THE ENUMERATION OF HEATED BACTERIAL SPORES

II. EXPERIMENTS WITH BACILLUS SPECIES

By W. G. MURRELL*, A. M. OLSEN†, and W. J. SCOTT*

[*Manuscript received January* 9, 1950]

Summary

Experiments with 13 strains of *Bacillus* isolated from canned foods have shown that, like *Clostridium* species, the germination of heated spores is affected by inhibitors present in the media. As with *Clostridium* species, susceptibility to inhibitors increases with the amount of heating to which the spores are exposed. The *Bacillus* strains, however, show more variation in the extent to which different strains are inhibited in a particular medium. There is also evidence of substantial differences in the amounts or types of inhibitors which are contained in different media. Some of the differences between media are due to variations in the concentrations of inhibitors which are adsorbed on charcoal, starch, or serum albumin, but some of the differences are attributable to inhibitors which are not adsorbed on charcoal. Treatment of nutrient agar with charcoal and subsequent removal of the charcoal is as effective in removing inhibitors as incorporation of the charcoal in the medium. The medium, therefore, is the principal or sole source of inhibitors. Attempts to demonstrate inhibitors in the inoculum were unsuccessful. It is unlikely that unsaturated fatty acids account for more than part of the observed inhibition. A suitable adsorbent should be incorporated in media used for evaluating the thermal destruction of *Bacillus* spores.

I. INTRODUCTION

In a previous paper (Olsen and Scott 1950) it was shown that the spores of several species of *Clostridium,* including *Cl. botulinum,* became progressively more sensitive to traces of inhibitory substances as the amount of lethal heating to which they had been subjected was increased. The number of heated spores capable of germinating and forming colonies increased when suitable adsorbents such as starch, charcoal, or serum albumin were incorporated in the nutrient medium. In this paper it will be shown that a similar phenomenon applies to spores produced by a number of *Bacillus* strains which have been isolated from spoiled canned foods. It will also be shown that the inhibitors concerned are derived mainly, if not entirely, from the various laboratory media commonly used for the cultivation and enumeration of these organisms.

II. METHODS

The methods generally were similar to those described in the previous paper (Olsen and Scott 1950) except for the following modifications. Spore suspensions were usually harvested from surface growths on various agar media.

* Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W.
† Division of Fisheries, C.S.I.R.O., Cronulla, N.S.W.

In the later experiments the temperatures of the oil bath were controlled more accurately, the nominal temperatures being realized within 0.05°C. Viable counts were made with the usual plating techniques, care being taken to observe the precautions described by Wilson *et al.* (1935). In addition to the media described in the previous paper, nutrient agar and dextrose tryptone agar were used. These were prepared according to the formulation given in the Difco Manual (1944).

The organisms studied included 10 strains isolated in these laboratories from spoiled canned foods and designated A21, A65, 196, 209, 256, 262, 320, 414, 1057, and 1541. Three strains of American origin (698, 1518, and HAX) were obtained through the courtesy of Dr. E. J. Cameron, N.C.A. Research Laboratories, Washington, U.S.A.

The activated charcoal used was a commercial product of animal origin, and the bovine serum albumin was partially purified by ammonium sulphate fractionation as described by McMeekin (1940). The albumin solution was dialysed against phosphate buffer, and additions of Seitz filtered material made in terms of the dry weight of protein in the solution.

III. Results

(*a*) *Experiments with Different Strains on Various Media*

As with *Clostridium* spores, the apparent susceptibility to inhibitors increases with the amount of heating to which the spores are exposed. A striking example of this appears in Figure 1, which shows results for *Bacillus* No. 262 grown on brain heart infusion agar. The only factor which contributes to the two widely different rates of destruction is that in one case the nutrient medium contained a supplement of 0.2 per cent. of soluble starch. The supplemented and control media both gave similar estimates of the spores which were viable before heating, but as the duration of heating and the associated spore mortality increased, the unsupplemented control medium became progressively less suitable for estimating survivors. It is perhaps worthy of comment that the highly erroneous data obtained with the control medium still approximate to a straight line when the logarithm of the number of survivors is plotted against the time of heating. Obviously arguments based on conformity to this type of destruction curve cannot be accepted as an indication that the data are reliable.

