Co., Inc.*

M. K. Brakke
*University of Illinois at Urbana-Champaign*

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# SECRETION OF α-AMYLASE BY RUMEX VIRUS TUMORS IN VITRO. BIOLOGICAL STUDIES[1]

L. G. Nickell and M. K. Brakke

THE SECRETION of extracellular enzymes by intact cells of higher plants apparently is a rare occurrence. One example of it is the secretion of an extracellular amylase by the tissue of a virus-induced tumor from the root of *Rumex acetosa* L. An amylase is secreted in sufficient quantities by this tissue to enable it to grow as well in vitro on a starch-containing medium as on one containing sucrose.

In the literature dealing with plant tissue culture there are few references to the utilization of starch as a carbon source by plant tissues grown in vitro. Some authors (Gautheret, 1945; Hildebrandt and Riker, 1949, 1950, 1953) state that starch supports growth poorly or not at all. In surveying carbon sources for the growth of virus tumor tissue from the root of *R. acetosa,* Nickell and Burkholder (1950) found soluble starch to be an excellent source of carbon for the growth of this tissue. A few preliminary experiments suggested that this utilization of starch was due to the presence of an extracellular enzyme. Brakke and Nickell (1951) demonstrated that the enzyme was an alpha-amylase secreted by the virus tumor tissue. This first report on the enzyme presented a microassay method which was used to study the enzyme's properties and which led to the characterization of the amylase. A second study presented evidence that plant hormones in concentrations up to and including 100 p.p.m. added to the substrate have no effect on the activity of this amylase (Brakke and Nickell, 1952).

The present report is concerned primarily with biological studies on the secretion of amylase: the rate of its production, comparison of virus tumor tissue and excised, virus-free roots from the host plant (*R. acetosa*), and factors affecting the secretion. Studies on other tissues confirming their inability to grow well on starch, in contrast to the virus tumor tissue, are also presented.

MATERIALS AND METHODS.—Spent liquid medium and extracts of tumors, prepared by macerating tumor tissue with a mortar and pestle, were clarified by filtration or centrifugation and assayed for enzyme by the method of Brakke and Nickell (1951). In some instances where the activity of the enzyme was too low to be measured accurately by this method, iodine was added to aliquots removed from the starch-containing medium at various intervals after introduction of the tumors. The optical density of the resulting complex was compared with that from control medium without tumors. This measured the cumulative action of all the enzyme secreted from the time the tissue was placed in the medium rather than the concentration of enzyme at a given time.

The methods and medium (no. 24) employed for growth studies were the same as those previously used for in vitro work with the *Rumex* virus tumor tissue (Burkholder and Nickell, 1949; Nickell, 1950; Nickell and Burkholder, 1950; Nickell et al., 1950). Growth is expressed in terms of the growth value which is the final wet weight of the experimental tissue divided by its original weight (approximately 30 mg. for the virus tumor tissue). Thus a growth value of one represents no growth. Values reported are averages of 5 replicate measurements of one experiment; all experiments were repeated at least once. The methods used in growth studies with the crown gall tissues were the same as those used for the virus tumor tissue except that the original weight of the crown gall tissues was approximately 50 mg. The basal medium used for the work with crown gall tissues was the low phosphate medium of Nickell (1951). The virus tissue used in this study was the same $R_1$ strain, isolated by L. M. Black in 1945, used in previous studies. This tumor was induced by the virus *Aureogenus magnivena* Black in the roots of the host plant *R. acetosa.*

The crown gall tissues used were periwinkle (*Vinca rosea* L.) tissue isolated by Dr. P. R. White in 1945, tobacco (*Nicotiana tabacum* L.) and cactus (*Opuntia monacantha* Haworth) tissues isolated by Dr. G. Morel, sunflower (*Helianthus annuus* L.) petiolar tissue isolated by Dr. A. C. Braun in 1946 and sunflower stem tissue isolated by Dr. R. S. de Ropp in 1946. Samples of all these tissues, except the periwinkle, were obtained from Dr. A. C. Braun in 1950. These tissues were all free of the bacterium, *Agrobacterium tumefaciens* (Smith and Townsend) Conn., which induced the original tumors. Extensive tests using a wide variety of cultural conditions failed to show any bacteria in extracts from the tissues used in this work.

