Spontaneous extracellular synthesis of DNA released by frog auricles

Autor(en): **Anker, Philippe / Stroun, Maurice**

Objekttyp: **Article**

Zeitschrift: **Archives des sciences [1948-1980]**

Band (Jahr): **30 (1977)**

Heft 2

PDF erstellt am: **14.09.2024**

Persistenter Link: https://doi.org/10.5169/seals-739825

SPONTANEOUS EXTRACELLULAR SYNTHESIS OF DNA RELEASED BY FROG AURICLES

BY

Philippe ANKER [1] and **Maurice STROUN** [2]

We are grateful to Professor G. Bernardi and 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.

We thank Miss J. Henri for her excellent technical assistance.

This work was supported by grants from La Ligue Suisse contre le Cancer, Hoffman-La Roche and the O. J. Isvet Fund.

RÉSUMÉ

Les oreillettes de grenouilles relâchent, *in vitro*, un complexe contenant du DNA. Il a été démontré que ce processus d'excrétion n'est pas en relation avec les morts cellulaires mais est réglé par un mécanisme homéostatique. La structure relâchée contenant le DNA possède des propriétés particulières. Quand un précurseur phosphorylé du DNA est ajouté au surnageant acellulaire de la culture, le DNA recouvré du milieu est marqué. Ce marquage est dû à une incorporation réelle du précurseur radioactif et non à un simple attachement comme le démontrent les données obtenues selon la technique du plus proche voisin (nearest neighbour). En effet, si l'on ajoute au surnageant du TTP marqué au ^{32}P en α et que l'on hydrolyse le DNA extracellulaire en deoxyribonucléotides 3′, on retrouve de la radioactivité sur les quatre nucléotides. Bien que le type exact de synthèse n'ait pu encore être déterminé, la possibilité d'un enzyme terminal qui ne ferait qu'ajouter des nucléotides en bout de chaîne a pu être éliminée par des traitements comparatifs à la DNase et à la phosphodiestérase de venin de serpent qui montrent que la radioactivité est située tout au long de la chaîne. L'incorporation de précurseurs est inhibée par la DNase, la RNase et la pronase. Cette synthèse extracellulaire du DNA dans le surnageant n'est pas affectée par le taux de morts cellulaires survenues auparavant lors de la culture. La courbe de renaturation du DNA synthétisé acellulairement montre une absence de gènes répétitifs.

SUMMARY

Frog auricles were shown to release, *in vitro*, a complex containing DNA. It has also been reported that the release process is unrelated to cell death and is regulated by an homeostatic mechanism. Some properties of this extracellular DNA were investigated. When a phosphorylated precursor was added to the cell free supernatant, the DNA recovered from the medium was labeled.

---

[1] Division d'Oncohématologie de la Faculté de Médecine, Hôpital Cantonal, Genève, Switzerland.

[2] Département de Physiologie Végétale de la Faculté des Sciences, Université de Genève, Switzerland.

Evidence that DNA labeling represented true precursor incorporation and not simple attachment was obtained from nearest neighbour analysis data. When $_{\alpha}{}^{32}$P TTP was added to the supernatant and the labeled DNA completely hydrolyzed to 3′ deoxyribonucleotides, radioactivity was found in all four nucleotides. While the exact kind of synthesis cannot be determined at this stage, the possibility of a terminal transferase system in which the enzyme would merely add a nucleotide at the end of the chain was eliminated since comparative digestion with DNase and venom phosphodiesterase showed that labeling was located along the whole length of the chain. Precursor incorporation into the DNA was inhibited by DNase, RNase, pronase. This extracellular synthesis in the supernatant was not affected by cell death rate which had occured previously in the culture. The renaturation curve of the extracellular DNA synthesized in the cell free medium showed a lack of gene reiteration.

INTRODUCTION

Frog auricles were shown to release, *in vitro*, a complex containing DNA (1, 23, 26). This release is governed by an homeostatic mechanism and is not related to cell death (23, 26).

Spontaneous release of DNA seems to be a general phenomenon in eukaryotic systems since other organisms such as non stimulated (4, 5, 6) or stimulated (15, 16, 17, 18) lymphocytes also excrete DNA. An homeostatic system also ensures a constant extracellular DNA concentration in non stimulated human lymphocytes (5).

Furthermore, a spontaneous incorporation of labeled DNA precursors was observed in the cell free culture media of frog auricles (23). Similar results were obtained later with the supernatant of lymphocytes (4, 6). The fact that the radioactivity could not be removed from the DNA in spite of very drastic purification procedures, argued in favour of a true DNA synthesis and against an artefact such as precursor attachement. Moreover, the incorporation of DNA precursors seemed template and enzyme dependent as shown by its inhibition by DNase and pronase. These arguments, however, were not completely convincing and were, as was pointed out, indirect proofs of a synthetic process.

In this paper, we present evidence that the incorporation of phosphorylated precursors into the DNA previously released in the supernatant by frog auricles reflects a real cell free synthesis. Preliminary results concerning the type of extracellular synthesis taking place are also discussed.

MATERIAL AND METHODS

Auricle Culture and Separation of the Supernatant

Frog auricles were sterily extracted as described previously (26). Two hundred auricles per series were incubated for varying time periods in 80 ml of fresh Ringer in presence of antibiotics (26). In some experiments the medium was regularly renewed in order to have enough supernatant, as we had previously found (23, 26)

that similar amounts of DNA were excreted at each change of medium. The auricles were removed and the supernatant submitted to different centrifugations (26). As a control for 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.

Labeling

^{3}H TTP (26 Ci/mmole or 47 Ci/mmole, labeled in the methyl group) ^{3}H dATP (14,5 Ci/mmole), ^{3}H dCTP (25 Ci/mmole), or ^{3}H dGTP (12 Ci/mmole) was added to the cell free supernatant or to the auricles in culture for various periods of time at a final concentration of 1 μCi/ml. In some experiments ^{3}H TdR (10 Ci/mmole, labeled in the methyl group) was added to the auricles in culture. All these compounds with a purity of 97 to 99% were purchased from the Radiochemical Center, Amersham, England.

