THE RELATION OF THE COMPOSITION OF THE CULTURE MEDIUM TO THE FORMATION OF ENDOSPORES BY AEROBIC BACILLI.

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(With Plate V containing Figs. 1–4.)

An adequate summary of the literature dealing with bacterial spores has recently been prepared by Cook (1932). A study of this review at once makes it obvious that neither the nutritive conditions requisite for practically complete sporulation, nor those which inhibit this physiological process almost entirely, have ever been thoroughly determined. Thus Osborne (1890), working with *Bacillus anthracis*, found that sporulation was, apparently, most marked when the organism was cultivated on a 1 per cent. agar medium containing 1 or 0·5 per cent. beef extract. When lower concentrations of beef extract were employed (0·2 to 0·02 per cent.) growth was somewhat meagre, and spores were entirely absent. Henrici (1928) obtained quite different results. He observed that, when *B. cohaerens* was inoculated on agar slants of normal composition and others containing one-quarter the amount of peptone and meat extract, spore formation proceeded more rapidly in the media of lower nutritive value than in the full strength media, regardless of the size of seeding. Thus, after 72 hours' incubation, 56 to 65 per cent. of spores were present in the more concentrated medium, depending on the concentration of the initial inoculum. The dilute medium, under similar conditions, contained from 84 to 92 per cent. of spores. According to Henrici, sporulation "appears earlier in the heavily seeded cultures and in the more dilute media, because those cultures do not have so long a period of vegetative reproduction; that is, because the resting phase of the culture appears earlier."

Cook (1931) studied spore formation in *B. subtilis*. He found that, when this organism was cultivated in tryptic digest of casein, sodium chloride, in relatively high concentrations, apparently exerted no unfavourable effect on sporulation. Spores were formed on a large number of the media studied, including tryptic digests of casein, peptone, and inorganic salt media containing glycerol or sodium lactate as sources of carbon. Apparently spore formation took place both on fluid media, and media made solid by the addition of agar, though autolysis seemed to take place to a greater extent in the fluid media. Glucose almost entirely inhibited sporulation, due, probably, to the rapid

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cessation of growth resulting from abundant acid formation. Cook's results do not permit of any definite conclusion being drawn with regard to the possibility of there being any well-defined nutritive requirement for sporulation.

Williams (1929), during a study on the heat resistance of the spores of *B. subtilis*, made the interesting observation that this bacillus formed more spores on a simple peptone medium than on a casein digest medium. And later (1930–1) he showed that the percentage of spores formed by *B. subtilis* when grown in 5 per cent. peptone water was much lower than when the same organism was cultivated in 0·5 per cent. of peptone water. Thus, after 10 days' incubation at 37° C., 916 out of 1000 cells were spores when the bacillus was grown in 0·5 per cent. peptone water, while in 5 per cent. peptone water only 115 out of 1000 cells were found to be spores. He concluded that a comparative paucity or depletion of the nutrient material is more important in promoting the formation of spores than an actual accumulation of metabolites.

Experimental.

The following experiments were devised in order to determine the conditions necessary for almost complete spore formation, and those required for entire or partial inhibition of this physiological process.

Cultures. The following cultures have been employed.

B. mesentericus (I)	Isolated during a previous investigation. See Harrison *et al.* (1930).
B. subtilis (I)	Isolated during a previous investigation. See Harrison *et al.* (1930).
B. subtilis (II)	Nat. Coll. of Type Cultures, No. 85.
B. megatherium	,, ,, ,, No. 654.
B. mesentericus (II)	,, ,, ,, No. 2589.
B. anthracis (II)	,, ,, ,, No. 109.
B. anthracis (I)	Dept. of Pathology, Cambridge.
B. mycoides	,, ,, ,,

Methods. In order to make the conditions as uniform as possible all the media mentioned in the following series of experiments have been employed as agar slants, 2 per cent. of agar being used, and the media being sloped in approximately 5 c.c. amounts in $5 \times {}^{5}$ inch test-tubes. In each instance the medium has been inoculated by transferring a small amount of a 24-hour-old Hartley agar culture of the organism under investigation on the tip of a platinum needle, and streaking it along the length of the culture medium. Experience has shown that no increase in spore formation occurs after 7 days, and generally sporulation has been found to be complete after 3 days' incubation at 37° C. Therefore, in all experiments the cultures have been incubated at 37° C. for 7 days before the percentage of spores has been determined. In view of the fact that a permanent preparation was found desirable the spores have been stained by Dorner's technique (Society of American Bacteriologists,

1926). This method has been found extremely satisfactory in that both spores and vegetative cells can readily be counted. All counts given are reported as the percentage of spores of the total count of twenty representative microscopic fields, not less than 200 cells being counted in any case. Due to the greater degree of contrast the photomicrographs have all been taken from films prepared by spreading a mixture of one drop each of a sterile, saturated aqueous solution of nigrosine and a thick aqueous suspension of the bacillus on a slide.