The various *Bacillus* strains tested have shown considerable differences in their susceptibility to inhibitors, some, like No. 262, showing large increases when adsorbents such as starch were incorporated in the medium, whereas others, such as the National Canners' Association test organism *Bacillus* No. 1518, show only a small response. All 13 strains tested have, however, shown consistent evidence of increased germination of heated spores when inhibitors were removed by starch or charcoal. Some strains have shown a marked response to thioglycollate as well as to starch, and for some organisms the combined effects of thioglycollate and starch have been more than additive. Some

results obtained with four strains inoculated into four basal media are given in Table 1. Comparisons are based on aliquots of the same spore suspensions and of the same batch of basal medium.

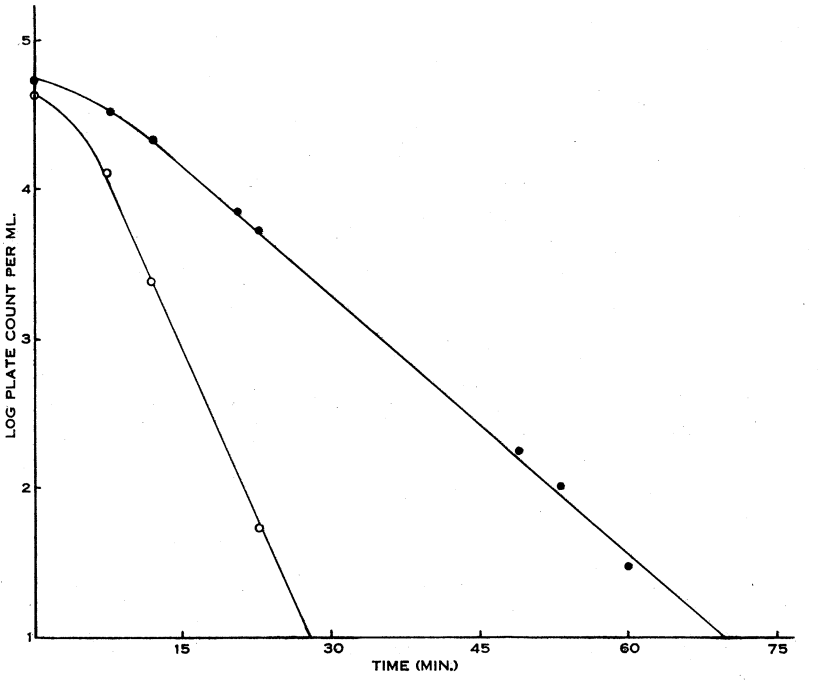

Fig. 1.—Destruction of *Bacillus* No. 262 spores in M/15 phosphate buffer, pH 7.0, at 115°C. as measured on brain heart infusion agar with and without 0.2 per cent. soluble starch.

O Medium without starch. ● Medium with 0.2 per cent. added starch. Plates incubated for 2 days at 55°C.

It is evident from the data in Table 1 that there are considerable differences between strains and between media. All strains reveal some increases due to the starch supplement, especially on the more complex infusion media. The effects are greatest with strain 262 and least with strain 1518. The effect of thioglycollate is more variable, being marked for strain 262, small for strain 1518, and absent for strains 320 and A21. The addition of both starch and thioglycollate results in remarkable increases for strain 262, a small response for strain 1518, and no increase over the values for starch alone with the remaining two organisms.

(*b*) *Comparative Effects of Starch, Charcoal, and Serum Albumin*

The addition to the medium of starch, charcoal, or serum albumin has been shown by Pollock (1947) to promote the growth of *Haemophilus pertussis* and by Olsen and Scott (1950) to increase the germination of heated *Clostridium* spores. Tests were made of the comparative effects of these three substances on the germination of *Bacillus* spores, and the results with strain 320 are given in Table 2.

