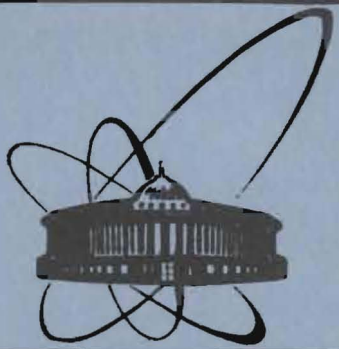

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THREE METHODS TO DETERMINE THE YIELDS OF DNA DOUBLE-STRAND BREAKS

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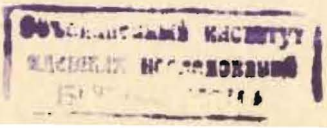
Today correlation between the yield of DNA double strand breaks (DSB) and cell viability after exposure to ionising radiations[1-4] is universally acknowledged and puts forward the task to develop adequate methods for determination of DNA DSB occurrence frequency. Now there is a traditional method of sedimentation of DNA after lysis of irradiated cells in the neutral sucrose density gradient[5]. This method is based on the ground which limits its application to some extent. For example, freely sedimenting DNA appears only as a result of bad damages by very high doses of irradiation (over 200 Gy), and statistically reliable sedimentation profiles for data processing and determination of the number of DNA DSB can be obtained only at a low speed of ultracentrifuging (up to 10000 rpm), which takes 17-24 hours depending on the irradiation dose. More difficulties arise when one determines the yield of DSB after exposure to a radiation with high LET, since many small fragments of DNA are there. All this, in our opinion, shows convincingly that more reliable and less labor-consuming methods should be found for determination of the DNA DSB yield.

This paper deals with two methods which we first suggested for the above purpose.

The first method is sedimentation of DNA fragments released under ionising irradiation due to DSB[6]. This method was used earlier for investigations of chromatin supercoil subunits (method 1). In paper[6] the molecular weight (M_0) and volume of supercoiled subunit have been determined. On introducing these parameters as known values, it becomes possible to determined the yield of DNA DSB by comparison of the theoretical and experimental curves which show dependence of the released amount of DNA on the radiation dose F(D).

The second method is sedimentation of DNA-membrane complexes (DMC) in the neutral sucrose density gradient with ethydium bromide (method 2). Up to now the method has been applied for investigations of DNA highest organization in chromatin[7,8,9] and - in some cases[10] - for determination of the yield of DNA single-strand breaks (SSB). In Ref.[10] sedimentation behaviour of DMC at high irradiation doses is described for the first time and possibility to use this method for determination of the DNA DSB yield is discussed.

Below are given the first results of determination of the DNA DSB yield for cells affected by ionising radiations with different physical characteristics, obtained by the two methods. The results are compared with the data obtained by the traditional method of sedimentation (method 3).



MATERIALS AND METHODS

The technique of cultivating, tagging by ^{3}H-thymidine, and treating the Chinese hamster cells V79-4 as well as conditions of their irradiation by ^{137}Cs γ-rays, heavy ions (at the accelerator U-200, JINR, Dubna) and neutrons (in the biophysical channel of the IBR-2 reactor, JINR, Dubna) is described in detail in papers [11,12]. Here in Table 1 we shall list only compositions of lysis mixtures, sucrose solutions, and centrifuging conditions for samples in the three discussed methods. Examples of sedimentation profiles obtained by methods 1 and 3 are given in Ref. [11].

Table 1

	Method 1	Method 2	Method 3
Sucrose solution	5-20% of sucrose 2 M NaCl 0.01 M Na-citrate 0.2 ml of 60%-solution of sucrose with saturated CsCl are put on the bottom of the test tube pH 9-9.5	5-20% of sucrose 1 M NaCl 0.01 M Tris-HCl 0.01 M EDTA 6 or 30 μg/ml of ethydium bromide pH 7 - 7.5	5-20% of sucrose 1 M NaCl 0.01 M Tris-HCl 0.01 M EDTA pH 7 - 7.5
Lysis solution	0.2% sarcosyle 0.08% Na-deoxycholate, 2 M NaCl, 0.01 M Na-citrate 0.02 M EDTA pH 9 - 9.5	1% SDS 0.2% Na-deoxycholate, 0.05 M Na-citrate 0.05 M EDTA pH 7 - 7.5	1% SDS 0.2% Na-deoxycholate, 0.05 M Na-citrate 0.05 M EDTA pH 7 - 7.5
Speed of ultracentrifuging	20000 rpm	30000 rpm	9000 rpm
Time for ultracentrifuging	3 hours	0.5-1 hours	17-24 hours

