I. QUANTITATIVE DETERMINATION OF THE AMOUNT OF DNA PER NUCLEUS BY INTERFERENCE MICROSCOPY

CECILIA BIBBIANI, ROBERTO TONGIANI, and MARIA PIA VIOLA-MAGNI

From the Istituto di Patologia Generale, University of Pisa, Italy. The present address of the authors is Via Roma 55, 56100 Pisa, Italy

ABSTRACT

A method for the determination of the DNA content of isolated nuclei of different ploidy has been developed. It is based on measurement of the nuclear dry mass, with an integrating microinterferometer, before and after DNase treatment. The values found are slightly low, because, as indicated by biochemical determinations, consistently 5% to 8% of DNA is not extracted by DNase under these conditions. The average DNA values thus obtained for diploid and tetraploid nuclei of adult rat liver are 7.7 and 15.6 pg (10^{-12} g), respectively. Definite advantages of this procedure are: i) comparisons with biochemical determinations to give DNA values for each class of ploidy, ii) comparisons with histophotometry of the Feulgen dye-DNA complex to give absolute values instead of arbitrary units.

INTRODUCTION

Several attempts to measure the amount of DNA per nucleus by means of interference microscopy have been reported in the literature (1–4).

In some of these studies the nuclear dry mass was determined before and after trichloroacetic acid (TCA) hydrolysis (2, 3) or DNase digestion (4), and the difference between the two measurements was assumed to correspond to the DNA content per nucleus.

However, in all the studies reported so far, the possibility that the procedure used to extract DNA also could cause the loss of other materials from the nuclei has not been convincingly ruled out.

This paper is devoted to a careful study of the various steps of a procedure involving nuclear DNA digestion with DNase and microinterferometric determinations of the amount of the extracted material in order to rule out possible causes of error inherent both in the enzymatic hydrolysis of DNA and in the measuring apparatus.

MATERIALS AND METHODS

Adult Albino Italico rats, fed with Randoin and Causeret's diet (5), were used. About 100 mg of liver were homogenized in Barnes, Esnouf, and Stocken's solution (6) with a Philpot and Stanier's homogenizer (7) and centrifuged at 1,100 *g* for 5 min in order to isolate the cell nuclei. Stock No. DN-C and Stock No. DN-100 DNase (Sigma Chemical Co., St. Louis, Mo.) were used for DNA digestion.

DNase Digestion

A: The liver homogenate was washed with Barnes et al. solution in a centrifuge refrigerated at 0°C (1,100 *g*, 5 min.). The sediment containing the nuclear fraction was resuspended in the same medium (about 3 ml; pH 7.1–7.2) and divided into

three fractions: i) the first fraction was used to determine the dry mass of untreated nuclei; ii) the second fraction was diluted with an equal volume of Barnes et al. medium in which the DNase was dissolved (1 mg/ml). The nuclear suspension was incubated at 37°C, and stirred every 5 min. After half an hour, the nuclear suspension was quickly cooled to 0°C in order to stop the reaction and was centrifuged three times, and washed in Barnes et al. solution at 0°C (1,100 *g*, 5 min.).

After the third centrifugation, the sediment was resuspended and the dry mass of the digested nuclei was determined. The release of nucleotides was assayed by measuring the absorption of the three supernatants at $\lambda = 256$ mμ.

iii) The third fraction was diluted with an equal volume of Barnes et al. medium and incubated at 37°C for 30 min with DNase inactivated by treatment at 90°C for 10 min (pH 5) as described by Gillespie, Hapuer, and Hartzell (8). In this way the DNase activity was completely destroyed, whereas the RNase and other enzymes remained active. At the end of the incubation, the same procedure was followed as in ii), in order to evaluate the loss of material nonspecifically caused by the procedure. In some experiments cystein was added to the medium to a final concentration of 0.05 M in order to inhibit the possible proteolytic activity of DNase (9). This substance is without effect on the specific activity of DNase (10). Moreover, the proteolytic activity of DNase was tested by measuring the azo dye released from a suspension of Azocoll.[1]

B: In these experiments the same general procedure as in A was followed, except that the nuclei were suspended in a solution made up of 7 parts of anhydrous glycerol and 3 parts of Barnes et al. fluid (v/v). The DNase was dissolved in Barnes et al. solution and thereafter added to anhydrous glycerol. In these experiments the material was centrifuged three times at 9,500 *g* for 5 min, because of the high viscosity of the medium.

