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INTRODUCTION

Having for a goal to study the syntheses and degradations of nucleic acids in the course of the germination and sporulation of B. subtilis, it seemed necessary to us to proceed first of all with the study of different types of ARN in the mature spore itself. If in fact numerous works treat the composition of sporal envelopes or of catabolic enzymes present in the spore (bibliography in Murrell, 1961 (7) and in Halvorson, 1962 (5), the composition in ARN of this one was relatively little studied. The interest in such studies comes from the fact that, if the spore is metabolically inert, it is, apart from its germination, rapidly capable of taking up macromolecular syntheses, protein in particular. Thus the question posed is to know if certain factors of protein synthesis are present in the spore, and which ones, and how do those that would be absent from it reappear at the time of germination.

One knows that, relative to vegetative forms, spores are poor in ARN [(Fitz-James, 1955 (4)]. Woese and coll. (1960 (9)) have demonstrated, by analytical centrifugation, the presence of ribosomes in the spores, that is, ribosomes 50 S and 70 S, and the absence of ribosomes 30 S and 100 S; they also observed that the majority of ARN in the spore is under an undefined shape. The analysis, by centrifugation prepared in drop of sucrose, raw extracts of spores uniformly marked by uracil-^{14}C or by ^{32}P, has also shown that the majority of ARN sediment spores, even in the presence of magnesium at $10^{-2}M$, at less than 30 S (Balassa, 1963 [2]). In this article we will study more precisely the nature of these ARN.

In former analyses carried out by centrifugation in drop of sucrose of purified ARN (Balassa, 1963 [1]) led us to conclude that the sporal ARN

are composed of soluble ARN (4 S), of ribosomal ARN (16 and 23 S), representing only a small fraction, and of an important fraction of polydispersed ARN, distributed around 8 S, and that could represent, we would say, either "forerunners or derivitives of ribosomal ARN", or "a family of stable ARN messengers" (Balassa, 1963 [1]), ulterior studies having besides rendered the first interpretation more probable (Balassa, 1963 [2]). Later on, however, Doi and Igarashi (1964 [3]), utilizing another method of extraction and analyzing ARN by chromatography on columns of methylated albumin, found only ribosomal and soluble type ARN.

These results suggest that our preparations had been partially diminished; we have thus decided to re-examine the nature of sporal ARN.

MATERIAL AND METHODS

PREPARATION OF THE SPORES. --The mutant Ind^-_{168} of the Marburg rootstock of Bacillus subtilis was utilized. The sporulated cultures were obtained in a complex medium (Schaeffer and coll., 1963 [8]) : for 1 liter of medium : nutritive bouillon Difco, 8 g; $MgSO_4, H_2O$, 0.25 g; KCl, 1 g; pH ajusted to 7.1-7.5. After autoclavage, one adds $Ca(NO_3)_2$ for 10^{-2}M; $MnCl_2$ for 10^{-5}M and $FeSO_4$ for 10^{-6}M. The spores were treated with lysozyme (Sigma, 100 ug/ml) in presence of versene 10^{-2}M, centrifuged, washed three times in distilled water, put back into suspension again in physiological water, heated from one to three hours at 60^0 and conserved at 4°C or at -20^0 C. In order to avoid eventual contaminations, the suspensions of spores are heated again at 80° for ten minutes before their use. Several stocks of spores thus were prepared; they were utilized in the three months.

RADIOACTIVE SPORES. -- In order to obtain radioactive spores, uracil-^{14}C (0.5 uCi/ml, 10 mCi/mM) or of phosphate-^{32}P (30 uCi/ml) were added to cultures in exponential growth, still being able to carry out at least four doublings before the beginning of sporulation.

EXTRACTION OF ARN. -- Washed spores, resuspended in acetate tampon (sodium acetate, 0.01 M; NaCl 0.1M at pH 5.0) are mechanically grinded. A mixture of 20 ml in spore suspension, in acetate tampon enclosing 0.5 p. 100 of duponol and 200 ug/ml of polysulfate of dextrane with 10 ml of phenol at 90 p. 100, 2 ml of suspension at 2 p. 100 of activated bentonite and 30 g of glass balls (diameter 0.11-0.12 mm), is agitated for three minutes in a vibrator apparatus (B. Braun Apparatebau, Melsungen, Germany), in refreezing by detent of CO_2. The grinded material, additioned by 20 ml of phenol and by 10 ml of acetate tampon is energetically agitated for forty minutes at 2° C before gathering up the watery phase by centrifugation.

ARN is precipitated two times by the addition of two volumes of alcohol at --20°C, dissolved in acetate tampon, treated with activated bentonite (200 ug/ml), centrifugated again and finally congealed. Certain extracts undergo, before the addition of bentonite, a treatment by DNase (Worthington, 10 ug/ml, in tris tampon 0.025M at pH 7.0, in the presence of $MgSO_4$ 10^{-2}M, for an hour at 0°).