RESULTS

Method 1. After lysis of irradiated cells by means of sarcosyle it is possible to separate slowly sedimenting released fragments and rapidly sedementing DNA in DMC, since a thick stratum of 60% solution of sucrose with CsCl prevents them from falling down to the bottom of the test tube. One can judge about the amount of each part of DNA by the ratio of total radioactivities in both peaks. It is well seen from sedimentation profiles [11] that a larger amount of DNA released from DMC corresponds to a higher irradiation dose. Values of F(D) obtained in this way for γ-radiation are shown in Fig.1. A solid line is the calculated curve in very good agreement with experimental data ($M_0 s = 1.08 \cdot 10^{-2}$ Gy^{-1}, where M_0 is the molecular weight of a supercoiled subunit, s is the yield of DNA DSB). On introducing $M_0 = 3.98 \cdot 10^{-15}$g determined for the same cells by sedimentation of supercoiled DMC in the sucrose density gradient with ethydium bromide, one gets the value of the DNA DSB yield equal to $2.7 \cdot 10^{12}$ Gy^{-1} g^{-1}.

Figures 2a and 2b show experimental data for ^{12}C ions with LET 250 keV/μm. Solid lines are theoretically calculated functions F(D) for three different DSB frequencies: $4.8 \cdot 10^{12}$, $6.6 \cdot 10^{12}$ and $8.9 \cdot 10^{12}$ Gy^{-1} g^{-1}; in each case a lower curve corresponds to the subunit volume 0.2 μm in diameter and an upper curve is for the subunit volume 0.3 μm in diameter, their values determined in [6]. Comparison of experimental and theoretical data reveals that the best agreement for low irradiation doses is observed at $s = 6.6 \cdot 10^{12}$ Gy^{-1} g^{-1}, and at $s = 4.8 \cdot 10^{12}$ Gy^{-1} g^{-1} for high irradiation doses.

Method 2. In Fig.3 the DMC relative sedimentation velocity (RSV) is plotted versus the γ-irradiation dose. In our paper [10] assumptions were made as to reasons for this behaviour of all parts of the curve. It was mentioned, among other things, that higher RSV in the γ-irradiation dose range 50-550 Gy and lower RSV at doses over 550 Gy are due to a larger number of DNA DSB and, correspondingly, a larger amount of free DNA. Those assumptions led us to the conclusion that RSV of the DNA-containing agregate could be calculated in the first approximation as follows:

$$rS = A \cdot rS_{desp.} + B \cdot rS_{d.s.} + F \cdot rS_{fragm.},$$

where A is amount of subunits with RSV $rS_{desp.}$ and despiralised because of SSB, but without DSB of DNA; B is amount of destroyed subunits which lost fragments of DNA because of DSB, their RSV is $rS_{d.s.}$; F is amount of free DNA with sedimentation velocity $rS_{fragm.}$. On the basis of obvious assumptions that

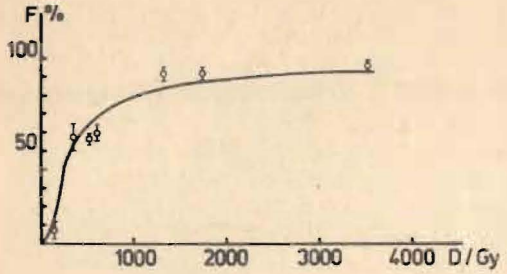


Fig.1. Amount of released DNA vs γ-irradiation doses. Abscissae: doses in Gy. Ordinates: amount of released DNA in per cent. The curve is calculated by Eq.(2) in [6], O - experimental data.

