***Bacillus subtilis* Spores labelled with Radiophosphorus**

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SUMMARY: *Bacillus subtilis* was grown in surface culture on media containing about 0.6 mC./ml. of ^{32}P as phosphate, and harvested after 2 days. The spore suspension was heated at 60° for 1½ hr. and washed by centrifuging and resuspending in water six times. The fresh suspension contained about 2×10^9 spores/ml., of which 20 % were viable. The radioactivity was about 0.05 mC./ml. of which more than 95 % was carried by the spores. The radioactivity was sufficient for 10^3 spores to be readily detected and assessed by conventional Geiger-Müller counting methods. These highly radioactive suspensions are particularly useful in studying the respiratory retention of bacterial aerosols.

A number of investigations have been reported in which micro-organisms have been labelled with radio-isotopes by growing them in media containing the isotope in a chemical form suitable for assimilation. In those experiments the level of radioactivity in the media was generally low, of the order of a few micro-curies/(mC.) ml. It seemed likely that the concentration of radioactive material could be considerably increased without serious loss in the yield of labelled micro-organisms. Because of the probable usefulness, in various studies, of micro-organisms labelled to the highest practical degree of radioactivity, we decided to investigate the use of media of much higher activity than any previously reported.

Bacillus subtilis was chosen for this work because of the relatively high resistance of spores to radiation damage and because *B. subtilis* is a robust and easily handled organism. We used a locally selected strain that has the general characteristics of *B. subtilis* and forms spores freely at 37° in 2 days. Radiophosphorus (^{32}P) was an obvious choice: phosphorus is readily assimilated from simple inorganic phosphates and forms 2–3 % by weight of the spore, and the isotope emits hard β -rays that are easily counted.

The purpose of this paper is to describe the preliminary work, to give the method finally selected, and to note some properties and uses of the highly radioactive spore suspensions obtained.

Radiation dosage

The biological effects of heavy radiation dosages on spores and on resting suspensions of vegetative organisms have been extensively studied (Lea, 1947), but nothing appears to have been previously reported on growing organisms in comparable radiation fluxes. It is of interest to calculate the β -radiation dosages affecting our *B. subtilis* during growth and in subsequent storage.

Note on units of dosage

The roentgen (r.) is the unit of radiological dose and is defined in terms of the ionizing power of X- or γ -radiation. For radiation other than X- or γ -radiation, the roentgen-equivalent-physical (rep.) is used, and is defined as

the radiological dose which produces the same energy absorption in tissue as 1 r. of X-rays or γ -rays. The millirep (m.rep.) is a useful smaller unit.

The organisms were grown on solid medium of activity up to about 600 mC./l., the incubation period being about 45 hr., and the harvested spore suspensions were stored for various periods.

Surface dosage on active medium

We are indebted to A. C. Chamberlain, Atomic Energy Research Establishment, Harwell, for a modification of the treatment of this case given by Parker (1948).

The dosage on the surface of a radioactive material (so long as it is composed of light elements) is half what it is in the middle. Parker gives for a submerged object

$$\text{Dosage rate} = 2.6 CE/\rho \text{ m.rep./hr.},$$

where C = activity in $\mu\text{C./l.}$; for our most active media this is about $6 \times 10^5 \mu\text{C.}$ E = average β -ray energy; for ^{32}P this is about 0.7 Mev. (Million electron volts); ρ = density; for our media this is 1.06 g./c.c. The dosage rate is therefore

$$\frac{1}{2} \frac{2.6 \times 6 \times 10^5 \times 0.7}{1.06} = 5.15 \times 10^5 \text{ m.rep./hr.}, \text{ or } 515 \text{ rep./hr.}, \text{ and the total dosage}$$

in the 45 hr. incubation period is $515 \times 45 = 2.3 \times 10^4 \text{ rep.}$

Self-irradiation in the spore suspension

The freshly harvested spore suspensions from medium of activity about 600 mC./l. give about 25×10^7 counts/min. from 20 ml. Since the Geiger-Müller (GM) counter is about 10 % efficient, the number of disintegrations/min./ml. is

$$\frac{25 \times 10^7}{20} \times 10 = 1.25 \times 10^8$$

1 $\mu\text{C.} = 2.2 \times 10^6$ disintegrations/min. and the activity of the suspension is therefore

$$\frac{1.25 \times 10^8}{2.2 \times 10^6} = 57 \mu\text{C./ml.}, \text{ or } 57 \times 10^3 \mu\text{C./l.}$$

