Zeitschrift: Archives des sciences [1948-1980]
Herausgeber: Société de Physique et d'Histoire Naturelle de Genève
Band: 30 (1977)
Heft: 2

Artikel: Spontaneous release of newly synthesized DNA from frog auricles
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DOI: https://doi.org/10.5169/seals-739823

SPONTANEOUS RELEASE OF NEWLY SYNTHESIZED DNA FROM FROG AURICLES

BY

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We are grateful to Professor G. Bernardi and Professor A. Bernardi (Biologie Moléculaire, Université de Paris 7) for the interest they took in this work and for having allowed us to learn in their laboratory some of the techniques necessary to achieve this work.

This work was supported by La Ligue Suisse contre le Cancer and the O. J. Isvet Fund.

RÉSUMÉ

Les oreillettes de grenouille relâchent du DNA *in vitro*. Une fois isolé du complexe libéré dans le milieu de culture, on obtient un DNA purifié comme le montre sa courbe d'absorption en UV, sa réaction aux colorants spécifiques du deoxyribose et sa sensibilité à la DNase.

Le DNA libéré dans le surnageant ne semble pas dû à des cellules mourantes ou mortes mais à un mécanisme homéostatique. En effet: (a) La même quantité de DNA est retrouvée dans le milieu après un temps d'incubation court ou long. (b) Quand les oreillettes sont transférées dans un nouveau milieu plusieurs fois de suite, on retrouve une quantité similaire de DNA extracellulaire dans chacun des surnageants successifs. En revanche, si les oreillettes sont sorties et remises dans le même milieu on n'observe aucune augmentation dans la quantité de DNA extracellulaire. Ceci suggère un mécanisme de régulation actif indépendant de tout effet mécanique. Le complexe contenant du DNA présent dans le milieu à la suite de l'incubation d'une série d'oreillettes empêche la libération de DNA par une nouvelle série d'oreillettes alors que le DNA purifié de grenouille dans le milieu n'inhibe pas le processus de sécrétion.

Le fait que l'activité spécifique du DNA excrété est nettement plus forte que celle du DNA cellulaire après une période de marquage de longue durée ne s'explique que par le relâchage préférentiel du DNA nouvellement synthétisé dans la cellule.

Les courbes de renaturation indiquent que le DNA excrété est très complexe et consiste surtout de séquences uniques.

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SUMMARY

Frog auricles released DNA *in vitro*. Once purified from the complex appearing in the supernatant, this DNA exhibited typical characteristics as shown by its UV absorption curve, its deoxyribose coloration, and its sensitivity to DNase.

The DNA appearing in the supernatant does not seem to be due to dead or dying cells but is due to an homeostatic mechanism, since: (a) The same amount of DNA was found in the medium whether the incubation was long or short; (b) When the auricles were transferred to a new medium several times in a row, a similar amount of extracellular DNA was isolated from each of the successive supernatants, whereas if the auricles were removed and put back in their original medium no increase in the amount of extracellular DNA was observed, suggesting an active regulatory mechanism independent of a mechanical effect. Purified frog DNA does not inhibit the release, but the presence of the DNA-complex resulting from a previous incubation of auricles in the medium decreases dramatically the amount of released DNA.

The higher specific activity of the released DNA, compared with that of the cellular DNA after a long labeling period, can be explained by a preferential release of the newly synthesized DNA.

The renaturation curves indicate that the DNA excreted is highly complex and is composed mainly of unique sequences.

INTRODUCTION

Frog *(Rana esculenta)* auricles *in vitro* were shown to release a complex containing DNA (1, 21, 22). This was not released from dead or dying cells since *a)* after 24 hr of culture, all cells were alive as shown by autoradiographs of auricles pulsed with ^{3}H-uridine at the end of incubation; *b)* the same amount of DNA was found in the medium whether the incubation lasted 2, 4 or 24 hours, instead of a predicted increasing amount of DNA with time which would be expected if cells were dying; *c)* the constant renewal of extracellular DNA observed with each change of medium suggested an active, homeostatic control mechanism.

Molecular weight estimation obtained by chromatography on DEAE cellulose (8) showed that the released DNA was composed mainly of fractions from 1×10^5 to $1{,}5 \times 10^6$ Daltons. The released DNA annealed specifically to unlabeled cellular DNA trapped on filters (10), apparently as well as newly synthesized cellular DNA.

However, at that time the extracellular DNA could not be totally purified since although sensitive to DNase it was not destroyed as thoroughly as the cellular DNA and while its sugars could be coloured to some degree by indole, some unpurified components prevented reaction to diphenylamine (22).