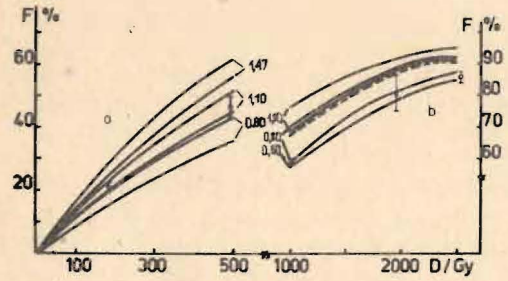


Fig.2. Amount of released DNA vs the dose of irradiation by ^{12}C ions. Abscissae: doses in Gy. Ordinates: amount of released DNA in per cent. Solid curves are calculated by Eq.(2) from [6] for s = $4.8 \cdot 10^{12}$, $6.7 \cdot 10^{12}$, $8.9 \cdot 10^{12}$ Gy^{-1} g^{-1} at two volume diameters - 0.2 and 0.3 μm - for each s; - experimental data; a - low dose range (irradiation under normal conditions), b - high dose range (irradiation under enoxic conditions).

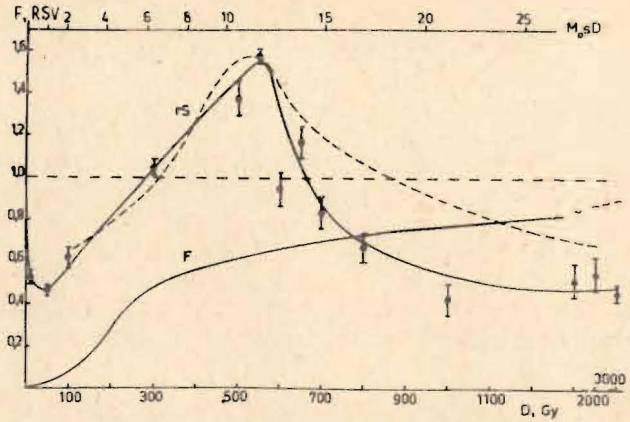


Fig.3. Relative sedimentation velocity of DMC and amount of free DNA vs γ-irradiation doses. Abscissae: doses in Gy. Ordinates: DMC RSV and amount of free DNA in relative units. The upper part of abscissae shows an average of DNA DSB per a subunits.

at 550 Gy A = 0 (all subunits have DSB) and the mean value of $rS_{fragm.}$ is 0.3 we obtain $rS_{d.s.}$ = 3.8. Results of these calculations are shown in Fig.3 by a dotted curve with the maximum at the 550 Gy dose. In fact, $rS_{fragm.}$ (and consequently $rS_{d.s.}$)) depends on the irradiation dose, but in general our assumptions on the role of DNA DSB in increase of the RSV curve in the 50-550 Gy dose range are obviously true.

Method 3. Experimental results on determination of DNA DSB by a traditional sedimentation method are usually presented as dependence of $1/M_n$ (M_n is an average molecular weight of DNA fragments) on the irradiation dose. The incline of these straight lines allows one to determine the following values of the DNA DSB yield: under normal conditions for ^{137}Cs - $3.2 \cdot 10^{12} Gy^{-1} g^{-1}$, ^{12}C - $7.8 \cdot 10^{12} Gy^{-1} g^{-1}$, ^{16}O - $6.7 \cdot 10^{12} Gy^{-1} g^{-1}$; under anoxic conditions (a region of high doses) for ^{12}C - $3.2 \cdot 10^{12} Gy^{-1} g^{-1}$, ^{16}O - $2.7 \cdot 10^{12} Gy^{-1} g^{-1}$.

For better obviousness the DNA DSB yields obtained by the three methods are summed up in Table 2.