TABLE 1

RESPONSE OF FOUR STRAINS OF *BACILLUS* TO VARIOUS SUPPLEMENTS ON FOUR BASAL MEDIA

Figures represent plate counts per ml. after 2 days at 50°C. for strains 1518, 262, and 320 and after 2 days at 30°C. for strain A21

Basal Medium	Supplement *	Strain 1518		Strain 262		Strain 320		Strain A21	
		0 min.	35 min. at 115°C.	0 min.	60 min. at 115°C.	0 min.	12 min. at 115°C.	0 min.	40 min. at 100°C.
Nutrient agar pH 7.9	*A*	97,000	44	3,000	< 1	28,000	25	6,800,000	90
	B	101,000	59	8,000	225	39,000	74	7,500,000	137
	C	94,000	71	6,000	194	39,000	24	6,700,000	59
	D	112,000	104	8,000	1200	67,000	98	4,700,000	24
Brain heart infusion agar pH 7.8	*A*	–	32	13,000	76	20,000	4	9,000,000	27
	B	–	61	9,000	700	24,000	37	7,500,000	152
	C	–	51	11,000	710	13,000	6	7,500,000	24
	D	–	93	13,000	2000	34,000	30	7,800,000	90
Pork infusion agar pH 8.0	*A*	50,000	< 1	2,000	1	39,000	57	8,200,000	91
	B	50,000	< 1	5,000	100	68,000	100	7,800,000	108
	C	52,000	15	5,000	19	98,000	54	8,500,000	110
	D	50,000	72	10,000	705	93,000	78	8,400,000	98
Dextrose tryptone agar pH 7.9	*A*	142,000	64	9,000	128	15,000	3	8,100,000	20
	B	138,000	77	10,000	680	19,000	24	7,900,000	85
	C	138,000	78	12,000	650	12,000	6	8,600,000	12
	D	128,000	90	14,000	1250	19,000	25	8,700,000	76

* Supplements added: *A*—none; *B*—0.1% soluble starch; *C*—0.01M Na thioglycollate; *D*—0.1% starch + 0.01M Na thioglycollate.

All three substances increase the numbers of heated spores which form colonies and, if this is expressed in terms of the apparent rate of destruction, the supplemented media indicate significantly lower rates of death than does the control medium. There is a suggestion that, in the amount added, charcoal may be slightly more effective than starch, but there is no indication

TABLE 2

EFFECT OF SOLUBLE STARCH, SERUM ALBUMIN, AND ACTIVATED CHARCOAL ON THE GERMINATION OF HEATED *BACILLUS* SPORES (STRAIN 320) IN BRAIN HEART INFUSION AGAR pH 6.70 ± 0.03

Figures represent plate counts per ml. after 5 days at 50°C.

Medium Supplement	Time of Heating at 110°C.					K110°†	S.D.
	0 min.*	5 min.	20 min.	40 min.	60 min.		
None	1,400	32,000	1,420	56	< 1	0.0786	0.0045
Starch 0.1%	1,800	34,000	4,600	400	23	0.0567	0.0014
Albumin 0.2%	2,600	34,000	–	500	35	0.0532	0.0015
Charcoal 0.2%	2,850	30,000	5,000	870	72	0.0464	0.0021
Starch 0.1% + albumin 0.2%	3,000	50,000	3,700	510	50	0.0520	0.0042
Starch 0.1% + charcoal 0.2%	4,300	38,000	4,600	560	44	0.0522	0.0020
Albumin 0.2% + charcoal 0.2%	2,300	34,000	5,200	540	61	0.0505	0.0010
Starch 0.1% + albumin 0.2% + charcoal 0.2%	–	–	4,600	490	54	0.0483	0.0002

* Viable count at 0 min. low due to dormancy, maximum count being reached after approximately 5 min. at 110°C.

† Figures for 0 min. omitted in calculation of rate of destruction. Differences necessary for significance, 0.0076 ($p = 0.05$) and 0.0106 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in proportion to the number of replicates on which it was based.

that the combined effects of any pair or of all three substances are any greater than the effect of any one of the adsorbents. The indication is, therefore, that starch, charcoal, and serum albumin all act in a similar way by adsorbing from the medium the same type(s) of inhibitory substance(s).