In some experiments the following hydrolases and DNA synthesis inhibitors were added to the cell free supernatant before labeling: (a) 200 μg/ml of DNAse II, 2 μg/ml or 200 μg/ml of RNAse boiled for 20 minutes before use, (b) 2 mg/ml of pronase previously subjected to autodigestion for 8 hr at 37° C, (c) 30 μg/ml of Actinomycin D.

After DNA extraction, radioactivity was counted in the presence of the suitable phosphor in a Beckman Tri-Carb liquid scintillation counter. In order to exclude the possibility that, after extraction, the radioactivity carried by the purified DNA could be ascribed to a radioactive contaminant, perchloric acid hydrolyzed ^{3}H DNA (previously labeled with ^{3}H TTP) was chromatographed (20) showing that radioactivity migrated with the thymine spot only. The same controls were performed with all tritiated precursors used.

In experiments designed for nearest neighbour analysis the cell free supernatant or the auricles in culture were labeled for 12 hours with $_{\alpha}{}^{32}$P TTP (50 Ci/mmol) at a final concentration of 1 μCi/ml. The radioactivity was counted without the addition of a scintillating medium.

DNA extraction

DNA was extracted from both the auricles and the supernatant according to the method of Marmur (14) until the first ethanol precipitation where the solutions were loaded on hydroxyapatite columns (7).

In order to eliminate any labeled precursor or any labeled impurity adsorbed on the DNA, further purification was carried on. The fractions containing DNA, as indicated by UV absorption at 258 nm, were dialysed for 24 hr against water. KCl was added to a concentration of 3 M and the solution was loaded on a second hydroxyapatite column which was eluted with the phosphate buffers described

above. All buffers were adjusted to 3 M KCl. The eluted double-stranded DNA was peletted by ultracentrifugation for 16 hr at 45,000 rpm. The DNA was resuspended in the suitable solvent, depending on the subsequent characterization procedure.

As a control, labeled Ringer solution was subjected to the same extraction procedure at the end of which no material and no radioactivity were recovered.

Characterization of the DNA Synthesized in the Cell Free Medium

The specific activity of the DNA was calculated and expressed in cpm/μg, the amount of DNA being determined on a Beckman DB spectrophotometer by UV absorption at 258 nm and by deoxyribose colorations (9, 10).

To prove that the labeling reflected a real incorporation and was not due to some adsorption, a simplified nearest neighbour analysis (12) was performed. The true nature of precursor incorporation can be confirmed by this technique. In a real synthetic reaction, when $_{\alpha}{}^{32}$P 5′TTP is provided to the cell free supernatant this ^{32}P (the innermost phosphate) becomes the bridge between the TMP and its nearest neighbour nucleotide containing one of the four bases. The ^{32}P is thus linked in position 5′ to the thymidine moiety and in position 3′ to its nearest neighbour. The purified DNA is then hydrolysed in 5′, yielding 3′ monophosphates. If the synthetic reaction has taken place the ^{32}P will have switched from the TTP to its nearest neighbour and all four monophosphates will be labeled. After the extraction of ^{32}P DNA, samples of 10 μg were suspended in 100 μl of a solution containing 45 μl of buffer (Sodium Acetate pH 4.5, 0.01 M, EDTA 0.01 M), 15 μl H_2O, 25 μl Spleen DNase (2 mg/ml) and 20 μl of Spleen phosphodiesterase (1 mg/ml). This solution was incubated at 37° C in a capillary tube for 1 hr and was then spotted on a thin layer chromatographic sheet (TLC Ready plastic sheets 1440 Pei/LS 254, Schleicher and Schuell AG, Feldbach, Germany). The chromatography was performed in a tank saturated with a solvent mixture of (v/v) isobutyric acid, water, Ammonia and EDTA 0.1 M at pH 4.6 (50:29.4:2.1:0.9). The migration of the monophosphate nucleotides was checked by UV absorption at 254 nm and compared to the migration of control 3′ monophosphate nucleotides. The location of the radioactivity was determined by exposing an X-ray film on the chromatograph. After development it was checked that UV absorption spots and radioactivity coincided. The monophosphate spots were counted in a liquid scintillation counter and the percentage of radioactivity on each nucleotide was calculated.

DNA labeled with ^{3}H TTP was used for the following characterization procedures: (a) Enzyme sensitivity was tested by acid precipitation (19) following digestion for 2 hr at 37° C with 100 μg/ml splenic DNase II or with 100 μg/ml of pancreatic RNase in which the DNase had been inactivated by heating at 90° C for 15 min, or with 500 μg/ml of pronase previously submitted to autodigestion for 8 hr at 37° C. The DNA was also treated for various periods of time with snake venom

phosphodiesterase as described by Lieberman *et al* (13). The kinetics of digestion were compared to those obtained with pancreatic DNase. (b) The molecular weight was estimated by zonal centrifugation on linear gradients of 5 to 20% sucrose in the presence of sedimentation markers (23 S, 16 S and 5 S RNA, Miles Laboratory, Slough, England). (c) DNA labeled in the supernatant with ^{3}H TTP was hybridized against an excess (ratio 1 to 1.000) of non-labeled frog auricle DNA. Moreover, renaturation kinetics of labeled cellular DNA and extracellular ^{3}H DNA labeled in the cell free supernatant were compared. Because of the low specific activity of the cellular DNA after labeling with ^{3}H TTP, the cellular ^{3}H DNA was obtained from cells labeled with ^{3}H thymidine. C_0t curves were performed according to the method described by Britten and Kohne (8).