The nuclei, suspended either in aqueous or glycerol medium, appeared free of cytoplasmic remnants when observed in the interference microscope. The parenchymal nuclei were easily distinguished from the nuclei of other types of cells present in the liver (Kupffer cells, endothelia, etc.) on the basis of their shape, their chromatin distribution, and the presence and appearance of the nucleoli.

Microinterferometric Measurements

The suspension of nuclei was diluted in such a way that no more than one nucleus for a microscopic field could be observed (× 1000).

[1]Calbiochem AG, Lucerne, Switzerland.

The dry mass determinations were performed with a microinterferometric integrating apparatus (11) which employs a Smith's interference microscope "double focus-shearing system" (Vickers Ltd., Croydon, England).

In the apparatus, by means of a mica plate, the microscope field is divided into two halves in which the light is polarized in different planes forming an angle $\beta = 112°$ with each other. Two holes drilled in a metallic plate, placed above the microscope goniometer analyzer, delimit a region in each half of the microscopic field. One region is used as "measuring field" and the other one as a "reference field."

When an object is moved into the measuring field, the light intensity of the latter changes in proportion to the optical density of the object and to the ratio of object area to measuring field area. The optical path difference (OPD) measurement is performed by rematching the light intensities of the measuring field and the reference field. In this way the apparatus gives directly the object OPD integrated over a field of known area, according to the equation (11):

$$\cot x^\circ = \frac{(2/a - 1)\ \mathrm{cosec}\ r^\circ + \cot r^\circ - \cot \beta}{1 + \cot \beta \cot r^\circ - \cot \beta\ \mathrm{cosec}\ r^\circ} \qquad [1]$$

where: x° is the half of the integrated OPD of the object, expressed in degrees, times the ratio object area/measuring field area; a is the ratio object area/measuring field area; r° is the object OPD and β is the reference retardation (112° in the apparatus employed).

The nuclear dry mass (M) was calculated according to the equation:

$$M = \frac{2\ x^\circ \cdot \lambda}{360} \cdot \frac{A}{100\ \alpha} \qquad [2]$$

where: x° is the half of the nuclear integrated OPD in degrees; λ is the wavelength of the monochromatic light used (= 546 mμ); A is the area of the measuring field, and α is the specific refractive increment (0.0018 in aqueous medium [12], and 0.00103 in 70% glycerol[2]). If λ and A are measured in μ and in μ^2, respectively, M is expressed in pg (= 10^{-12}g).

[2]The specific refractive increment of a substance is defined by the Gladstone and Dale's formula (14):

$$\alpha = \frac{n_s - n_m}{100\rho} \qquad [3]$$

where n_s and n_m are the refractive indexes of the substance and of the medium, respectively, and ρ is the specific density of the substance. Since n_s and concentration for protein ($\rho = 1.27$) are 1.568 and 127 g/100 ml respectively (ref. 12; p. 79), and n_m of 70% glycerol was found to be 1.4335, the value of

α is calculated to be 0.00175 for DNA in water solution at $\lambda = 546$ mμ (12, 13). Since the DNA in aqueous nuclei is about 30% of the total dry mass, the value of α for the nuclei containing DNA would be equal to 0.001785 according to Gladstone and Dale's formula (14); however, since the DNA is contained in the nuclei as a nucleoprotein, it is not unlikely that its value is much closer to that of proteins. This is the case for lipids, the α value of which is 0.0015 when they occur as "droplets" and 0.00178 when they occur as lipoproteins (13). For this reason, it seems safe to use an α value of 0.0018 which may, at the most, give an error of 1% in defect in the determination of the dry mass. Accordingly, the α value of 0.00103 for nuclei in 70% glycerol has been used both before and after DNA digestion. It must be noted that the possible uncertainties about the exact value of α for a given biological object may give, at most, an error of 5% in the absolute measurement of mass (15), but do not severely affect the difference between the dry mass measurements on which the present method is based.