We have now been able by a new method of extraction to purify more thoroughly the released DNA and bring new evidence for the homeostatic system which governs DNA release and which is independent of cell death. Furthermore we show that there is a preferential release of unique sequences of the newly synthesized cellular DNA.

MATERIALS AND METHODS

Sterilely extracted frog auricles were kept at 20° C in aerated Ringer solution containing 200 μg/ml of penicillin and 200 μg/ml of colimycin. Two hours of incubation were sufficient to remove all blood cells. Two hundred auricles per series were further incubated for varying time periods in 80 ml of fresh Ringer (in presence of antibiotics). In some experiments the medium was regularly renewed. This medium was either fresh, enriched or not with purified frog DNA, or was the supernatant in which frog auricles had previously been in culture. After incubation, the auricles were removed and the supernatant submitted to centrifugations, first at 12,000 rpm for 20 minutes and then at 50,000 rpm for up to 12 hr to remove any cellular debris, the DNA-complex remaining in the supernatant. As a control to sterility, aliquots were tested before and after the experiment. Controls without antibiotics were performed to be sure that the results were not altered by these compounds. DNA was prepared according to the method of Marmur (12) until the first ethanol precipitation and then loaded on hydroxyapatite columns (5). Eluted double-stranded DNA was pelleted by ultracentrifugation for 16 hrs at 45,000 rpm.

The following studies were performed on the DNA extracted from the cells and from the supernatants. (I) Their amount was determined by UV absorption and by deoxyribose colorations (7, 9). The absence of protein contamination was controlled (11). (II) The DNA was subjected to treatments with DNase I, DNase II, pancreatic RNase and pronase. (III) The molecular weight was estimated by zonal centrifugation on linear gradients of 5 to 20% sucrose or, in order to compare the specific activities of the fractions of different molecular weights, by diethylaminoethyl-cellulose paper pulp chromatography with discontinuous elution (8). (IV) The DNA was also characterized by renaturation curves and by DNA-DNA hybridization. C_0t curves were performed according to the method described by Britten and Kohne (6).

Labeled released DNA was obtained by adding methyl-^{3}H-thymidine (26 Ci/mmole, 10 μCi/ml) to the frog auricle cultures for various periods of time before transfering the auricles to a cold Ringer solution where the released ^{3}H-DNA was recovered.

Cell viability of the frog auricles was controlled by determining the pulsation rate at the beginning and at the end of the experiments. Control auricles were also labeled with ^{3}H-uridine during the last hour of the different incubation periods and labeled cells counted on autoradiographs showing 100% of labeled cells. Moreover, the pellets of the different supernatants were examined after the 12,000 rpm and after the 50,000 rpm centrifugations for the presence of cells or cell fragments. As can be expected from a syncitium whole or lysed cells are not shed in the medium.

Indeed microscopic examination of the pellets reveals no complete cell and very few fragments which, even if each fragment were counted as a cell, could not represent more than 1×10^{-6} of the total population.

RESULTS

The material released from the auricles can be considered as DNA since: (I) it has a typical UV absorption maximum at 258 nm and a minimum at 230 nm; (II) its amount, determined by UV absorption, is fully confirmed by the deoxyribose reactions; (III) while more than 95% of it is digested by both DNases tested, it is insensitive to RNase and pronase.

The molecular weight of the released DNA is not as homogeneous as that of the cellular DNA (Fig. 1).

