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MACROMOLECULAR SYNTHESES IN THE COURSE OF GERMINATION OF B. SUBTILIS SPORES

II. -- REGULATION

by G. BALASSA and G. CONTESSE

(Microbic Physiology Service,
Institute of Physico-Chemical Biology
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INTRODUCTION

In a previous article (5) cytological and bio-chemical events, characteristic of the germination of spores in complex medium, were described. kinetic peculiarities of the synthesis of ARN and of proteins incited us to specify the regulation mechanisms of these syntheses, by studying the effect of amino acids on the one hand, of chloramphenicol on the other, on the synthesis of these macromolecules.

MATERIAL AND METHODS

The rootstock utilized is still the mutant Ind-$_{168}$ of the Marburg rootstock from Bacillus subtilis. The preparation of spores, the complex medium and the methods utilized were described elsewhere (4, 5). The minimum medium of germination is the M 63 medium (for 1 liter : KH_2PO_4, 13.6 g; $MgSO_4$ $7H_2O$, 0.2 g; $(NH_4)_2SO_4$ $7H_2O$, 2 g; $FeSO_4$ $7H_2O$, 0.005 g, ajusted to pH 7.2 by KOH), completed by L-tryptophane (50 ug/ml), by L-alanine (50 ug/ml), and after sterilization, by glucose (at 0.5 %). In certain experiments with the incorporation of uracil or of radioactive valine, these non marked substances were added from the beginning of germination (5 ug/ml of each) in order to avoid changing the composition of the medium in the course of the experiment. For the incorporation of ^{32}P, the concentration was reduced from it to 10^{-3} M, the medium being plugged by Tris (10^{-2} M to pH 7.2).

Several mixtures of amino acids were used. One, of 18 amino acids, contained all of the amino acids except the two already present; in the

other, of 17 amino acids, valine was omitted, finally a third, of 9 amino acids, contained: arginine, aspartate, glutamate, histidine, isoleucine, leucine, methionine, phenylalanine and proline. The two first ones have generally always been added for 10 ug/ml of each amino acid, the last one for 20 ug/ml. The amino acids (L shape, for analysis) originate from Sigma or from Nutritional Biochemical Co. Chloramphenicol (Hoffmann-Laroche) was utilized at a concentration of 100 ug/ml.

RESULTS

I. --- EFFECT OF AMINO ACIDS ON GERMINATION

1 GERMINATION IN SYNTHETIC MEDIUMS. --- In the previous article, germination was studied in complete medium, that is to say in optimal conditions that end in its evolution at its maximum speed. This study is completed here by the analysis of germination in different synthetic mediums and by research of the role that various substratum can play there, not indispensable to growth.

First of all we have followed the changes of the optical density of a suspension of spores in the course of germination in synthetic mediums of various compositions.

In the minimum medium (containing glucose, L-alanine and tryptophane) the initial phase of germination manifests itself normally by a drop in the optical density and by the loss of the spores' refractivity, but the active phase (5) doesn't take place: the bacteria do not emerge from sporal layers and the optical density remains constant for seven hours (fig. 1). In the same medium, containing the 18 amino acids however, the active phase goes on normally and leads to the return to growth. The stimulation of the active phase by the 18 amino acids is the same, whether these be present, each at a concentration of 5 or of 50 ug/ml. The mixture of 9 amino acids (see METHODS) gives a comparable or slightly inferior stimulation (fig. 1a and b). On the contrary the addition of amino acids by groups of 3 or of 4 allows only a very slow germination, whose speed depends upon the amino acids chosen (fig. 2). Arginine, glutamic acid and aspartic acid are the exception, however: the addition of one of them provokes a notable stimulation (fig. 1 b), the mixture of aspartate + glutamate has an even more marked effect; finally the aspartate + glutamate + arginine + asparagine + glutamine mixture is sometimes almost as efficacious as the mixture of 18 amino acids. Woese (25) described an important stimulation by aspartate and glutamate; he observed however (personal communication) variable responses with the preparation of spores employed. In conclusion, no amino acid is thus specifically required for germination, but each one of them possesses its own capacity of accelerating it.

GERMINATION OF SPORES. --II. REGULATION

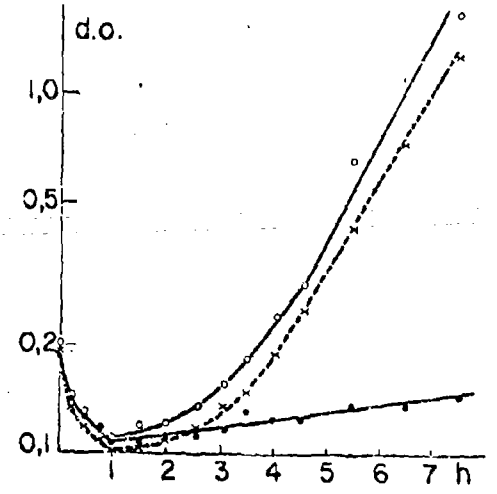

Fig. 1 a.

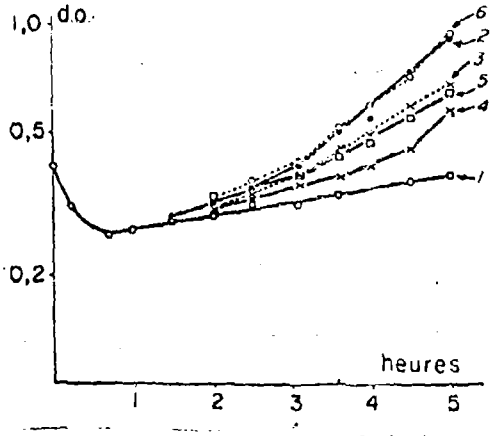

Fig. 1 b.

Fig. 1. --- Effect of amino acids on germination.
The evolution of the optical density of cultures is followed in the course of the germination of spores in minimum medium without addition (a —•—, b 1) or containing the following amino acids : 18 amino acids (a —○—, b 2), 9 amino acids (see METHODS: a --- X---, b 3), glutamate (b 4), glutamate + aspartate (b 5), glutamate, aspartate, arginine, glutamine and asparagine (b 6). During the ninety first minutes all of the curves come together. The addition of aspartate or of arginine alone gives the same results as that of glutamate.