RESULTS

Nature of Extracellular DNA Synthesis

Figure 1 which is an autoradiograph of a thin layer chromatography of extracellular DNA labeled with $_\alpha{}^{32}$P TTP shows that after digestion of the DNA with splenic DNase and splenic phosphodiesterase, all four 3′ monophosphates were labeled. After all purification procedures were completed the extracellular DNA had a specific activity of 221 cpm/μg while when the auricles themselves were labeled with the same precursor the cellular DNA had a specific activity of only 42 cpm/μg. Chromatography of digested cellular DNA as expected also yielded four labeled 3′ monophosphates. But with such a low specific activity due to poor precursor uptake by the cells, the nearest neighbour analysis is probably not representative of the total cellular syntheiss. Thus all comparisons of the percentage of radioactivity on the four spots between cellular and extracellular DNA would be spurious.

It should be stressed that unlabelled orthophosphate added in vast excess with the $_\alpha{}^{32}$P TTP to the acellular medium does not decrease the specific activity of the DNA synthesized acellularly.

Table I shows that the released DNA was labeled when one of the four specific triphosphate precursors was added to the cell free supernatant. In addition, it also indicates that the specific activity of the DNA was not decreased by the presence of an excess of unlabeled triphosphate precursor carrying a different base, in this case ^{3}H thymidine triphosphate with 1×10^4 fold excess of unlabeled deoxyguanosine triphosphate.

Maximum labeling was obtained in about 1 hr as can be seen on Table II. Moreover, the specific activity of the DNA was the same whether the cells were labeled immediately after centrifugation or 12 hr later. In both cases, however, the amount of DNA recovered from the medium was the same.

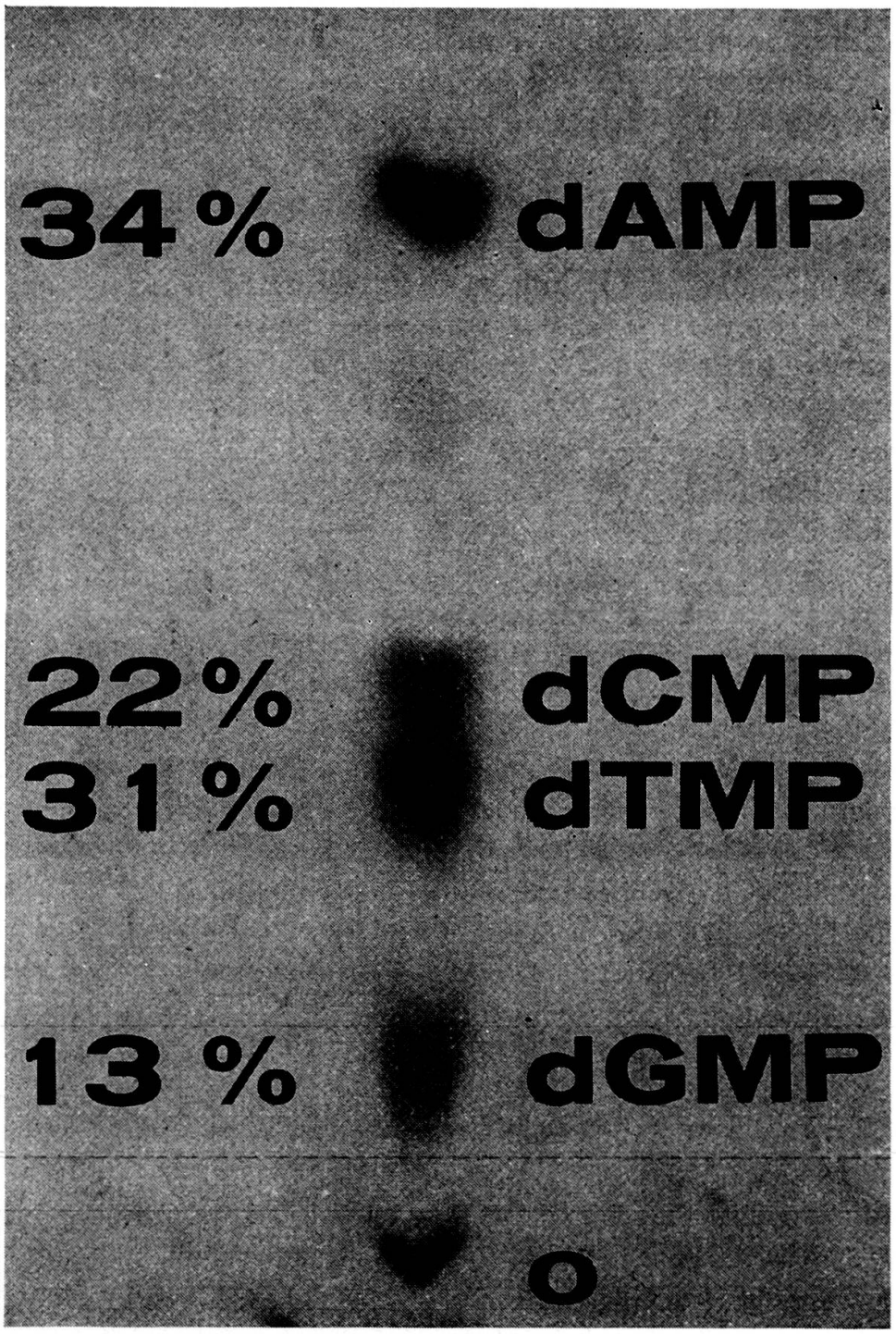

FIG. 1. — *Autoradiography of a thin layer chromatograph of extracellular DNA labeled with* $_{\alpha}{}^{32}P$ *TTP in the cell free medium and digested into 3′ monophosphates.* 300 frog auricles were incubated in 150 ml of Ringer solution for 4 hr at 37° C. The culture was then centrifuged. The cell free supernatant was labeled for 12 hours with $_{\alpha}{}^{32}P$ TTP (50 Ci/mmole) at a final concentration of 1 μCi/ml. After extraction the DNA was digested with splenic DNase and splenic phosphodiesterase and chromatographed. The amount of radioactivity present on the spots corresponding to the different 3′ monophosphates was determined and expressed as percentages of total radioactivity. These percentages are indicated on the left side of the photograph.

