BIOCHEMICAL DATA ON NUCLEASE ENZYME FROM TOBACCO CALLUS TISSUE

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The method of plant-tissue cultivation is extensively used in our days for solving problems of theory and practice in a variety of fields in plant physiology. The callus tissue of tobacco *(Nicotiana tabacum)* could be called one of the most frequently used object for this. Extensive research was conducted for a more and more detailed exploration of the growth and metabolic processes of the callus tissue (Skoog and Robinson 1950, Jablonski and Skoog 1954, Linsmaier and Skoog 1965). The examinations, which aimed at clearing up the laws of plant growth and, especially, the way of action of the hormonal factors, were of particular importance. The author has set himself the task to determine such characteristics of the tobacco callus in the field of biochemical properties become better known by now as regards higher plants (seedlings and parts of plants). In view of the significance attached to more thorough information on the metabolic background of the growth processes, this refers, in the first place, to some important enzymes. Therefore the author has conducted a number of experiments for a biochemical analysis of certain properties of the enzymes decomposing nucleic acid, so that rather scanty fundamental information can be completed. He refers to the general data, relying on which nuclease enzymes seem to have an important function in growth regulation (Truelsen 1967, Pilet 1970, Pilet and Braun 1970, Caldogno et al. 1968, Lontai 1971, Vetter 1971). His examinations were aimed at obtaining data to serve as a biochemical basis for further experiments of physiological character. Depending on these, he intends then to study the control mechanism of plant growth.

Material and method

In the experiments, the author grew a culture of the callus of *Nicotiana tabacum* on a modified variation of the medium applied by Mu-

rashige and Skoog (1962). The explanate of 200 mg raw weight was placed on the aseptic culture medium. The cultivation dishes were kept under the natural alternations of daylight and night, at 18 – 24° C.

For the biochemical examination, 0.1 M of extract was made of the callus tissue in an acetate buffer of pH = 5, in a Potter – Elvehjem homogenizer, at continuous cooling. The extract was centrifuged for 30 minutes in a Janetski K – 24 type centrifuge at 15 000 rpm and 0° C. The examinations were conducted on the obtained supernatant (applying various dilutions).

Ribonuclease activity is characterized following a given incubation, relying on the increase of optical density (OD) to be measured at 260 $m\mu$, according to the earlier methods of the author (Vetter 1971).

For the Sephadex column-chromatographic experiments a 35×1.5 cm. water-coated column was used. It was produced on a fibreglass layer, of Sephadex G – 100, upon 3 days of pre-swelling. For swelling, equilibration and elution an acetate buffer of 0.02 M pH = 5 was used. For the examinations on the column, the tobacco tissue was rubbed off in a cooled mortar in the above acetate buffer, which contained 17% saccharose, 0.1% cysteine and 0.1% citric acid. The obtained thick homogenate was centrifuged at 0° C, then 2 ml of it were carried over to the column. The volume of the fractions was, as a rule, 86 drops (approx. 2.5 ml). When determining the molecular weight, the author followed the principles of Bagi and Farkas's (1967) method. The valuation of the fractions obtained from the gel column he did in the following way.

Desoxyribonuclease activity was determined by a spectrophotometric method, relying upon the quantity of the acid-soluble components to be measured at 260 $m\mu$. The reaction mixture contained 1 mg DNA, acetate buffer and the enzyme to be measured. The activity was measured at 40 °C, upon incubation for 80 minutes, in the way as described with the determination of ribonuclease, however, activity was measured without further dilution.

The peroxidase determinations were performed in aliquot samples taken from fractions resulting from the Sephadex column, founded on the author's methods described earlier (Vetter 1971). Activity was characterized by the increase in intensity of the brown colour come about as a result of enzyme activity during the time unit (change in OD per minute).