Other experiments show that glucose is required for the accomplishment of the active phase itself in the presence of all of the amino acids. If it is omitted or replaced by citrate, germination is very much slowed down; if it is replaced by glycerol, it is done with a half-hour of retard. In other respects, in the presence of glucose and amino acids, the addition of four principal bases of ARN, of citrate or of acetate does not change the speed of germination.

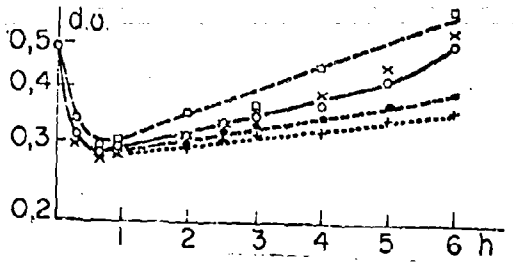

Fig. 2. --- Effect of certain amino acids on germination.
Germination in minimum medium, containing 20 ug/ml of the following amino acids : —O— his, tyr and phe; —x— thr, pro, met and cys, ---O--- leu, lys and ser, ---+--- val, gly, ser and isoleu. In another culture ---□--- , containing all of the amino acids, glucose was replaced by sodium citrate.

2 HETEROGENEOUS GERMINATION OF SPORES IN ABSENCE OF AMINO ACIDS. --- As we have seen, after a period of latency from at least four to six hours, the optical density of a suspension of spores begins to augment even in absence of amino acids (fig. 1 a). In order to determine if it is a question of a slow germination retarded by all of the spores, or else if a small fraction of spores can enter into the active phase in absence of amino acids, a suspension of heated spores was stretched out on three mediums containing agar : the minimum medium, with or without the addition of amino acids, and the complex medium. The number of colonies (table ?) identical on the complex medium and on the minimum medium containing the amino acids is, on the contrary, ten times weaker than the minimum medium. Thus, although the incapacity to germinate without amino acids is permanent in the majority of spores, a certain fraction (about 10 % of spores) can escape there, however.

GERMINATION OF SPORES. --- II. REGULATION

TABLE I. --- Heterogeneous Germination of spores

Origine des spores :	Milieu d'étalement		
	Complexe	Minimum + acides aminés	Minimum
	Nombre de colonies		
Milieu complexe	6.10⁸	5,5.10⁸	7,6.10⁸
Milieu synthétique :			
carence de carbone	1,3.10¹⁰	1,0.10¹⁰	1,9.10⁸
carence d'azote	1,3.10¹⁰	1,3.10¹⁰	3,1.10⁸

3 SYNTHESIS OF ADN, OF ARN AND OF PROTEINS IN THE COURSE OF GERMINATION IN SYNTHETIC MEDIUM. --- Before finding out which is the biochemical mechaniam of the effect of amino acids on germination, the synthesis of these macromolecules was followed in synthetic medium, with or without the addition of 18 amino acids, and compared to that observed in complete medium (5). In the presence of amino acids (fig. 3 a and b), the kinetics observed recall those described in complex medium; the synthesis of ARN begins immediately after the initial phase and and still preceeds here that of proteins from about ten to twenty minutes. That of ADN (not represented) begins around one hundred minutes, and growth becomes exponential only towards the one hundred twentieth minute. As in the complex medium (see the preceeding article), we have measured the rates of incorporation of uracil and of radio-active valine, added at different times of the germination. The relationship of rates of synthesis of ARN to rates of synthesis of proteins diminishes at the beginning of germination, then passes by a minimum before stabilizing itself; this confirms the displacement between the two syntheses.

If the germination takes place in the absence of amino acids, the speeds of incorporation of uracil and of valine are first of all, for twenty to forty minutes, the same as in the presence of amino acids, the initial setback of the incorporation of valine is still observed here. Next, these incorporations, weaker than in the presence of amino acids, remain linear for more than three hours (fig. 3 b), while the relationship of rates of incorporation of uracil and of valine oscillates around minimum values, measured in the medium containing amino acids.

In conclusion, independently of the presence of amino acids, the synthesis of ARN begins, at a very weak rate, before that of proteins, and the two syntheses are accelerated for about thirty minutes. Next

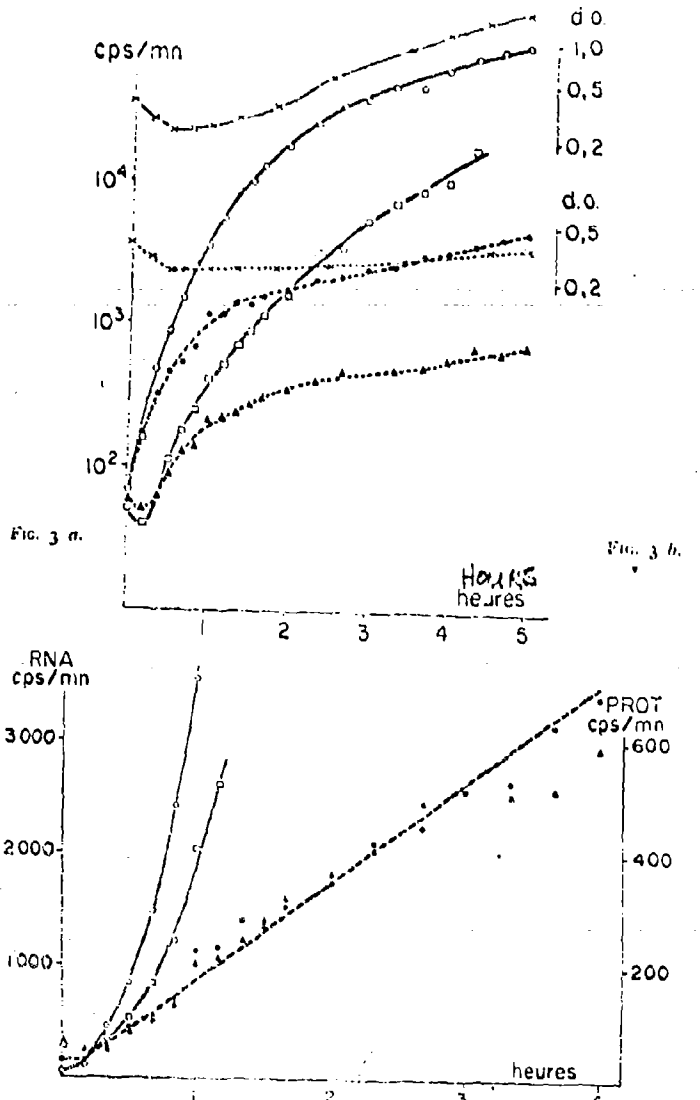

Fig. 3 a.