TABLE I

Specific activity of DNA labeled with various precursors in the cell free supernatant of frog auricles in culture

Five successive cultures of 200 auricles in 70 ml of Ringer solution were incubated for 4 hr each at 20° C. The cultures were pooled, centrifuged and the supernatant divided in 5 parts. Each sample was then incubated for 2 hr with one of the four labeled deoxyribonucleoside triphosphates indicated on the table at a final concentration of 1 μCi/ml. An excess (1×10^4 fold) of unlabeled dGTP was added with the ^3H TTP to the fifth sample. All 4 precursors were adjusted to the same specific activity (12 Ci/mmole). The DNA was then extracted and its specific activity was determined.

Labeling	Specific activity of extracellular DNA labeled in absence of auricles (cpm/μg)
^3H thymidine triphosphate	675
^3H deoxyadenosine triphosphate	538
^3H deoxyguanosine triphosphate	702
^3H deoxycytosine triphosphate	629
^3H thymidine triphosphate with 1×10^4 fold excess of unlabeled deoxyguanosine triphosphate	631

TABLE II

Amount and specific activity of DNA labeled for various periods of time with ^3H TTP in the cell free supernatant of frog auricles in culture

Five successive cultures of 200 auricles in 70 ml of Ringer solution were incubated for 4 hr each at 20° C. The cultures were pooled, centrifuged and the supernatant divided in 6 parts. In a), b), c), d), e), ^3H TTP (1 μCi/ml, 47 Ci/mmole) was added immediately after centrifugation and in f) ^3H TTP was added 12 hr later. The DNA of each series was extracted immediately at the end of the labeling and its amount and specific activity determined.

Time of labeling	Extracellular DNA	
	Amount (μg)	Specific activity (cpm/μg)
Labeling immediately after centrifugation		
a) 1 min	18	87
b) 10 min	16.5	168
c) 30 min	17.5	388
d) 60 min	19	427
e) 10 hr	17	451
Labeling 12 hr after centrifugation		
f) 60 min	19.5	404

As indicated in Table III (A and B), the supernatant of auricles submitted to hypotonic death did not yield much more extracellular DNA in the first incubation medium than a normal culture of auricles. If the medium was changed, practically no DNA was found in the following incubation medium and none at all in the third and fourth supernatants of the dead auricles. The specific activity of the DNA labeled extracellularly was about 2 times lower in the supernatant of dead auricles than in the supernatant of living auricles. In Table III (C) one can see that when auricles are homogenized in the same amount of medium and subsequently centrifuged at 50,000 rpm the amount of DNA recovered from the supernatant is not significantly more important than in the supernatant of living auricles (Table III (A)).

TABLE III

Amount and specific activity of DNA labeled with ^3H TTP in the cell free supernatant of auricles in culture incubated under normal conditions or after hypotonic death or after homogenization

600 auricles were divided into three parts: (A) One part was cultured in 70 ml of Ringer solution at 20° C for 4 hr in normal conditions. (B) The second part was killed by incubation in distilled water before being put in 70 ml of Ringer solution at 20° C for 4 hr. (C) The last part was homogenized in 70 ml of Ringer solution. All three series were centrifuged and the supernatant labeled for 2 hr with ^3H TTP (1 μCi/ml, 47 Ci/mmole). In series A and B, the medium was renewed 3 times and their supernatant labeled for 2 hr. The DNA was extracted from the supernatants and its amounts and specific activity determined.

Auricle treatment	Extracellular DNA		
	Amount (μg/culture)		Specific activity (cpm/μg)
A) normal conditions			
1 st incubation	17,6		430
2nd incubation	20	72,1	393
3rd incubation	16,3		415
4th incubation	18,2		523
B) hypotonic deaths			
1st incubation	25		208
2nd incubation	3	28	227
3rd incubation	0		0
4th incubation	0		0
C) homogenized auricles	22		191

Table IV shows that DNase II, RNase, and pronase strongly inhibited the incorporation of ^3H TTP while the amount of DNA recovered remained unaltered. Trace amounts of RNase (2 μg/ml) were shown to be an ineffective inhibitor thus

establishing that the strong inhibition induced by DNase could not be due to the presence of small quantities of RNase. Actinomycin D did not block the incorporation of ^{3}H TTP.

TABLE IV

Inhibition by different compounds of the incorporation of ^{3}H TTP into the DNA of the cell free supernatant of auricles in culture

Five successive cultures of 200 auricles in 70 ml of Ringer solution were incubated for 4 hr each at 20° C. The cultures were pooled, centrifuged and the supernatant divided in 6 parts. Various inhibitors (DNase, RNase, pronase, and Actinomycin D) were added to the supernatant samples which were incubated for 2 hr before a 30 min labeling with ^{3}H TTP (1 μCi/ml, 47 Ci/mmole). The control was treated in the same way except that the inhibitor was omitted. The DNA was then extracted and its amount and specific activity determined.