Table 2

DNA DSB yields (in $Gy^{-1} g^{-1}$) obtained ny three methods

Type of radiation	Method 1	Method 2	Method 3
^{137}Cs - γ	$2.7 \cdot 10^{12}$	$1.5 \cdot 10^{12}$	$3.2 \cdot 10^{12}$
^{12}C, 250 keV/μm (normal conditions)	$6.7 \cdot 10^{12}$		$6.7 \cdot 10^{12}$
^{12}C, 250 keV/μm (anoxic conditions)	$3.6 \cdot 10^{12}$		$3.2 \cdot 10^{12}$
^{16}O, 510 keV/μm (normal conditions)			$6.7 \cdot 10^{12}$
^{16}O, 510 keV/μm (anoxic conditions)			$2.4 \cdot 10^{12}$

DISCUSSION

A traditional method of determination of the DNA DSB yield, being good in many aspects, has a number of drawbacks. It can be applied only at high irradiation doses, and requires radioactive indicator fagging, long centrifuging measuring of radioactivity level of gradient fractions, data processing in the computer. Method 1, though includes some stages similar to method 3 (tagging, gradient fractionation, radioactivity counting), allows however a high speed of ultracentrifuging and

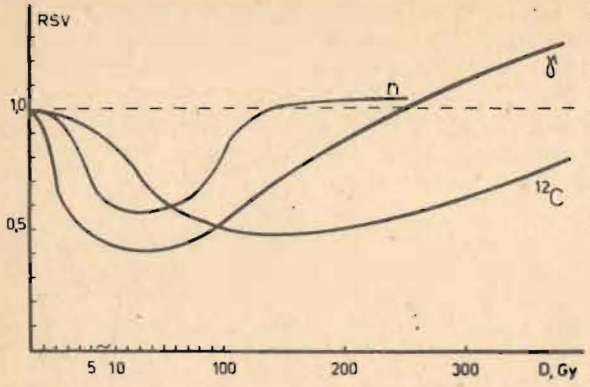


Fig.4. Relative sedimentation velocity of DMC vs dose of γ-radiation, neutrons and ^{12}C ions. Abscissae: doses in Gy. Ordinates: DMC RSV in relative units.

does not require a complicated data processing, which permits one to obtain results much easier and quicker. However, method 2 is, in our opinion, the most promising. It can be used in the 50–550 Gy dose range, allows a high speed of ultracenrifuging (30000 rpm) at minimum time. Measurements of the aggregate sedimentation distance is a very simple procedure which does not require a costly equipment. In Fig.4 the first results obtained by method 2 are presented as curves of DMC RSV dependences on doses of radiations with different physical characteristics. Calculations of DNA DSB yields on the basis of these curves will be published.

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Эрцгребер Г., Лапидус И.Л. E19-85-202

Три метода определения выхода двунитевых разрывов ДНК

Обсуждается возможность определения выхода двунитевых разрывов ДНК клеток китайского хомячка V79-4 методами нахождения доли ДНК, высвобождаемой в результате образования разрывов, и определения относительной скорости седиментации ДНК-мембранных комплексов при воздействии ионизирующих излучений с разными физическими характеристиками. Результаты анализа сравниваются с данными, полученными традиционным методом седиментации в нейтральном градиенте плотности сахарозы. Обсуждается сравнительная характеристика методов. Выходы двунитевых разрывов ДНК, определенные указанными независимыми методами, хорошо согласуются, что открывает возможность изучения индукции и репарации двунитевых разрывов с помощью более простых и надежных методов.

Работа выполнена в Лаборатории ядерных проблем и Лаборатории нейтронной физики ОИЯИ.

Препринт Объединенного института ядерных исследований. Дубна 1985

Lapidus I.L., Erzgräber G. E19-85-202

Three Methods to Determine the Yield of DNA Double-Strand Breaks

A possibility of determining the yield of DNA double-strand breaks in cells of the Chinese hamster (V79-4) by finding the amount of DNA released as a result of breaks and by determining the relative sedimentation velocity of DNA-membrane complexes affected by ionising radiations with different physical characteristics is discussed. Results of the analysis are compared with the data obtained by a traditional method of sedimentation in the neutral sucrose density gradient. Comparative characterisation of the methods is discussed. The yields of DNA double-strand breaks determined by the suggested independent methods are in good agreement, which opens possibilities of studying induction and repair of double-strand breaks by means of simpler and more reliable methods.

The investigation has been performed at the Laboratory of Nuclear Problems and the Laboratory of Neutron Physics, JINR.

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