The tryptic casein digest "stock" broth has been prepared by the method of Cole and Onslow (1916), and the tryptic meat digest broth following the technique developed by Hartley and Hartley (1922). Unless otherwise stated all media have been adjusted to *p*H 7·4 (colorimetrically) previous to use.

The amino nitrogen has been determined by the formol titration method of Brown (1923), the determination being made prior to the addition of agar to the medium.

Experiment 1.

A preliminary study of spore formation by six strains in four media was made. The following media were employed:

(1) Casein digest diluted 1: 3 with 0·5 per cent. NaCl.

(2) Inorganic medium (Stephenson, 1930) plus 2 per cent. of Witte peptone[1].

(3) 1 per cent. Witte peptone, 0·5 per cent. "Lemco" beef extract and 0·5 per cent. NaCl.

(4) Hartley's beef digest.

Inoculations, incubation and spore counts were made by the technique already described; the results are given in Table I.

Table I. *Spore formation by six strains of bacilli on four different media.*

	Percentage of spores formed in medium			
Culture	1	2	3	4
B. mesentericus (I)	<0·1	70	60	6
B. subtilis (I)	5	80	80	6
B. subtilis (II)	0	77	71	<0·1
B. megatherium	0	69	85	0
B. mesentericus (II)	0	0	<0·1	0
B. mycoides	0	0	0	0

It is at once apparent from a study of the results given in Table I that the last two strains are practically asporogenous. Subsequent experiments have shown that neither of these cultures, nor one of the strains of *B. anthracis*, has formed any appreciable proportion of spores on any of the media studied. In the case of the first four cultures spore formation is relatively abundant on media 2 and 3, while few or no spores are formed on the other two media. There appeared to be two possible explanations of this fact: (1) that intermediate products of protein hydrolysis are necessary for spore formation, or

[1] Witte peptone was shown by Berman and Rettger (1918) to have a very low amino nitrogen content, and, as will be seen, my experiments confirm their findings.

(2) that amino acid-rich media will not permit spore formation. A second experiment was conducted in order to study these possibilities.

Experiment 2.

Eight different media were prepared as follows:

(1) Casein digest "stock" broth diluted 1 : 3 with 0·5 per cent. NaCl.
(2) ,, ,, ,, 1 : 15 ,, ,,
(3) ,, ,, ,, 1 : 30 ,, ,,
*(4) ,, ,, ,, 1 : 30 ,, ,,
(5) Hartley's beef digest broth undiluted.
(6) Hartley's beef digest broth diluted 1 : 5 with 0·5 per cent. NaCl.
(7) ,, ,, ,, 1 : 10 ,, ,,
*(8) ,, ,, ,, 1 : 10 ,, ,,

* 1 per cent. of Witte peptone added to (4) and (8).

The amino nitrogen content of these media was determined by the technique already described, and is given in Table II. Inoculation, incubation and determination of the number of spores formed were carried out in the usual manner, and the results appear in Table II.

Table II. *The effect of dilution of the medium and the addition of peptone on spore formation by eight strains of bacilli.*

	Percentage of spores present in medium							
Culture	1 (208)*	2 (43)	3 (20)	4 (37)	5 (101)	6 (21)	7 (11)	8 (26)
B. mesentericus (I)	<0·1	96	98	85	6	97	99	71
B. subtilis (I)	3	89	97	77	15	96	98	76
B. subtilis (II)	0	91	96	56	<0·1	97	97	52
B. megatherium	0	87	98	63	0	92	97	78
B. mesentericus (II)	0	0	0	0	0	0	0	0
B. mycoides	0	<0·1	1	<0·1	0	<0·1	2	<0·1
B. anthracis (I)	<0·1	14	12	<0·1	15	53	86	78
B. anthracis (II)	0	0	0	0	0	0	0	0

* The figures in brackets represent the amino nitrogen in mg. per 100 c.c. of medium.