Inhibitors	Extracellular DNA	
	Amount	Specific activity (cpm/μg)
Control	16	483
Splenic DNase (200 μg/ml)	14.5	68
Pancreatic RNase (2 μg/ml)	13.4	457
Pancreatic RNase (200 μg/ml)	16.2	71
Pronase (2.000 μg/ml)	13.4	62
Actinomycin D (30 μg/ml)	17	473

Characterization of ^{3}H DNA Synthesized in the Cell Free Medium

The purified ^{3}H DNA was sensitive to both pancreatic and splenic DNAse. After 12 hours of digestion, over 90% of the acid-insoluble radioactivity became acid soluble. Figure 2 shows that with venom phosphodiesterase the acid insoluble

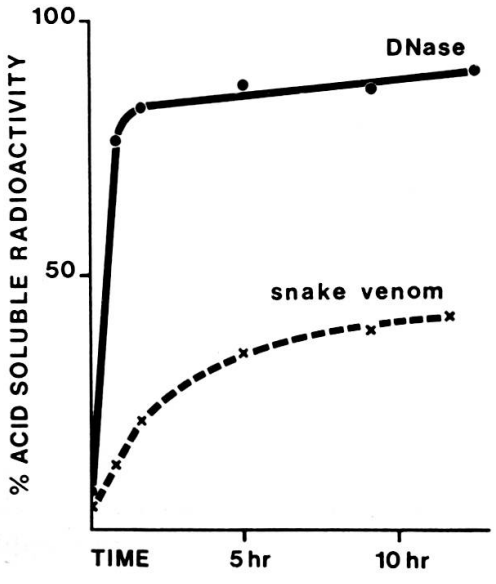

FIG. 2. — *Snake venom phosphodiesterase and pancreatic DNase digestion of extracellular ^{3}H DNA previously labeled in the cell free medium with ^{3}H TTP. Ordinate:* Percentage of total cpm rendered acid soluble. *Abcissa:* Time of digestion. ^{3}H DNA treated with snake venom phosphodiesterase (2 μg/ml) ×- - - - × ^{3}H DNA treated with pancreatic DNase (2 μg/ml) ●——●.

radioactivity became gradually soluble while with pancreatic DNAse a plateau was rapidly reached. On the other hand, pronase or RNAse had no effect on the purified molecules.

After heat denaturation over 90% of the radioactive DNA was eluted from hydroxyapatite by 0.12 M phosphate buffer.

After sucrose gradient centrifugation, DNA synthesized in the cell free medium banded in two main peaks, one at 11 S and the other at 7 S (Figure 3).

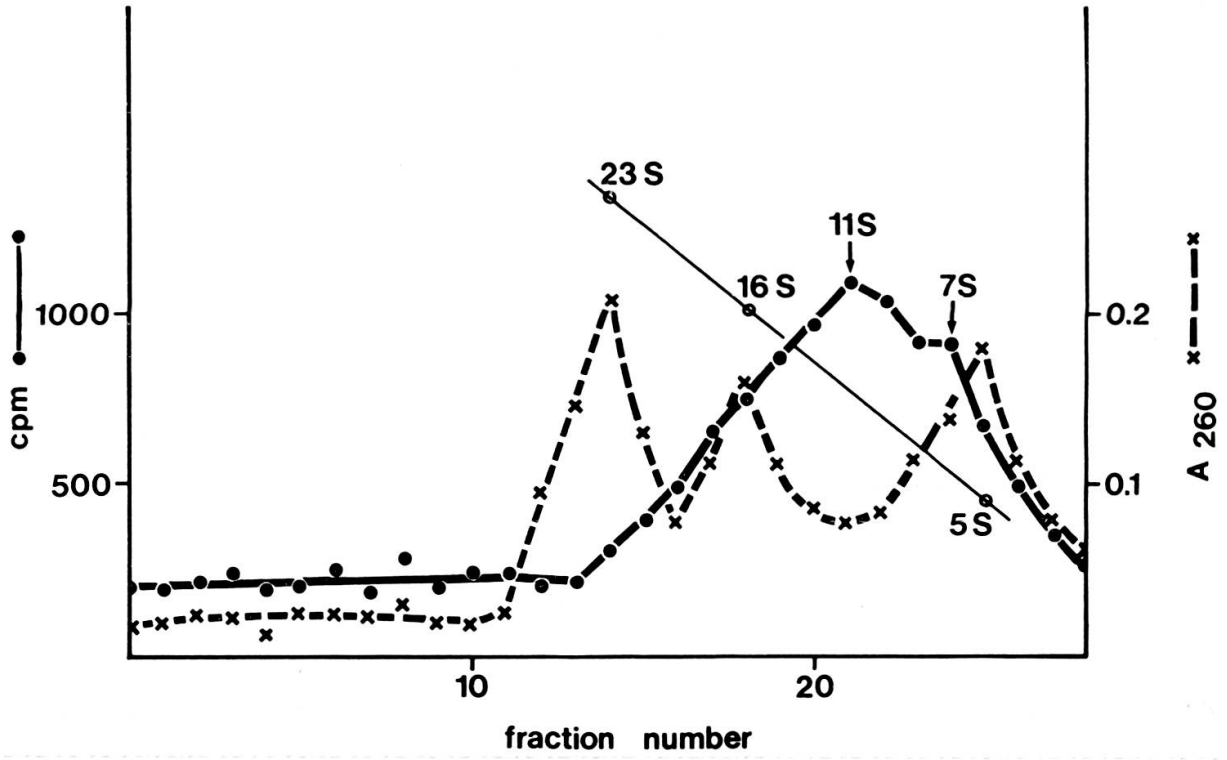

FIG. 3. — *Sucrose gradient centrifugation of extracellular ^3H DNA labeled in the cell free medium with ^3H TTP.* Five μg of ^3H DNA were purified from a cell free supernatant mixed with 40 μg of 23 S, 16 S and 5 S unlabeled reference RNA. The samples were layered on 5 ml linear gradients of 5-20% sucrose in 0.015 M NaCl and 0.0015 M sodium citrate, and centrifuged in an SW 50.1 rotor at 45,000 rpm at 20° C for 2 hr 30 min.

Figure 4 shows the renaturation curves of cellular ^3H DNA, and of extracellular ^3H DNA labeled in the cell free supernatant. The cellular ^3H DNA presents a characteristic curve of frog cellular DNA which starts to fall down already at low C_0t, reaching the half C_0t value at approximately C_0t 50. On the other hand, the extracellular ^3H DNA is stable until about C_0t 50 where the curve drops suddenly.

