RIBONUCLEASE AND DESOXYRIBONUCLEASE ACTIVITIES IN NORMAL AND REGENERATING BONE MARROW HOMOGENATES*

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INTRODUCTION

It has generally been observed that changes in the growth state of a tissue are parallelled by changes in the quantities of the tissue nucleic acids[1-6]. This is true also when single cells are examined; the growing blast cells of the bone marrow, for example, show high cytoplasmic concentrations of pentose nucleic acids that decrease continuously during cell maturation and cessation of growth[7,8].

It may be postulated that the level and metabolism of nucleic acids in the cell are enzymically regulated[9]. This paper describes the ribonuclease (RNase) and desoxyribonuclease (DNase) activities in homogenates of normal and regenerating chicken bone marrow. Activity changes have been found which could be correlated with changes in the growth state of the bone marrow.

EXPERIMENTAL

Young hens, of a strain of White Leghorn (Edo) weighing about 1.5 kg, were used. Before and during the experiments they had free access to a standardized chicken food (Fors).

The regeneration of the bone marrow was brought about by an intramuscular injection of a 1% phenylhydrazine-HCl solution at a dose of 50 mg per kg of body weight. After 72 hours the growth rate was maximal[5].

The bone marrow was immediately taken out after exsanguination of the chicken, and frozen in a beaker immersed in a dry-ice-alcohol mixture. After thawing, the material was homogenized in glass-distilled water for four minutes with a Bühler homogenizer. The tissue concentration was about 10%. Precautions were taken to keep the temperature of the homogenate close to 0° C.

The nuclease activities were determined as described earlier[10]. The RNase activities are expressed as the increase in acid-soluble phosphorus during 60 minutes at 37° C per unit of total tissue nitrogen or tissue desoxyribonucleic acid phosphorus (DNAP). The DNase activities, for reasons discussed elsewhere[10], were expressed per 1 mg total tissue nitrogen or per 20 μg DNAP. As substrates, purified, commercial (Baker) yeast ribonucleic acid (RNA) or desoxypentose nucleic acid (DNA), prepared according to HAMMARSTEN[11], were used. Acetate buffers of 0.2 M were used up to pH 5.7 and 1/15 M phosphate buffers above this pH. The pH values of the reaction mixtures were measured with a Radiometer pH meter 3.

An aliquot of the homogenate was taken for nucleic acid determinations. These compounds were extracted according to the method of SCHNEIDER[12], and the quantitative determinations were done colorimetrically[13,14] with a Beckman quartz spectrophotometer, using a 1 cm cell. Every determination, including the extraction procedure, was run in duplicate.

The nitrogen determinations were done in duplicate by the micro-Kjeldahl technique[15].

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Manipulations and centrifugations (M.S.E. refrigerated centrifuge) were carried out at temperatures close to o° C. All reagent solutions were freshly prepared daily.

The cytological investigations were made according to the technique referred to earlier[5].

The material was statistically treated according to analysis of variance[16].

RESULTS

Nucleic acids

The pentose and desoxypentose nucleic acids have been determined in twelve normal and in seven regenerating bone marrows. Table I gives the values expressed per mg total tissue nitrogen and the PNAP/DNAP quotients. Despite considerable scatter of the values in the normal and regenerating groups the increase of PNAP/DNAP in the regenerating group is statistically significant ($0.001 < P < 0.01$). This is in accordance with earlier results obtained for bone marrow[17], regenerating rat liver[5] and human placenta[4,6].

The great scatter of the PNAP/DNAP quotients may be due partly to a somewhat varying admixture of the red blood cells, which in the hen are nucleated and contain a high proportion of DNA (PNAP/DNAP quotient 0.35).

TABLE I

PENTOSE AND DESOXYPENTOSE NUCLEIC ACIDS IN NORMAL AND REGENERATING BONE MARROW

	Hen	*PNAP/mg total N*	*DNAP/mg total N*	*PNAP/DNAP*
Normal	1	11.70	19.16	0.61
	2	7.57	21.33	0.35
	3	11.18	18.98	0.59
	4	10.78	20.20	0.53
	5	17.53	19.12	0.92
	6	11.12	26.32	0.42
	7	15.80	28.98	0.55
	8	15.02	25.07	0.60
	9	9.35	21.67	0.43
	10	12.15	21.60	0.56
	11	14.04	24.62	0.57
	12	12.36	17.16	0.72
Regenerating	1	23.13	27.38	0.85
	2	16.60	23.95	0.69
	3	21.41	18.41	1.16
	4	20.67	23.58	0.88
	5	24.74	33.02	0.75
	6	20.70	27.57	0.75
	7	9.08	11.66	0.78

RNase activity

Optimum activity is at pH 7.5 when the enzyme is not activated. The RNase activities were determined in five normal and five regenerating marrows. The figures are given in Table II. The RNase activities are higher for the regenerating bone marrows than for normal tissue. This increase is more pronounced when the activities are calculated per mg tissue nitrogen than when they are referred to the tissue DNAP. For results of statistical analysis see Table III.