Fig. 3 b.

Fig. 3. --- Synthesis of ARN and of proteins.
a) Incorporation of uracil-^{14}C (○,●) and of valine-^{14}C (△,□) is measured in the course of germination in minimum medium with (—) or without (---) the 18 amino acids. Y = optical density. b) The same results are represented in linear coordinates.

they are made, in absence of amino acids, only at a constantly weak speed, without augmentation of optical density nor emergence of bacteria.

4 SYNTHESIS OF ARN AFTER TRANSFER IN POOR MEDIUM ("shiftdown"). --- The control exercised by amino acids on the synthesis of ARN was revealed in three highly studied situations in bacteria in growth; either in the course of a deficiency in an auxotrophic mutant in its required acid, or after transfer of the bacteria from a medium rich in amino acids to a minimum medium or vice-versa (reviews in 10, 18). From such transfers, to which we will conserve their English designation of shift-down and shift-up, respectively, were carried out in the course of germination.

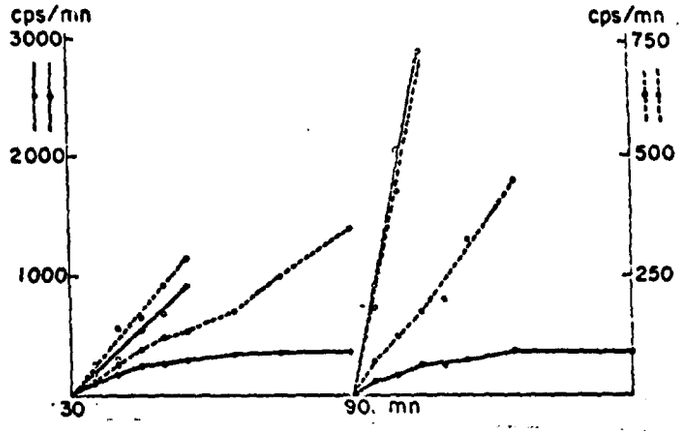

Fig. 4. --- Snythesis of ARN and of proteins after transfer in poor medium (shift-down).

Spores in germination for thirty or ninety minutes in minimum medium containing the 17 amino acids are filtrated and resuspended in the same medium with (O) or without (C) the amino acids. The incorporation of uracil-^{14}C (----) and of valine-^{14}C (—) is measured.

If one transfers bacteria, at 30 or at 90 min. of germination, from a medium containing all of the amino acids, to a medium that was deprived of them (shift-down), one declares, as with the bacteria in growth, that the synthesis of ARN, immediately slowed down, is stopped rapidly, while that of proteins diminishes only gradually (fig. 4). The inhibition of synthesis of ARN is less noticed at 30 min. than at 90 min.

5 EFFECT OF THE ADDITION OF AMINO ACIDS (" shift-up"). ---
We have seen that in the absence of amino acids the germinated spores incorporated uracil at a weak rate, constant for hours. The addition of amino acids doubles immediately the rate of synthesis of ARN (fig. 5). This initial stimulation is carried out in less than a minute, it also takes place in the presence of chloramphenicol. Next, the synthesis of ARN is rapidly accelerated just as in the experiments where germination goes on entirely in the presence of amino acids. However, this last acceleration is not observed in the presence of chloramphenicol. These

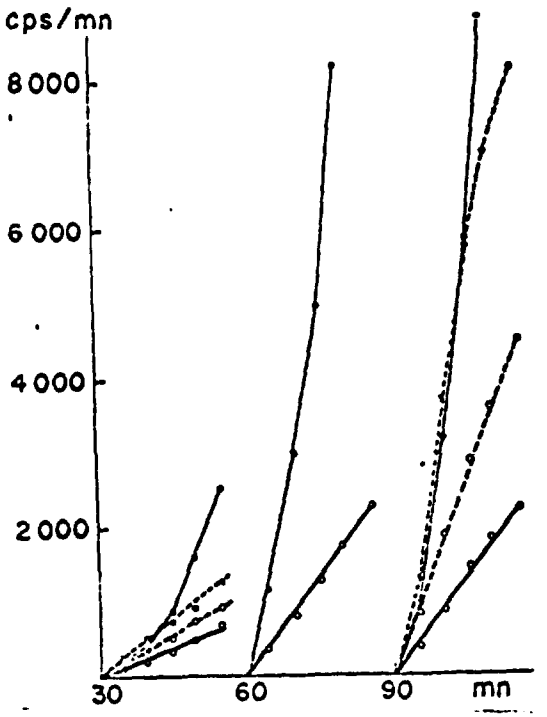

Fig. 5. --- Synthesis of ARN after transfer in rich medium (shift-up).
Spores in germination in minimum medium receive, at the indicated time, uracil-^{14}C alone (O), with a mixture of 17 amino acids (●), with (---) or without (-) chloramphenicol.

experiments indicate that : 1 the synthesis of ARN is immediately stimulated by the amino acids, even in the absence of proteic syntheses; 2 the initial stimulation, although relatively weak (two times more), permits, however, after the formation of new proteins, the acceleration of all of the syntheses and the normal germination of bacteria. The well-known control of synthesis of ARN by amino acids is thus revealed also in the course of germination.

6 GERMINATION OF SPORES OBTAINED IN SYNTHETIC MEDIUM. --- In the preceeding experiments, the spores utilized were obtained in complex medium, rich in amino acids. Now, it is known that the composition of bacteria (in particular their content in biosynthesis enzymes of amino acids) and consequently their reaction to transfer in a poor medium, depend on their origin; one thus had to ask himself if the effect observed of amino acids on germination was independent of the sporulation medium. Experiments were repeated with spores formed in an exhausted minimum medium either in glucose, or in azote. They showed that the effect of amino acids on the evolution of the optical density and the synthesis of ARN does not depend on conditions in which the spores are formed. The fraction of spores, capable of forming colonies on minimum medium (table I), is also the same in the three lots of spores.