EXPERIMENTAL RESULTS.—*Secretion of amylase by Rumex virus tumor tissue grown in vitro.*—Time studies showed a rapid secretion of amylase which was detectable very soon after placing tumor tissue in liquid medium. The amount of secreted amylase in a given flask increased rapidly at first, but usually remained almost constant after the first 1–2 days. This suggested either that (a) destruction was rapid or that (b) the secretion decreased or ceased after the first day or two.

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Figure 1 shows the relative amounts of amylase present in 8 flasks after various time intervals. Values are expressed as per cent of the 2-day amount which has been set at 100. The curve is that which would result if there were a constant rate of production and if destruction were by a 1st order process with a half life of 0.7 days. However, the measured half life varied from 10 to 30 days in unpurified, unbuffered liquid medium from which the tumor tissue had been removed. Hence it appears that destruction was not responsible for the leveling off of enzyme concentration. Rather the rate of production must have decreased rapidly after the first few days. In most cases the tumor tissues did not grow well in the liquid medium, probably because of lack of oxygen since they were usually largely submerged. When returned to a solid medium the tumor tissues resumed their normal rate of growth. It is possible that the decrease in rate of enzyme production and slow rate of growth were both due to a low respiration rate.

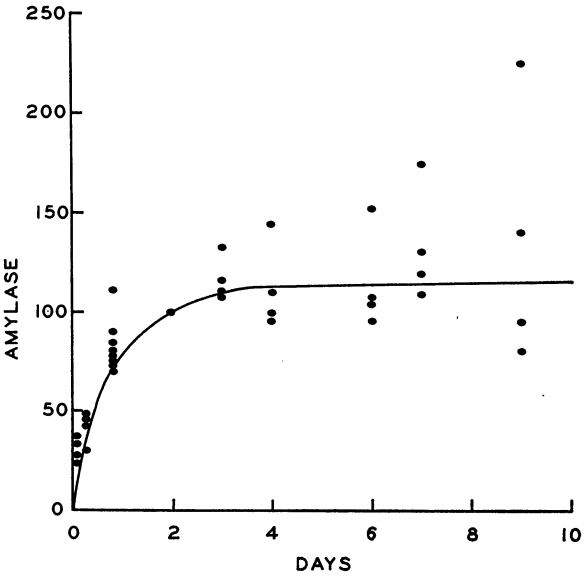
The concentrations of amylase in tumor tissue and in surrounding medium for several experiments are given in table 1. Ratios of the total amount of amylase in the medium to that in the tissue are also presented. For example, as reported in the 6th line of this table, after one day there was one-third as much total amylase in the medium as in the tumor, i.e., one-fourth of all the amylase present had been secreted. If cellular breakdown were to account for such an amount of enzyme, over one-fourth of the cells would have had to break down within 24 hr. after the tumor tissue was placed in the medium. However, all the tissue was in excellent condition and showed no evidence of cellular breakdown. In experiments lasting longer, the total amount of enzyme in the medium was as high as 8 times that in the tissue. Such a level would be impossible on the basis of cellular breakdown.

*Growth studies of Rumex virus tumor tissues on*

TABLE 1.—*Amounts of amylase in virus tumor tissue and medium*

| Time in days | Amylase concentration: tumor tissue | Amylase concentration: medium | wt. of medium / wt. of tumor tissue | total amylase in medium / total amylase in tumor tissue |
|---|---|---|---|---|
| | Medium containing sucrose | | | |
| 2 | 15.0[a] | 5.1[b] | 3.8 | 1.3 |
| | 14.3 | 3.3 | 4.0 | .9 |
| | 12.3 | 3.9 | 3.5 | 1.1 |
| | 12.4 | 5.1 | 3.3 | 1.3 |
| 9 | 11.1 | .41 | 25.0 | .8 |
| | Medium containing soluble starch | | | |
| 1 | 15.3 | 3.7 | 1.3 | .3 |
| 2 | 14.9 | 3.4 | 1.3 | .3 |
| 9 | 4.4 | 4.9 | 6.2 | 7.5 |
| | 14.6 | 5.5 | 7.2 | 2.7 |
| | 10.6 | 10.0 | 4.7 | 4.5 |
| 5 | 5.3 | .36 | 25.0 | 1.7 |
| 8 | 8.5 | .33 | 25.0 | 1.0 |
| 11 | 7.2 | .29 | 25.0 | 1.1 |
| 15 | 7.7 | .31 | 25.0 | 2.3 |
| 17 | 2.6 | 1.6 | 4.7 | 2.9 |
| 28 | 1.8 | 1.4 | 6.4 | 4.8 |

[a] Mg. starch hydrolysed/hr./g. tissue.
[b] Mg. starch hydrolysed/hr./ml. medium.