Experimental results and their valuation

The temperature dependence of ribonuclease activity: From among the approximation methods for biochemically characterizing the enzymes, first the temperature dependence of the activity of the enzyme isolated from the callus culture was examined. In this experiment the ribonuclease activity of the supernatant prepared from the standard material was studied by incubating the substrate and the enzyme at various tempera-

tures for the same time (which was identical with the 40 minutes applied by the author at any time), then the activity was determined in the way described earlier. The optical density (OD) to be measured at 260 mμ at identical dilutions was given as unit. The results of the series of experiments about the temperature dependence of the ribonuclease activity of the callus culture are shown in Figure 1. It is clearly to be seen in the

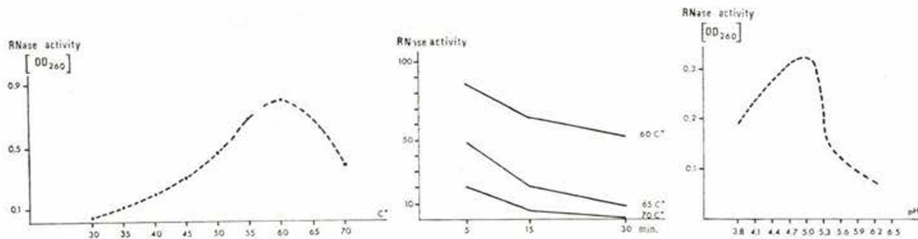

Figure 1. Changes of the RNase activity of tobacco callus as a function of temperature
Figure 2. Changes of the RNase activity of tobacco callus (in per cent of the control) as a function of duration (minute) of thermal treatment at 60°, 65° and 70° Celsius
Figure 3. Changes of the RNase activity of tobacco callus as a function of pH

curve representing the changes of activity, that till it has reached maximum temperature (this ensues at 60° C), activity rises evenly, then, after having attained the maximum value, it falls steeply, conform to the rate of denaturalizing. The observed relatively high (60° C) optimum may be called general among the plant enzymes, also similar or higher optimum temperatures have been found with the ribonucleases.

Heat resistance: In the next series of experiments, the author studied the heat resistance of the enzyme. Thermal treatments were applied for 5, 15 and 30 minutes at 60, 65 and 70° C, then the remaining enzyme activites were determined in the usual way. The obtained data were related to the activity of the untreated enzyme, considering it as 100%. As indicated by the said data (Figure 2), treatment at 60° decreased activity to 50% at most, that at 65° C to 10%, while that at 70° C to 0%. Again, within the applied temperatures, activity showed a linear decrease with the increase of the period of the treatment. Thus the author could find that a 30 minutes treatment at 70° C completely stopped activity.

The pH dependence of enzyme activity: As generally known, enzymes exert their activity only in a certain interval of pH. Generally, the curve of activity represented as a function of pH bears the character of an optimum curve. Actually, the effect of pH is a compound one, thus it affects the maximum reaction rate, the formation of the substrate enzyme complex and the stability of the protein. This latter effect is the main contributor to forming the optimum curve. Still, among the biochemical examinations one may find so-to-say at any time also the value of the optimum pH, which, consequently, serves with information rather regarding the stability of pH. The examinations were performed by

Table I.

Characteristics of a few vegetal nuclease enzymes (substrate specifity, optimum pH, decomposition type, molecular weight)

Object	Substrate specificity	Optimum pH	Decomposition type	Molecular weight	Literary source
Physarum polycephalum	RNA, DNA	4.0	Endonuclease	31 000	BRAUN and BEHRENS 1969
Avena sativa leaf	poly I, poly A, poly U		Endonuclease		WYEN et al., 1969
YEAST		7.4 – 7.6	Endonuclease		NAKAO et al. 1968
Avena sativa leaf	RNA, DNA	5.5 – 6.0	Endonuclease	33 000	WYEN et al., 1971
Cucumis sativus seedling		5.6		12 600	KADO, 1967
Cycas revoluta endospermium	RNA	4.7			HARA et al., 1969
Cycas revoluta pollen	RNA	5.7			HARA et al., 1970
Bacillus subtilis	RNA	5.5 – 5.7			YAMASAKI et al., 1970
Zea mays seed ..	RNA	5.0	Endonuclease	23 000	WILSON, 1968
Zea mays root ..	RNA	5.4 – 7.0	Endonuclease	17 000	WILSON, 1968

three-tenth units, in a way that also the pH of the substrate starting the reaction (RNA) was separetely adjusted to the corresponding value. As shown by the obtained data, (Figure 3), the maximum activity was found at a value of pH = 5.0, while in the rising (and thus highly acid) stage the enzyme was markedly more stable; between 5.0 and 6.5 values of pH, a considerable decrease in activity was to be observed. Relying on *Table I*, the data obtained for the optimum pH values can be compared with the ribonuclease and nuclease enzymes of various objects.

