ACTA CHEMICA SCANDINAVICA 7 (1953) 721–734

Studies on Ribonuclease and Desoxyribonuclease Activities in Homogenates from Human Placenta

SAM BRODY

Department of Pathology, Karolinska Institutet, Stockholm 60, Sweden

The biological importance of the nucleic acids has stimulated the interest in the enzymes related to these compounds. Numerous investigations on the mechanism of action and the kinetics of these enzymes have been carried out. The subject has been reviewed recently [1,2]. However, attempts to establish a correlation between the developmental stage of a tissue and the activities of these enzymes, have not led to any positive results.

In connection with an investigation on the quantitative changes of nucleic acids during the growth of the human placenta [3,4,19], it was considered desirable also to carry out some analyses on the ribonuclease (PNase) and desoxyribonuclease (DNase) activities of this tissue at different stages of development. The object of the present study is to find out whether there exists a correlation between the enzyme activities and the developmental stage of the tissue.

MATERIALS AND METHODS

Tissue preparation. Placentas from different stages of pregnancy were obtained at abortions provoked on social-medical indications according to Swedish law, and at parturitions. All the patients were in good health. The age of the placentas was estimated from the over-all (crown-heel) length of the foetus, applying Haase's rule (quoted from Wylie and Amidon [5]).

Immediately after removal the placentas were freed from the amnion membrane and the decidua. The remaining foetal placenta was cut into small pieces, washed in glass-distilled water and frozen in a beaker immersed in a dry ice-alcohol mixture. The preparation was immediately brought to the laboratory and homogenized in glass-distilled water for four minutes in a Bühler homogenizator. The tissue concentration was about 10 per cent. Precautions were taken to keep the temperature in the homogenate close at 0° C. This homogenate was used for the quantitative assay of the enzyme activities, and the determinations were begun within an hour after removal of the placentas.

Substrates. Commercial yeast nucleic acid was used as the original substance of the substrate for the PNase determinations. All the preparations were purified in the same way. The crude preparation was dissolved in a 10 % sodium chloride solution by adding some concentrated NH_3 to pH 6—6.5. The concentration of the nucleic acid was about 3 to 4 per cent. The solution was dialyzed against running tap water (about + 10° C) for 20 days and against frequently changed distilled water (+ 4° C) for two days. The nucleic acid was then precipitated by adding three volumes of absolute alcohol and a few ml of a 10 % sodium acetate solution. The precipitate was washed a few times with absolute alcohol and dried with ethyl ether. The physico-chemical data of the preparation used in this study are summarized in Table 1. The extinction values are calculated by the method of Chargaff and Zamenhof [6].

In the determinations of DNase activity desoxyribonucleic acids (D.N.A.) from calf thymus was used as substrate. It was prepared by Hammarsten's [7] method. The only modification was that at the last precipitations with absolute alcohol a few ml of a 10 % (w/v) sodium acetate solution were added instead of a sodium chloride solution. The physico-chemical data of the preparation used in this study are summarized in Table 1.

For convenience the substrates are referred to as D.N.A. and yeast nucleic acid, but it is understood that it is always the neutralized salt of the nucleic acids to which reference is made.

General procedure for the determination of enzyme activities. The PNase and DNase activities were measured by estimating the release of acid-soluble substances containing pentose and desoxypentose sugar. The colorimetric determinations were carried out as described earlier [8,9]. By running correspondent determinations of acid-soluble substances containing phosphorus, the correlation between phosphorus and sugar was evaluated. By this means the enzyme activities can be expressed in terms of the number of micrograms of acid-soluble phosphorus. The main reason why the colorimetric sugar determinations were preferred throughout the study is that the tissue and substrate blanks, in the DNase determinations being zero, are considerably lower than the blanks when using the phosphorus determination procedure, owing to the relatively greater amount of acid-soluble tissue phosphorus. The sensitivity of the colorimetric methods and their convenience also contributed to this choice.

Determination procedure for the PNase activity. The homogenate, containing between 2 mg and 3.5 mg of total nitrogen per ml, was diluted 25 times with glass-distilled water. Duplicate determinations were made with two amounts of tissue. 0.5 ml of a 1/15 *M* phosphate buffer (pH 7.40—7.45) and 0.5 ml of a 1 % purified yeast nucleic acid solution

Table 1. Physico-chemical data of the substrates used.

