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Polynucleotidases in Animal Tissues

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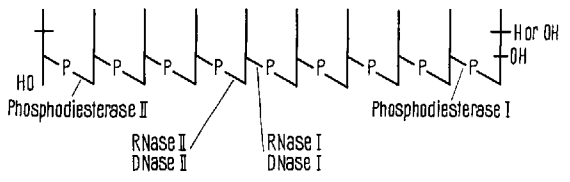
The term polynucleotidases is intended to include both endonucleases and exonucleases, the latter frequently referred to as phosphodiesterases[1]. Interest in these enzymes arose initially as a result of their presence in the digestive tract, and the first to be extensively purified and characterized was bovine pancreas ribonuclease[2], which will be referred to as alkaline RNase II. A number of other ribonuclease activities have been described in diverse tissues, and a few of these have demonstrated sufficiently different properties and modes of action compared to the pancreatic ribonuclease that they may be considered unique biochemical entities[3]. Pancreatic secretions also contain a potent deoxyribonuclease, DNase I, whose counterpart in other tissues has been sought with some success[4]; additionally, such investigations have revealed the presence of another deoxyribonuclease, DNase II, possessing sufficiently different properties to warrant a conclusion that it likewise is a unique biochemical entity[5]. Furthermore, two distinct phosphodiesterases, one from snake venom, PDase I, and one from calf spleen, PDase II, are distinguishable from the ribonucleases and deoxyribonucleases through the ability of the phosphodiesterases to liberate mononucleotides only from the ends of polynucleotide chains[6,7]. For purposes of clarification in subsequent discussions the names which will be used for the several enzymes, together with their sites of attack on polynucleotide chains, have been integrated into a single schema, shown in the Figure.



It is becoming apparent that the intact tissues of mammals[8] and birds[9] regularly possess a number of these polynucleotidases. The value of studies on their existence and properties therefore extends, beyond the potential and demonstrated use of such enzymes to probe the finer details of nucleic acid structures, to a question of their significance in cell reproduction and in differentiation of cells within and between tissues[10]. In order to clarify the methodology by which these enzymes may be distinguished from one another in analyses performed on tissue or cell homogenates, it will be necessary to review some of the unique and functional characteristics of each of the enzymes in turn.

Alkaline Ribonuclease II

This term is used to distinguish the pancreatic-type enzyme from other ribonucleases, particularly from the acid RNase II which is found in pancreas and other tissues[11,12]. Alkaline RNase II has its counterpart in tissues other than pancreas – such as liver, kidney and spleen – although the precise composition of the enzyme molecule in all cases may not be identical[13]. An examination of Table I will reveal the essential characteristics of this ribonuclease, and the differences between it and the other similar enzymes. Although it is not apparent in extracts of pancreas, an endogenous inhibitor normally accompanies the enzyme, which effectively masks its activity to a high degree in liver and to detectable degrees in other tissues[14]. This inhibitor is unstable under the acid conditions (0.25 *N* H_2SO_4, 4°, 1 h) in which the enzyme itself is unaffected; the destruction of the inhibitor is therefore an

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Table I

	Metal ion	pH optimum	Acid stability	Heat stability	Base specificity	Subcellular distribution	Endogenous inhibitor	Primary product
1. Alkaline RNase II	None	7.0–8.3	high	high	Py-3'-P	soluble	strong	2',3'-cyclic-P
2. Acid RNase II	None	4.8–5.8	high	low	Py-3'-P	lysosomal	moderate	2',3'-cyclic-P
3. RNase I	Mg^{++}	7.2–7.6	nil	low	none	nuclear	low	5'-P

essential preliminary step in the detection of the enzyme[13]. It is a relatively basic protein of molecular weight about 13,700. The enzyme hydrolyzes only those internucleotide bonds adjacent to the 3'-phosphoryl group of pyrimidine bases, so that RNA, poly-C and poly-U are hydrolyzed to yield small fragments, whereas poly-A is not[13].