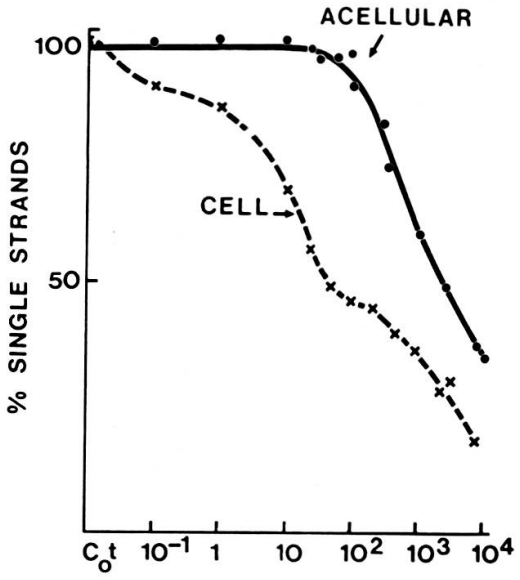

FIG. 4. — *Comparison of the renaturation kinetics of cellular ^3H DNA and extracellular ^3H DNA labeled in the cell free supernatant.* Cellular ^3H DNA was purified from frog auricles labeled with ^3H thymidine for 8 hr. Extracellular ^3H DNA was purified from cell free supernatants of auricles in culture, labeled with ^3H TTP for 8 hr. The ^3H DNA was sheared to 7 S size and separated in different aliquots containing up to 30 A/ml. They were 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 for up to 300 hr. The sealed tubes were removed at desired intervals and frozen at 20° C until processed. Percentage of renaturation 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 ^3H DNA versus C_0t.

Figure 5 shows that the DNA labeled in the cell free supernatant hybridizes with unlabeled cellular DNA only at a high C_0t value (5×10^3).

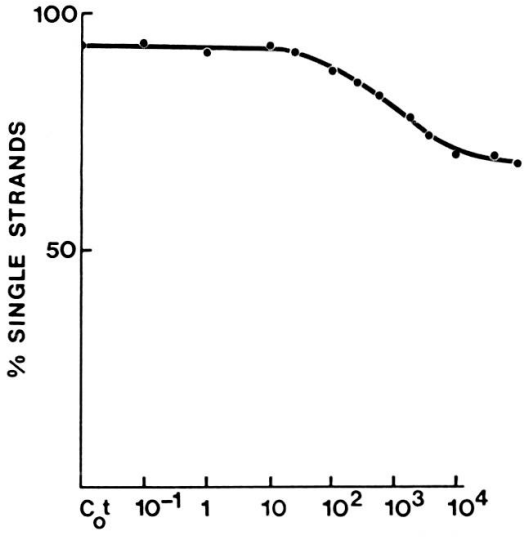

FIG. 5. — *C_0t curve of extracellular ^3H DNA labeled in the cell free supernatant and hybridized with an excess of non labeled cellular DNA.* Extracellular ^3H DNA was purified from cell free supernatants of auricles in culture labeled with ^3H TTP for 8 hr. The ^3H DNA was added to non labeled frog cellular DNA in a mass ratio of 1 : 1,000. This mixture was submitted to the same treatments as those indicated in Figure 4.

DISCUSSION

Nature of the Extracellular DNA Synthesis

The labeled material extracted from the cell free supernatant can be considered to be DNA because of its sensitivity to all DNases used and its insensitivity to RNase and pronase. The molecule is further shown to be double-stranded by the

fact that after heat denaturation over 94% of the radioactive DNA previously eluted from hydroxyapatite by 0.48 M phosphate buffer is freed by 0.12 M phosphate. Its molecular weight is not homogeneous and part of the DNA is quite small with however an important fraction sedimenting at 11 S.

The results obtained by the nearest neighbour analysis when $_{\alpha}{}^{32}$P TTP is added to the medium show that the precursors have really been incorporated and that a simple adsorption process can be excluded.

Thus the ^{3}H DNA obtained after a labeling with ^{3}H TTP instead of $_{\alpha}{}^{32}$ P TTP can also be considered as the result of a true synthetic reaction. Indeed, both precursors are 5′ TTP, the only difference being the position of the isotope on the molecule.

The low incorporation observed in the extracellular DNA, could suggest that this small synthesis is induced by traces of enzymes and DNA released into the medium from damaged cells during the experimental manipulations. This trivial explanation however seems to be ruled out by the following observations. (a) The same amount of DNA and the same incorporation of precursor into this DNA were found in the medium whether the incubation lasted 30 min or 10 hr (Table 2). One would expect the extracellular concentration and the amount of labeled precursors incorporated to increase with time if the phenomenon were due to leakage by dying cells. (b) When the auricles have been killed by distilled water (Table III) or homogenized, the amount of DNA recovered from their supernatant is not much higher than the amount found in the supernatant of a normal culture while the specific activity is about twice lower in the water killed or homogenized series. Now, in these series 100% of the cells are killed or mechanically disrupted, whereas in the normal series all cells seem to be alive since they incorporate ^{3}H uridine in the last hour of a 24 hr incubation (26). Since the extracellular incorporation of ^{3}H TTP is lower when 100% of cells have been killed or homogenized than when the auricles are in good condition, the synthesis observed in the medium of normal auricles could hardly be attributed to dead cells. Moreover, when the medium is renewed only living cells still release DNA synthesizing structures. (c) Furthermore the renaturation curves which will be discussed later show that the DNA synthesized in the cell free medium is qualitatively different from the DNA synthesized in the cells.

The exact kind of synthesis cannot be determined at this stage.