Substance	N/P	*E* max	*E* min	ε(P)max	ε(P)min	ε(P) 280	pH
D.N.A.	1.69	260	231	8 045	3 198	5 097	6.0
P.N.A.	1.56	258	228	8 199	2 980	4 217	6.2

N = nitrogen; P = phosphorus; *E* max, *E* min = wave length in mμ for maximal and minimal extinction; ε (P) = atomic extinction coefficient with respect to phosphorus.

were used. The total volume of the reaction mixture was 2 ml and the final * pH 7.55—7.60. The determinations were carried out in a Warburg apparatus with a constant temperature water bath at 37° C under continuous shaking of the reaction vessels. The yeast nucleic acid was placed in the side-arm and added after temperature equilibrium had been attained. The incubation time was 60 minutes. The reaction was stopped by adding 2 ml of an ice-cold solution of 0.25 % (w/v) uranyl acetate in 10 % (w/v) trichloroacetic acid (T.C.A.) [10]. The tubes were allowed to stand at + 4° C for one hour and were then centrifuged. An aliquot of the centrifugate was used for the colorimetric sugar determinations. Appropriate tissue and substrate blanks were carried out for all analyses. The activity is expressed as the increase in acid-soluble phosphorus during 60 minutes per mg of total tissue nitrogen or per μ g of tissue D.N.A. phosphorus (D.N.A.P.); see Discussion.

Determination procedure for the DNase activity. The determinations were carried out with five amounts of tissue, using from 0.1 ml to 1.0 ml of homogenates, containing 2 mg to 3.5 mg of total nitrogen per ml. 0.5 ml of a 0.2 *N* acetate buffer (pH 4.95—5.00) and 1 ml of a 1 % (w/v) D.N.A. solution were used. The volume was made up to 3.5 ml and the final pH was 5.0 to 5.1. The determinations were carried out in the Warburg apparatus for 180 minutes at 37° C. The reaction was stopped by adding 1.5 ml of an ice-cold 20 % (w/v) T.C.A. solution. After standing at + 4° C for about 15 minutes the tubes were centrifuged. An aliquot of the centrifugate was used for the colorimetric sugar determinations, which were made immediately. Tissue and substrate blanks were zero. The activity is expressed as the increase in acid-soluble phosphorus during 180 minutes and is given for a point on the concentration-activity curve, which corresponds to an amount of tissue, containing 1 mg of total nitrogen or 20 μg of D.N.A.P.

Other determination procedures. Tissue D.N.A.P., after having been extracted by the method of Schneider [11], was determined as described earlier [9]. Every determination, including the extraction procedure, was run in duplicate.

The duplicate nitrogen determinations were done by the micro-Kjeldahl technique [12]. Phosphorus was determined according to Teorell's [13] modification of Fiske and Subba-Row's [14] method. Manipulations and centrifugations (M.S.E. refrigerated centrifuge) were carried out as close to 0° C as possible. All the solutions were prepared daily.

The colorimetric determinations were done in a Beckman quartz spectrophotometer, using a 1 cm cell. The pH values were electrometrically controlled with a Radiometer pH-meter 3.

EXPERIMENTAL

Prior to the adoption of determination procedures for the PNase and DNase activities in placental homogenates, the influence of a variety of factors on these activities had to be studied.

1. Determination of PNase activity

Influence of pH. The effect of hydrogen ion concentration is demonstrated in Fig. 1. The optimum is situated near pH 7.6. The inhibitory effect of the borate buffer should be noted [15].

* By final pH is meant the pH value of the whole reaction mixture at the beginning of the reaction. In order to avoid losses it was measured in a duplicate mixture.

Table 2. Relationship between homogenate concentration and PNase activity.

Amount of homogenate in mg total nitrogen	Activity per μg D.N.A.P.
0.028	28.6
0.045	30.2
0.056	29.4
0.090	29.4
0.112	29.3
0.180	31.6
0.280	30.0
0.450	23.2
0.560	19.3

Activity expressed as increase in μg of acid-soluble phosphorus during 60 minutes. D.N.A.P. = desoxypentose nucleic acid.