7 ABSENCE OF DEVELOPMENT IN MINIMUM MEDIUM. --- We have just seen that in minimum medium the active phase of germination does not take place, despite a weak synthesis of ARN and of proteins. Although incapable of assuring germination, these syntheses could nevertheless permit, after the addition of amino acids, an ulterior accelerated germination. Nothing of the kind however: if one follows the evolution of the optical density (fig. 6), or that of syntheses of ARN and proteins (fig. 7), one declares that even a preincubation which is prolonged (one hundred twenty minutes) in absence of amino acids does not accelerate ulterior germination. (A weak acceleration after an incubation of thirty minutes is explained by the fact that the initial phase is accomplished, in a certain number of spores, only during this incubation). Woese (25) ended in similar conclusions.

II. --- EFFECT OF CHLORAMPHENICOL ON SYNTHESIS OF ARN.

1 STUDIES IN COMPLEX MEDIUM. --- Specific inhibitor of the synthesis of proteins, chloramphenicol allows the study of the relations between synthesis of ARN and that of proteins. In this goal, we have utilized it in the course of germination.

Synthesis of proteins (measured by the incorporation of valine-^{14}C in the fraction, insoluble in TCA at 5 %) is inhibited at least at 95 %

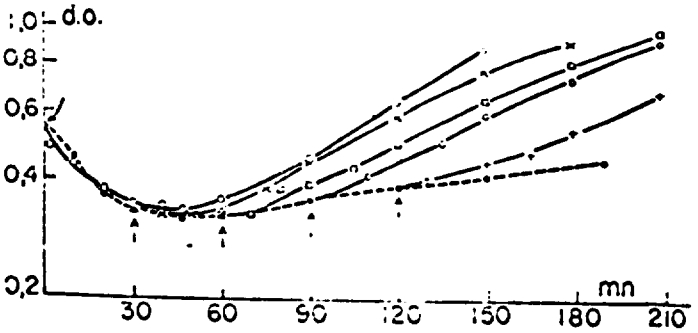

Fig. 6. --- Effect of a retarded addition of amino acids on germination.

To spores in germination in minimum medium one adds a mixture of 17 amino acids (---○---trial). Addition after zero (○), thirty (x), sixty (□), ninety (○) and one hundred twenty minutes (÷) of incubation.

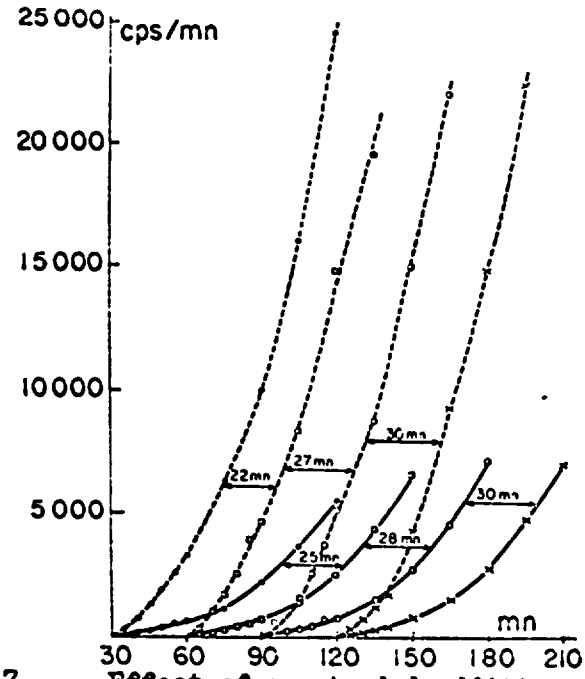

Fig. 7. --- Effect of a retarded addition of amino acids on ARN synthesis.

Germination as in experiment of figure 6. Uracil-^{14}C (---) or of valine-^{14}C (—) are added, at the same time as the amino acids, after thirty (○), sixty (□), ninety (○) and one hundred twenty minutes (x) of incubation. The figures indicate the displacement of curves, in minutes.

by chloramphenicol, added in the course of germination. As for ARN, added at the zero cycle, the chloramphenicol allows a synthesis of weak and linear ARN to exist (fig. 8). When it is added later, but still prematurely, a weak quantity of ARN is formed at the same rate as in the absence of antibiotic, then the synthesis is slowed up brusquely, and continues at a reduced rate. Three parameters of synthesis of ARN can thus be determined for each time of addition of chloramphenicol : 1 the initial linear rate; 2 the quantity of ARN synthetized at this rate (measured by the "fracture level" of curves); and finally 3 the rate of

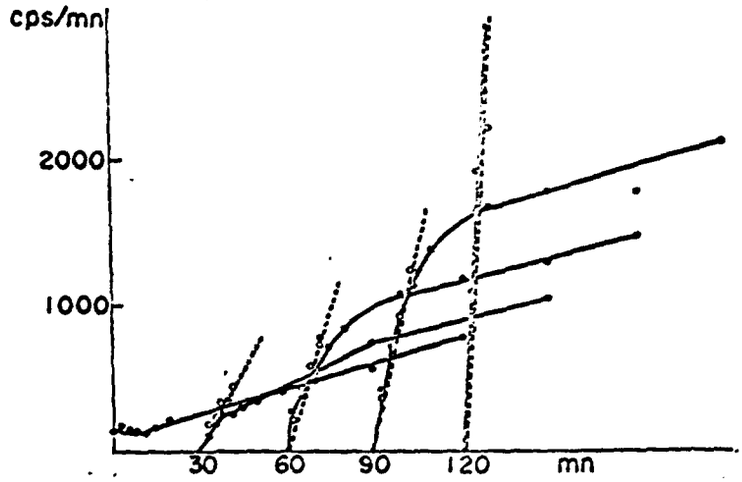

FIG. 8. --- Synthesis of ARN in presence of chloramphenicol.

Germination in complex medium. In the times indicated, uracil-^{14}C is added alone (---o---) or with chloramphenicol (-c-).

residual synthesis, constant for several hours. One sees (fig. 8) that the "fracture level" augments rapidly in the course of germination, on the contrary the residual rate, established in the presence of chloramphenicol, is the same as the antibiotic added at zero cycle or at ulterior cycles, and augments only if the addition of chloramphenicol is very late.

It is probable that the "fracture level" reflects the quantity of a supposed ribosomal protein, present at the time of addition of the antibiotic and utilized in the course of the synthesis of ARN (see DISCUSSION).