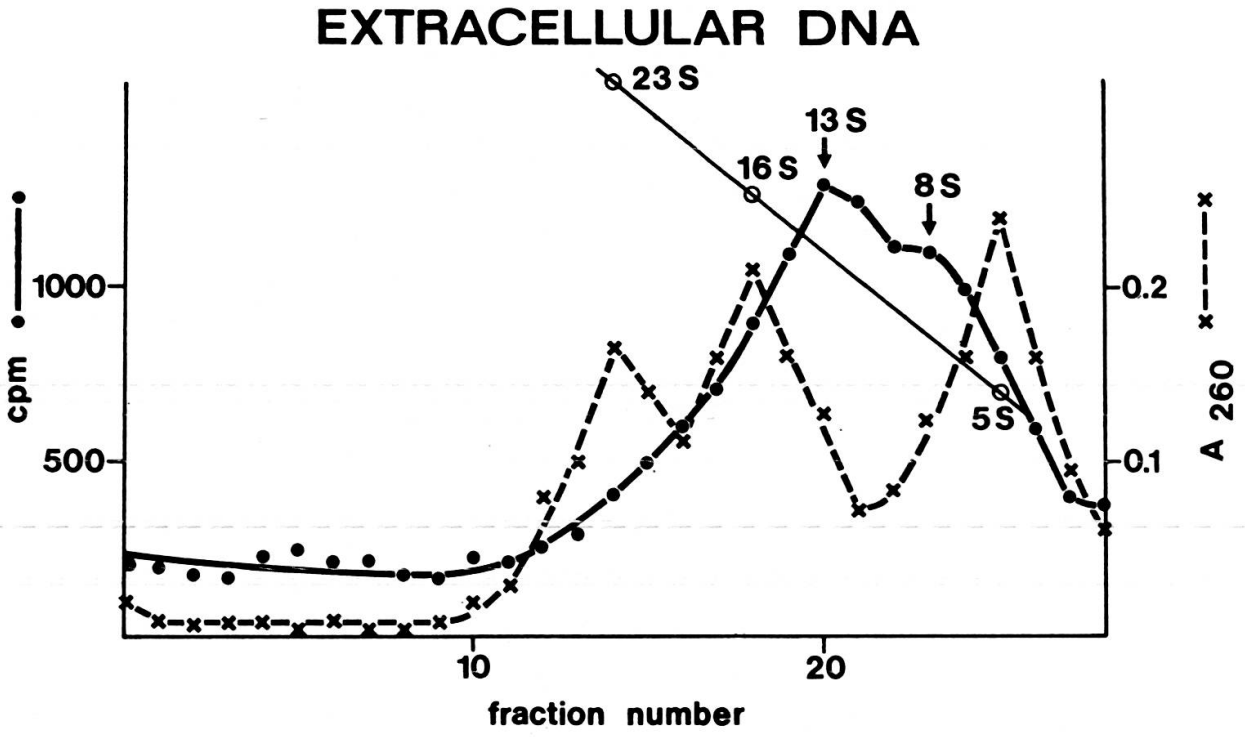

FIG. 1. — *Sucrose gradient centrifugations of cellular and extracellular frog auricle ^3H-DNA.* Extracellular and cellular DNA were obtained by incubating auricles with 20 μCi/ml of ^{3}H-thymidine (40 Ci/mmol) for 6 hours. The labeled auricles were then transfered to a new medium for 4 hours. 5 μg of ^{3}H-DNA purified from frog auricles or from their culture medium were mixed with 40 μg of 23 S, 16 S and 5 S unlabeled reference RNA. The samples were layered on 5 ml linear gradients of 5-20% sucrose in 0,015 M NaCl and 0,0015 M lodium citrate and centrifuged at 45,000 rpm at 20° C for 2 hr 30 min.