Owing to radioactive decay, the mean activity over a period of 14 days will be about $42 \times 10^3 \mu\text{C./l.}$ Using the formula given above, the dosage rate over this period will be $2.6 \times 42 \times 10^3 \times 0.7 = 7.6 \times 10^4 \text{ m.rep./hr.}$ (The density is taken as 1.0 g./c.c.) The total dosage is therefore $7.6 \times 10^4 \times 10 \times 24 = 2.6 \times 10^7 \text{ m.rep.}$ or $2.6 \times 10^4 \text{ rep.}$ in 14 days. This is probably an underestimate, since the ^{32}P is mostly contained within the spores which settle out and increase the concentration of activity in their vicinity. Summarizing these calculations we may say that the organisms receive a dosage of about $2.3 \times 10^4 \text{ rep.}$ during the incubation period and probably more than $3 \times 10^4 \text{ rep.}$ during 14 days storage.

The literature gives median lethal dosages (M.L.D.) of, for example, $4 \times 10^3 \text{ rep.}$ (β -radiation) for *Bacterium coli*, and $1.1 \times 10^5 \text{ rep.}$ for *B. mesentericus* spores (Lea, Haines & Bretscher, 1941). If the *B. subtilis* used here be assumed to spend 1 day of the incubation period in vegetative multiplication, it probably received two or three M.L.D. during this period; this is in agreement with the

low yield of spores: namely, about 40 % of that on inactive medium (see below). It was also found that only 20 % of the harvested spores were viable as shown by the ratio of viable to total (optical density) count (see Table 3). When the spores were examined by a technique developed by Pearce & Powell (1951), this was confirmed, and the further interesting observation was made that a number of the spores that were non-viable in the sense of being unable to form macroscopic colonies did, however, germinate and might achieve one or two divisions before growth ceased and lysis took place. This suggests the formation of 'lethal mutants' as described by Lea (1947). The decrease in viable count due to self-irradiation is shown in Table 1, which compares suspensions grown on media of various degrees of radioactivity.

Table 1. *Influence of storage after harvesting on viable counts of B. subtilis spore suspensions grown on CCY agar containing various amounts of ^{32}P : viable counts are expressed as percentages of original counts on day of harvesting*

Suspension no.	Radioactivity of medium (mC./l.)	Days storage									
		1	2	3	6	8	10	15	20	30	49
12	605	98	88	69	65	54	43	30	22	7	4
14	60.5	99	106	108	102	102	102	103	—	—	—
15	0	109	103	111	103	108	107	108	109	102	97

Safety precautions

The precautions taken in handling the radioactive materials followed established practice, but a short description may be helpful to workers unfamiliar with this field. Surgical gloves and gowns were used for all operations with highly radioactive material. A consignment of, say, 120 mC. of $\text{Na}_2\text{H}^{32}\text{PO}_4$ in 10 ml. of water was received in an ampoule, which was opened by blow-pipe flame. The contents were transferred to a sterile test-tube by means of a Pasteur pipette and rubber bulb, and 20 mC. lots were distributed into bottles containing 30 ml. of medium by means of a graduated pipette with rubber bulb. Reliance was placed on rapid handling to reduce radiation dosage, and film badges on the wrist showed that the dosage received was below the limit of tolerance.

The glass culture bottles used were only 2–3 mm. thick, and metal shields were therefore used during incubation. Subsequent operations such as harvesting and washing spores were done with bulb-operated pipettes, and reliance was again placed on rapid handling rather than shielding to avoid radiation injury. The bench top was protected by metal trays lined with filter-paper. Gloves, gowns and bench were monitored after the operation, not only for reasons of health but also to detect contamination which might affect experimental material. We have found that normal bacteriological techniques are well suited to this kind of work. Bottles containing expended media, and glassware used in handling strong suspensions, were placed in a metal container and stored for several months until the activity had decayed to a tolerable level. Other glassware was rinsed before being returned for normal washing up.

Preliminary observations

Medium. CCY agar (Gladstone & Fildes, 1940) gave the best yield of spores; experiments with defined media, undertaken to give more controllable total phosphorus content, were abandoned when it was found that the yields of spores were always much lower than on CCY. The normal method of preparation of the CCY agar was modified by omitting the glycerophosphate, so as to diminish the content of non-radioactive phosphorus. This modification did not decrease the spore yield.