Acid Ribonuclease II

Some degree of uncertainty still exists regarding the actual existence of this enzyme, because its catalytic activity and properties may be mimicked by a mixture of alkaline RNase II and PDase II. At pH values about 6.0, the alkaline RNase II is still partially active, producing products bearing free 5'-hydroxyl end groups, and PDase II is active at such pH values, liberating nucleoside-3' phosphate residues from the products of the action of the RNase II. Thus, the impression is gained that a mixture of enzymes is one activity which liberates nucleoside-3' phosphate residues from high molecular weight RNA.

To date, most methods of purification for this enzyme involve procedures which lead to contamination of the enzyme preparations with either alkaline RNase II[9] or PDase II[12]. Nevertheless, results which demonstrate that the acid RNase II can be freed of PDase II, but not of alkaline RNase II[9], permit the conclusion that the acid enzyme truly produces oligonucleotides from RNA, and not mononucleotides – as had frequently been supposed in the past. The enzyme appears to be specific for pyrimidine-3' phosphoryl bonds, since poly-A is not hydrolyzed by a preparation of acid RNase II containing some alkaline RNase II[9]. Although acid RNase II cannot hydrolyze poly-C[12], the lack of reaction is probably not a result of the inability to hydrolyze the bond connecting cytidine-3' phosphate residues to adjacent bases, as in RNA, but rather results from the highly ordered structure[15] of poly-C at pH 5.5. The ordered structure of poly-C at the low pH renders it uniquely resistant also to alkaline RNase II[9], which is known to hydrolyze cytidine-3' phosphate bonds at higher pH.

Ribonuclease I

The unique properties of this enzyme allow more definite statements to be made about its identity. Originally described in nuclei from guinea-pig liver[16], it can be shown to be present in the nuclear fraction of diverse tissues from many sources[9,17]. The rationale for assigning its name derives from its similarity of pH and cation requirements to other enzymes such as DNase I and PDase I, as well as from its formation of products with 5'-phosphate end groups. Purification procedures for the enzyme appear to be greatly influenced by the presence of cathepsins in the cell homogenate, and the numerous difficulties encountered in reproducing a purification protocol have not yet been brought under proper control. The enzyme hydrolyzes all polyribonucleotides without regard to base sequence and since poly-A is rapidly attacked, the RNase I is clearly differentiated from alkaline RNase II.

Phosphodiesterase I

This enzyme was originally described as a component of the venom of various snakes[18], and has been found extremely useful in studies of the structure of ribonucleates, deoxyribonucleates, nucleotide coenzymes and similar compounds bearing phosphodiester bonds[19]. It is active at high pH values, with an optimum at pH 9.2, and requires divalent cations such as magnesium for maximum activity. Although nonspecific with respect to the nucleotide base, the enzyme shows an absolute specificity for a nucleoside-5' phosphoryl residue having an exposed 3'-hydroxyl group[20]. Therefore, substrates for this enzyme include oligonucleotides with a free 3'-hydroxyl function[21], coenzymes such as NAD, FAD, UDPG[17], and relatively novel materials of biological or synthetic origin

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such as ApppA[22] and GppG[23,24]. The systematic name of PDase I derives from the requirement for a free 3'-hydroxyl group and the liberation by the enzyme of nucleoside-5' phosphate residues from the diverse substrates on which it acts. The counterpart enzyme which is found in animal tissues has similar requirements for activity and substrate specificity, and is localized in the microsomal fraction[25]. Since the activity of the enzyme is but little affected by the substituent attached to the 5'-phosphate residue of the substrate, it is possible to synthesize a number of derivates of nucleoside-5' phosphates. Of these, *p*-nitrophenyl thymidine-5' phosphate has proved to be a particularly useful substrate for studies on the distribution of this enzyme in diverse materials, as well as for detailed kinetic studies of the enzyme itself. The properties of the enzyme and its substrate specificity combine to permit an unequivocal demonstration of the presence of this enzyme in mixtures containing other polynucleotidases.