Tissue concentration. To establish the relationship between homogenate concentration and enzymic activity the experiment summarized in Table 2 was undertaken. A rather wide tissue concentration range is determinable and it covers safely the homogenate concentrations used.

Incubation time. The relationship between activity and incubation time is dependent on the concentration of the homogenate used. Under the present experimental conditions the linearity between enzymic activity and time is sustained for more than 60 minutes. In order to check that every experiment was conducted within the appropriate time and tissue concentration all analyses were carried out in duplicate with two amounts of tissue.

Stability of the PNase activity in the homogenate. No decrease in PNase activity was observed after 12 hours. After 24 hours the activity had decreased by about 15 per cent.

Digestibility of the substrate. The digestibility of different batches of yeast nucleic acid, purified in the same way, varied considerably. The same preparation was therefore used throughout this study.

Substrate concentration. The effect of the substrate concentration on the PNase activity is demonstrated in Fig. 2. Five mg of yeast nucleic acid were considered adequate for the PNase determinations. An increase in substrate concentration brought about a considerable increase in blank values.

2. Determination of DNase activity

Influence of pH. Fig. 3 shows a rather sharp activity peak at pH 5.0 for a 12 week old placenta. For a full term placenta maximum activity is situated near pH 5.5. This displacement of the pH maximum was found to be correlated to the age of the placenta. Before the sixteenth to eighteenth week of pregnancy maximum was invariably situated in the range of pH 5.0. At the middle of pregnancy the activities at pH 5.0 and pH 5.5 were about the same. In full term placentas maximum was situated near pH 5.5.

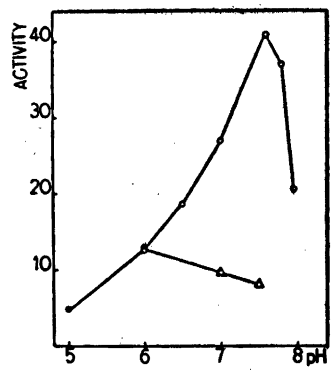

Fig. 1. Effect of pH on the PNase activity in placental homogenate. Activity expressed as increase in acid-soluble phosphorus per μg of tissue D.N.A.P. per 60 minutes.

●——● *0.2 N acetate buffer.*
○——○ *1/15 M phosphate buffer.*
△——△ *0.2 M boric acid, 0.05 M sodium tetraborate.*

Influence of magnesium. It was noted that magnesium inhibited the placental DNase activity at pH 5.0. Fig. 4 shows the effect of inhibition at different concentrations of magnesium.

Tissue concentration. Fig. 5 shows some DNase activity curves from homogenates of placentas of varying ages, where the activity has been plotted against the amount of homogenate expressed as μg of tissue D.N.A.P. In the concentration range chosen, all the curves show a lag phase in the rate of formation of acid-soluble enzymic hydrolysis products, but from a certain point all the curves are linear.

Because of the configuration of the homogenate concentration curves and for the sake of comparison between different placentas, the activities were calculated for a tissue nitrogen or tissue D.N.A.P. value common to all the placentas.

Incubation time. Fig. 6 demonstrates the time-activity curve for placental DNase activity. This curve also exhibits a lag phase. Because of this fact and of the relatively weak DNase activity in full term placentas an incubation time of 180 minutes was used.

Stability of the DNase activity in the homogenate. A fairly rapid inactivation of the DNase activity was observed at pH 5.0. The activity eight hours after the homogenization procedure was 65 per cent of that immediately after homogenization. In contrast to this finding the activity at pH 5.5 showed an increase during the same period, amounting to 47 per cent. After 22 hours, however, the activity at this pH had also decreased by 20

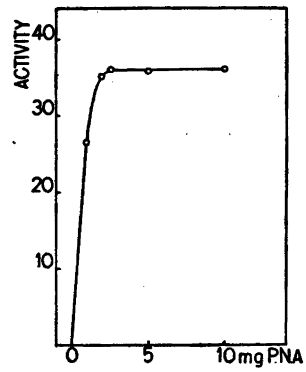

Fig. 2. Effect of substrate concentration on the PNase activity in placental homogenate. P.N.A. = yeast nucleic acid.