The following experiment is destined to show the exhausting of this protein in the presence of chloramphenicol. A culture is exposed to the antibiotic between 60 and 90 min. of germination, this duration being sufficient so that the synthesis of ARN attains its "fracture level" (see fig. 8). It is then filtrated, washed, resuspended and divided into several portions. The one serves in observing the resumption of the synthesis of ARN, to the others chloramphenicol is added again after various times of incubation (fig. 9). The curves thus obtained recall those of figure 8; they show that the capacity of bacteria to accumulate ARN in the presence of chloramphenicol, reduced in the course of incubation to thirty minutes with the antibiotic, reappears when the bacteria are resuspended in the new medium, and augments as in the spores at the beginning of their germination.

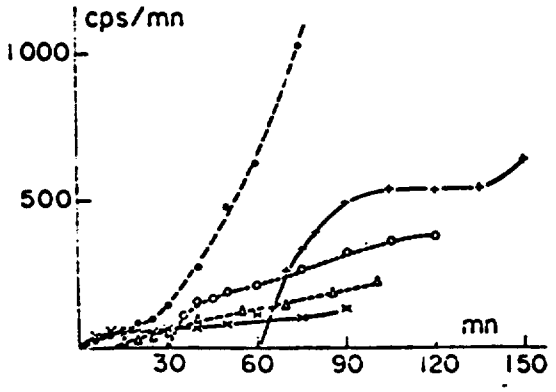

FIG. 9. --- Treatment repreated in chloramphenicol.

Germination in complex medium. At sixty minutes chloramphenicol is added to the culture, that is next filtrated at ninety minutes, washed and resuspended in the same volume of new medium. Uracil-^{14}C is added either alone, immediately after filtration (---○---), or with chloramphenicol, zero (x), fifteen (△), thirty (○) and sixty (+) minutes after filtration (time scale begins after filtration).

It is known that ARN, accumulated by bacteria in the presence of chloramphenicol, are metabolically unstable and degrade themselves, at least in certain conditions, when one takes away the antibiotic (21). This is equally true during germination; however, the kinetics of degradation are extremely variable according to the germination medium, the moment of the addition of chloramphenicol, the time of incubation and

the medium utilized after treatment. An example is given in figure 10 : spores, having germinated for ninety minutes in complex medium are exposed for thirty supplementary minutes to chloramphenicol in the presence of uracil-^{14}C; they are then filtrated, washed and resuspended in three different mediums : synthetic medium containing amino acids, complex medium, and finally complex medium containing actinomycine. In the three suspensions a great part of radioactive ARN initially present is degraded (let us note however that this degradation is always slower than that of messager ARN 5).

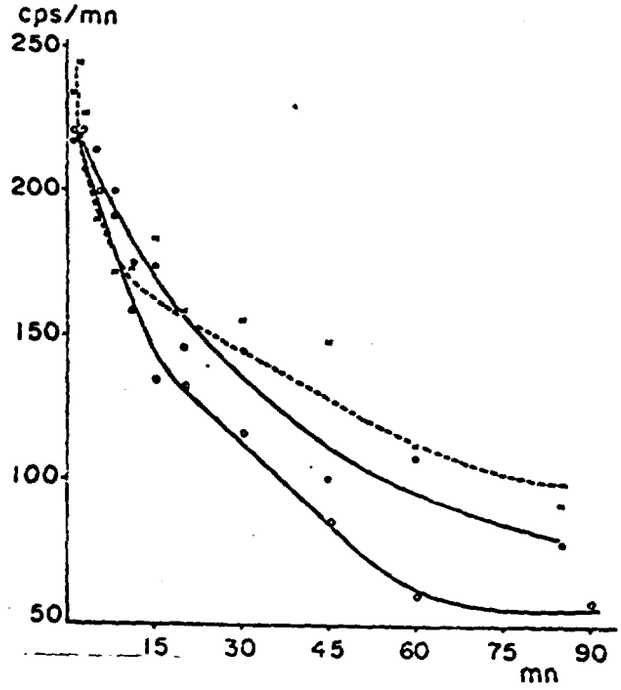

FIG. 10. --- Degradation of ARN formed in presence of chloramphenicol.
Germination in complex medium. At ninety minutes, uracil-^{14} and chloramphenicol are added together to the culture, that is next filtrated at one hundred twenty minutes, washed and re-suspended either in synthetic medium containing all of the amino acids (X), or in complex medium, with (O) or without (●) actinomycin (time scale begins after filtration).

2 STUDIES IN SYNTHETIC MEDIUM. --- The effect of chloramphenicol on the three parameters of synthesis of ARN was also studied in synthetic mediums. In the presence of amino acids, the curves (fig. 11) are qualitatively identical to those obtained in complex medium (fig. 8). On the contrary, if the amino acids are omitted, one observes a marked stimulation of the initial synthesis by chloramphenicol (fig. 5). Such a situation, already noted in bacteria in growth (20) is generally attributed to the effect of amino acids which are accumulated in the absence of proteic synthesis. Stimulation by chloramphenicol is weaker, besides, than that provoked by the addition of amino acids; it is all the more strong when the addition of antibiotic is later, which suggests that the capacity of synthetizing amino acids, weak at the beginning, augments during germination.

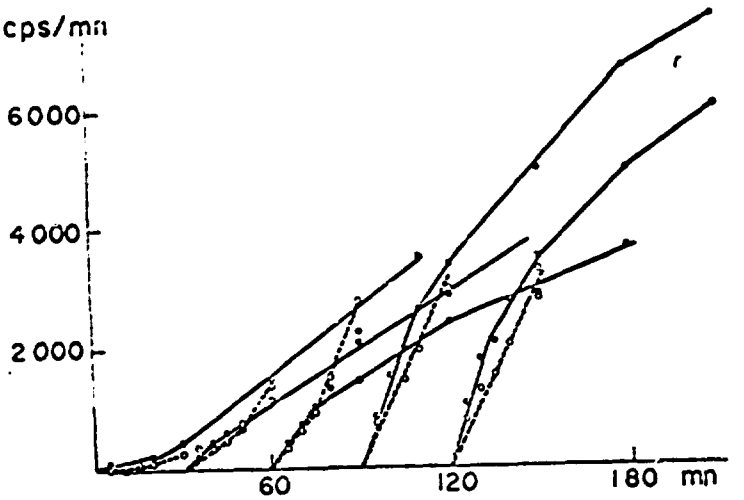

FIG. 11. --- Synthesis of ARN in minimum medium in presence of chloramphenicol (- -).