The same technique that was adopted in this investigation has earlier been used for the determination of RNase activities at different stages of growth in the human

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TABLE II

MITOTIC FREQUENCIES* AND NUCLEASE ACTIVITIES IN NORMAL AND REGENERATING BONE MARROW HOMOGENATES

	Hen	Mitotic frequency	RNase activity		Non-activated DNase activity		Mg^{++}-activated DNase activity	
			per µg DNAP	per mg N	per 20 µg DNAP	per 1 mg N	per 20 µg DNAP	per 1 mg N
Normal	1	0.3 ± 0.10		-	31.0	30.0	53.5	51.2
	2	0.2 ± 0.05	5.0	125	16.0	17.5	43.0	45.6
	3	0.4 ± 0.10	5.5	103	-	-		-
	4	0.2 ± 0.08	5.9	120	-	-	44.4	44.8
	5	0.7 ± 0.15	7.6	145	-	-	68.1	65.2
	6	0.2 ± 0.05	3.9	102	12.0	18.0	45.0	59.0
	7	0.3 ± 0.10	-	-	-	-	42.6	61.7
	8	0.3 ± 0.10	-	-	-	-	97.0	121.0
	9	0.2 ± 0.05	-	-	-	-	-	-
	10	0.3 ± 0.10	-	-	-	-	-	-
	11	0.2 ± 0.08	-	-	-	-	56.1	69.2
	12	0.5 ± 0.15	-	-	-	-	69.1	59.5
Regenerating	1	1.4 ± 0.20	5.5	152	-	-	40.0	55.0
	2	1.1 ± 0.20	7.1	171	30.0	50.0	40.0	48.0
	3	2.5 ± 0.25	10.9	290	87.0	74.0	113.0	104.1
	4	1.8 ± 0.20	9.2	217	65.0	80.0	73.0	86.5
	5	2.5 ± 0.30	6.8	224	35.0	50.0	65.0	108.0
	6	2.0 ± 0.20	-	-	-	-	67.0	65.5
	7	2.2 ± 0.25	-	-	-	-	87.0	50.6
Normal blood**		0		-	0	-	0	-
Immature blood**		0		-	5.0	-	2.5	-

* The mitotic frequencies are estimated on stained paraffin-sections and calculated according to the Poisson distribution.

** The nuclease activities in the blood expressed per g fresh blood.

TABLE III

RESULTS OF STATISTICAL ANALYSIS OF DIFFERENCES BETWEEN NORMAL AND REGENERATING BONE MARROWS

Factor of comparison	Statistical significance
Mitotic frequency	$0.001 < P < 0.01$
RNase per µg DNAP	$0.05 < P < 0.2$
RNase per mg total N	$0.001 < P < 0.01$
Non-activated DNase per 20 µg DNAP	$0.05 < P < 0.2$
Non-activated DNase per 1 mg total N	$0.001 < P < 0.01$
Mg^{++}-activated DNase per 20 µg DNAP	$P > 0.2$
Mg^{++}-activated DNase per 1 mg total N	$P > 0.2$
PNAP/DNAP	$0.001 < P < 0.01$

placenta[10]. With this technique no changes in activity during normal growth were found. In other experiments using a differently prepared substrate, a considerable decrease of the RNase activity was found during the decrease of the growth activity in this organ[9]. Thus, the substrate is of considerable importance. FINAMORE[18], studying the growth of *Rana pipiens*, found cyclic changes in the RNase activity during the early embryonic development, changes that were supposedly synchronous with various phases of growth and division.

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DNase activity

The bone marrow from chickens showed in general high DNase activities. The optimum is near pH 5.7. At this pH, the addition of Mg^{++} causes inactivation. On the other hand, at more acid pH values, Mg^{++} causes an activation, which can be seen in Fig. 1. Furthermore, it was noticed that different concentrations of Mg^{++} in the reaction mixture were necessary for obtaining maximal activation at different pH values. At pH 5.2 the final Mg^{++} concentration for maximal activation was $1 \cdot 10^{-2} M$, and at pH 4.7, $2 \cdot 10^{-2} M$. Fig. 2 gives a graphical summary of these results. It may be mentioned that the Mg^{++} concentration in the bone marrow homogenates was about $10^{-4} M$.