Agar. Shredded agar (British Drug Houses Ltd., Kobe No. 1) gave the best yield of spores. 'Oxoid' powdered agar gave an increased uptake of ^{31}P and ^{32}P /spore, but the total yield of spores was so much lower that the utilization of the ^{32}P was less efficient than with the shredded agar (Table 2).

Table 2. *Influence of type of agar on yield of spores when B. subtilis was grown on normal and radioactive CCY*

Sus- pension no.	Radio- activity (mC./l.)	Agar	Spores/bottle ($\times 10^{-10}$)			GM count/ bottle ($\times 10^{-7}$)	GM count/ 10^5 spores		^{32}P utilized (%)
			Total	Viable	Viable (%)		Total	Viable	
29	0	Shr.	9.0	8.0	89	—	—	—	—
18	0	Ox.	1.8	1.6	89	—	—	—	—
23	0	Ox.	3.0	1.9	63	—	—	—	—
26	0	Ox.	2.52	2.06	82	—	—	—	—
32	0	Ox.	2.16	1.48	69	—	—	—	—
30	59.5	Shr.	8.4	3.6	43	3.96	47	108	7.7
31	59.5	Ox.	1.8	0.804	45	2.38	132	296	4.6
19	60.2	Ox.	2.4	0.796	33	2.57	107	323	4.8
12	605	Shr.	3.6	0.816	23	32.7	910	4000	6.3
16	635	Ox.	1.8	0.091	5	7.12	398	7720	1.3

Films of suspensions from shredded agar showed complete spore formation, but those from 'Oxoid' agar only about 30 %. Shr.=shredded agar; Ox.= 'Oxoid' powdered agar.

Method of cultivation. Surface culture on 30 ml. solid medium in flat 8 oz. bottles was preferred. The bottles facilitated handling of the highly radioactive medium, and the radiation dosage in surface culture is one-half that in liquid medium.

Radiation shielding. The radiation dosage during the incubation period was of the order of the M.L.D. for vegetative organisms. If this dosage could be diminished without affecting other conditions, the number of organisms growing and surviving to form spores should be increased. It was thought that a layer of unglazed porcelain lying on the upper surface of the medium would substantially reduce the β -radiation without interfering with the transport of nutrient material to organisms inoculated on the upper surface of the porcelain. It was found, however, that the yield of viable spores obtained when using inactive media was so much diminished, in a variety of test assemblies, that the method was not worth pursuing.

Cellophan-covered medium. The initial water soluble activity of the harvested spore suspensions is high, necessitating tedious repeated washings. It was

thought that growth on cellophan covering the agar might decrease this activity by enabling the spores to be stripped cleanly from the medium, but it was found that the yield of spores was halved without any advantage being gained.

Quantity of medium. Volumes of 30 ml. medium/8 oz. bottle were used in the first experiments. It was thought that this might be an unnecessarily large volume, so that the spore yield was limited by surface area rather than by available nutrients; some economy in radiophosphorus might therefore be obtained by use of less medium. Experiments with inactive medium showed, however, that decrease of the volume of medium below 30 ml./bottle gave decreased spore yield/ml. medium.

Phosphorus content of medium. The ^{32}P was added as Na_2HPO_4 , a readily utilizable form of phosphorus. If the phosphorus-31 in the medium were less readily available, the ^{32}P would be taken up preferentially. If the ^{31}P were present in as readily available a form as the ^{32}P , then the proportion of the radiophosphorus taken up by the spores would be the same as the proportion of inactive phosphorus taken up. Some experimental results bearing on this are given.

(i) A bottle of CCY agar containing 4.77 mg. total phosphorus gave a spore suspension that contained, after washing, 0.522 mg. phosphorus. The uptake of phosphorus was therefore $(0.522/4.77) \times 100 = 10.9\%$ of that present initially in the medium. The proportion of ^{32}P taken up from media of low activity was about 15 % (see Table 3). This indicates that the uptake of the two isotopes was similar, but that part of the ^{31}P was probably in a less readily utilizable form. Inorganic phosphates are more readily utilizable than more complex organic compounds.