Germination in medium containing all of the amino acids. In the times indicated, uracil-^{14}C is added alone (---○---) or with chloramphenicol (-●-).

We finally asked ourselves how amino acids, added to the minimum medium at the same time as chloramphenicol, would affect the values of the three parameters in question. The experiment shows (fig. 12) that they augment all three.

3 REVERSION OF THE EFFECT OF CHLORAMPHENICOL. --- When chloramphenicol is added in the beginning of germination, it hinders all visible manifestations of the active phase, even though a slow but significant synthesis of ARN subsists. The situation recalls that of germination in

absence of amino acids. Again this time one can ask himself if this synthesis (or other reactions being carried out at the same time as it) permits to accelerate, once the antibiotic is taken away, the ulterior germination of the spore.

The experiment (fig. 13) shows that it is nothing of the kind : the incorporation curves of uracil and of valine are the same ones that the culture had been or not previously treated with chloramphenicol. That shows us moreover, that added at the beginning of germination, chloramphenicol does not have a toxic effect persisting on the ulterior biosynthetic capacities of treated bacteria.

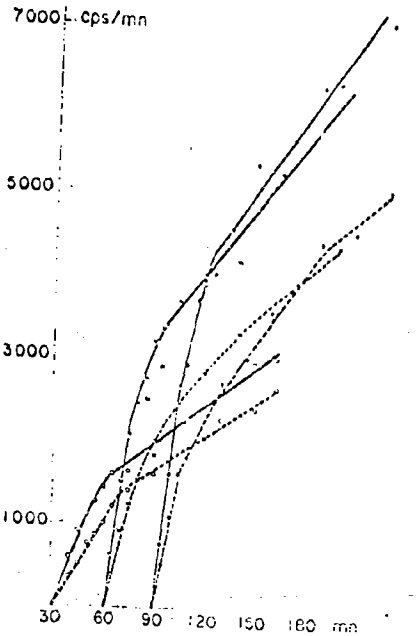

FIG. 12. --- Effect of amino acids on ARN synthesis in presence of chloramphenicol.
Uracil-^{14}C and chloramphenicol are added either alone (---), or with a mixture of 17 amino acids (—), at thirty (○), sixty (×) and ninety (⌀) minutes of germination in minimum medium.

On the contrary, if the antibiotic is added later, a toxic effect appears; after resuspension in fresh medium, synthesis of ARN and of proteins begins again, but slowly (fig. 14). This effect is accentuated with time; one knows besides that bacteria in the vegetative phase, treated with chloramphenicol, pass by a long latent period before continuing their growth. Let us notice finally that this toxic effect affects the synthesis of ARN and that of proteins almost in the same proportions.

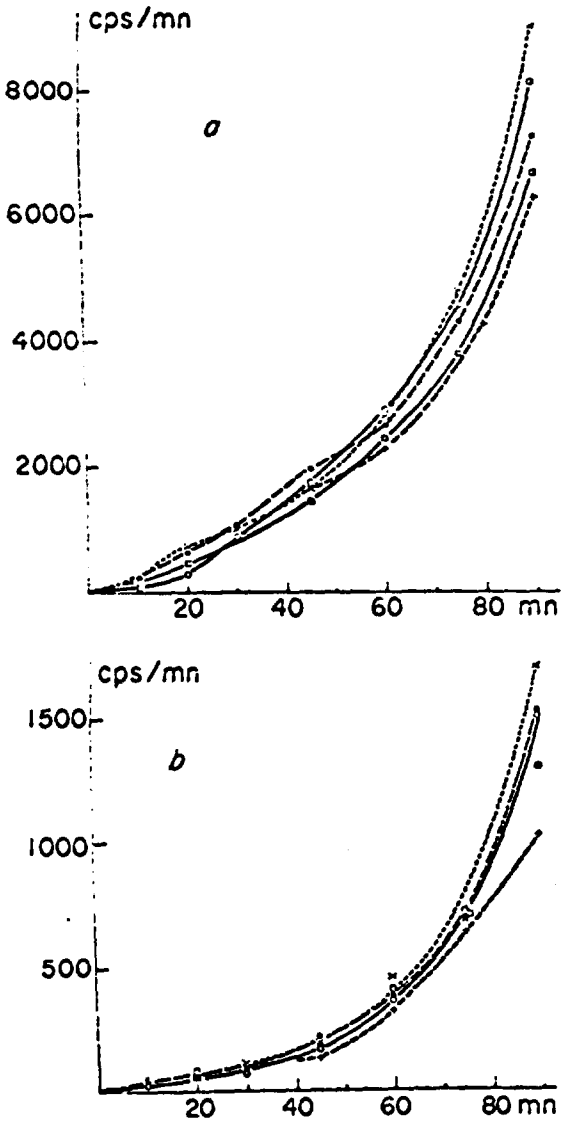

FIG. 13. --- Reversion of the effect from chloramphenicol : precocious treatment.
Spores are incubated in complex medium, in presence of chloramphenicol, for fifteen (○), thirty (x), sixty (□) or ninety (+) minutes, they are then filtrated, washed and resuspended in a complex new medium. (Test without chloramphenicol : ○.) After filtration, the incorporation of uracil-^{14}C (a) and of valine-^{14}C (b) is measured.

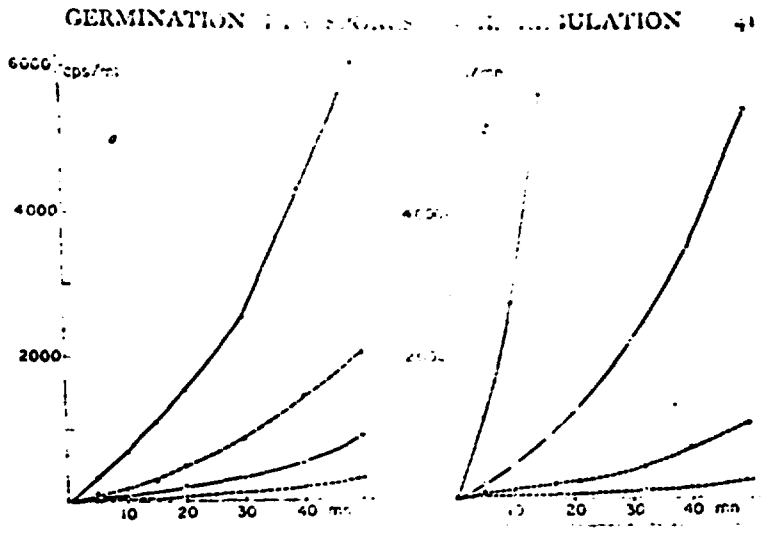

FIG. 14. --- Reversion of effect of chloramphenicol : tardy treatment.