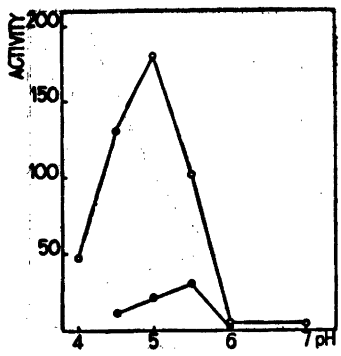

Fig. 3. Effect of pH on the DNase activity in placental homogenate. Activity expressed as increase in acid-soluble phosphorus per 20 μg of tissue D.N.A.P. per 180 minutes.
0.2 N acetate buffer pH 4.0—6.0
1/15 M phosphate buffer pH 6.0—7.0
12 week old placenta.
full term placenta.

per cent compared with the initial value. It should be noted that the activity at pH 4.5 showed principally the same pattern as that at pH 5.0 and that the pH 6.0 value principally resembled that at pH 5.5. No corrections for the decrease in activity at pH 5.0 have been made.

Substrate concentration. The influence of the substrate concentration is demonstrated in Fig. 7. 10 mg of D.N.A. were considered adequate for the DNase activity determinations.

3. Inhibitor experiments

Some experiments were carried out to demonstrate the presence of an inhibitor in homogenates from human placenta against crystalline DNase. The reactions were run at pH 7.0, using a 0.1 *N* veronal-acetate buffer, and at pH 5.0, using a 0.1 *N* acetate buffer. Crystalline DNase, obtained from Worthington Chemical Corp., was used in a concentration of 30 μg per ml at pH 5.0 and 3 μg per ml at pH 7.0. The enzyme was stabilized by 0.25 per cent gelatin [16]. The DNase was activated by magnesium, which in the reaction mixture had a concentration of 0.01 molarity. Besides 0.25 ml of a 0.140 *M* magnesium sulphate solution and 0.5 ml of the DNase solutions, the same volumes were used as those quoted for the placental DNase activity determinations. The incubation time was 30 minutes.

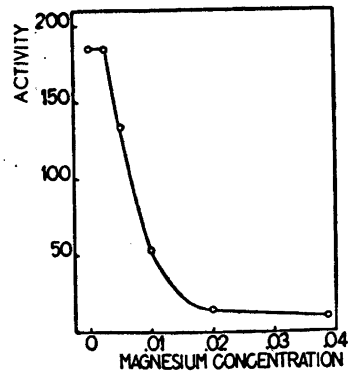

Fig. 4. Effect of magnesium on the DNase activity in placental homogenate. Magnesium concentration expressed as final molarity.

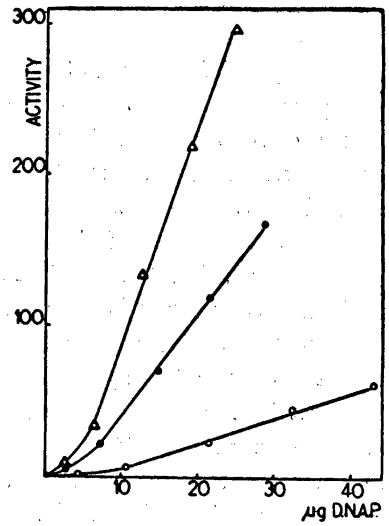

Fig. 5. Relationship between tissue concentration and DNase activity in placental homogenate. The amount of tissue expressed as µg of D.N.A.P. Activity expressed as increase in acid-soluble phosphorus per 180 minutes.

△——△ *11 week old placenta.*

●——● *20 week old placenta.*

○——○ *40 week old placenta.*

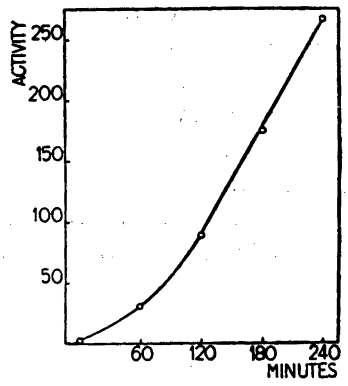

Fig. 6. Rate of formation of acid-soluble phosphorus by incubation of D.N.A. with placental homogenate. Activity expressed as increase in acid-soluble phosphorus per 20 µg of tissue D.N.A.P.