Effect of metal ions: The author also conducted examinations for finding out the effect of various metal ions on the activity of the enzyme. The enzyme preparate to be examined was produced of the callus culture grown on the basic medium in the earlier described way, then it was

incubated in the reaction mixture together with a suitable quantity of metal ions. The concentration of the metal ions referred to the reaction mixture of 4 ml was at all times: 1×10^{-3}; 1×10^{-4}; 1×10^{-5} M. The applied substances were $ZnSO_4$; $CuCl_2\times2H_2O$; $MgCl_2\times6H_2O$; $FeCl_3\times6H_2O$ $CoCl_2\times6H_2O$; $MnCl_2\times4H_2O$; $NiCl_2\times4H_2O$; KCl. The obtained data were given in percent of the control not containing metal ions, the value of the said control was taken as 100% *(Table II)*. From the Table it appears

Table II.

Effect of metal ions on the RNase activity of tobacco callus, compared with the untreated control (100%)

Concentration mol per litre	Metal ions							
	K^+	Cu^{2+}	Zn^{2+}	Mg^{2+}	Co^{2+}	Ni^{2+}	Mn^{2+}	Fe^{3+}
1×10^{-5}	103	93	97	93	100	98	106	97
1×10^{-4}	100	45	96	98	96	92	102	78
1×10^{-3}	103	28	67	93	85	76	100	53

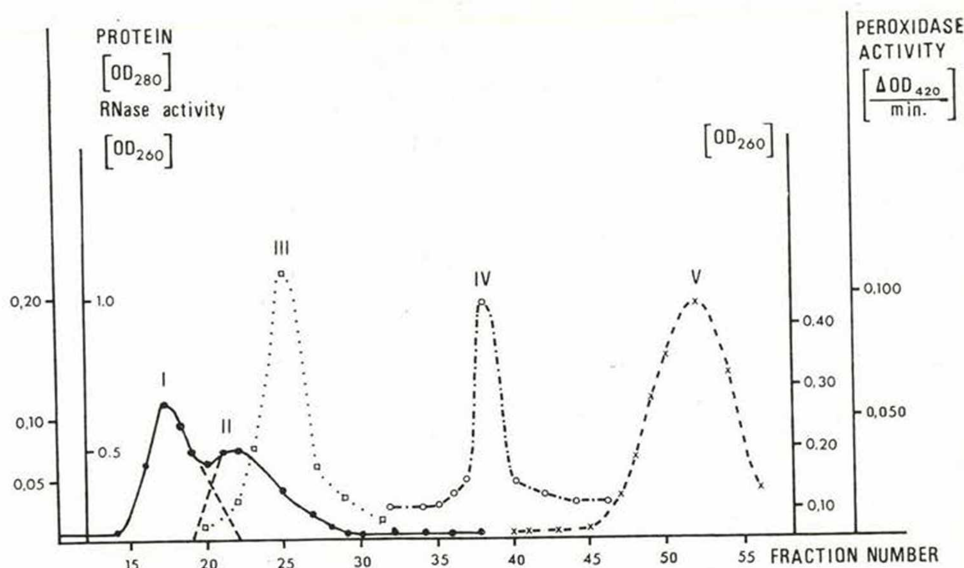

Figure 4. Elution diagram of the standard substances used for determining the molecular weight on a Sephadex G – 100 column. γ globulin (I), albumin (II), measured relying on OD_{280}; CTP (V), measured relying on OD_{260}; peroxidase (III) and pancreas RNase (IV), measured relying on the activity of aliquot samples of the fractions. Substances applied: 3 mg γ globulin in 1 ml saccharose buffer, 9 mg albumin, 0.05 mg peroxidase, 0.4 mg pancreas RNase, 0.7 mg CTP

that K^+, Mg^{2+}, Mn^{2+} are practically ineffective, with the increase of concentration the other metals (Co^{2+}; Mn^{2+}; Ni^{2+}) call forth an increasing inhibition of activity, while Fe^{3+} and Cu^{2+} proved to have the most intensive inhibiting effect, — did iron decrease activity but to 53%, and Cu^{2+} but to 28%.