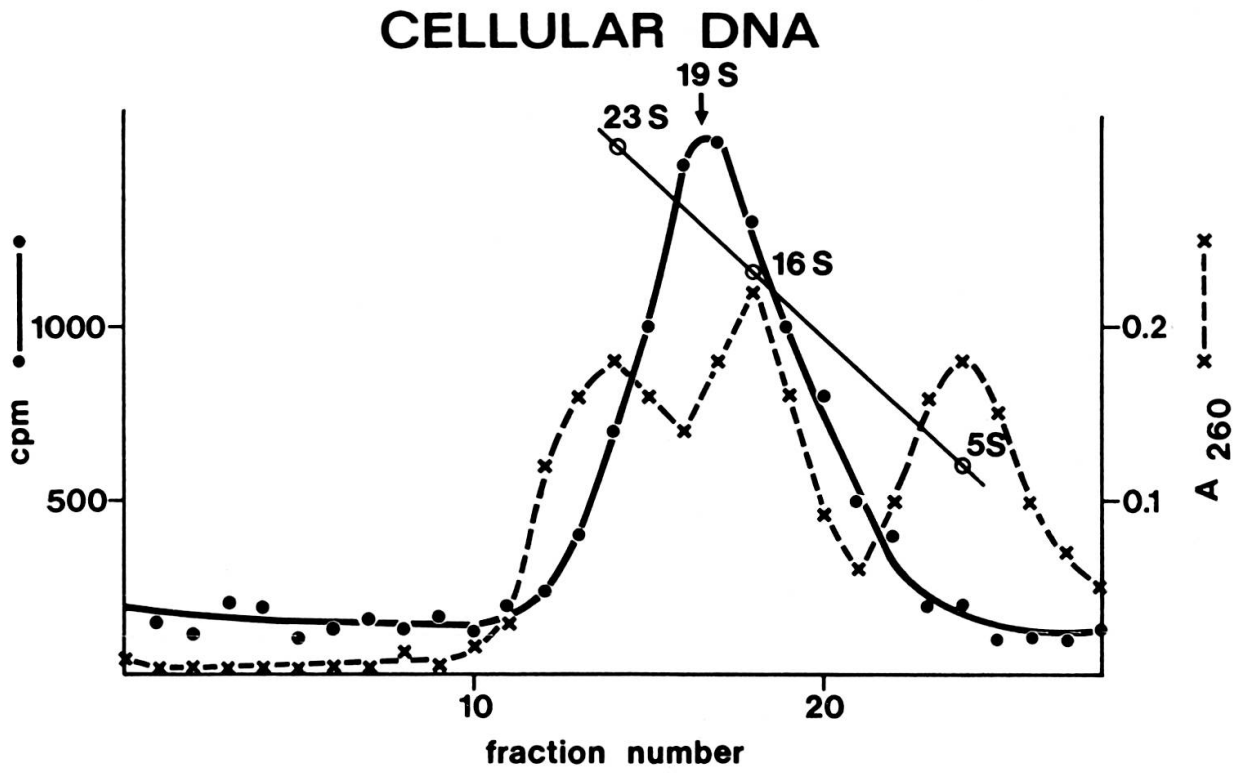

Table I (a and b) shows that between 1 and 2% of the bulk cellular DNA is recovered in the supernatant whether the incubation lasted 24 hours or 4 hours. Similar quantities of released DNA are found at each change of fresh medium (Table I b) whereas when the auricles are taken out of their medium and put back in the same medium there is not significant increase of extracellular DNA (Table I c).

TABLE I

Amount of DNA extracted from frog auricles and from their culture medium after various periods of incubation

The auricles were cultured either continuously or in successive periods with medium renewal.

Time of incubation	Amount of DNA (μg)			% of released DNA		
	cellular	extracellular				
a) 24 h.	1,360	27		1.9		
b) 4 h. (1st incubation)		26		1.7		
4 h. (2nd incubation)		23.5	74.4	1.5	4.8	
4 h. (3rd incubation)	1,491	24.9		1.6		8.4
4 h. (4th incubation)		17.8	128	1.2		
4 h. (5th incubation)		16.5	53.6	1.1	3.6	
4 h. (6th incubation)		19.3		1.3		
c) 6 successive 2 h. incubations (auricles resuspended each time in the same medium)	1,482	30		2		

As can be seen in Figure 2 the change of medium is favourable to the survival of the auricles.