Phosphodiesterase II

The enzyme originally detected in the mitochondrial fraction of spleen homogenate has its counterpart in other tissues[8,26]. A variable proportion of this enzyme is found in the supernatant fractions of homogenates of the various tissues, possibly as a result of its liberation from the mitochondria or from lysosomes. The pH optimum is about 5.9, and no divalent ions are required – that is to say, the enzyme is active in the presence of $10^{-2}M$ EDTA. The products of hydrolysis of oligonucleotides are nucleoside- or deoxynucleoside-3' phosphate residues[27], and no evidence points to the involvement of a 2',3'-cyclic phosphate intermediate in the hydrolytic mechanism catalyzed by this enzyme. Indeed, there may be an enzyme-product intermediate in the form of nucleoside-3' phosphoryl-enzyme complex, since transfer of nucleoside-3' phosphate to available 5'-hydroxyl functions occurs at high substrate concentrations, resulting in the synthesis of oligonucleotides larger than the substrate[28,29]. The name PDase II derives from the specificity and properties of the enzyme, in that activity occurs in the presence of EDTA, the substrates must bear a free 5'-hydroxyl function, and the products are nucleoside-3' phosphates. The milieu in which this enzyme functions, and the products which it produces, are therefore related to those of RNase II and DNase II.

As in the case of PDase I, it has proved possible to utilize suitably substituted deoxynucleoside phosphates as substrates, and the properties of the enzyme together with its ability to hydrolyze *p*-nitrophenyl thymidine-3' phosphate[29] permit an unequivocal demonstration of the presence of this enzyme in mixtures containing other polynucleotidases. It should be mentioned that attempts to measure the activity of PDase II using oligonucleotides may be equivalent to the procedure using *p*-nitrophenyl thymidine-3' phosphate, but that attempts to measure ribonucleases must be performed with high molecular weight RNA because crude commercial RNA is a mixture of oligonucleotides of a size readily attacked by PDase II. This situation had led to occasional confusion in the earlier literature between 'acid RNase' and the PDase II[30].

Deoxyribonucleases I and II

It is not possible at the present time to define a set of conditions under which the two DNases may be assayed in cell extracts with confidence in the significance or reproducibility of the results. Although DNase I is active at pH 6–7 in the presence of $10^{-2}M$ magnesium and manganese ions (depending on the substrate concentration)[31] it is accompanied by one or two inhibitors with relatively undefined stability characteristics and apparently also by an activator which is similarly of uncertain distribution and properties[32,33]. The enzyme activity in extracts is unstable as a result of the interplay of one or more of these factors – possibly together with an actual instability of the enzyme molecule itself. A similar situation appears to exist for the DNase II, although even less is known of the endogenous inhibition or inactivation factors[9,33]; it may be distinguished from DNase I by its low pH optimum of 4.7 and its lack of requirement for magnesium above $10^{-3}M$, but in mixtures of the two enzymes there are not as yet the well-documented exclusive characteristics such as allow the RNases to be distinguished.

In spite of the limitations on the interpretation of results which a dearth of data on DNases might impose, we have proceeded to examine cells and tissues for the ribopolynucleotidases in the expectation that something might be learned of their utility in cellular metabolism of RNA species.

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Survey Procedures

As was pointed out above, it is necessary to treat homogenates of tissue with acid in order to destroy the inhibitors of the RNase II enzymes, but such treatments will completely inactivate the RNase I, PDase I and PDase II; therefore, a homogenate of a tissue must be separated into two parts, one of which receives the acid treatment and is subsequently neutralized prior to assay for the RNase II's, while the other receives no acid treatment and is utilized directly in the other polynucleotidase assays. In the case of the acid-treated preparation, it is possible to satisfy the requirement that the preparation possess no activity against *p*-nitrophenyl thymidine-5′ phosphate (the substrate for PDase I) nor activity against *p*-nitrophenyl thymidine-3′ phosphate (the substrate for PDase II). The surviving RNases may be measured by the rate at which they liberate acid-soluble fragments from high molecular weight RNA at pH values of 7.8 (alkaline RNase II) and at pH 5.5 (acid RNase II). A mixed buffer of acetate-phosphate-*Tris* is most convenient for both assays[13]. A correction may be made for the fraction of the activity of the alkaline RNase II which is expressed at the lower pH in the assay for the acid RNase II. This is accomplished by heat treatment of a part of the acid-treated mixture, which destroys the acid RNase II[13] and permits the subsequent measure of the activity of the alkaline RNase II at pH 5.5. The latter value is subtracted from the value for the acid RNase II obtained prior to heat treatment.