Biochemical Measurements

The amount of DNA per nucleus was evaluated with a biochemical technique. The nuclear suspension prepared as described above was treated according to the procedure of Schneider (16) and Steele, Okamura, and Busch (17), and the amount of DNA was evaluated by the diphenylamine reagent according to Burton's procedure (18).

The number of nuclei present in the nuclear fraction was determined by counting in a Bürker chamber. Then, the average DNA content per nucleus was calculated by dividing the total amount of DNA by the number of nuclei. Furthermore, the relative percentage of nuclei of different ploidy was determined on smears, prepared from the same fractions, fixed with ethanol and stained with the Feulgen method. In this way it was possible to calculate the average DNA content of the nuclei belonging to each class of ploidy.

DNase Activity Assay

In order to assay the DNase activity, a solution of highly polymerized calf thymus DNA (sodium salt, Sigma Type 1) in Tris buffer (pH 7.2) (200 μg/ml) was incubated at 37°C (30 min) with various concentrations of DNase ranging from 7.8 μg/ml to 500 μg/ml final conc.; an equal volume of cold perchloric acid (0.5 N) was added and the solution was centrifuged for 15 min at 8,500 *g*.

The sediment was hydrolyzed at 70°C and the DNA not digested by DNase was chemically determined.

The assay was repeated on the isolated nuclei fraction by using various concentrations of DNase and different periods of incubation.

RESULTS

Nuclei Suspended in Aqueous Medium

The dry mass of nuclei was determined by microinterferometry after the treatments described in i), ii), iii) (see Methods), and the values were expressed in 10^{-12} g. Column A of Table I gives the total insoluble dry masses of nuclei before the DNase treatment; Column B, the dry masses of nuclei after the enzymatic digestion at 37°C for 30 min; and Column D, the dry masses of nuclei after incubation with inactivated DNase.

The difference between the values of Columns A and B of Table I (dry mass of nuclei before and after treatment with DNase) are reported in Column C. This difference corresponds to the amount of material lost from the nuclei because of the action both of DNase and of other enzymes possibly contaminating the DNase, as well as of the treatment (incubation at 37°C).

The difference between the values of Columns A and D of Table I (dry mass before and after treatment at 37°C for 30 min with inactivated DNase) are reported in Column E. This difference corresponds to the amount of material lost as a consequence of the action of enzymes possibly contaminating the DNase (essentially RNase) and/or of the incubation at 37°C.

In this way it is possible to evaluate the amount of material lost from the nuclei independently of the DNase digestion: by subtracting this amount from the difference in weight of the nuclei examined before and after the DNase digestion, a third value is obtained which represents the amount of material lost from the nuclei as a consequence of the specific DNase action (Column F, Table I).

In order to exclude the action of proteases, the dry mass lost from the nuclei after DNase digestion was determined in a solution containing 0.05 M cystein which inhibits the action of proteolytic enzymes (9). No difference can be observed with

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α, under our experimental conditions, is equal to 0.00106.

For the nucleus in water an average value of α equal to 0.0018 instead of 0.00185 as for pure protein was adopted. Accordingly, the α value for the nucleus in 70% glycerol was assumed to be equal to 0.00103.

TABLE I

Amount of DNase-Sensitive Material per Nucleus, Determined by Microinterferometry and Expressed in 10^{-12} g ± S.E.M., in the Liver of Albino Italico Rats

In each experiment 20 diploid and 20 tetraploid nuclei were examined. Nuclei suspended in Barnes, Esnouf, and Stocken solution.