(*c*) *The Response to Charcoal Supplements added to Different Basal Media*

If a number of media are all nutritionally adequate for spore germination and growth of a particular organism, any variation in their ability to promote the germination of spores may well be due to variations in the concentration and type of sporostatic substances which they contain. If these inhibitory

substances are capable of adsorption on charcoal, then it might be expected that a sufficient concentration of added charcoal would bring each medium to the same level of efficiency in promoting spore germination. This question was examined with *Bacillus* No. 320 and the results of adding various amounts of charcoal to five basal media are shown in Figure 2.

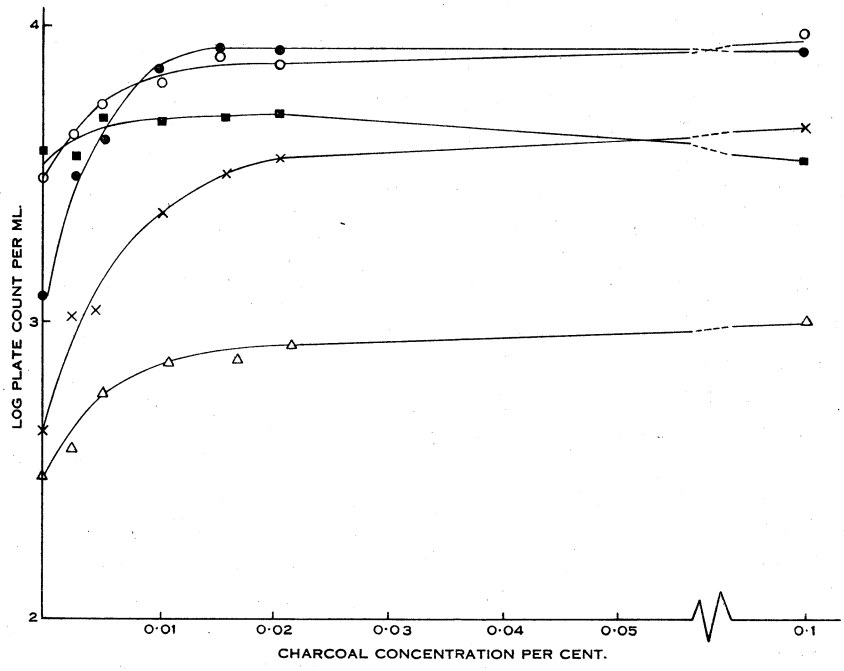

Fig. 2.—Effect of different concentrations of charcoal added to five media on the estimates of *Bacillus* No. 320 spores after 31 minutes at 110°C.

O Nutrient agar. Mean pH 6.6. ● Pork infusion agar. Mean pH 6.4. ■ Dextrose tryptone agar. Mean pH 6.5. × Brain heart infusion agar. Mean pH 6.3. △ Peptic digest agar. Mean pH 6.4 Plates incubated 5 days at 50°C. Initial population 35,000 per ml. approx.

The curves show how the estimates of the spores surviving heating for 31 minutes at 110°C. increase with increasing charcoal concentration in each medium. It will be noted that only two of the five media, namely nutrient agar and pork infusion agar, show signs of reaching the same maximum estimate in the presence of charcoal. For the other three media it is clear that even greater concentrations of charcoal will not cause the same number of spores to germinate and produce colonies. It is of interest to note that in the absence of added charcoal the two comparatively simple media, nutrient agar and dextrose tryptone agar, gave considerably greater estimates than the more complex infusion and peptic digest media. The improvement following the addition of charcoal was, however, greatest for nutrient agar and pork infusion agar, least for dextrose tryptone agar, and intermediate for brain heart infusion. The peptic digest medium proved quite unsuitable for enumerat-

ing viable spores of this organism even when supplemented with 0.1 per cent. of charcoal. It may be added that all media except the peptic digest gave comparable estimates of the spores viable prior to heating.