The determination of molecular weight: Data on the elution of enzymes of known molecular weight were compared with the elution characteristics of the unknown protein molecule, making use of the connection which prevails between the size of the molecule (approximately the molecular weight) and the fraction number on the Sephadex gel. According to these, namely, the connection between the logarithm of molecular weight and the fraction number is linear.

For determining molecular weight, the author used, as standard substances: γ globulin (Mw: 160 000), bovine serum albumin (Mw: 67 000), horse radish peroxidase (Mw: 40 000), pancreas ribonuclease (Mw: 12 700) and CTP (Mw: 1 500), respectively. The elution curve of the standard substances is presented in Figure 4. Having eluted the substance to be determined under the same circumstances, the author got the point necessary for the determination of the unknown molecular weight. The data of the standard substances gave the calibration curve to be seen in Figure 5. From the Figure it appears that between the logarithm of the molecular weight and the fraction number, the connection is actually linear. Curiously, the substance of the heaviest molecular weight: γ globulin, is somewhat outside the straight line. This agrees with the earlier data (Bagi and Farkas 1967), and can be called theoretical. Its cause lies in the circumstance that the molecular weight 160 000 of γ globulin is somewhat over the molecular-weight solving capacity of Sephadex G – 100 (the deviation is shown by a dashed line in the Figure). The values sought for could be read off the calibration curve, cut out at fraction numbers 36 – 37. In view of the approximating character of the method, this value proved to be between 15 000 – 17 000.

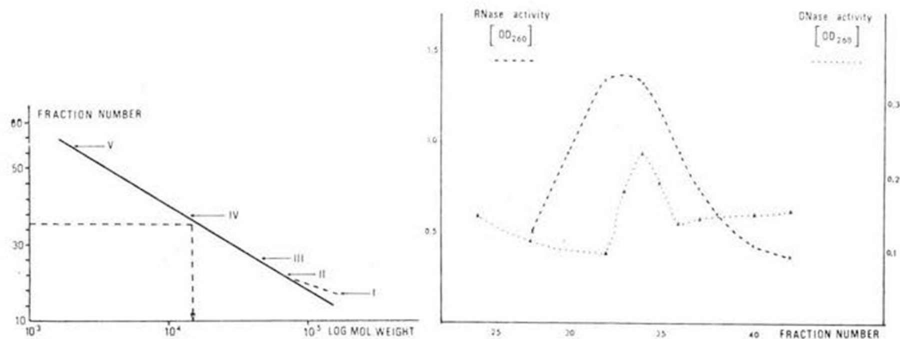

Figure 5. The calibration curve applied for determining the molecular weight. Globulin: I, albumin: II, Peroxidase: III, Pancreas RNase: IV, CTP: V

Figure 6. Determination of the RNase and DNase activity of tobacco callus nuclease on a Sephadex G – 100 column. Ribonuclease: – – – –, Desoxyribonuclease: ....