The method described here is essentially that which we have formerly utilized (Balassa, 1963[1]), however supplementary precautions are taken to avoid any degradation of ARN. Besides the addition of dextrane polysulfate, it appears to us important on the one hand to freeze very hard the suspension in the course of grinding and, on the other hand, to carry out the purification of ARN very rapidly beginning with the not too concentrated solutions, after a very energetic agitation in the presence of phenol. The method of Doi and Igarashi (1964[3]) differs from ours by the mode of grinding the spores and by the presence, in the course of grinding, of magnesium (5.10^{-2}M).

ANALYSIS OF ARN. -- The purified ARN are analyzed by centrifugation in drop of sucrose (5 to 20 p. 100, in acetate tampon). The samples deposited on the gradiant are centrifuged eighteen hours at 22 500 t/min. in a rotor Spinco SW 25. After piercing the bottom of the tubes, ARN in totality is measured in the fractions gathered up by its absorption at 260 mu and radioactivity is measured after precipitation in cold TCA at 5 p. 100. In certain experiments a part of each fraction is treated by the RNase (Worthington, 10 ug/ml, thirty minutes at 37°).

Soluble ARN is purified as described in the text, its acceptance activity of amino acids is measured according to Monier (1962[6]), with the help of a supernatant from a fresh extract of B. subtilis in exponential growth.

RESULTS

1 SEDIMENTATION OF ARN. --- While the method of extraction previously utilised (Balassa, 1963[1]) would give up polydispersed preparations of ARN, the improved technique described here above, conducted by "classical" profiles of sedimentation showing 3 picks corresponding to ribosomal ARN (23 S and 16 S) and soluble (4 S). From such profiles was obtained after grinding, with or without addition of eroding bacterii, small quantities of uniformly marked spores at ^{32}P (fig. 1) or at uracil-^{14}C (fig. 2). The radioactivity of each fraction can be entirely solubilized by RNase : it is well incorporated in ARN. From other preparations of ARN, originating from the grinding of more important quantities of non-radioactive spores and analyzed by their absorption at 260 mu, presented profiles of analogous sedimentation profiles (fig. 3). One must thus conclude that the sporal ARN are constituted, in majority if not in totality, by ribosomal

and soluble ARN.

According to Doi and Igarashi (1964-3), the presence of magnesium in very high concentrations (5.10^{-2}M) is required during the grinding of spores. The preparations of figures 2 and 3 were obtained in the absence of magnesium, that of figure 1 in its presence. Other experiments show also that, in a general way, the presence of magnesium during grinding does not change the sedimentation of obtained ARN. However, in certain

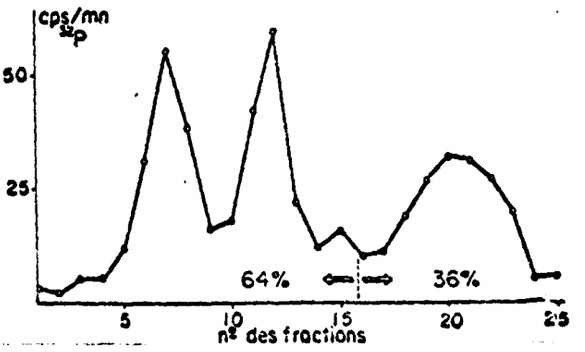

FIG. 1. --- Profile of sedimentation of ARN of uniformly marked spores to ^{32}P.
The extraction of ARN and centrifugation conditions are described in the text.

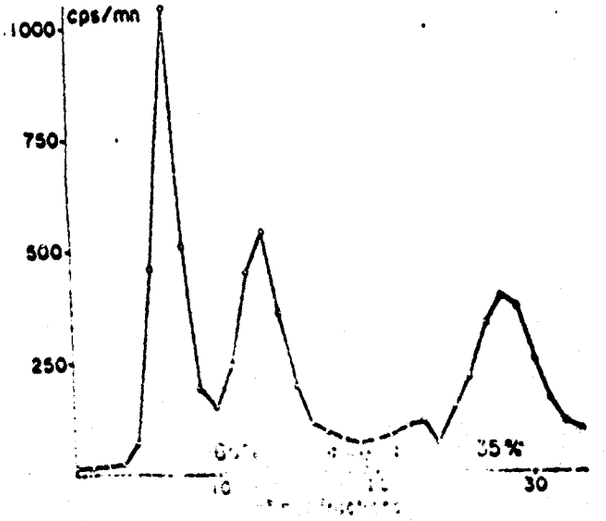

FIG. 2. ---Profile of sedimentation of ARN of spores uniformly marked with uracil-^{14}C.

experiments, ARN with profile of "polydispered" sedimentation, or presenting a supplementary pick between 6 and 12 S, were obtained, while ARN prepared parallely, but after grinding in presence of 5.10^{-2} M of magnesium acetate, showed a more classical profile. It seems thus that the presence of Mg can hinder the partial degradation of sporal ARN, if these are not sufficiently protected during their extraction.