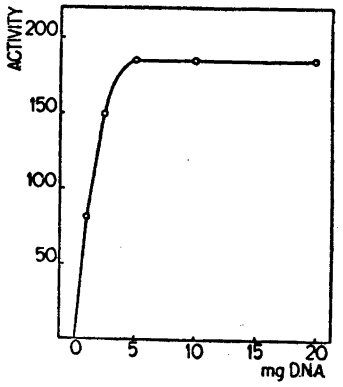

Fig. 7. Effect of substrate concentration on the DNase activity in placental homogenate. Activity expressed as in Fig. 3. D.N.A. = desoxypentose nucleic acid.

RESULTS

PNase activity. Table 3 summarizes the figures for the PNase activity in homogenates from placentas of different ages. When the activity is calculated per mg of total tissue nitrogen, the values show some variability between different placentas, but seem to be approximately constant throughout development. This approximate constancy is also observed when the enzymic activity is calculated per μg of tissue D.N.A.P.

DNase activity. Table 4 is a condensation of the analytical results concerning DNase activity in homogenates from placentas at different phases of development. The activity, expressed per 20 μg of tissue D.N.A.P., shows a marked decrease during the aging of the placenta. The activity of a 10 week old placenta is about ten times that of a full term placenta. If the activity is expressed per 1 mg of total tissue nitrogen there is also an indication of such a decrease, but the correlation to the age of the placenta is not as well established as when the tissue D.N.A.P. is used as a basis of reference.

Inhibition of crystalline DNase. The inhibitor experiments demonstrated the presence of an inhibitor against crystalline DNase. Small amounts of homogenate, corresponding to about 0.2 mg of total tissue nitrogen, caused an inhibition of 70 per cent. The inhibitor exerted its effect at pH 7.0. At pH 5.0 the reverse was observed, the crystalline DNase being activated by placental homogenate with about 100 per cent. Under these conditions of the experiment the DNase which was incubated at pH 5.0 in the presence of placental homogenate, had an activity that was about 20 per cent of that of the pure crystalline DNase activity at pH 7.0. The problem will be further studied.

Table 3. PNase activity during development of placenta.

Age in weeks	PNase activity/mg N	PNase activity/μg D.N.A.P.
10	323.9	36.5
11	419.0	41.4
13	354.8	27.3
14	342.7	25.5
17	364.8	25.3
20	493.4	37.8
40	501.3	39.7
40	478.0	33.1

Activity expressed as increase in μg of acid-soluble phosphorus during 60 minutes. N = total nitrogen; D.N.A.P. = desoxypentose nucleic acid; Every value the mean of duplicates.

Table 4. DNase activity during development of placenta.

Age in weeks	DNase activity/1 mg N	DNase activity/20 μg D.N.A.P.
10	68	217
11	90	226
13	73	133
13	100	183
14	64	110
14	82	195
17	111	131
20	69	108
20	42	113
40	10	22
40	11	23
40	12	20

Activity expressed as increase in μg of acid-soluble phosphorus during 180 minutes. N = total nitrogen; D.N.A.P. = desoxypentose nucleic acid phosphorus; The activity values calculated from the regression lines, see text.

STATISTICAL ANALYSIS

The error of the method has been calculated from differences of individual pairs of values according to the following formula (the error dependent on the level of the substance measured)

$$k = \sqrt{\frac{\sum w^2}{2n}}$$

where k = the relative error (coefficient of variation)

$$w = \frac{x-y}{\frac{1}{2}(x+y)}$$ (x and y duplicate determinations)

n = number of pairs.

For the calculation of the error of the method for the DNase determination procedure the curved part of the line (Fig. 5) has been omitted throughout the material by leaving out the first point of observation. To each placenta a straight line has been fitted to the remaining points by the method of least squares [17,18], each point being given the same weight. The residual variance has been determined by standard methods. With the method used for the

Table 5. Accuracy of determination methods used revealed by statistical analysis.