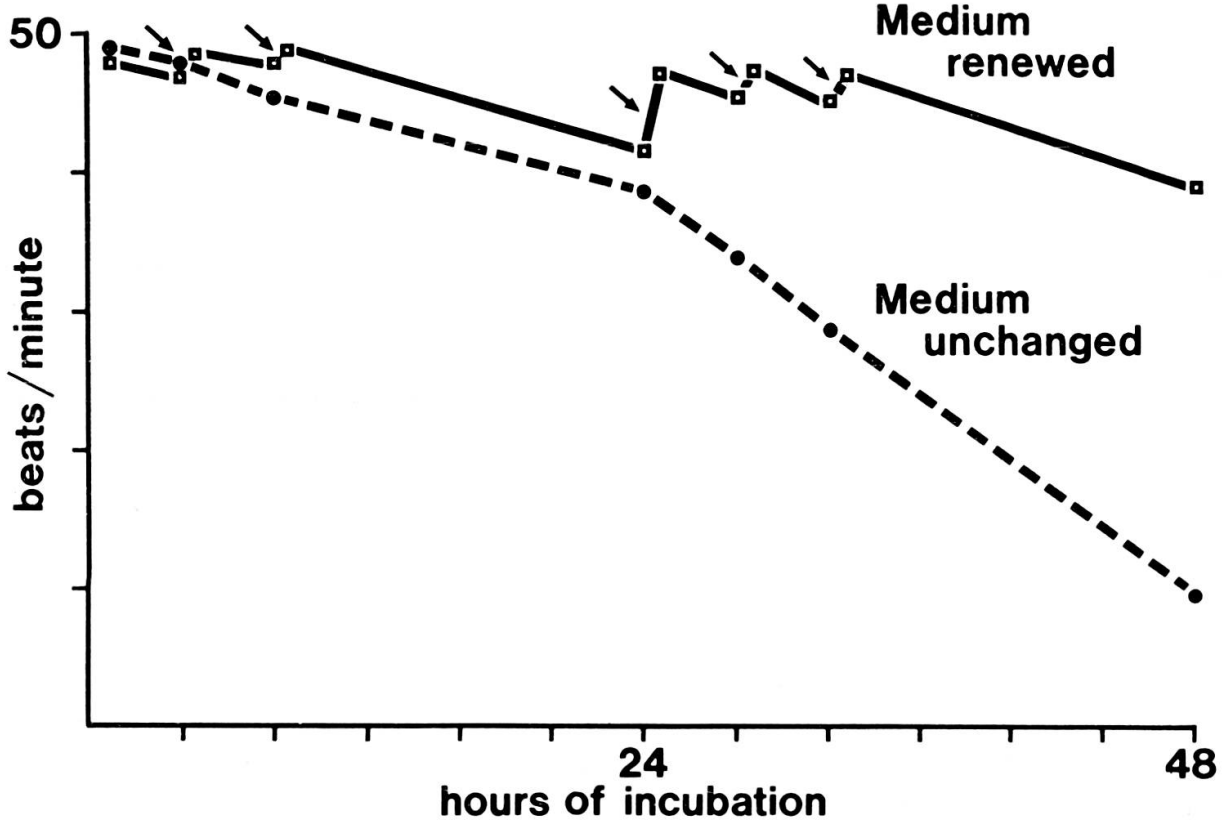

FIG. 2. — *Contraction rythm of frog auricles in culture with or without medium renewal.* Ten auricles per series were incubated in 20 ml of Ringer solution. In one series □——□ the medium was renewed at the different times showed by arrows. In the other series ●- - - -● the medium was not changed. Data plotted on the ordinate represent mean rhythm of ten auricles.

Purified frog DNA added to the medium does not inhibit at all the release of DNA while the DNA-complex in the medium resulting from a previous incubation of auricles decreases by 80% the amount of extracellular ^{3}H-DNA (Table II).

Table III shows that whole auricles release more DNA than wounded auricles. The same amount of DNA is recovered in the medium of whole living auricles or in the supernatants of auricles killed by hypotonicity or by homogenization.

Specific activity of the released DNA depended on the length of the labeling of the auricles before they were transfered to cold Ringer solution. About the same specific activity was found for the cellular DNA and for the released DNA after a short period of labeling while values far exceeding cellular DNA specific activity were observed on extracellular DNA released by auricles labeled for a long period (Table IV).

TABLE II

Radioactivity of DNA released by frog auricles labeled for 8 hours in Ringer solution and subsequently transfered to different cold media (fresh or already containing DNA) or released DNA

The amount of radioactivity was counted after purification of the DNA released in the different cold media. Total radioactivity (cpm) is presented, the specific activity (cpm/μg) being meaningless in the series in which additional purified DNA or non-extracted released DNA was present before the addition of the labeled auricles.

Cold meduim	Total radioactivity (cpm)	% of cpm in relation to the control
Fresh medium (control)	8,400	100
Medium with 100 μg/ml of purified frog DNA	8,700	103
Supernatant of medium in which frog auricles had been incubated for 4 hr	1,724	20

TABLE III

Amount of DNA in the cell free supernatant of auricles in culture incubated under normal conditions or after hypotonic death or after homogenization

800 auricles were divided into four parts: (A) One part was cultured in 80 ml of Ringer solution at 20° C for 4 hr in normal conditions. (B) The second part consisted of auricles cut in four (but still beating slowly) and cultured as in (A). (C) The third part was killed by incubation in distilled water before being put in 80 ml of Ringer solution at 20° C for 4 hr. (D) The last part was homogenized in 80 ml of Ringer solution. All four series were centrifuged. The DNA was extracted from the supernatants and its amounts determined.

Auricle treatment	Amount of extracellular DNA (μg/culture)
(A) normal conditions	21,3
(B) cut in four	13,4
(C) hypotonic death	26
(D) homogenized auricles	28,2

TABLE IV

Specific activity of the cellular and extracellular DNA in relation to the time of labeling with ^{3}H-thymidine

Frog auricle cultures were labeled with ^{3}H-thymidine (10 μci/ml) for a short or a long period before the auricles were transfered to cold Ringer solution. The cellular and extracellular DNA (of the 2nd incubation medium) were extracted and the amount and specific activity were determined.