From the data given in Table II it is quite evident that, with the exception of the apparently almost asporogenous races, namely *B. mesentericus* (II), *B. mycoides* and *B. anthracis* (II), spore formation bears an almost direct relationship to the available amino nitrogen content of the medium. Peptone, apparently, does not greatly influence sporulation, except that it brings about some decrease in the percentage of spores formed, probably because it raises the amount of available nutrient material. It is therefore certain that large peptone molecules are not necessary for spore formation.

It is interesting to note that the vegetative cells are never absent in the stronger media, and are never completely lysed. On the other hand, as far as ordinary microscopic observation could show, the spores in the dilute media were always free from cell material. Photomicrographs of both *B. subtilis* (II)

and *B. megatherium* were prepared from cultures on dilute and strong casein digest media and are reproduced in Plate V.

The fact that one strain of *B. anthracis* did not form large numbers of spores in the dilute casein digest media cannot be explained. The same culture formed relatively large numbers of spores in diluted beef digest medium.

In order to show that the above effect was due to a direct dilution of the available nutrient material, and not to any other effect, such as dilution of some inhibiting unknown factor present in the broth, the following experiment was carried out.

Experiment 3.

An inorganic salt solution of the following composition was prepared: KH_2PO_4, 0·5 per cent.; $MgSO_4.7H_2O$, 0·05 per cent.; NaCl, 0·1 per cent. and $FeCl_2$, trace; the constituents being dissolved in distilled water. With this solution as diluent four different media were made containing 0·5, 0·25, 0·125 and 0·05 per cent. respectively, of each of the following nutrients: glycine, alanine, leucine, glutamic acid, tryptophane and asparagine. The amino acids were neutralised where necessary with sodium hydroxide solution. The customary technique of inoculation, incubation and determination of the number of spores present was followed, and the results are given in Table III.

Table III. *Spore formation in media containing varying concentrations of amino acids and a constant proportion of inorganic salts.*

	Percentage of spores present in medium			
Culture	1 (279)*	2 (139)	3 (71)	4 (28)
B. mesentericus (I)	0·5	32	89	97
B. subtilis (I)	2	29	85	98
B. subtilis (II)	0	21	94	99
B. megatherium	0	3	28	87
B. mesentericus (II)	0	0	0	0
B. mycoides	0	0	0	0

* These figures represent amino nitrogen in mg. per 100 c.c.

A study of the figures given in Table III shows that, with the exception of the two apparently asporogenous strains, sporulation depends on the amount of amino acid or other available nutrient material present in the medium. The effect of various single amino acids and asparagine was next studied in a further experiment.

Experiment 4.

A basic medium consisting of casein digest diluted 1 : 30 with 0·5 per cent. NaCl was prepared, and with this as a control six different nutrient substrates were prepared each containing 2 per cent. of one of the following compounds: $(NH_4)_2HPO_4$, asparagine, glycine, alanine, tryptophane and glutamic acid. These media were inoculated, incubated and the percentage of spores present in each determined following the customary technique. The results are to be found in Table IV.

Table IV. *The effect of the addition of various substrates on spore formation in casein digest medium diluted* 1 : 30.

Culture	Percentage of spores in medium containing: Control (no added substance)	2% of $(NH_4)_2HPO_4$	2% of glycine*	2% of asparagine	2% of alanine	2% of tryptophane	2% of glutamic acid
B. mesentericus (I)	99	93	98	7	61	83	84
B. subtilis (I)	98	80	†	0	75	94	83
B. subtilis (II)	99	98	82	0	44	81	70
B. megatherium	98	†	†	0	‡1	16	15

* Growth was very meagre, or apparently absent, in the media containing glycine, but spore formation does not appear to be stopped.

† No growth, or practically no growth.

‡ This figure is only approximate; long filaments made the estimation of the actual number of spores impossible.

From the figures given in Table IV the following conclusions may be drawn: (1) Sporulation is almost complete in the control dilute casein digest medium. (2) Ammonium salts, except in one instance in which growth is almost entirely inhibited, do not hinder spore formation. (3) Asparagine in all cases markedly inhibits or entirely represses spore formation. (4) Glycine, in spite of the fact that it retards growth in two cultures and inhibits it in the others, does not exert any adverse influence on sporulation. (5) The amino acids alanine, tryptophane and glutamic acid all inhibit the formation of spores to a greater or lesser degree, depending on the culture employed.