Substance	n	Relative error in single determination
Total nitrogen	28	± 1.4
D.N.A.P.	30	± 1.7
PNase activity	16	± 5.9
DNase activity	36	± 7.1

D.N.A.P. = *desoxypentose nucleic acid.* n = *number of determinations.*

determination of DNase activity the error in a y-determination (y = activity) should be proportional to the y-value. This assumption has been confirmed by plotting the mean deviation (square root of the residual variance) against $\bar{y}$ (mean y-value) for each line. Under these conditions the mean deviation from a straight line related to the mean y-value gives an estimate (Q) of the relative error. The mean relative error (coefficient of variation = $\bar{Q}$) has been determined according to the formula

$$\bar{Q} = \sqrt{\frac{\sum Q^2}{n}}$$

where n = is the number of lines.

The results of the statistical analysis of the material are summarized in Table 5.

DISCUSSION

From the experiments described here it is evident that a correlation can be demonstrated between the developmental stage of the placenta and the DNase activity (calculated with the tissue D.N.A.P. as a basis of reference) in the corresponding homogenate. No such correlation was established for the PNase activity.

It has been demonstrated that the average D.N.A.P. content per nucleus in the placenta is constant during different stages of development [19], as calculated from the D.N.A.P. contents and cell counts on suspensions of isolated nuclei. The calculation of the enzymic activities per μg of D.N.A.P. thus gives a relative expression for the average per tissue unit. The D.N.A.P. content of a tissue sample gives an estimation of the total number of units in it and seems to be a better expression for the active mass than is, for instance, total nitrogen and wet or dry weight.

The homogenates used in this investigation are suspended in distilled water. Examination by phase contrast microscopy reveals almost complete disintegration of whole cells and nuclei. The mitochondria, however, are not disrupted by the mechanical homogenization procedure, but it is considered that these particles disintegrate in distilled water [20], which may be the case especially after previous freezing and thawing. Studies on the cellular distribution of DNase and PNase activities have revealed that the mitochondrial fraction is endowed with enzymic activity. The extent of this activity varies considerably in different tissues and according to the methods used [21–23]. In calf thymus cells [24] the mitochondrial DNase activity is 12 per cent of that of the whole homogenate and the mitochondrial PNase activity 23 per cent, in mouse liver [25] the corresponding figures are 73 per cent and 58 per cent.

It should be emphasized that the complete disintegration of the structural formations of the cell, which certainly play an important rôle in connection with intracellular enzymic activity, means that what is measured is not the average *in vivo* intracellular enzymic activity but the average maximal activity under the conditions chosen.

The DNase activity of a young human placenta has its pH optimum in the range of 5.0 and is inhibited by magnesium (see foregoing). This is in sharp contrast to the observations on the enzyme, which is prepared from pancreatic tissue and available in crystalline form. This enzyme exerts its maximal activity between pH 6 and 7 and requires magnesium for activation [16,26–29]. A broad pH spectrum for the optimal DNase activities from different organs has been demonstrated [30]. The inhibitory effect of magnesium has also been demonstrated for spleen [31] and calf thymus [24] DNase activity. The optima for these enzymic activities are also situated near pH 5.

The initial, non-linear, increase in the rate of formation of acid-soluble enzymic degradation products (Fig. 6) is in agreement with observations by Kunitz [29] on crystalline pancreas DNase. The fact that the initial lag phase is more marked when increasing the substrate concentration, is thought to be due to the higher viscosity of the more concentrated D.N.A. solutions [29].

The observed decrease in DNase activity during the development of the placenta can be brought about by a decrease in the enzyme concentration of the tissue, a decrease of an activator of the enzyme, an increase of an inhibitor against the enzyme, or by a combination of all these factors. Zamenhof and Chargaff [32,33] reported the existence of a specific DNase inhibitor which was found in yeast cells. Inhibition of crystalline DNase from beef pancreas by a great variety of both normal and neoplastic tissues has been demonstrated [34–37].

The present investigation has shown that a strong inhibition of crystalline pancreas DNase is brought about by homogenates from human placenta at pH

7.0 but that the activity is increased at pH 5.0, both findings being in agreement with the results obtained by Henstell and Freedman [38] on extracts of whole blood or bone marrow. These authors hold that the degree of inhibition of crystalline DNase is correlated to the maturity of the cells and discuss the possibility of this inhibitor to control cell maturation and development. It is questionable, however, if any conclusions regarding the regulation of tissue DNase activity may be drawn from experiments on the inhibition of crystalline pancreas DNase by tissue homogenates or extracts.