Time of labeling	Amount of DNA (μg/culture)		Specific activity (cpm/μg)	
	cellular	extracellular	cellular	extracellular
Experiment 1 1st incubation (3 hr) in presence of ^{3}H thymidine followed by a 2nd incubation (4 hr) without label.	627	13,5	187	235
Experiment 2 1st incubation (16 hr) in presence of ^{3}H thymidine followed by a 2nd incubation (4 hr) without label.	644	13	277	2,700

Figure 3 shows that all fractions over 5×10^5 Daltons have a much higher specific activity in the released DNA than in the cellular DNA.

The renaturation curve of the cellular DNA (Fig. 4 a) starts to fall down at low C_0t value already while the released ^{3}H-DNA falls down only when 30 to 50% of the cellular DNA is already renatured. The hybridization curves (Fig. 4 b) indicate that both released and newly synthesized cellular ^{3}H-DNA present an important homology with non-labeled cellular DNA. However, the released ^{3}H-DNA curve seems to reach a plateau after 1/2 C_0t whether the mass ratio of labeled released DNA to unlabeled cellular DNA was 1:100 or 1:400.

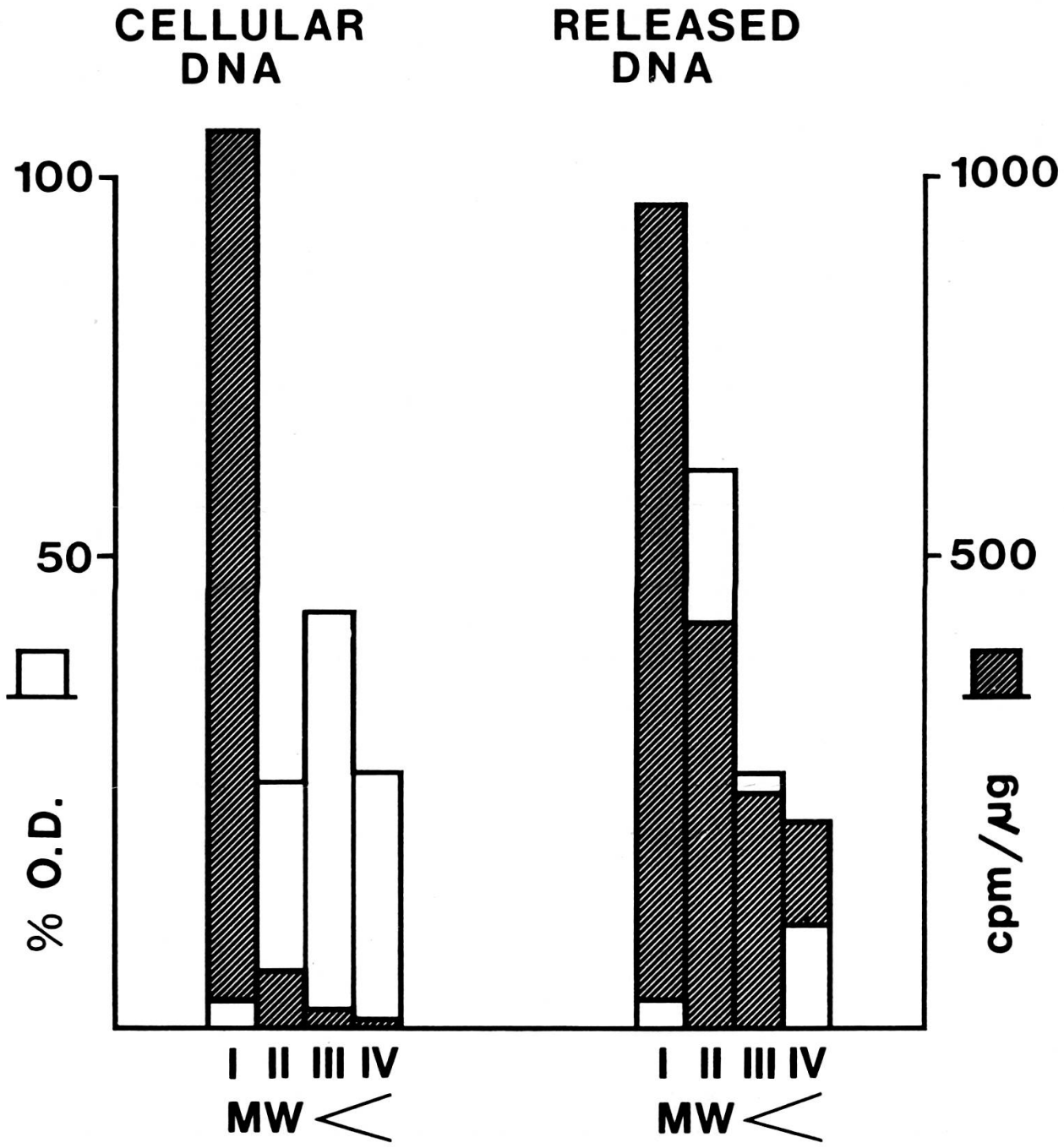

FIG. 3. — *Chromatographic profiles of cellular and extracellular DNA.* Abcissa: In white, amount (expressed in percent) of DNA measured by UV absorption. In black, specific activity (cpm/µg) of the DNA. Ordinate: Fractions of increasing molecular weight, (I) up to 5×10^5 Daltons, (II) from 5×10^5 to $1{,}5 \times 10^6$ Daltons, (III) from $1{,}5 \times 10^6$ to 4×10^6 Daltons, (IV) over 4×10^6 Daltons.