These results support strongly the theory that spore formation is directly dependent on the amount of available nutrient material present in the medium, and tend to discredit the idea that spore formation may be retarded because of the toxic effect of high concentrations of nutrients on actual growth. Thus glycine, for example, inhibits growth markedly, yet does hinder the formation of spores in cultures in which growth is evident. The effect of sucrose, as carbon source, on spore formation was next studied.

Experiment 5.

Casein digest broth was diluted 1 : 30 with 0·5 per cent. NaCl, 2 per cent. of freshly precipitated calcium carbonate and 2 per cent. of agar, and finally varying concentrations of sucrose (2, 1, 0·1 and 0 per cent.) to make four different media were added. The agar slopes were prepared in this case so that the carbonate was evenly distributed throughout the medium. The final *p*H of these media was 7·8 (colorimetrically). Inoculation, incubation and estimation of the percentage of spores formed were carried out as usual, the results of the experiment being recorded in Table V.

It is evident from the data given in Table V that sucrose tends to inhibit spore formation, the effect being more marked in higher concentrations of this sugar, and varying greatly with each culture. Naturally the possible retardation of sporulation as a result of acid production is not entirely removed due to

the somewhat slow diffusion of acid which probably takes place in such a solid medium. It would appear, however, that the effect of sucrose is merely due to its availability to the organism; least spore formation occurring in the cultures which utilise this carbon source most readily. It must also be noted that spore

Table V. *The effect of varying concentrations of sucrose on the formation of spores in a dilute casein digest medium.*

Culture	Percentage of spores in medium containing 2 % sucrose	1·0 % sucrose	0·1 % sucrose	No sucrose
B. mesentericus (I)	71*	68*	86	84
B. subtilis (I)	93	89	98	97
B. subtilis (II)	14	29	74	89
B. megatherium	6*	5*	53	88

* In these media the *p*H at the conclusion of the experiment was approximately 7·4, while it remained at about 7·8 in the other cultures.

formation is not quite as marked in the presence of carbonate as it is in its absence (compare medium containing no sucrose, Table V, with medium 3, Table II). Thus it would appear that spores are not quite as readily formed in a medium which is strongly buffered at about *p*H 7·8. The formation of spores in a medium in which the source of nitrogen was ammonium phosphate was next studied.

Experiment 6.

A medium having the following composition was prepared: Sucrose, 0·1 per cent.; $(NH_4)_2HPO_4$, 0·1 per cent.; NaCl, 0·1 per cent.; $MgSO_4.7H_2O$, 0·05 per cent.; KH_2PO_4, 0·5 per cent. and $FeCl_2$, trace. A "control" medium without any nitrogen source was also prepared.

The formation of spores on this medium was studied first after direct inoculation from a 24-hour-old Hartley agar culture of the organism, and secondly after seven consecutive transfers at 24-hour intervals on the above medium. The methods of inoculation, incubation and determination of the percentage of spores formed were identical with those used in all previous experiments. The results are given in Table VI.

The results given in Table VI show undoubtedly that spores are formed

Table VI. *Spore formation in an "inorganic" medium with sucrose as source of carbon.*

Culture	Percentage of spores: Direct inoculation	After seven successive transfers	Control medium: no amm. phosphate
B. mesentericus (I)	96	96	No growth
B. subtilis (I)	92	71*	,,
B. subtilis (II)	98	99	,,
B. megatherium	83	0†	,,

* Very small spores, staining faintly. † Extremely good mucilaginous growth.

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readily on an inorganic medium. The lack of spores after seven successive transfers and incubation in the case of *B. megatherium* is interesting, and as the growth is excellent it would appear again that the transfer of this bacillus in the presence of sucrose has enhanced its ability to utilise the sugar as carbon source, thereby hindering spore formation as a result of cultivation in the presence of abundant available nutrient material. In *B. subtilis* (II) the results are similar, only the effect is not so pronounced.

Discussion of Results.

While conditions such as *p*H, temperature, salt concentration, etc., may play some rôle in spore formation, the results obtained in the above series of experiments show that, undoubtedly, the major factor controlling the formation of spores by aerobic bacilli when cultivated under aerobic conditions on the surface of solid agar media is the amount of available nutrient material present in such media. Thus in a medium containing relatively large amounts of available nutrient materials few or no spores are formed. On the other hand, in a medium of low nutritive content, such as a medium containing only small amounts of amino acids, sporulation is abundant, or almost complete. At present no really adequate explanation can be given for these results. It is suggested, in accordance with Henrici's statement already cited, that in a "rich" medium growth is so rapid that the cells perish before spore formation has had time to take place. It would appear possible that the proteolytic enzymes produced in the more dilute medium are stronger than those produced in concentrated media, thereby accounting for the fact that practically nothing but free spores are to be observed in media of low nutritive content. This remains to be proved. In general the results obtained serve to emphasise the marked morphological and physiological changes which may be brought about in bacterial cultures as a result of cultivation in varying concentrations of nutrients.