Examination of substrate specificity

With the enzymes decomposing plant nucleic acids – and, in general, with nuclease enzymes of what ever origin –, the data referring to the enzyme substrate and/or to the place, way and possible preference of the decomposition afford most important information. The author could undertake to perform but the simplest of such examinations, namely to find out whether the enzyme was also capable to decompose DNA; in other words: if it also had DNase activity. For deciding the question it seemed expedient to elute a relatively concentrated tissue homogenate on a Sephadex column. The fractions obtained were tested for RNase- and DNase activity, For the determination of DNase activity, the auth or used DNA of great molecular weight as a substrate, produced from chicken blood and – counting on an eventual low activity – raised the time of incubation to the double (80 minutes). The results of the elution are presented in Figure 6. Only the part of the fractions affecting the examination was stressed in the Figure, the low values of DNase activity were represented on a highly enlarged scale. It could be found that the fraction solving DNA of the tobacco callus homogenate was arranged in the same fractions as the enzyme decomposing RNA, and was possibly identical with it. In the biochemical respect it is perhaps more correct to talk, instead of RNase, about a nuclease equally capable of decomposing RNA and (even if in a much lesser measure) DNA. Otherwise the enzyme can be understood as a nuclease not specific to sugar. In the author's opinion it is not impossible either, that with a finer method, the enzyme decomposing DNA can be separated from RNase.

The type of decomposition called forth by the enzyme: For verifying the decomposition type of the tobacco callus nuclease, the following series of experiments was conducted. Founded on the way in which they decompose the substrate, the nuclease enzymes are ranged with two groups: 1. Endonucleases, which start decomposing within the molecule and the decomposition productes are mainly oligonucleotides and 2. Exonucleases: they decompose from the ends of the molecules and the products are mainly mononucleotides.

For deciding the type of decomposition, the author used the Sephadex technique, elaborated relying on B i r n b o i m's (1966) method.

In the discussed experiment the reaction mixture to be examined was kept at 40° C for 0; 60; 90 and 120 minutes. When the incubation period had passed, aliquot samples were taken and, having them kept at a temperature of 100° C for 2 minutes, the enzyme reaction was stopped. For deciding if the thermal treatment did not call forth hydrolysis, or some change in extinction, also the thermally treated RNA was separately eluted.

The results of the experiments are indicated in Figure 7. The control curve represents the elution of a mixture of RNA- and CTP content. It indicates that RNA gives a relatively flat curve, which finds its expla-

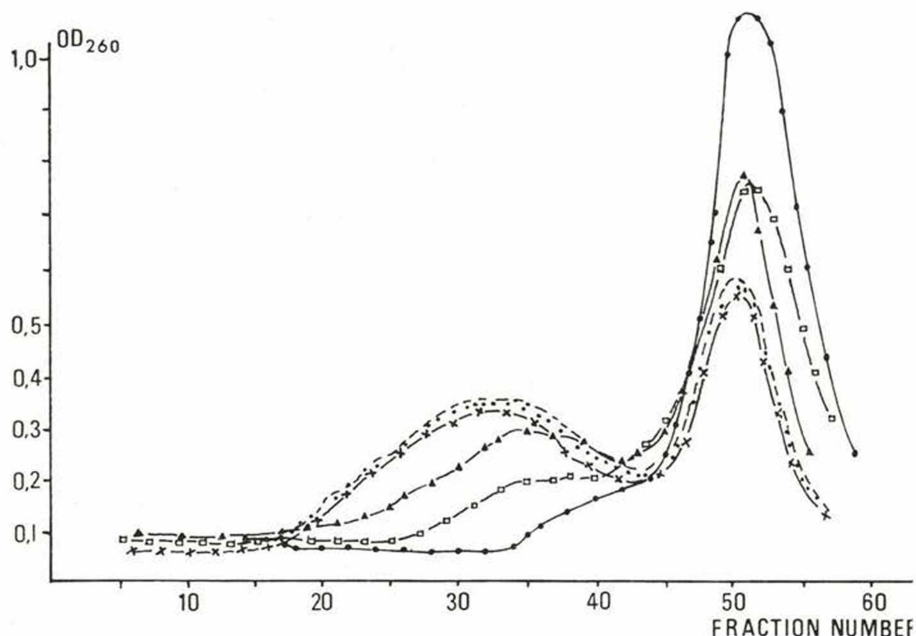

Figure 7. Examination of the decomposition type of callus nuclease, following various durations of RNA decomposition (0; 60; 90; 120 minutes) by means of elution on a Seph adex G−100 column. The quantity of un-decomposed RNA and the various pieces split off are indicated relying on the OD_{260} values of the fractions, as a function of the fraction number. Control: ....; 0 minute: − − − −; 60 minutes: △ △ △ △; 90 minutes: □ . . □ . . □ . . □ . .; 120 minutes: ● ● ● ●; thermally treated: × × × ×