The auricles were labeled for 8 hours and transfered to cold Ringer solution for 4 hours. The DNA was extracted from the auricles and from the 2nd incubation.

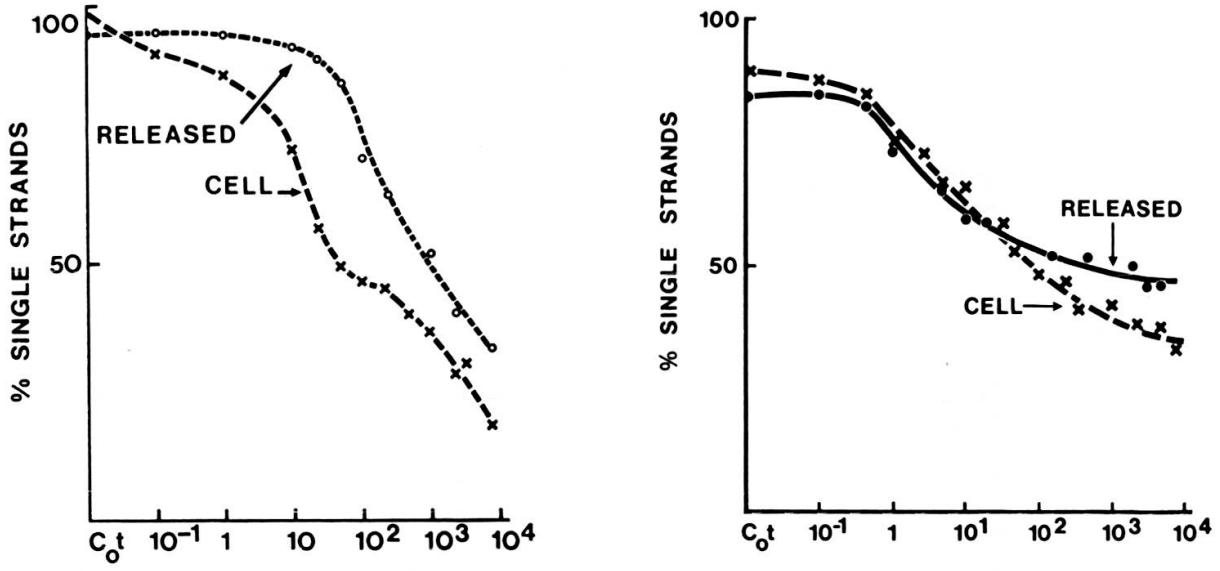

FIG. 4. — *C_0t curves of cellular and released DNA of frog auricles.* A. Comparison of the renaturation kinetics of frog auricle ^{3}H-DNA, and of ^{3}H-DNA released from previously labeled cells. B. Comparison of C_0t curves of frog auricles ^{3}H-DNA, and of ^{3}H-DNA released from previously labeled cells hybridized with an excess of non labeled cellular DNA (mass ratio of *1 : 100*).
Cellular ^{3}H-DNA or released ^{3}H-DNA were obtained after frog auricles had been labeled for 8 hrs with ^{3}H-thymidine.
The DNA was sheared to 7 S size and separated in different aliquots. It was denatured in a silicone oil bath at 120° C for 15 min. and cooled rapidly on ice. DNA solutions previously maintained in 0.03 M phosphate buffer were adjusted to 0.12 M of the same buffer and incubated in sealed disposable micropipets at 60° C. The sealed tubes were removed at desired intervals and frozen at 20° C until processed. Percentage of renaturation or hydridization was determined by applying each sample to an individual 1 cm water-jacketed column containing 2 cm of hydroxyapatite maintained at 60° C. The samples were eluted with 16 successive 1 ml fractions of 0.12 M phosphate buffer followed by 16 successive 1 ml fractions of 0.48 M phosphate buffer. The radioactivity of each fraction was counted by liquid scintillation. Data are plotted as percentage of single strands of ^{3}H-DNA versus C_0t (moles/liter x sec.).