The failure of the charcoal supplement to equalize the performance of the different media would seem to be most probably due to the presence of inhibitory substances which are not adsorbed by charcoal. The alternative that complex media such as brain heart infusion or peptic digest medium are lacking in essential nutrients seems less likely in view of the ability of these media to support the growth of a great variety of bacteria with complex nutritional requirements.

(d) The Removal of Inhibitors from Media by Adsorption with Charcoal

If the adsorbents are able to bind the inhibitory substances sufficiently firmly to prevent their anti-bacterial action it should be possible to remove the adsorbent with the inhibitors still attached. This has been examined with brain heart infusion and nutrient agars treated with activated charcoal.

In the first experiment the brain heart infusion agar, with and without 0.2 per cent. charcoal, was compared with a medium prepared by treating the double strength broth moiety with 0.4 per cent. charcoal, removing the charcoal by filtering the autoclaved mixture through sintered glass, and aseptically adding the filtered broth to the separately autoclaved agar. Appropriate control treatments with the charcoal remaining in the medium were included. The results are shown in Table 3. The estimates of surviving spores indicate different apparent rates of destruction depending on the medium used. The medium from which the charcoal was removed (*E*) shows a lower death rate than the appropriate control (*C*), but higher than when the charcoal remained in the medium (*D*). There is no suggestion that separate autoclaving of the broth and agar fractions has had any significant effect on the properties of the medium (treatments *A* and *C*; *B* and *D*).

The second experiment was carried out with nutrient agar to which was added 0.2 per cent. charcoal either before or after autoclaving. Portions of each medium were centrifuged under sterile conditions at 50°C., and the charcoal-free supernatant compared with the uncentrifuged portion with the charcoal remaining in the medium. The control medium received no treatment with charcoal. Table 4 summarizes the results, which show that all four media treated with charcoal gave significantly lower "rates of destruction" than the control medium. There is no difference between the media from which the charcoal was removed by centrifuging and those in which it remained, indicating that the medium is the principal source of the inhibitors adsorbable on charcoal. That the charcoal did, in fact, adsorb a considerable quantity of material was shown by washing the charcoal precipitate in boiling water, drying at 110°C., and reweighing. The 2.00 g. of dry charcoal originally added to one litre of medium had increased to 2.61 g. After extraction with dilute HCl and NaOH the dry weight was 2.33 g. and this was further reduced to 1.98 g. after 24 hours' extraction with methanol.

Comparison of the experiments in Tables 3 and 4 provides further confirmation of the superiority of the nutrient agar for enumerating spores of *Bacillus* No. 320, the apparent rate of destruction when this medium is the index of survival being less than half the rate shown by brain heart infusion agar. This is, however, partly due to the difference in pH of the media, the value of pH 6.4 for the nutrient agar being close to the optimum for this strain.

TABLE 3

EFFECT OF CHARCOAL ADSORPTION OF BRAIN HEART INFUSION AGAR ON THE ESTIMATES OF HEATED SPORES OF *BACILLUS* NO. 320

Figures represent plate counts per ml. after 5 days at 50°C.

	Treatment of Medium*	Time of Heating at 110°C. 0 min.‡	10 min.	20 min.	30 min.	40 min.	K110°†	S.D.
A	Brain heart infusion agar control	860	3,100	660	111	44	0.0632	0.0035
B	Brain heart infusion agar + 0.2% charcoal	2,180	12,300	4,000	1,600	430	0.0480	0.0017
C	Brain heart broth + agar (mixed after autoclaving)	920	5,000	1,250	300	51	0.0659	0.0023
D	Brain heart broth with 0.4% charcoal + agar (mixed after autoclaving)	1,790	8,400	2,960	930	310	0.0477	0.0008
E	Brain heart broth with 0.4% charcoal removed + agar (mixed after autoclaving)	960	7,700	2,140	560	136	0.0569	0.0011

* Media *A* and *B*: pH 7.3; *C*, *D*, and *E*: pH 7.5.

‡ Viable count at 0 min. low due to dormancy, maximum count being reached after approximately 5 min. at 110°C.