No. exp.		A*	B	C (A–B)	D	E (A–D)	F (C–E)
1	2n	23.46 ± 0.50	13.56 ± 0.31	9.90	21.30 ± 0.42	2.16	7.74
	4n	42.22 ± 0.71	21.84 ± 0.46	20.38	37.50 ± 0.61	4.72	15.66
2	2n	22.32 ± 0.44	12.48 ± 0.36	9.84	20.46 ± 0.45	1.86	7.98
	4n	41.28 ± 0.79	21.90 ± 0.28	19.38	37.20 ± 0.55	4.08	15.30
3	2n	23.16 ± 0.39	13.32 ± 0.50	9.84	20.88 ± 0.49	2.28	7.56
	4n	41.40 ± 0.62	21.60 ± 0.43	19.80	37.08 ± 0.58	4.32	15.48

*A, dry mass of nuclei before treatment with DNase; *B*, dry mass of nuclei after treatment with DNase; *C*, total amount of the nuclear dry mass lost by the treatment (37°C × 30 min) and DNase action; *D*, dry mass of nuclei after incubation with DNase inactivated at 90°C × 10 min; *E*, amount of the nuclear dry mass nonspecifically lost by treatment (37°C × 30 min); *F*, amount of the nuclear dry mass specifically lost by the DNase action.

TABLE II

Comparison Between the Amounts of Material Lost from Liver Cell Nuclei After DNase Treatment with and without Cystein

Nuclei suspended in Barnes, Esnouf and Stocken solution. The values are expressed in g x 10^{-12} ± S.E.M. and represent the average of 20 determinations.

0.05 M cystein	A	B	C (A–B)	D	E (A–D)	F (C–E)
2n without	24.36 ± 0.40	14.70 ± 0.33	9.66	22.36 ± 0.42	2.00	7.66
with	24.15 ± 0.34	14.17 ± 0.34	9.97	22.05 ± 0.33	2.10	7.87
	t = 0.3987	t = 1.059		t =0.578		
	0.7 < P <0.6	0.3 < P <0.2		0.6 < P <0.5		
4n without	46.30 ± 0.50	26.56 ± 0.46	19.74	42.31 ± 0.50	3.99	15.75
with	46.09 ± 0.55	26.35 ± 0.50	19.74	42.00 ± 0.53	4.09	15.65
	t = 0.2830	t = 1.002		t = 0.4323		
	0.8 < P <0.7	0.4 < P <0.3		0.7 < P <0.6		

For the meaning of A–F see Table I.

respect to the nuclei digested without cystein addition (Table II). Also, the Azocoll assay does not show a release of the dye after DNase incubation, whereas a clear-cut dye release is observed after digestion with pronase. Therefore, it can be concluded that the DNase used at the concentration of 500 μg/ml is practically free from proteolytic activity.

The values of the nuclear dry masses are distributed in well defined groups, which represent classes of different ploidy (3); a similar distribution, though obviously around smaller average values, is present also after DNase treatment (Fig. 1). By subtracting the mean value of the groups of nuclei weighed after the treatment with DNase from the value of the corresponding groups of nuclei weighed before the treatment, it is possible to determine the amount of DNase-sensitive material lost respectively from the diploid nuclei, from the tetraploid nuclei, and so on.

The values obtained in this way are distributed into two groups: in one group the value is double

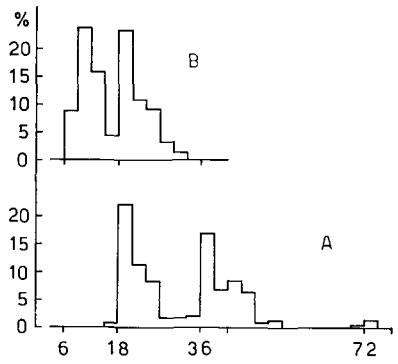

FIGURE 1 Histograms of dry mass of liver nuclei before (*A*) and after (*B*) digestion with DNase. 250 nuclei were measured. Abscissa: nuclear dry mass expressed in 10^{-12} g; Ordinate: percentage of nuclei.

that in the other group (Table I, Column F). It can be seen that the diploid nuclei lose 7.76 × 10^{-12} g and the tetraploid ones 15.48 × 10^{-12} g of DNase-sensitive material. The same results were obtained with addition of 0.05 M cystein to the nuclear suspension (Table II).