One can nevertheless eliminate the possibility of a terminal transferase system in which the enzyme would merely add a nucleotide at the end of the chain. Indeed digestion of the DNA with venom phosphodiesterase shows that the acid insoluble radioactivity becomes gradually soluble following a slower curve than when digested with pancreatic DNase. If the labeled nucleotides were added at the end of the chain, a much larger amount of radioactivity would be found in the acid soluble fraction after the first hours of digestion. The synthesis inhibition observed after RNase treatment would not fit with a transferase system either.

An unscheduled DNA synthesis as described in DNA repair process appears unlikely since the sensitivity of the acellular synthesis to RNase is in contradiction with what could be expected with a repair enzyme which does not, to our knowledge, require the presence of RNA. The hybridization curves indicate that only part of the released DNA is synthesized extracellularly as discussed later. One does not see why a special part of the released DNA rather than another would be subject to repair synthesis.

The inhibition of precursor incorporation by RNase suggests either some sort of replicative synthesis where an initiator RNA (27) is necessary or an RNA directed DNA synthesis (28).

The absence of synthesis inhibition by Actinomycin D would suggest a reverse transcriptase system. However, the possibility that DNA directed DNA synthesis primed by RNA cannot be discarded since a negative result with Actinomycin D could be due to different reasons, such as DNA coating. This interpretation is enforced by the high inhibition observed even with low concentrations (0.7 μg/ml) of Actinomycin D treatment in the acellular synthesis of the DNA released by human lymphocytes (6). Whatever the role of the extracellular RNA might be, the presence of RNA in the medium is not surprising since we have previously shown that a polyribonucleotide is released by eukaryotic cells at the same time as the DNA (1, 3). Moreover, release of RNA has also been reported in another laboratory (11).

The fact that precursor incorporation occurs without the addition of any component other than one single labeled precursor, implies that the released DNA is accompanied by a stock of necessary precursors and enzymes amongst which is a DNA polymerase. It is well known that enzymes are excreted by cells. We have previously reported furthermore that a DNA-dependent RNA polymerase accompanies the DNA released by bacteria (21, 24). In the present study, an enzyme dependent system is already suggested by the inhibition of precursor incorporation by pronase. It is unfortunately not possible to quantify this enzymatic reaction since substrates and enzymes cannot be dissociated at this stage. Another difficulty arises from the fact that we seem to be dealing with a multienzymatic system. This point is suggested by the turnover to which the released DNA seems subjected, implying the presence of both a polymerase system as well as DNA hydrolases. Indeed, the incorporation of ^{3}H TTP reaches a plateau very rapidly whether the cell free supernatant is labeled immediately after centrifugation or only 12 hr later while the amount of DNA recovered remains the same.

A turnover of the extracellular DNA would explain the apparent low incorporation of radioactive precursors, which are in competition with excreted cold precursors and nucleotides resulting from a breakdown process. It is therefore not possible to estimate the amount of DNA synthesized.

Characterization of the Acellular DNA

The high half C_0t value of the renaturation curve shown by the ^3H DNA synthesized in the supernatant is about the same as that of the cellular DNA and indicates a high complexity. The absence of renaturation of the extracellular ^3H DNA until about C_0t 50 contrarily to what happens with the cellular ^3H DNA, shows a lack of gene reiteration in the acellularly labeled ^3H DNA. The low percentage of hybridization observed between unlabeled cellular DNA and ^3H DNA synthesized in the cell free supernatant implies (a) either random synthesis of DNA in the acellular medium or (b) faulty replication or (c) foreign nature of a part of the DNA synthesized. (a) A random synthesis can be discarded on the basis of the nearest neighbour analysis data which shows a very striking deviation from the nucleotide frequencies which would be expected to be even for the four nucleotides if their arrangement were completely at random (12). Furthermore, the addition of a cold precursor carrying another base, for example dGTP, to the acellular medium does not decrease the specific activity of the ^3H TTP labeled DNA. It would be expected in random synthesis that the different precursors would compete for incorporation while in a template dependent synthesis, incorporation of the different precursors follows the template requirements. (b) It is possible that we are in presence of faulty replications due to *in vitro* conditions. (c) The foreign nature of part of the DNA synthesized in the cell free medium cannot definitively be excluded. Of course, the possibility of a direct contamination by a bacteria or a mycoplasm is rules out by the complexity of the acellularly labeled ^3H DNA, as shown by the C_0t renaturation curves, without mentioning the sterility of the supernatants. Besides, the medium is centrifuged before labeling at 50,000 rpm for up to 12 hr and in such conditions even small mycoplasms or viruses would be pelleted out. It is, however, still possible that some integrated DNA of foreign origin such as a provirus (29) or a bacterial DNA derived from transcession (2, 21, 22, 24, 25) might have been released with the cellular DNA and that this foreign DNA can be preferentially synthesized in the extracellular medium. In this case, the extracellular synthesis of DNA could have some pathological function.

The high C_0t value at which hybridization occurs, points out a preferential extracellular synthesis of unique sequences of the cell genome. Indeed, this hypothesis is supported by the absence of reiteration observed in the renaturation curve of the extracellular ^3H DNA and might explain, if one assumes that all sites are saturated, the low hybridization between cellular DNA and extracellularly labeled DNA. It thus appears that only a small part of the released DNA which was shown to present an important homology with the cellular DNA (26) is synthesized in the cell free supernatant. This preferential synthesis in the extracellular medium of what could be structural genes seems to be a general phenomenon since we have also recently found a spontaneous synthesis of unique DNA released from lympho-

cytes (6). This extracellular synthesis of released DNA from lymphocytes or from auricles presents exactly the same characteristics except for the sensitivty to Actinomycin D. The exact nature of this synthesis as well as its biological or pathological function(s) has still to be determined.