† Figures for 0 min. omitted in calculation of rate of destruction. Differences necessary for significance, 0.0066 ($p = 0.05$) and 0.0095 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in inverse proportion to its variance.

It is also evident that adsorption of the complete nutrient agar medium and removal of the charcoal by centrifuging has been more effective in removing inhibitors than adsorption of the broth moiety of brain heart infusion. This may be due to the greater concentration of inhibitors in brain heart infusion (see Fig. 2), or to the agar itself being a significant source of inhibitors, as was

found by Ley and Mueller (1946) in experiments with the gonococcus. These authors found that exhaustive methanol extraction effectively removed inhibitors of a fatty acid nature from the agar.

TABLE 4

THE EFFECT OF CHARCOAL ADSORPTION OF NUTRIENT AGAR ON THE ESTIMATES OF HEATED SPORES OF *BACILLUS* NO. 320

Figures represent plate counts per ml. after 5 days at 50°C. pH of media 6.40 ± 0.05

	Treatment of Medium	Time at 110°C. 6 min.	31 min.	56 min.	$K110°$*	S.D.
A	Nutrient agar control	23,500	3,740	565	0.0323	0.0002
B	Nutrient agar + 0.2% charcoal (added before autoclaving)	33,600	18,400	4,760	0.0166	0.0038
C	Nutrient agar + 0.2% charcoal (added after autoclaving)	34,600	19,000	4,970	0.0188	0.0033
D	As for *B*, but charcoal removed before using medium	37,000	18,000	5,340	0.0168	0.0014
E	As for *C*, but charcoal removed after 3 hours at 50°C.	36,600	19,200	5,160	0.0165	0.0033

* Differences necessary for significance, 0.0102 ($p = 0.05$) and 0.0160 ($p = 0.01$). In the calculation of K and its error the mean count at each time was weighted in inverse proportion to its variance.

If simple synthetic media are prepared and solidified with methanol-extracted agar it should be possible to produce inhibitor-free media in which starch or charcoal supplements would be without effect. Experiments in this direction have been unsuccessful, considerable effects of starch or charcoal still being observed in media containing only simple substances and methanol-extracted agar. In these synthetic media some samples of charcoal were markedly toxic, this being presumably due to inhibition by metals in the charcoal as the toxic effects were reduced by extraction with mineral acid or 8-hydroxyquinoline, or by incorporating 1.0 p.p.m. of the latter substance in the medium. Charcoal prepared from sucrose lacked this toxic property.

The possibility exists that the inoculum itself is a significant source of inhibitors, but treatment of heated spore suspensions by exposing them to charcoal in cellophane sacs or by mixing with charcoal and subsequent removal

of the adsorbent has failed to show any inhibitors in the inoculum. Such evidence is, however, of a negative character and does not eliminate the possibility that the heated spores themselves may, at times, carry substances which are inimical to their germination.

IV. Discussion

The experiments provide further evidence that there are considerable technical difficulties in the detection and enumeration of *Bacillus* spores that have been subjected to lethal conditions of heating. Some of the observations are without any obvious explanation and simply raise questions without solving them. It is believed, however, that such effects as the response to combined supplements of thioglycollate and starch (Table 1) are of sufficient magnitude to establish their importance, even as unexplained facts.

It is clear that, as the period of heating is increased, the surviving spores become increasingly sensitive to inhibitors in the medium. It is not known, however, whether this is due to a progressive "heat injury" which causes the spores to become more sensitive to inhibitors, or whether the most heat-resistant spores in a suspension are inherently the most susceptible to inhibitors. Whatever the cause, however, the fact remains that the addition of a suitable adsorbent to a particular medium may cause it to indicate a significantly lower rate of destruction than that revealed by the control, unsupplemented medium. In general, the unheated spores show little or no evidence of susceptibility to inhibitors, at least in the concentrations in which they occur in common media. This is true even for organisms such as *Bacillus* No. 320 where only 2 to 5 per cent. of the unheated spores may germinate unless the spores are subjected to an appropriate "heat activation" treatment.