Nuclei Suspended in 70% Glycerol

The values of the total dry mass of nuclei suspended in 70% glycerol (Table III) are consistently higher than those of the nuclei suspended in aqueous medium, since in 70% glycerol the nuclei retain their content of soluble material (19, 20). These values are in good agreement with those obtained from nuclei freeze-dried and suspended in anhydrous glycerol, a medium in which the nuclei do not lose any material (21).

The amount of DNase-sensitive material determined in the nuclei suspended in 70% glycerol is equal to that determined in the nuclei suspended in aqueous medium (Tables I, III).

Biochemical Results

The amount of DNA determined in the homogenates is 2.68–2.90 μg/mg of tissue; after digestion with DNase it is 0.14–0.20 μg/mg of tissue, which corresponds to 5–8% of the total DNA content. The digestion of DNA by DNase was confirmed by measuring the absorption of the supernatant fractions at λ = 256 mμ. The absorption is high in the first supernatant fraction, small in the second, and practically absent in the third one. (See Methods). These results indicate that three washings were sufficient to free the nuclei from the digestion products. The values obtained for the nuclei after treatment at 37°C with inactivated DNase are the same as those obtained for the nonincubated nuclei.

The average value of DNA per nucleus determined biochemically on the basis of the counts of the nuclei present in the nuclear fraction ranges between 15 and 19 × 10^{-12} g.

Recently, with the same method, Iype et al. (22) obtained values of DNA content per liver nucleus ranging between 12.1 and 19.7 × 10^{-12} g. Other values reported in the literature are similar or slightly smaller (22).

In each experiment the percentage of nuclei belonging to each class of ploidy was determined on the basis of histophotometric measurements. According to the ploidy class distribution, the amount of DNA per diploid nucleus could be calculated. It corresponds to 7.8 – 8.2 × 10^{-12} g, in full agreement with the microinterferometric measurements (Table IV).

TABLE III

Amount of DNase-Sensitive Material per Nucleus, Determined by Microinterferometry and Expressed in 10^{-12} g ± S.E.M., in the Liver of Albino Italico Rats

In each experiment 20 diploid and 20 tetraploid nuclei were examined. Nuclei suspended in 70% glycerol.

No. exp.	A	B	C (A–B)	D	E (A–D)	F (C–E)
1 2n	36.76 ± 0.36	26.98 ± 0.35	9.78	34.51 ± 0.33	2.25	7.53
4n	72.02 ± 0.61	51.60 ± 0.63	20.42	67.72 ± 0.55	4.30	16.12
2 2n	36.83 ± 0.41	27.22 ± 0.31	9.61	34.88 ± 0.39	1.95	7.66
4n	71.06 ± 0.55	52.92 ± 0.66	18.14	68.58 ± 0.56	2.48	15.66

For the meaning of A–F see Table I.

TABLE IV

AMOUNT OF DNA PER NUCLEUS DETERMINED BY BIOCHEMICAL METHOD

The diploid value was calculated according to the nuclear percentage of each ploidy class.

No. exp.	Histophotometric measurements				Average DNA per nucleus	DNA per diploid nucleus
	% 2n	% 4n	% 8n	% 16n	g × 10^{-12}	g × 10^{-12}
1	40	50	10	—	15	8.3
2	22	52	26	—	18	7.8
3	20	52	26	2	19	8.1

DNase Assay

The activity of DNase was assayed with respect to its concentration (Figs. 2 *a*, *b*) and to the time of incubation (Fig. 3). A solution of DNase four times less concentrated than that used in our experiments (500 μg/ml) is more than sufficient to digest almost completely a solution of DNA (200 μg/ml) (Fig. 2 *a*). The same result is obtained on the liver homogenates (Fig. 2 *b*).