The part of the homogenate not treated with acid will contain all the polynucleotidases, but the RNase II enzymes will not normally hydrolyze poly-A under conditions in which the RNase I is active. RNase I can be shown to be responsible for activity observed under assay conditions for it, if a control is included in the series having an addition of EDTA in excess of the magnesium ion, that is about $10^{-2}M$. Any slow rate of hydrolysis of poly-A in the presence of EDTA may be due to an extremely high concentration of alkaline RNase II in the homogenate, since a very slow rate of hydrolysis of purine internucleotide bonds is observed with crystalline pancreas RNase II[34].

The phosphodiesterases may be assayed independently by use of the *p*-nitrophenyl esters appropriate to each. For some years there was little to suggest that any activity might be present in tissue homogenates other than the well-characterized phosphodiesterases I and II. However, during studies on the activity which leads to the hydrolysis of *bis*(*p*-nitrophenyl) phosphate in liver extracts, it was observed[35] that some concentrated fractions of liver homogenate supernatant hydrolyzed *p*-nitrophenyl thymidine-5′ phosphate in the presence of EDTA at pH 5–7. Further, studies on plant tissue extracts[36] have revealed the presence of an activity against *p*-nitrophenyl thymidine-5′ phosphate at low pH in the presence of EDTA also, which appears to be a property of the nucleotide pyrophosphatase. Work is in progress to establish whether the enzyme in animal tissues is, as suspected[37], also a nucleotide pyrophosphatase. Thus far, it appears that nucleotide pyrophosphatases do not possess polynucleotidase activity[38], and therefore they may be ignored for the time being in the context of this article.

Polynucleotidases in Tissues versus Cells in Culture

The data in Table II indicate the levels of enzyme activity in fresh tissue homogenate versus homogenate of primary cell line derived directly from the tissue (Part A). It is apparent that PDase I and the RNase II

Table II. Polynucleotidase activities of tissue and cell extracts (results in μmoles of product/h/mg protein)

(A)	Rat kidney tissue		Rat kidney cell line	
	Observed	% control added[a]	Observed	% control added[a]
PDase I	5.7	105	< 0.10	98
PDase II	0.42	102	4.0	100
RNase I	0.86	96	2.0	90
Alkaline RNase II	41	90	5.1	95
Acid RNase II	6.0	103[b]	0.55	95[b]

(B)	Human cervical carcinoma		Cultured carcinoma cell line	
	Observed	% control added[a]	Observed	% control added[a]
PDase I	0.52	100	< 0.05	100
PDase II	0.41	94	< 0.10	102
RNase I	0.72	92	1.4	105
Alkaline RNase II	18	104	0.1	100
Acid RNase II	7.7	104[b]	< 0.05	100[b]

(C)	Mouse lymphoma cells (5178-Y)	Cultured beating chick heart cells
	Observed	Observed
PDase I	< 0.05	1.9
PDase II	0.11	0.31
RNase I	0.96	0.18
Alkaline RNase II	0.10	9.3
Acid RNase II	0.06	0.14

[a] Purified enzymes in each case were added to the assay mixture to establish whether endogenous inhibitors were present in excess. Little evidence is in favor of such an interpretation. [b] Acid RNase II added as a control was contaminated with alkaline RNase II, but free of PDase II.

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enzymes are much lower in the cultured cells, whereas PDase II and RNase I are elevated. Such changes are not altogether characteristic of cultured cells, however, since other primary cell lines do not show elevated PDase II levels. Of a number of cell cultures examined so far, the common characteristic appears to be: a drastic decrease in PDase I and a maintenance of RNase I; apart from these, the levels of the other enzymes do not appear to vary in a predictable manner.