That starch, charcoal, and serum albumin owe their effects to adsorption of inhibitors is shown by the fact that all three substances produce similar effects when added singly or in combination, by the shape of the curves relating the response to the concentration of added charcoal, and by the removal of inhibitors from the medium along with the charcoal. The latter result shows also that the medium is, in fact, the principal source of the inhibitors. Although the various media all show evidence of inhibitors which are adsorbed on charcoal, the results shown in Figure 2 rather strongly suggest that some media contained inhibitors which were not adsorbed.

The present experiments furnish no evidence regarding the nature of the inhibitors although it is probable that long chain unsaturated fatty acids are involved. Traces of these acids have been shown by a number of workers to inhibit the growth of a variety of organisms including species of *Haemophilus, Lactobacillus, Mycobacterium,* and *Neisseria,* the inhibition being reversed by one or more of the adsorbents used in these experiments. Wynne and Foster (1948) and Foster and Wynne (1948) have shown that germination of the spores of *Cl. botulinum* was inhibited by traces of unsaturated fatty acids although the growth of vegetative cells was unaffected by much higher concentrations. These authors found no inhibition of the germination of unheated

spores of four species of *Bacillus* by unsaturated fatty acids. Murrell (unpublished data) has, however, found that the susceptibility of *Bacillus* spores to unsaturated fatty acids increases with the duration of heating. Although it is likely that these fatty acids do account for at least part of the inhibitory properties of the media, more direct evidence is needed to demonstrate whether or not they are the only substances of importance. In this connexion it should be remembered that starch, charcoal, and serum albumin are each able to combine with many substances other than fatty acids. Schuhardt *et al.* (1949) have recently reported that certain peptones contain an anti-brucella factor which can be adsorbed on charcoal. The substance is not a fatty acid, but is believed to be an oxidized polypeptide or amino acid.

It is obvious that many important problems relating to the germination of heated spores still remain unanswered. Studies of spore germination in a chemically defined, inhibitor-free environment should enable the optimum conditions for germination to be defined, and lead to more reliable and precise techniques for evaluating rates of thermal destruction. Meanwhile, there is need for considerable care in selecting a suitable medium for these studies, and it would seem advisable to supplement the medium with a suitable adsorbent and to confirm the estimates with at least one other basal medium.

V. Acknowledgment

The authors are indebted to Mr. G. Ferris, Section of Mathematical Statistics, C.S.I.R.O., for the statistical calculations.

VI. References

Difco Manual (1944).—7th Ed. (Difco Laboratories Inc.: Detroit.)

Foster, J. W., and Wynne, E. S. (1948).—Physiological studies on spore germination, with special reference to *Clostridium botulinum*. IV. Inhibition of germination by unsaturated C18 fatty acids. *J. Bact.* **55**: 495-501.

Ley, H. L., Jr., and Mueller, J. H. (1946).—On the isolation from agar of an inhibitor for *Neisseria gonorrhoeae*. *J. Bact.* **52**: 453-60.

McMeekin, T. L. (1940).—Preparation and purification of serum and plasma proteins. II. Crystallization of a carbohydrate-containing albumin from horse serum. *J. Amer. Chem. Soc.* **62**: 3393-6.

Olsen, A. M., and Scott, W. J. (1950).—The enumeration of heated bacterial spores. I. Experiments with *Cl. botulinum* and other species of *Clostridium*. *Aust. J. Sci. Res.* B **3**: 219.

Pollock, M. R. (1947).—The growth of *Haemophilus pertussis* on media without blood. *Brit. J. Exp. Path.* **28**: 295-307.

Schuhardt, V. T., Rode, L. J., Foster, J. W., and Oglesby, G. (1949).—An anti-brucella factor in peptones. *J. Bact.* **57**: 1-8.

Wilson, G. S., *et al.* (1935).—The bacteriological grading of milk. Med. Res. Coun. Gr. Brit.—Spec. Rep. Ser. No. 206.

Wynne, E. S., and Foster, J. W. (1948).—Physiological studies on spore germination with special reference to *Clostridium botulinum*. I. Development of a quantitative method. *J. Bact.* **55**: 61-8.