Up to the present comparatively little work has been done on the relationship between PNase and DNase activities and the developmental stage of a tissue. An approach to the problem has been made by Greenstein and Jenrette [39], Greenstein [40-42], Greenstein and Stewart [41] and Greenstein and Thompson [43]. In a series of investigations on these enzymic activities in normal and neoplastic tissues these workers examined the PNase and DNase activities in adult resting and regenerating rat liver and in hepatomas and foetal livers of the same species. These authors, however, were not able to correlate the activities to the physiological state of these different tissues. When comparing the DNase activities of induced tumors and the normal tissue of origin no consistent results were obtained.

With respect to the constancy of the PNase activity during different stages of cellular development the present investigation confirms the results obtained by Greenstein and co-workers [39-43]. It may be emphasized, however, that all these investigations, including the present one, have been carried out with yeast nucleic acid as a substrate. The possibility that experiments with organ-specific, highly polymerized P.N.A. might give other results cannot be ruled out.

The DNase activity measurements in the investigations by Greenstein and co-workers [39-43] were done by the viscosimetric technique. This fact, however, does not necessarily exclude a comparison between these investigations and the present one. Laskowski [44] has presented evidence indicating that probably the same enzymic activity is measured, when estimating the decrease in viscosity as when determining the amount of acid-soluble enzymic degradation products of the D.N.A. All the determinations by Greenstein and co-workers, however, were run at pH 7. Moreover, the measurements were made on a water extract which was allowed to stand at + 5° C for 18 hours. A considerable inactivation of the enzyme during this time cannot be ruled out (see under Experimental).

Lately, Allfrey and Mirsky [23] have brought some evidence of a correlation between the DNase activity of a tissue and its D.N.A. turnover, measured as the incorporation of N^{15}. The enzymic determinations were run near pH 5. From these 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 or regeneration". However, comparative studies made by these workers on enzymic activities of foetal and adult tissues or young, adult and regenerating rat livers have not brought any direct support of this hypothesis.

In a recent investigation [45], using the same technique as that presented in this paper, a correlation between the growing state, expressed by the mitosis frequency, and the DNase activity was established in normal and regenerating bone marrow from hens. The results from these studies are thus in principal agreement with those obtained for placental tissue. In the homogenates from bone marrow activation of the DNase activity in the range of pH 5.0 by magnesium was observed, but a considerable activity was present also without addition of magnesium. A full account will be given in a report to be published.

SUMMARY

1. Methods based on the determination of acid-soluble nucleic acid degradation products containing pentose and desoxypentose have been worked out for the determination of PNase and DNase activities in human placental homogenates.

2. The DNase activity in a young placenta has its pH optimum in the range of pH 5.0 and is inactivated by magnesium at this pH.

3. This DNase activity has been measured in homogenates of placentas of different ages. A correlation between enzymic activity (calculated with tissue D.N.A.P. as a basis of reference) and the developmental stage has been established. The activity of a 10 week old placenta is ten times that of a full term placenta.

4. The PNase activity has its pH optimum near pH 7.6.

5. This PNase activity is approximately constant throughout development.

To Professor E. Hammarsten and Dr. Bo Thorell I want to acknowledge my indebtedness for their constructive criticism and helpful suggestions I wish also to express my deep gratitude to Professor K. Myrbäck for valuable discussion.

I am also indebted to Dr. Nils Blomqvist for useful suggestions on statistical problems. To Mrs. M. Thorell, who made the drawings, I want to express my deep thanks.

REFERENCES

1. Laskowski, M., in Sumner, J. B., and Myrbäck, K. *The Enzymes*, Academic Press Inc., New York 1952.
2. Jordan, D. O. in *Ann. Rev. Biochem.* **21** (1952) 209.
3. Brody, S. *Exptl. Cell Research* **3** (1952) 702.