The minimum time required by a solution of DNase (500 μg/ml) to digest the DNA present in the liver homogenates is 15 min (Fig. 3). After this time, only a small amount (5–8%) of DNA (DNase resistant) remains.

DISCUSSION

Reliability of the Measurements

The amount of DNA per nucleus has been determined by means of the microinterferometric apparatus devised by Tongiani (11). The validity of the results obtained is dependent on i) the accuracy of the OPD measurements provided by the apparatus, and ii) the exactness of the α values used in equation [2].

As far as the accuracy of OPD measurements is concerned, it has been shown on theoretical and experimental grounds (11) that the range of error of the apparatus, when $\beta = 112°$ (see eq. [1]), is between 0 and ±5% over a range of central OPD 0° – 115° in spherical objects, as the ratio measuring field area/object area changes from 1 to 10 (11).

In our experimental conditions, the range of central OPD of the nuclei was 80°–100°, and the ratio measuring field/nuclei areas lay between 6 and 10. It follows that the range of error affecting the total OPD measurements was about ± 2% (ref. 11; see Table I), a percentage of error equal to or smaller than that claimed for other apparatus (23–27).

Concerning the α values, the error due to the use of the same value of α in equation [2] for nuclei with and without DNA is no more than 1%, as discussed in Methods.

It should be noted that these two errors may

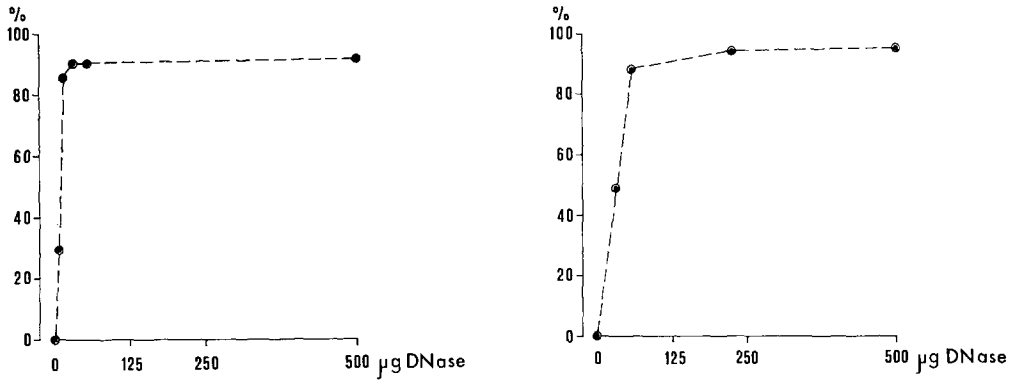

FIGURE 2 *a:* Assay of DNase activity on a solution of DNA Sigma Type 1 (200 μg/ml); Abscissa: different DNase concentrations/ml. Ordinate: digested DNA as per cent of the initial amount. *b:* Assay of DNase activity on liver nuclei homogenates (average DNA concentration 150 μg/ml). Abscissa: different DNase concentrations/ml. Ordinate: digested DNA as per cent of the initial amount. Incubation time 30 min at 37°C in Barnes et al. medium. DNA determination by the diphenylamine reaction.

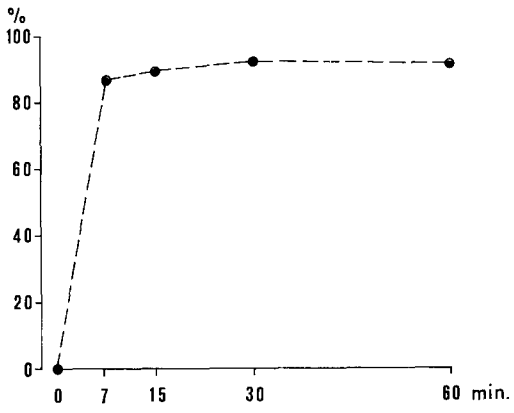

FIGURE 3 Assay of DNase activity (500 μg/ml) on liver nuclei homogenates (average DNA concentratoin 150 μg/ml) as a function of different times of incubation. Abscissa: times of incubation at 37°C in Barnes et al. medium. Ordinate: digested DNA as per cent of the initial amount. DNA determination by the diphenylamine reaction.

not necessarily add to each other in any given experiment. It can be concluded that the maximum error which can affect the dry mass difference determinations may be at most of the order of ± 2.5%.