Summary.

1. Spore formation in eight typical members of the genus *Bacillus* has been studied.

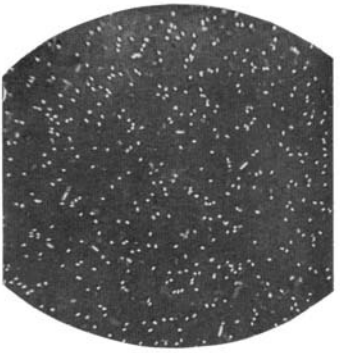
2. Three of these strains, including one species of *B. anthracis*, have been found to be practically asporogenous under the experimental conditions. In general the following statements hold good for the sporogenous races studied.

3. Spore formation is almost, or entirely, inhibited by cultivation on media rich in amino acids, such as tryptic digests of casein or meat. Similar inhibition results following cultivation on a medium containing reasonably high concentrations of a mixture of amino acids and asparagine.

4. When such media are suitably diluted with standard inorganic salt solutions the percentage of spores formed is greatly increased, and frequently at least 99 per cent. of spores are formed if the dilution is sufficiently high.

5. When simple nitrogenous compounds such as amino acids are added to a dilute casein digest medium in which sporulation is almost complete, a definite decrease in the percentage of spores present is observed. Asparagine, which is

1



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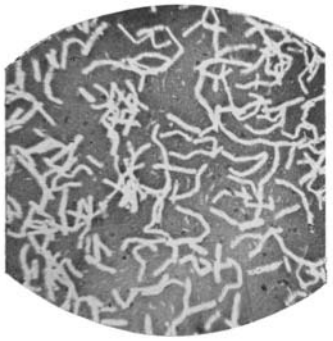

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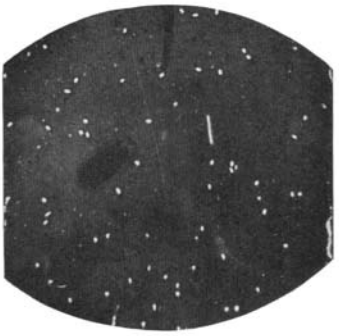

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probably readily assimilated, apparently completely hinders spore formation in most cases. Other amino acids do not exert so pronounced an effect, and ammonium phosphate does not appreciably inhibit the formation of spores.

6. The fact that the addition of glycine suppresses growth markedly when it is added to a dilute casein digest medium, but does not appreciably hinder sporulation, suggests that the formation of spores is not due to any toxic effect of added compounds, or compounds already present in the medium.

7. Sporulation is almost complete in a "synthetic" medium in which low concentrations of ammonium phosphate and sucrose represent the sources of nitrogen and carbon, respectively. However, frequent transfers in such a medium may inhibit spore formation partially or entirely in certain instances. This effect probably depends upon the enhanced ability of the culture in question to utilise sucrose as a source of carbon when cultivated constantly in its presence.

8. It is concluded, from the above data, that endospore formation in aerobic bacilli bears an inverse relationship to the amount of available nutrient material present in the culture medium.

I am indebted to Prof. Sir F. G. Hopkins and Miss M. Stephenson for their constant encouragement during the progress of this work. My thanks are due to Mr Pirie of this Department who kindly furnished me with several of the amino acids employed, and to Dr Miles of the Department of Pathology for his kindness in supplying me with certain of the cultures.

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EXPLANATION OF PLATE V.

Photomicrographs of cultures after one week at 37° C.
Note absence of spores in Figs. 1 and 3 and almost complete sporulation in Figs. 2 and 4.

Fig. 1. *B. subtilis* (II) culture on casein digest agar diluted 1 : 3.
Fig. 2. *B. subtilis* (II) culture on casein digest agar diluted 1 : 30.
Fig. 3. *B. megatherium* culture on casein digest agar diluted 1 : 3.
Fig. 4. *B. megatherium* culture on casein digest agar diluted 1 : 30.

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