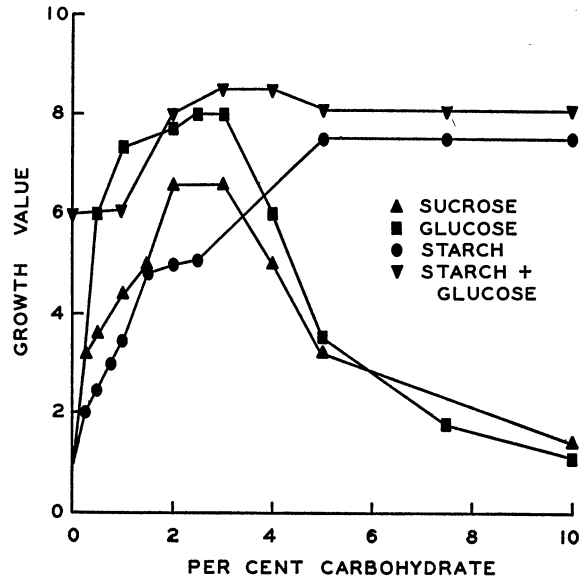




Fig. 1–2.—Fig. 1. Amount of amylase in liquid medium at various times after introduction of virus tumor tissue. Curve represents values which would result if enzyme production were at a constant rate and if its destruction were by a 1st order process with a half life of 0.7 days.—Fig. 2. Effect of various concentrations of certain carbon sources on the growth of *Rumex* virus tumor tissue.

*solid media.—Comparison of various sugars, dextrins, and starches as carbon sources.*—The data in fig. 2 show that this tissue grew well on solid media containing various concentrations of soluble starch, sucrose, glucose, or glucose plus soluble starch. Glucose and sucrose showed a depressant effect at the higher concentrations whereas soluble starch did not. At the 2 per cent level (concentration at which most of the work was done) glucose was slightly better than sucrose which in turn was somewhat better than soluble starch. However, as the concentration was increased, the two sugars clearly depressed the growth rate, whereas growth became better with starch. At 5 per cent starch the growth rate reached a maximum which stayed steady up to 10 per cent. At these concentrations the sugars did not support good growth.

It is apparent that at the lower concentrations glucose was utilized better than sucrose. Because of the effective utilization of low concentrations of glucose and of high concentrations of starch, it was decided to test media containing 0.5 per cent glucose in addition to various concentrations of soluble starch. As shown in fig. 2 the growth values started out at the level of 0.5 per cent glucose and increased as the concentration of starch increased to a value slightly higher than that for starch alone. This extra growth was probably supported by the glucose shortly after time zero while the secreted amylase was hydrolyzing starch.

Growth of the virus tumor tissue was good also on solid media containing 2 per cent of any of the following: corn starch, corn dextrin, potato starch or potato dextrin. Growth on the dextrins was slightly better than on the corresponding starches. Color of the experimental tissue was greener on the starches and dextrins than on sucrose or glucose.

*Utilization of starch as a carbon source by crown gall tissues grown in vitro.—Growth on solid media.*—Table 2 shows the growth of 4 crown gall tissues on the low phosphate basal medium containing 2 per cent soluble starch compared with their growth on this medium containing 2 per cent sucrose. Growth of the virus tumor tissue on medium no. 24 containing either soluble starch or sucrose is also given. When growth on starch is expressed as per cent of the growth on sucrose, it is seen that the crown gall tissues did not grow well when starch was employed as the sole source of carbon.

*Starch hydrolysis in liquid media by crown gall tissues.*—The poor growth of crown gall tissues on starch-containing medium suggests that they secrete less starch-hydrolyzing enzyme than does the virus tumor tissue. In order to check this hypothesis, tissues which had been growing on agar slants were placed in flasks containing 10 ml. of liquid low-phosphate medium with soluble starch as the carbon source. The hydrolysis of starch was determined after 1 and after 3 days by measuring the optical density of the starch-iodine complex formed from the medium. Table 3 shows the small amount of starch hydrolyzed by crown gall tissues after 1 day or even after 3 days. *Rumex* virus tumor tissue hydrolyzed 5, 10, 22 and 65 per cent of the starch in 1, 3, 7, and 24 hr., respectively, under the same conditions. Assuming that all the tissues secreted enzyme at a constant rate and of the same activity, it can be calculated from the data that the crown gall tissues secreted enzyme 0.5–5 per cent as fast as did the virus tumor tissue.