nation partly in the application of Sephadex G − 100, partly in the circumstance that RNA is, actually, within a region of wide variety of molecular weights, consequently it is rendered impure by various pieces of RNA of lighter molecular weight. The curve of the thermally treated control demonstrates that the two minutes' thermal treatment does not cause any change affecting the experiments in the RNA molecule. A curve exactly coincident with the control was also obtained from the reaction mixture of 0 minute. The effect of the decomposition of the enzyme is impressively reflected by the curves obtained following reaction for 60, 90 and 120 minutes. On the other hand: the vertex of the RNA curve is being gradually flattened and shifted from left to right, that is, the decomposition lasting for a longer time is continually decreasing the number of particles of heavier molecular weight. On the other hand, the vertex of CTP, corresponding with the mononucleotides and smaller oligonucleotides keeps increasing, and the maximum of the 120 minutes' reaction is two times as great as that of the control. Relying upon the form of the curves it could be found that the decomposition was probably of endonuclease type.

By way of appraising the biochemical data, there were several valuable observations to be made. The temperature dependence of the activity of the nuclease enzyme (Figure 1) shows the optimum curve to be expected, the optimum is found to be near 60° C. The relatively high point of temperature is interesting, although, with some other plant ribonucleases the data to be found in the literature give account on values of approximately the same character, moreover, even on higher ones. Thus, for instance, in case of ribonuclease isolated from barley root, the optimum temperature is round 70° C (Nakagiri et al. 1968) and similarly round 70° C with lemon leaf ribonuclease (Kessler and Monselise 1959). Thermal treatment called forth a decrease of various degree in the enzyme activity (Figure 2). By increasing the temperature and time, the activity can be stopped altogether.

According to the author's data on the effect of pH, it was pH = 5 which proved to be the optimum value. This one — compared with findings gained with other objects *(Table I)* — falls within the interval of the most frequent values of pH. Consequently, in this regard the callus enzyme does not differ from „average" plant nucleases.

The data about the effect of metal ions modifying activity revealed an inhibiting effect of Co^{2+}, Zn^{2+} and Ni^{2+}, further a most intensive one of Fe^{3+} and, especially, Cu^{2+}. Remarkably, these findings of the author agree with the data described with barley roots and oat leaves, respectively (Nakagiri et al. 1968, Wyen at al. 1971).

The determination of molecular weight carried out on the gel column gave an approximate value of 15—17 000. Curiously, this quite significantly differs from the value obtained the tobacco leaf homogenate ribonuclease (Bagi and Farkas, 1967).

When applying the two substrates it apperaed that the enzyme of the callus tissue homogenate also showed a slight DNase activity. Therefore, it seems more proper to call it nuclease, having regard to the circumstance that the decomposition seems to be of a character not specific to sugar. Owing to the rather slight DNase activity, there is a possibility, that the nuclease vertex, seeming uniform, might include a special enzyme fraction.

From the experiment carried out concerning the decomposition type of the enzyme a so-called endonuclease-type decomposition seems probable (Figure 7).

Summary

The author set as the aim of his biochemical examinations to determine some fundamental data connected with the enzyme of the tobacco callus decomposing nucleic acid. Thus he examined the dependence of the activity of the enzyme on temperature, its stability against thermal treatment, the effect of pH on its activity, as well as the effect of some

metal ions modifying that activity. The data obtained in this way were, in general, of a tendency identical with those known with the plant nucleases.

As found by means of an approximating method, the molecular weight of the enzyme was about 15 – 17 000.

As shown by the experiment regarding the substrate, the callus enzyme was capable of decomposing in the first place RNA and, to a small degree, DNA. According to the data obtained regarding the type of decomposition, it can be called an endonuclease.

Serving with a characterization of the nuclease enzymes, significant in the regulation of vegetal growth, the author's examinations may afford a suitable basis for further physiological experiments.

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