4. Brody, S. *Acta Chem. Scand.* **7** (1953) 507.
5. Wylie, B., and Amidon, B. F. *Am. J. Obstet. Gynecol.* **61** (1951) 193.
6. Chargaff, E., and Zamenhof, S. *J. Biol. Chem.* **173** (1948) 327.
7. Hammarsten, E. *Biochem. Z.* **144** (1924) 383.
8. Brody, S. *Acta Chem. Scand.* **7** (1953) 495.
9. Brody, S. *Acta Chem. Scand.* **7** (1953) 502.
10. MacFadyen, D. A. *J. Biol. Chem.* **107** (1934) 297.
11. Schneider, W. C. *J. Biol. Chem.* **161** (1945) 293.
12. Pregl, F. *Die Quantitative Organische Mikroanalyse.* Berlin 1917.
13. Teorell, T. *Biochem. Z.* **230** (1931) 1.
14. Fiske, C. H., and Subba Row, Y. J. *J. Biol. Chem.* **66** (1925) 375.
15. Zittle, C. A. *Federation Proc.* **6** (1947) 500.
16. Mc Carty, M. *J. Gen. Physiol.* **29** (1946) 123.
17. Fisher, R. A. *Statistical Methods for Research Workers.* Edinburgh and London 1936.
18. Bonnier, G., and Tedin, O. *Biologisk Variationsanalys,* Stockholm 1940.
19. Brody, S. *Acta Obstet. Gynecol. Scand.* **32** (1953) Suppl. **6.**
20. Dounce, A. L., in Sumner, J. B., and Myrbäck, K. *The Enzymes,* Academic Press Inc., New York 1952.
21. Schneider, W. C., and Hogeboom, G. H. *Cancer Research* **11** (1951) 1.
22. Allfrey, V., Stern, H., Mirsky, A. E., and Saetren, H. J. *J. Gen. Physiol.* **35** (1952) 529.
23. Allfrey, V., and Mirsky, A. E. *J. Gen. Physiol.* **36** (1952) 227.
24. Brown, K. D., Jacobs, C., and Laskowski, M. *J. Biol. Chem.* **194** (1952) 445.
25. Schneider, W. C., and Hogeboom, G. H. *J. Biol. Chem.* **198** (1952) 155.
26. Kunitz, M. *J. Gen. Physiol.* **24** (1940) 15.
27. Fischer, F. G., Böttger, I., and Lehmann-Echternacht, H. *Hoppe-Seylers Z. physiol. Chem.* **272** (1942) 246.
28. Laskowski, M., and Seidel, M. K. *Arch. Biochem.* **7** (1945) 465.
29. Kunitz, M. *J. Gen. Physiol.* **33** (1950) 363.
30. Siebert, G., Lang, K., and Corbet, A. *Biochem. Z.* **320** (1950) 418.
31. Maver, M. E., and Greco, A. E. *J. Biol. Chem.* **181** (1949) 861.
32. Zamenhof, S., and Chargaff, E. *Science* **108** (1948) 628.
33. Zamenhof, S., and Chargaff, E. *J. Biol. Chem.* **180** (1949) 727.
34. Dabrowska, W., Cooper, E. J., and Laskowski, M. *J. Biol. Chem.* **177** (1949) 991.
35. Mc Carty, M. *J. Exptl. Med.* **90** (1949) 543.
36. Cooper, E. J., Trautman, M. L., and Laskowski, M. *Proc. Soc. Exptl. Biol. Med.* **73** (1950) 219.
37. Henstell, H. H., Freedman, R. I., and Ginsburg, B. *Cancer Research* **12** (1952) 346.
38. Henstell, H. H., and Freedman, R. I. *Cancer Research* **12** (1952) 341.
39. Greenstein, J. P., and Jenrette, W. V. *J. Natl. Cancer Inst.* **1** (1941) 845.
40. Greenstein, J. P. *J. Natl. Cancer Inst.* **2** (1941/42) 357.
41. Greenstein, J. P., and Stewart, H. L. *J. Natl. Cancer Inst.* **2** (1941/42) 631.
42. Greenstein, J. P. *J. Natl. Cancer Inst.* **4** (1943/44) 55.
43. Greenstein, J. P., and Thompson, J. W. *J. Natl. Cancer Inst.* **4** (1943/44) 271.
44. Laskowski, M. *Arch. Biochem.* **11** (1946) 41.
45. Brody, S., Thorell, B., and Åkerman, L. *To be published.*

Received January 29, 1953.