Spores, germinated in complex medium for either thirty minutes (a), or seventy-five minutes (b), are next treated with chloramphenicol for thirty supplementary minutes, filtrated, washed and resuspended in a new complex medium. Incorporation of uracil-^{14}C (○) and of valine-^{14}C (x), added after filtration, is measured in the test cultures (—) and the treated cultures (----).

DISCUSSION

The rate of synthesis of ARN and proteins varies in the course of germination with time and with the composition of the medium. The question is raised thus to know what the mechanisms can be, susceptible of controling the speed of synthesis of these macro-molecules. We will envisage first the role played by the amino acids in ARN synthesis, next the effect of inhibition of proteic syntheses on the formation of ARN. We will try finally to analyze the relations between ARN syntheses and of proteins in the course of germination.

1 EFFECT OF AMINO ACIDS. --- Observations from numerous authors have shown that bacteria possess, during the active phase of germination, nutritive needs, different from those of the initial phase as well as from needs during exponential growth, and variable according to the type. These needs are most often satisfied by amino acids (see for example 23, 9, 25 and reviews in 17, 14). We have illustrated this need in

amino acids during the active phase of germination of B. subtilis. The observed effect does not arise from a need of some particular amino acid, even though each amino acid possesses a certain capacity to contribute to it, probably according to the facility with which it is converted into other amino acids. This implies that the spores are lacking or poor in certain enzymes of biosynthesis of amino acids. The differences concerning the stimulation by certain amino acids, according to the kinds or the spore preparations, could reflect variations in the content in these enzymes. The existence of 10 % of spores capable of germinating in the absence of amino acids is due seemingly to the presence of spores having conserved a greater quantity of these enzymes.

The effect of amino acids, we have seen, bears directly on the ARN synthesis, without the intermediary of new proteic synthesis. It appears thus that the particular nutritive needs of the active phase of germination can be attributed, at least in part, to the control exercised by the amino acids on the ARN synthesis. The hypothesis generally advanced to take into account the catalytic role of amino acids in ARN synthesis is that the non-charged soluble ARN would inhibite ARN synthesis (see reviews in 10, 18). This regulation by the amino acids exists well in B. subtilis, since ARN synthesis ceases brusquely after transfer of vegetative bacteria in a poor medium (unedited experiments); it was observed also during sporulation (3). In that which concerns germination, since spores contain biologically active soluble ARN (4), one can expect to find there still the same control. The experiments described in the present work fully confirm this hypothesis, explaining in this way the particular needs of the active phase of germination in amino acids.

It is surprising to declare that the medium in which the spores were formed has apparently no effect on their need of amino acids during germination. It seems thus that spores do not have "memory" of the sporulation medium and that their composition is relatively independent of it. However it is necessary to notice that sporulation begins only after exhaustion of the medium; the possibility remains thus that the metabolism of bacteria during sporulation is sensitively identical in the three mediums.

2 EFFECT OF CHLORAMPHENICOL. --- The inhibition of proteic syntheses by chloramphenicol has two effects on ARN synthesis. The first, immediate, manifests itself only in a medium deprived of amino acids : it concerns a stimulation attributed to the accumulation of free amino acids (20). The second effect, on the contrary, is the gradual inhibition and incomplete inhibition of ARN synthesis. The two effects are found again in the course of germination : actually, we observed in minimum medium a stimulation of the initial synthesis; on the other hand, in the presence of chloramphenicol the kinetics of ARN synthesis in the course of germination ressembles, in all of the studied mediums, that which Kurland and Maaloe (13) described in bacteria in growth, submitted to strong concentrations of chloramphenicol. According to these authors, chloramphenicol inhibites

synthesis of necessary protein (s) needed in the formation of ribosomes. When this protein, probably a ribosomal protein, is exhausted, ARN ribosomal synthesis is stopped, while soluble ARN synthesis continues at its normal rate, which explains the existence of a residual synthesis.

A supplementary presumption in favor of this interpretation was given in a preceeding publication (2), by the examination of synthetized ARN in the course of germination in presence of chloramphenicol. If this one is added at the zero cycle, the majority of the incorporated radioactivity is found in pick 4 S; if it is added later on, one still observes an elevated proportion of radioactivity in this pick, even though one finds some also in the ribosomal fractions. It is necessary however to recall that these experiments are submitted to criticisms previously formulated (4), concerning extraction and characterization of ARN.

The rate of residual synthesis remains constant in a given medium, no matter what the time of addition of chloramphenicol may be, and increases only when cellular division has begun. If it is true thus that residual synthesis is principally that of soluble ARN, its constant rate would indicate that the speed of synthesis of soluble ARN depends only on the quantity of ADN (or on the number of ARN formation sites) present in the cells (12).

If one accepts Kurland and Maaloe's interpretation (13); the "fracture level" observed would be an indirect measurement of the quantity of free "ribosomal" proteins, present in the cell at the time of the addition of the antibiotic. This hypothesis leads us thus to admit that rapid augmentation of the "fracture level" indicates the accumulation of a supposedly ribosomal protein. The spore would be deprived thus of this protein, whose preferential formation and accumulation in the course of germination would be a previous condition to the synthesis of ribosomes.

The addition of amino acids to minimum medium, containing chloramphenicol, increases at the same time the initial speed of ARN synthesis, the "fracture level" and the residual rate. Stimulation of initial and residual syntheses is in accord with the observations (8, 19, 25) that indicate that the control by amino acids reacts as well on ribosomal ARN as on soluble ARN. The increase of the "fracture level" remains unexplainable, however.

Finally, we have seen that the toxic effect of chloramphenicol, null at the beginning of germination, appeared progressively, the toxicity being all the more pronounced when the antibiotic is added later on. As the quantity of ARN, synthetized in the presence of chloramphenicol, increases also when germination progresses, it is possible, as that was observed in the 3C$_7$ mutant of E. coli (1), that this ARN is itself responsible for the observed toxic effect.