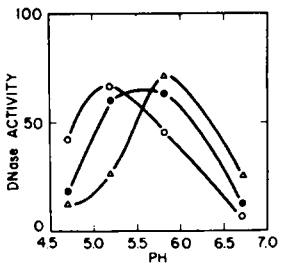

Fig. 1. Influence of Mg^{++} on the DNase activity at different pH values. △ without Mg^{++}, ● Mg^{++} concentration $5 \cdot 10^{-2} M$, ○ Mg^{++} concentration $1 \cdot 10^{-2} M$.

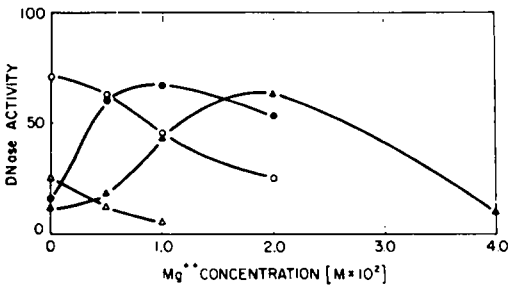

Fig. 2. Optimal Mg^{++} concentration for DNase activity at different pH values. ▲ pH 4.7, ● pH 5.2, ○ pH 5.8, △ pH 6.7.

When measuring the Mg^{++}-activated DNase activities, a zero order reaction was obtained in the range of the actual homogenate concentrations and incubation times. When determining the DNase activities without added Mg^{++}, a "lag" phase in the rate of formation of acid-soluble enzymic hydrolysis products was observed (Fig. 3). This phenomenon has been discussed earlier[10].

The Mg^{++}-activated DNase activities were determined in eight normal and in seven regenerating bone marrows. Non-activated DNase activities were measured in three normal and in five regenerating marrows. Both activities were determined at pH 5.2. Table II gives a summary of the results. The Mg^{++}-activated DNase activities were approximately the same in the two groups and exhibited great variations. The activities, measured without Mg^{++}, however, were invariably higher in the regenerating group. For results of statistical analysis see Table III.

In accordance with the present findings, a tissue DNase has earlier been observed with an optimal activity near pH 5[6,10,19,20]. At this pH, however, the addition of Mg^{++} caused an inhibition of the enzymic activity.

The homogenization procedure, used in these experiments, completely disintegrates cells and nuclei and probably also mitochondria[10]. By disintegration of the structures of the cell, the important role of the structural formations of the cell in connection with intracellular enzymic activity is affected and probably only an average enzymic activity is measured under the conditions used. To rule out the possibility that the presence of disintegrated tissue particles affected the enzymic activity by unspecific binding of the enzyme or by other means, the following experiment was undertaken. A homogenate was prepared; part was set aside for the determi-

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nation of the DNase and the rest centrifuged in a Spinco preparative ultracentrifuge at 40,000 × g for 30 minutes. The DNase activity was then determined in the supernatant. Table IV gives the results. It can be seen that almost all the enzymic activity is recovered in the supernatant.

TABLE IV

DESOXYRIBONUCLEASE ACTIVITY IN BONE MARROW HOMOGENATE AND SUPERNATANT*

	Without Mg^{++}				With Mg^{++}			
	*DNase activity***	*PNAP*	*DNAP*	*Total N*	*DNase activity***	*PNAP*	*DNAP*	*Total N*
Homogenate	26.2	6.77	10.55	1.2	57.8	7.18	11.12	1.4
Supernatant	26.3	0.45	1.27	0.4	45.0	0.24	1.35	0.5

* Supernatant obtained by centrifugation at 40,000 × g during 30 min.

** Enzymic activities determined at pH 5.25. All values are given per ml of homogenate or supernatant.

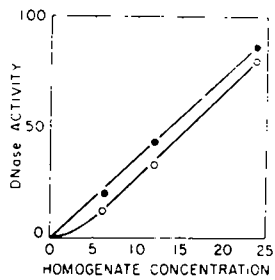

Fig. 3. Relationship between homogenate concentration, expressed as tissue DNAP, and DNase activity. ○ without and ● with Mg^{++} in the incubation mixture. Final Mg^{++} concentration $1 \cdot 10^{-2} M$.

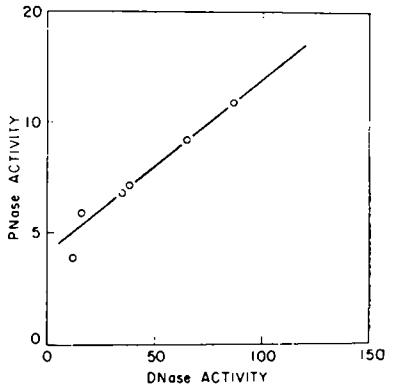

Fig. 4. Relationship between DNase and RNase activity in normal and regenerating bone marrows. DNase activity, measured without the addition of Mg^{++} and expressed per 20 μg tissue DNAP, RNase activity expressed per μg DNAP. For further explanation see text.