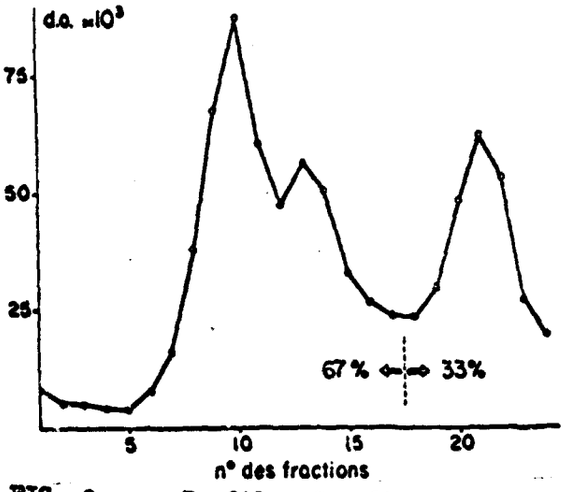

FIG. 3. --- Profile of sedimentation of ARN of spores, measured by their absorption at 260 mu.

2 The SOLUBLE ARN. --- In the preparations of sporal ARN that we have just described, about 35 p. 100 of ARN are found in pick 4 S corresponding to soluble ARN (fig. 1-3). Even in "polydispersed" preparations, the presence of soluble ARN can be brought to light, either by precipitation in NaCl 1M, or by chromatography on methylated albumin column (Balassa, 1963[2]; Doi and Igarashi, 1964[3]). In order to identify without ambiguity this fraction with transfer ARN, a functional test was carried out (in the laboratory of M. R. Monier).

A preparation of ARN was obtained by the method described. The presence in the extract of spores from a viscuous substance, presumably muco-peptidic, and a partial degradation of this preparation are doubtlessly responsible for the profile of polydispersed sedimentation and the weak yield obtained. The fraction, soluble in NaCl at 1 M, was purified by chromatography on DEAE-cellulose (Monier, 1962[6]).

When 300 ug of ARN sporal and soluble were incubated with a chloral hydrol-eat marked at ^{14}C and an enzymatic preparation originating from

B. subtilis in growth, 1 800 cps/min. were counted after precipitation under cold by the TCA at 5 p. 100. The figure corresponding in a control experiment carried out with soluble ARN of vegetative bacterid were 3 900 cps/min. Sporal soluble ARN possesses thus at least 40 p. 100 of the activity of soluble ARN from bacterid; taken into account the observed degradation, its initial activity could attain 100 p. 100.

DISCUSSION

When the ARN spore extracts of B. subtilis are analyzed by centrifugation on gradiant of sucrose, the obtained profiles put into evidence only ARN having sedimentation constants of 4, 16 and 23 S. When supplementary picks are observed, having neighboring constants of 8 S, they are due to a partial degradation of ribosomal ARN in the course of extraction; we are adopting finally on this point the opinion of Doi and Igarashi (1964[3]). These ARN, that represent 65 p. 100 of the total, have the same composition in basics as their homologues of vegetative bacterid (Doi and Igarashi, 1964[3]); however, they are not associated, in the spore, to normal ribosomal particles (Woese, 1960[9]), and perhaps it is necessary to see therein the cause of their particular sensitivity to degradation. A commentary imposes itself on the behavior, previously described, of partially degradated preparations on methylated albumin columns (Balassa, 1963[2]). While centrifugation showed a maximum of around 8 S, the majority of ARN of such a preparation was elected in two picks only around ribosomal ARN 16 S. This abnormal behavior is explained by the attachment, in high concentration of salt, of partially degradated ARN to normal ribosomal ARN, originating from vegetative bacterid added before the extraction (Hayes, personal communication).

35 p. 100 of ARN are represented by transfer ARN, characterized by their solubility in NaCl 1M, their sedimentation constant, their behavior on methylated albumin column and their biological activity. This proportion, very close to that found by Doi and Igarashi 3 (30 p. 100), is two times higher than in vegetative bacterid; we will return, in an article devoted to sporulation, on the mechanism of this enrichment.

Finally, neither the sedimentation profiles of non degradated preparations of sporal ARN, nor the trials of hybridation of this ARN with the denaturized bacterial AIN put into evidence the existence of a fraction of carrier type (Doi and Igarashi, 1963[3]; Balassa, 1963[2]).

RESUME

The results concerning the nature of RNA present in the spore dependont on the method of extraction, several methods were compared. One arrives at the following conclusions:

1. 35 % of the sporal RNA, as judged by physical characteristics (4 S) and function, is transfer RNA (the corresponding figure for bacteria is 17 %).

2. Ribosomal RNA (16 S and 23 S) constitutes 65 % of the sporal RNA. While in the vegetative form all of the ribosomal RNA is present in ribosomes, in the spore only about 5 % of the ribosomal RNA exists in this form, the remaining being present in a non-particulate form, highly sensitive to degradation.

3. The presence of an RNA of messenger type has not been revealed by sedimentation analysis or by hybridation tests.

* * *

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