REFERENCES

1) ANKER, P. and M. STROUN (1972). Spontaneous Release of Nucleic Acids by Living Bacteria and Cells of Higher Organisms. In: Abstracts of the VIIIth International Congress of Cell Biology, Brighton, p. 3. Sussex University Press.
2) —— and M. STROUN (1972). Bacterial Ribonucleic Acid in the Frog Brain after a Bacterial Peritoneal Infection. *Science*, *178*, 621-623.
3) —— M. STROUN, J. DESHUSSES, A. CATTANEO and J. HENRI (1973). Cell-free Extracellular Synthesis of a Polyribonucleic Fraction (PRN) after the Spontaneous Release of Nucleic Acids by Living Bacteria and by Cells of Frogs and Mammals. *Biochem. Soc. Trans. London*, *4*, 890-892.
4) —— M. STROUN and P. A. MAURICE (1975). Spontaneous Extracellular Synthesis of DNA Normally Released by Human Lymphocytes. Abstracts of the 11th International Cancer Congress, Florence, Italy, October 20 to 26, 1974, Vol. 2, p. 24. Amsterdam: Excerpta Medica.
5) —— M. STROUN and P. A. MAURICE (1975). Spontaneous Release of DNA by Human Blood Lymphocytes as Shown in an *in vitro* System. *Cancer Res.*, *35*, 2375-2382.
6) —— M. STROUN and P. A. MAURICE (1976). Spontaneous Extracellular Synthesis of DNA Released by Human Blood Lymphocytes. *Cancer Res.*, *36*, 2832-2839.
7) BERNARDI, G. (1965). Chromatography of Nucleic Acids on Hydroxyapatite. *Nature*, *206*, 779.
8) BRITTEN, R. J. and D. E. KOHNE (1968). Repeated Sequences in DNA. *Science*, *161*, 529-540.
9) CERIOTTI, G. (1952). Michrochemical Determination of Deoxyribonucleic Acid. *J. Biol. Chem.*, *198*, 297-303.
10) GILES, K. W. and A. MYERS (1965). An Improved Diphenylamine Method for the Estimation of DNA. *Nature*, *206*, 93.
11) KOLODNY, G. M., L. A. CULP and L. J. ROSENTHAL (1972). Secretion of RNA by Normal and Transformed Cells. *Exptl. Cell Res.*, *73*, 65-72.
12) KORNBERG, A. (1961). The Enzymatic Synthesis of DNA, pp. 21-26. London: John Wiley & Sons, Inc.
13) LIEBERMAN, M. W., R. N. BANEY, R. E. LEE, S. SELL and E. FARBER (1971). Studies on DNA Repair in Human Lymphocytes Treated with Proximate Carcinogens and Alkylating Agents. *Cancer Res.*, *31*, 1297-1306.
14) MARMUR, J. (1961). A Procedure for the Isolation of Deoxyribonucleic Acid for Microorganisms. *J. Mol. Biol.*, *3*, 208-218.
15) OLSEN, I. and G. HARRIS (1974). Uptake and Release of DNA by Lymphoid Tissue and Cells. *Immunology*, *27*, 973-987.
16) ROGERS, J. C., D. BOLDT, S. KORNFELD, A. SKINNER and P. VALERI (1972). Excretion of Deoxyribonucleic Acid by Lymphocytes Stimulated with Phytohemagglutinin or Antigen. *Proc. Natl. Acad. Sci. U.S.*, *69*, 1685-1689.
17) —— (1976). Characterization of DNA Excreted from Phytohemagglutinin-stimulated Lymphocytes. *J. Exp. Med.*, *143*, 1249-1264.
18) SARMA, D. S. R. and J. ZUBROFF (1973). Synthesis and Fragmentation of DNA in Phytohemagglutinin Stimulated Human Peripheral Blood Lymphocytes. *Immunol. Commun.*, *2*, 277-285.
19) SCHMIDT, G. and J. THANNHAUSER (1945). A Method for the Determination of Deoxyribonucleic Acid and Phosphoproteins in Animal Tissues. *J. Biol. Chem.*, *161*, 83-89.

20) SHARMA, P. R. (1974). Studies on the Mechanism of Action of 2-C-methylenemyo-inositol oxide. University of Geneva. Thesis No. 1654, pp. 19-21. Geneva: Edition Médecine et Hygiène.
21) STROUN, M. (1971). On the Nature of the Polymerase Responsible for the Transcription of Released Bacterial DNA in Plant Cells. *Biochem. Biophys. Res. Commun.*, *44*, 571-578.
22) —— and P. ANKER (1971). Bacterial Nucleic Acid Synthesis in Plants following Bacterial Contact. *Mol. Gen. Genet.*, *113*, 92-98.
23) —— and P. ANKER (1972). *In vitro* Synthesis of DNA Spontaneously Released by Bacteria or Frog Auricles. *Biochimie*, *54*, 1443-1452.
24) —— and P. ANKER (1973). Transcription of Spontaneously Released Bacterial Deoxyribonucleic Acid in Frog Auricles. *J. Bacteriol.*, *114*, 114-120.
25) —— P. ANKER, P. GAHAN, A. ROSSIER and H. GREPPIN (1971). *Agrobacterium tumefaciens* Ribonucleic Acid Synthesis in Tomato Cells and Crown Gall Induction. *J. Bacteriol.*, *106*, 634-639.
26) —— P. ANKER, P. B. GAHAN and J. HENRI (1977). Spontaneous Release of Newly Synthesized DNA from Frog auricles. *Arch. Sc. Genève*, *30*, 229-241.
27) SUGINO, A., S. HIROSE and R. OKASAKI (1972). RNA Linked Nascent DNA Fragments in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.*, *69*, 1863-1867.
28) TEMIN, H. M. (1970). Malignant Transformation of Cells by Viruses. *Perspectives Biol. Med.*, *14*, 11-26.
29) —— and S. MIZUTANI (1970). RNA-dependent DNA Polymerase in Virions of Rous Sarcoma Virus. *Nature*, *226*. 1211-1213.