The data indicate that some starch was hydrolyzed by the crown gall tissues, but, while a secreted amylase may have been responsible, the amount was so small that it would be difficult to prove whether or not it were an amylase.

*Lack of detectable enzyme secretion by excised roots of Rumex.*—Since the presence of such an efficient secretion of amylase had been shown for the *Rumex* virus tumor tissue, it was thought desirable to determine if the normal roots of the same plant secreted amylase. If not, the change from normal to neoplastic growth might have been accompanied by a qualitative change, a most significant point in the study of abnormal growth.

No amylase activity could be demonstrated by the assay of Brakke and Nickell (1951) in spent sucrose medium in which the roots had been growing nor in root extracts. As a more sensitive test, excised roots of *R. acetosa*, which had been subcultured in vitro for several years in low-phosphate, liquid medium containing sucrose, were placed in the same medium but with soluble starch as the sole carbon source. Iodine was added to aliquots removed from uninoculated control flasks and from experimental flasks at intervals over a 3-month period. There was no decrease in the optical densities of the starch-iodine complexes nor in the ratios of the optical densities at different wave lengths during the first 19 days. An optical density fall due to hydrolysis could have been counteracted by evaporation over the course of the experiment, but the ratios would have changed had hydrolysis oc-

TABLE 2. *Comparative growth of virus tumor tissue on solid medium No. 24 and crown gall tissues on solid low phosphate medium with sucrose or starch as the carbon source at the 2 per cent level.*

| Tissue | Growth Period (days) | Growth value | | Starch growth as % sucrose growth |
|---|---|---|---|---|
| | | Sucrose | Starch | |
| Crown gall tumors of | | | | |
| *Nicotiana tabacum* | 34 | 8.6 | 3.8 | 37 |
| *Helianthus annuus* (petiole) | 21 | 6.0 | 1.4 | 8 |
| *Helianthus annuus* (stem) | 21 | 8.3 | 1.6 | 8 |
| *Vinca rosea* | 21 | 4.2 | 2.1 | 34 |
| Virus tumor of | | | | |
| *Rumex acetosa* | 21 | 6.8 | 5.2 | 73 |

TABLE 3. *Starch hydrolysis in liquid medium by crown gall tissues.*

| Tissue | % conversion/gm. tissue 1 day | 3 days |
|---|---|---|
| Control | 0 | 0 |
| *Opuntia monacantha* | 0 | 0 |
| *Vinca rosea* | 11 | 39 |
| *Helianthus annuus* (stem) | 2 | 13 |
| *Helianthus annuus* (petiole) | 6 | 33 |
| *Nicotiana tabacum* | 6 | 33 |

curred. After 3 months the ratios of the optical densities of the starch-iodine complexes at 2 wave lengths indicated that 11 per cent of the starch had hydrolyzed in the flasks containing roots. No attempt was made to discover if this hydrolysis was due to an enzyme for the amount of enzyme necessary to cause 11 per cent hydrolysis in 3 months would be small indeed. At the end of the 3-months period, the roots were no longer viable. Apparently the starch could not be used as a carbon source and the excised roots died. Changes brought about by death of the tissues may have aided in the breakdown of starch.

It is concluded that no secretion of starch-hydrolyzing enzymes by the roots has been demonstrated. Since the medium contained phosphate, phosphorylase as well as amylase should have been detected if present.

*Effect of tannin on amylase activity.*—No amylase could be demonstrated in extracts of excised *Rumex* roots grown in vitro. Since work with *R. acetosa* plants grown in the greenhouse for virus studies had shown that this plant contains a rather large amount of tannin, it was thought important to test the effect of tannin on the action of amylase and to determine if tannin were present in the excised roots.

It was found that 0.02 per cent commercial tannic acid caused 50 per cent inhibition of the amylase activity while 0.1 per cent caused complete inhibition. Spent medium in which excised roots had grown did not inhibit amylase action, but extracts of macerated roots grown in vitro did inhibit the action of the enzyme. In testing the effects of root extracts, roots were macerated with twice their weight of water and the extract centrifuged 35 min. at 3400 r.p.m. Such an extract contained 1.4 mg. tannin per ml. according to the Folin-Denis test (Official Methods of A.O.A.C. 1950). Additions of this root extract to the reaction mixture to give 0.04 per cent tannin caused 50 per cent inhibition. Apparently, the level of tannin in the root extract, as determined by the Folin-Denis test, was high. However, the failure to demonstrate amylase in root extracts could have been due to the tannin of the roots.