DISCUSSION

As the amount of released DNA determined by UV absorption is fully confirmed by diphenylamine as well as by indole deoxyribose colorations, the quantitative results are now based on firm grounds.

The release of DNA is governed by an homeostatic system as evidenced by the constant amount of extracellular DNA recovered whether the incubation time is short or long. Moreover there is a constant renewal of extracellular DNA at each change of fresh medium. This repeated release of DNA seems subject to homeostasy rather than experimental stress since there is no significant increase of extracellular DNA when auricles are taken out of their medium and put back in the same medium. It should be stressed that auricles of which the medium is changed regularly survive much longer than auricles kept in the same medium. The possible role of a mechanical

stress seems also to be ruled out by the relatively long time (1 hr) necessary for extracellular DNA to reach its maximal concentration (22). The homeostasy is not regulated by the amount of DNA present in the medium but by the amount of DNA-complex in the medium. Indeed purified frog DNA does not inhibit at all the release of DNA while the DNA-complex in the medium resulting from a previous incubation of auricles decreases by 80% the amount of extracellular ^{3}H-DNA. Besides these arguments in favour of an active process there is no relation between cell death and extracellular DNA recovery. Indeed, wounded auricles sliced in four release less DNA than whole auricles in which all cells seem to be viable since they all incorporate ^{3}H-uridine into RNA during the last hour of the incubation periods and since no whole cells and very few fragments can be found in the pellet of the supernatants. Moreover, when 100% of the cells have been killed there is no significant increase of extracellular DNA. This shows that even if nuclei are lysed, the chromatin liberated is pelleted during the last centrifugation at 50,000 rpm. It should be remembered that the DNA is released in a complex containing not only proteins but also some light components since, before purification, the released DNA cannot be pelleted even after 12 hours of centrifugation at 50,000 rpm (4). The DNA recovered in the supernatant of the dead auricles might be membrane attached DNA in the process of being expelled.

The DNA expelled seems qualitatively different from the cellular DNA as shown by the specific activity and by the sequences with which it is composed.

The difference in the specific activity can be explained if one assumes that newly synthesized DNA is preferentially excreted. The similarity of the cellular and of the extracellular specific activities after a short period of labeling followed by the high increase of the specific activity of the extracellular DNA observed after a longer incubation time indicates that there is a certain delay between synthesis and release. This preferential release of the newly synthesized DNA is well illustrated by the comparative chromatographic profiles of cellular and released DNA which show that the released DNA cannot be a break down product of the cellular DNA since the fractions of high molecular weight have a much higher specific activity in the extracellular DNA than in the cellular DNA.

These new results on frog auricles confirm those which were recently observed in our work with unstimulated human lymphocytes showing the spontaneous release of DNA regulated by an active homeostatic mechanism (2, 3). As the released DNA is formed of newly-synthesized DNA and since auricles are composed of non-dividing cells, it is tempting to postulate that released DNA is metabolic DNA (14-16) which would consist of "extra copies of working genes" (16) and thus reflect the cell specialization. The released DNA being composed of unique sequences hence of structural genes which code for proteins according to Britten's hypothesis (6) support this view. We must add that in the present case, these extra-copies appear to be in limited number and should not be considered as what is usually called gene amplifi-

cation. All the different groups which worked on the released DNA from unstimulated (2, 3) or stimulated (13, 17, 18, 19) lymphocytes postulate that the extracellular DNA might have an immunological function. This speculation however, should not make us forget that the spontaneous release of DNA is not restricted to lymphoid cells but is a general biological event.

The present data on whole frog organs imply that before being excreted in the medium the DNA circulates from cell to cell. Indeed, the amount of DNA shed in the medium by frog auricles cannot be attributed only to cells in direct contact with the medium since there is not enough DNA in these cells to justify the amount of DNA recovered extracellularly. This estimation is even more striking if one bears in mind that the released DNA is composed preferentially of newly synthesized DNA without the reiterated sequences and represents but a fraction of the bulk cellular DNA.

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