3 THE ROLE OF RIBOSOMES AND RIBOSOMAL PROTEINS. --- ARN synthesis begins, we have seen, from the debut of germination and becomes exponential very quickly. Around 80 % of stable ARN, synthesized during this period, is constituted of ribosomal ARN, soon incorporated in the ribosomes (2). These last ones, almost entirely absent in spores, are rapidly synthesized during germination (27). Synthesis of proteins during this period is retarded about ten minutes as compared with that of ARN. This data suggests an exponential increase in number of ribosomes, whose synthesis would be thus autocatalytic : the formerly existing ribosomes would intervene in the synthesis of new ribosomes, necessary to the formation of proteins. The situation here described presents a certain analogy with that which one observes in bacteria lacking in magnesium : in this last case, it was shown that synthesis of ribosomes is autocatalytic (16).

We have seen elsewhere (5) that synthesis rates of three types of ARN remains in constant ratio between them during germination, this ratio being the same one as during growth. On the other hand, ARN synthesis, exponential from the beginning, begins with an extremely weak rate, even in complex medium. One can thus ask what is the factor which limits the synthesis of three ARN in the beginning of germination. All of the systems, invoked for the regulation ARN synthesis in bacteria in growth, are observed also in the course of germination : we have discussed the control by the amino acids, the role of a "ribosomal" protein and the autocatalytic synthesis of ribosomes. In that which concerns amino acids, although limiting in minimum medium, they cannot be called upon to explain the evolution of ARN synthesis in rich mediums. The presumed absence of "ribosomal" proteins takes into account quite well effects exercised by chloramphenicol; on the contrary, it would not know how to explain the limitation of ARN synthesis in the course of normal germination. In fact, the limitation due to the lack in these proteins cannot be of long duration since : 1 Syntheses of ribosomal and soluble ARN are between them in normal proportions from the onset of germination, 2 these proteins are accumulated rapidly. On the other hand, the principle limiting factor could concern the capacities of bacteria to synthesize ARN in general, for example the formation of forerunners' energy, or the activity of ARN-polymerase, etc. In this case, one would expect the specific inhibition of ribosomal ARN synthesis by chloramphenicol, at the debut of germination, permits, by compensation, a more rapid synthesis of other ARN and that, consequently, chloramphenicol cannot diminish the total synthesis of ARN during this period. However, such a compensation was not observed at any time; elsewhere, we have already pointed out that neither the contribution of energy due at the stopping of proteic synthesis, nor the addition of 4 bases stimulate ARN synthesis. Not one of the preceding regulation systems seems able to explain, to itself alone, the ensemble of facts observed.

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(1) We weren't able to take into account here articles that appeared on this subject in Spores III, Campbell (L. L.) and Halvorson (H. O.) eds. 1965. Amer. Soc. Microbiol.

Let us now recall that the two proprieties that particularly characterize the spore are : the insufficiency of ribosomes and the absence of a "ribosomal" protein. Ribosomes present seem to determine the speed of ribosomal ARN synthesis; it was suggested in other cases (6, 7, 24), that they intervene also in synthesis (or detachment) of messenger ARN. Nothing is opposed from this that such a mechanism plays in the formation of all the ARN : ribosomal, messenger, and soluble. To explain exponential synthesis of three types of ARN, in constant proportions in the course of germination, we propose thus the following modele : 1 the limited number of ribosomes determines at each moment the speed of synthesis of three types of ARN, 2 the formation of new ribosomes depends on previously existing ribosomes on the one hand, on a "ribosomal" protein, preferentially synthesized in the course of germination, on the other hand. This model also takes into account several observations :

a) The ten minute displacement observed between the kinetics of ARN synthesis and of proteins could be explained by time, necessary to the formation of complete and functional ribosomes beginning with ribosomal ARN (or its forerunner), to the attachment of the messenger ARN and to the synthesis of the polypeptidic chain itself. Besides, it is possible that the formation of complete ribosomes is slower in the germinated spores than in the vegetative bacteria, because of the insufficiency of "ribosomal" proteins.

b) The weak content in "ribosomal" proteins also explains Woese's observations (26) concerning the presence of a new type of ribosomes in the course of germination. These particles ressemble, besides, to incomplete particles formed in the presence of chloramphenicol (22).

c) Finally, if the syntheses during germination depend in the first place on the formation of ribosomes, itself necessitating the synthesis of ribosomal proteins, it is easy to understand that ARN synthesis in presence of chloramphenicol does not contribute at all to germination. It is also possible that the absence of germination in a medium deprived of amino acids is due from the fact that ribosomes do not form under these conditions, fault of the "ribosomal" protein.

Notice finally, that the study of germination permits to determine, indirectly it is true, the presence or the absence of certain constituents in the spore, intervening in macromolecular syntheses. The data gathered in the course of this work permit to set up the following balance sheet: probable presence of ADN-polymerase and enzymes that form the forerunners of ADN, but absence of the initiator protein from the ADN synthesis; presence of ARN-polymerase, enzymes of degradation of ARN, soluble ARN and activation enzymes, absence of the free "ribosomal" protein, and finally, absence of at least certain synthesis enzymes of amino acids.

SUMMARY

SYNTHESIS OF MACROMOLECULES DURING GERMINATION
II. --- REGULATION

A study of the regulation of RNA and protein synthesis during germination has led to the following conclusions :

1 Amino acids are required, even by a prototrophic strain, in order that the active phase of germination may proceed normally. In the absence of amino acids a slow turnover of RNA and protein takes place but germination does not continue beyond the initial phase.

2 The requirement for amino acids during germination is explained by their role in the regulation of RNA synthesis. This is shown by both shift-up and shift-down experiments. The amino acid effect which is immediate is observed even in the presence of chloramphenicol.

3 A detailed kinetic study of RNA synthesis in the presence of chloramphenicol suggests the spore lacks a protein; presumable ribosomal protein which is involved in the synthesis or the stabilisation of ribosomal RNA and which is synthesised preferentially at the beginning of germination. The hypothesis that the ribosomes play a role in the synthesis of all three types of RNA provides an explanation for both the autocatalytic rate of synthesis of these RNAs and for the constancy of their relative rates of formation.

In summary the spore is characterised by the absence of messenger RNA, and by its small content, relative to the vegetative form, of ribosomal protein, ribosomes, and amino acid synthesising enzymes.

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