Meaning of the Loss

In order to establish that the DNA content per nucleus corresponds to the difference between the dry mass of the nuclei determined before and that determined after incubation with DNase, it is necessary to verify the following conditions: i) the material lost from the nucleus is only DNA; ii) the DNase digests the whole DNA content of the nucleus.

i) The comparison between the dry mass of nuclei determined before and that determined after incubation with inactivated DNase (8) showed a loss of 2.1 pg per diploid nucleus and of 4.0 pg per tetraploid nucleus. This loss (not DNase dependent) may be due to the action of contaminating enzymes (RNase, etc.) and/or of the treatment (incubation at 37°C).

Since there is no significant difference between the nuclei incubated with and without cystein (Table II), any loss attributable to activity of proteases (either contaminating the DNase or present in the homogenized tissue) can be ruled out. Moreover, the possibility that the DNase used in the present experiments possesses some proteolytic activity can be ruled out by the results obtained with the Azocoll assay.

Appropriate controls (Ponenti and Viola-Magni, unpublished results) performed with a microbiological method show that no appreciable loss of histones from the nuclei occurs after incubation at 37°C for 30 min with DNase.

It must be concluded, therefore, that the only nonspecific loss was the one observed after incubation with inactivated DNase (8); thus, this nonspecific loss must be subtracted from the total loss observed after incubation with DNase, in order to determine the amount of nuclear material released by the specific action of the DNase.

ii) Zamenhof and Chargaff (28) have shown a "core" of DNA not sensitive to DNase that is equivalent to 7% of the total amount of DNA. Our biochemical determinations have shown that 5 – 8% of the initial amount of nuclear material stainable by the diphenylamine reagent (18) remained in the nuclei after digestion with DNase. The same percentage of DNase-insensitive DNA was found when a solution of the DNA Sigma type I was used as a substrate.

As a consequence, the values obtained by the difference between the nuclear dry mass calculated before and that calculated after digestion with DNase are approximate and the actual amount of DNA per nucleus is 5–8% greater than that found.

General Considerations

i) This procedure is applicable to every population of isolated nuclei. It seems especially useful for the study of: a) populations of cell nuclei having a different ploidy (as liver cell nuclei here examined), b) populations of cell nuclei of different types present in the same tissue (for example, glial and neuronal cell nuclei may be easily distinguished in nervous tissue [29]).

The values of dry mass of liver cell nuclei are well grouped in the histograms (Fig. 1) around a mean value according to the Gaussian distribution curve. These groups, the mean values of which are arranged in geometrical progression, represent classes of ploidy (3). With the procedure here described, the amount of DNase-sensitive DNA per liver cell nucleus has been calculated by the difference between the average values of dry mass of nuclei with the same ploidy, before

and after digestion with DNase. Therefore, the values reported in this paper are in fact the average amount of DNA per nucleus of a given ploidy.

If nuclei of unknown ploidy are present in the tissue examined, as for example in tumors, it is necessary to measure the amount of DNA for each single nucleus. In this case, the whole treatment with DNase must be done without removing the nuclei from the microscope field or done in such a way that the same nuclei can be found again.

iii) This method represents a definite advantage with respect to the biochemical procedure, which, generally, gives an average value of DNA per nucleus in a heterogeneous population of nuclei deriving from various types of cells, both parenchymatous and stromal.

CONCLUSION

It can be concluded that the procedure here described presents the following advantages with respect to other methods for the evaluation of the DNA content per nucleus: i) the values obtained are expressed in absolute units (10^{-12} g); ii) nuclei with different ploidy may be studied separately; (iii) various types of nuclei isolated from a tissue may be distinguished and analyzed independently of one another.

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