*Effect of sucrose and soluble starch on secretion of amylase.*—In order to determine if the secretion of amylase was affected by the kind of carbon source in the surrounding medium, experiments were set up in which soluble starch and sucrose were used in alternate flasks as the sole source of carbon in the synthetic liquid medium. The results of a typical experiment, table 4, show that there was no significant difference between sucrose and soluble starch in their effect on the secretion of amylase by this virus tumor tissue.

DISCUSSION.—The rapid increase in amylase concentration in liquid medium the first few days after introduction of *Rumex* virus tumor tissue supports the conclusion of Brakke and Nickell (1951) that this tissue secretes an α-amylase. The small amount of starch hydrolysis in the presence of the crown gall tissues indicates that the ability to secrete amylase may not be an exclusive property of the *Rumex* virus tumor tissue, but rather one which it has developed to an unusual degree.

Our methods did not detect any secretion of a starch-hydrolyzing enzyme by normal roots of *Rumex*. Even though there was a slight amount of hydrolysis of starch exposed to the roots for three months, it is not at all certain that this hydrolyses was due to a secreted enzyme since the roots died during the three months period. Although the roots contained tannin, which inhibits amylase activity, the surrounding medium did not contain enough tannin to inhibit added amylase. The important question as to whether or not the secretion of extracellular amylase is a characteristic of abnormal plant tissues remains unanswered.

An important matter to be kept in mind when evaluating such data as presented here is the relative amount of meristematic tissue in the test materials being compared. In the virus tumor tissue, a large majority of the total cells are meristematic, whereas the excised roots of *Rumex* have a rather low percentage of meristematic cells. As pointed out by Robinson and Brown (1952), the bulk of the enzymatic activity of roots occurs in a restricted area. It is not unreasonable, therefore, to believe that whatever starch-hydrolyzing enzymes might be present in excised roots of *Rumex* are primarily in a certain type of cell, which, in turn, is restricted to a small per cent of the total cell number. If so, it may be that the presence of the

TABLE 4. *Effect of carbon source on amylase secretion by Rumex virus tumor tissue in liquid medium.*

| Carbon source | Amylase concentration[a] 1 day | 2 days | 3 days | 7 days |
|---|---|---|---|---|
| Sucrose | 3.4 | 2.9 | 3.2 | 3.5 |
| Sucrose | 3.1 | 3.3 | 3.7 | 3.7 |
| Sol. starch | 2.1 | 2.9 | 3.5 | 3.8 |
| Sol. starch | 1.5 | 1.8 | 2.4 | 3.1 |

[a] Units represent mgm. starch hydrolyzed/hr./ml. medium.

enzyme in question could not be demonstrated because the techniques used were not sensitive enough.

The inability of the crown gall tissues to grow well on starch is due to their inability to secrete starch-hydrolyzing enzymes in sufficient amounts. With these tissues, no attempt was made to discover the number and type of enzymes involved in starch hydrolysis, nor to show if the enzyme involved was secreted or liberated by cell breakdown. In any event, the total hydrolytic action was of a lower order of magnitude than that caused by the virus tumor tissue.

SUMMARY

A study is presented of the secretion of $\alpha$-amylase by virus tumor tissue originally obtained from the root of a sorrel plant (*Rumex acetosa*). The data show that the release of amylase by this tissue cannot be due to cellular breakdown. In liquid medium secretion is rapid at first, then slows down or stops after 1–2 days. Reports in the literature that other plant tissues studied grow very little or not at all on starch is confirmed here for 4 different crown gall tissues. A comparison between these tissues and the virus tumor tissue is made by determining relative growth rates on solid medium containing starch and by a study of secretion of starch-hydrolyzing enzymes in liquid medium. Attempts to demonstrate amylase secretion by excised normal roots of *R. acetosa* were unsuccessful. The relative value of these unsuccessful attempts is discussed.

CHAS. PFIZER AND CO., INC.
BROOKLYN 6, NEW YORK
AND
DEPARTMENT OF BOTANY
UNIVERSITY OF ILLINOIS
URBANA, ILLINOIS

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