Bone marrow growth

The degree of regeneration, measured as the mitotic frequency of stained sections of the bone marrow samples, showed a considerable increase in the marrows that were regenerating after phenylhydrazine hemolysis. A correspondingly increased rate of production of red cells took place in accordance with earlier data[5].

Table II shows the correlation between the nuclease activities from four sources, representing different degrees of growth activity and the corresponding mitotic frequencies and enzymic activities. In the blood from normal chickens there are no mitotic cells and no measurable DNase activity. In the blood from regenerating animals there is an outflow of immature cells from the bone marrow. There are very few mitotic cells but a definitely measurable DNase activity. Normal bone marrow represents a "basal" mitotic state with a corresponding enzymic activity. Finally, regenerating bone marrow represents a rapidly growing tissue in which the highest nuclease activities are found.

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The RNase and DNase activities in the different bone marrow samples were rather well correlated with one another (Fig. 4).

The acid DNase activity has been studied by ALLFREY AND MIRSKY[21], who found a correlation between the DNase activity of a tissue and its DNA turnover, measured as the incorporation of ^{15}N. From experiments on a diversity of adult animal tissues these authors put forward the hypothesis that "there exists a possible connection between the DNase concentration of a tissue and its capacity for proliferation and regeneration".

Characteristically increased turnover rates have been found during the formation of proteins and polynucleotides in regenerating bone marrow and liver[3,5]. Incorporation of ^{15}N into the desoxyribose polynucleotides of regenerating bone marrow was higher than into those of normal tissue. There was also a definite increase of the incorporation into the ribosepolynucleotides[5], but to a much less extent. These investigations showed a correlation between the state of growth, measured as the mitotic activity, and the isotope contents of polynucleotides. Our experiments show a similar connection between mitotic frequencies and the nuclease activities.

Phenylhydrazine-induced regeneration of bone marrow thus causes a parallell increase in the incorporation of ^{15}N into the desoxyribose and ribosepolynucleotides and the DNase and RNase activities. It is furthermore of interest to note that in this material there is also a quantitative relationship between the magnitude of the increase of ^{15}N incorporation into the DNA and PNA and the relative increase in the corresponding enzymic activities. In the regenerating tissue the incorporation of ^{15}N into DNA-adenine and DNA-guanine was increased 160 and 170% and into PNA-adenine and PNA-guanine 20 and 50%. Table II and Fig. 4 show that the maximal observed increase in DNase activity is about 6-fold, the corresponding increase in RNase activity only about 2-fold. However, whether these relationships between nucleic acid turnover, as measured by ^{15}N incorporation, and enzymic activity, are merely coincidental is still not clear, especially as the pattern of ^{15}N incorporation, when studied in detail[22], is rather complicated and difficult to interpret in terms of growth and synthetic rates.

SUMMARY

1. Phenylhydrazine was used to induce regeneration of chicken bone marrow.

2. The mitotic frequencies were determined and showed up to a 10-fold increase in the regenerating tissues.

3. The pentose and desoxypentose nucleic acids were determined in the two types of tissue. A consistent increase in the PNAP/DNAP quotients was noted in the regenerating tissues.

4. The ribonuclease activity in the bone marrow homogenates has its pH optimum near 7.5. A correlation between the enzymic activity and the state of growth was found.

5. The desoxyribonuclease activity in the bone marrow homogenates shows a pH optimum near 5.7. The influence of magnesium at different pH values was studied. A good correlation between the desoxyribonuclease activity and the state of growth of the tissue was found.

6. The relationship between the metabolism of the pentose and desoxypentose nucleic acids and the corresponding enzymic activities is discussed.

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THE APPLICATION OF CHROMATOGRAPHIC METHODS TO STUDY THE INCORPORATION OF ^{32}P-LABELED ORTHOPHOSPHATE INTO THE PHOSPHATIDES OF RAT LIVER HOMOGENATES*

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In light of the newer paper and column chromatographic techniques for lipide fractionation[1-4] the study of the incorporation of labeled orthophosphate into the phosphatides of rat liver was undertaken. The results presented in this paper show that liver homogenates can carry out this latter process to a small extent and that the lipides which become most actively labeled resemble but are not identical to phosphatidic acids.[5] In contrast to *in vivo* studies[1,3,6], lecithin and phosphatidyl ethanolamine show very little incorporation in the *in vitro* system.

EXPERIMENTAL

Methods and reagents

Methanol, petroleum ether (b.p. 35°–60°), and chloroform which were used for lipide extraction and fractionation were Mallinckrodt 'analytical' reagents. Total lipide P was determined by a modified*** method of HARRIS AND POPAT[7] and ester analyses by the method of RAPPORT AND

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*** The method was modified by using half the amount of perchloric acid, molybdate and elon reagents and bringing the solution to a final volume of 10 ml.

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