Variations in the enzyme levels, which differ from those observed in the above case, may be seen in parts B and C of Table II. The PDase I levels in all but the beating chick heart cells are very low compared to the intact tissues, and the PDase II activities are not elevated. In some cases, the RNase II levels are extremely low, but separate experiments with HeLa cells have shown intermediate levels of RNase II enzymes. Further, the low PDase I activity in human cervical carcinoma tissue homogenates – shown in part B of Table II – are not characteristic of solid tumors; for example, early data on human liver tumor showed intermediate PDase I activity[8], and several analyses of PDase I levels in host liver and tumors (Morris hepatoma 7800, Reuber hepatoma) showed no consistent differences[39].

It is too soon to be confident about the significance of the results with the one cell line which had retained an intrinsic character of differentiation – the beating heart cells. It is noteworthy, however, that this culture alone among all the cell cultures examined so far, has retained an appreciable, although intermediate, level of PDase I. Results with homogenates of organs of very young mammalian embryos[10] (mouse, rat) and of chick embryos[9] have always revealed appreciable levels of PDase I; indeed, most assays are indistinguishable from tests on normal adult tissues. Thus, while other enzymes fluctuate in an apparently unpredictable manner, the RNase I is relatively unchanged between cells in organized tissue versus those in culture, whereas PDase I is usually not present in cultured cells.

It will be interesting to learn whether cells in culture which may be caused to differentiate (such as by virus 'transformation') are also caused to synthesize PDase I anew, or in some other way to alter the spectrum of polynucleotidases which they synthesize. The several polynucleotidases appear to have a great potential to influence the stability and functional capacity of various classes of RNA, and it may be fruitful to speculate that the reason for the wide variation in levels of all the enzymes (except RNase I) from one cell line to another arises from the lack of pressure on the isolated cell population to differentiate in culture. The consequence of a lack of stimuli to differentiate may be to permit the cells a randomized opportunity not to continue to synthesize one or several of the enzymes which are essential to the cells as they function in an organized tissue.

During the preparation of the above article, a series of publications has appeared from Strasbourg by BERNARDI et al. These workers have been able to isolate in high yield from spleen, the DNase II[40] and the acid RNase II[41]. For the former enzyme, no comments are made regarding the previously reported[42] presence in the purified DNase II of PDase II activity; nor has PDase II activity been assessed in the acid RNase II preparation by means of the *p*-nitrophenyl esters of the deoxynucleoside-3′ phosphates. If the highly purified endonucleases are still found to contain high activity against the *p*-nitrophenyl esters which are used as indicators of PDase II, this would cast grave doubts on the procedure for specific assays for these enzymes; however, it does not seem necessary to be so pessimistic at this time[43].

Résumé. Les organelles des Mammifères contiennent un grand nombre d'enzymes qui hydrolysent les acides nucléiques. Les enzymes qui agissent sur les acides désoxyribonucléiques étant accompagnées par quelques prohibiteurs et activateurs, on ne peut pas les évaluer avec certitude. Pour cette raison, la discussion ne porte que sur les enzymes ayant une certaine activité sur les acides ribonucléiques (alkaline RNase II, acide RNase II, RNase I, phosphodiestérase I, phosphodiestérase II). Les caractères servant à distinguer ces enzymes sont décrits ici. Elles renferment des substrats spéciaux et ont une inactivation sélective. Bien que les extraits des organelles renferment toutes les enzymes, les extraits des cellules cultivées in vitro n'en contiennent qu'une partie. Par hasard, une ou quelques activités peuvent faire défaut et la phosphodiestérase I est toujours déficiente. Cette exception s'observe dans le cas où les cellules ont des propriétés différenciées – comme les cellules musculaires du cœur – c'est à dire qu'elles continuent à produire de la phosphodiestérase I. Toutes ces cellules cultivées donnent continuellement la RNase I.

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