(ii) When glycerophosphate was added to CCY medium, the uptake of ^{32}P was much decreased, although the yield of spores was unchanged. This suggested at first that ^{31}P as glycerophosphate was more readily available than ^{32}P as phosphate, but when the addition of ^{32}P as glycerophosphate was tried the uptake was again lower than in the control suspension. It is probable that the decreased uptake of ^{32}P was due to there being a large excess of available phosphorus in the medium after the addition of glycerophosphate. The phosphorus in glycerophosphate is probably no more readily assimilated than in inorganic phosphate. The decreased uptake also found when radioactive glycerophosphate was added was probably due to the presence of inactive 'carrier' glycerophosphate, present after synthesis of the active material, and of which 6 mg. was added to each bottle.

Preferred method of preparation of labelled spore suspensions

The method selected for routine preparation of radioactive suspensions is as follows:

Thirty ml. CCY agar (without glycerophosphate) are melted in an 8 oz. 'medical flat' (medicine bottle $1\frac{1}{2}$ by $2\frac{1}{2}$ by $5\frac{1}{4}$ in., excluding neck) and about 20 mC. of $\text{Na}_2\text{H}^{32}\text{PO}_4$ in 1–2 ml. water added. The agar is allowed to set with the bottle lying on its side and the surface is then inoculated with 1.25 ml.

B. subtilis spore suspension containing about 2×10^8 spores. The bottle is incubated in this position at 37° , in a metal shield to screen β -radiation, for about 45 hr. The growth is washed off with 20 ml. distilled water into a 1 oz. screw cap bottle, shaken by hand with glass beads to break up clumps, and heated at 60° for $1\frac{1}{2}$ hr. Water soluble ^{32}P is removed from the suspension by centrifuging and resuspending in water six times, after which the activity of the sixth supernatant fluid is less than 5 % of the activity of the whole suspension.

Table 3. *B. subtilis* spore suspensions grown on CCY agar containing ^{32}P

Suspension no.	Radio-activity (mC./l.)	Spores/bottle ($\times 10^{-10}$)		Viable (%)	GM count/bottle ($\times 10^{-7}$)	GM count/ 10^5 spores		^{32}P utilized (%)
		Total	Viable			Total	Viable	
15	0	9.6	9.4	98	—	—	—	—
29	0	9.0	8.0	89	—	—	—	—
35	0	9.0	7.82	87	—	—	—	—
2	40.0	—	6.72	—	5.06	—	75	14.6
9	42.0	4.8	4.48	93	4.59	96	102	15.2
30	59.5	8.4	3.6	43	3.96	47	108	7.7
38	58.1	6.4	4.36	68	7.1	111	163	14.3
14	60.5	7.8	4.52	58	5.8	74	128	11.1
33	250	4.8	2.4	50	17.9	374	747	8.4
3	320	—	1.64	—	14.9	—	910	5.4
34	571	5.0	1.06	21	26.1	522	2460	5.4
37	581	4.0	1.03	26	25.5	638	2460	5.1
28	595	4.8	0.96	20	26.2	546	2730	5.1
4	638	—	1.08	—	21.8	—	2020	4.0
12	605	3.6	0.816	23	32.7	910	4000	6.3

The activity of the medium is based on the activity of the radioactive phosphate solution as given by the A.E.R.E., Harwell. For convenience in subsequent calculations, both this and the GM count of the suspension have been corrected for decay to one day after inoculation of the bottle: that is, half way through the incubation period. All counts are in pulses per minute and were taken in equipment of about 10 % counting efficiency.

The total number of spores/ml. is estimated from optical density measurements. We found that these measurements were reasonably reliable both by determinations on fully viable non-radioactive suspensions and by microscopical examination which showed no abnormal proportion of debris. The viable count was made by the plate-dropping technique of Miles & Misra (1938).

Radioactive counts were done either with known volumes dried on aluminium planchettes and presented to an end-window counter (GM 2 or GM 4; General Electric Co.), or with M 6 liquid counters (Veall, 1948; 20th Century Electronics Ltd.). The end-window counter was particularly useful for checking the decreases of activity in the supernatants during washing. The liquid counter was used for determinations in connexion with the experimental use of active suspensions, so that direct comparisons might be made between suspension activity and experimental results obtained with liquid preparations.

Table 3 gives details of suspensions prepared by the routine method at various levels of radioactivity, including control suspensions prepared on non-radioactive medium.

The following points are worth comment: (a) the *total* yield of spores was progressively decreased with increasing radioactivity of medium to about 40 % of the yield on non-radioactive medium; (b) the yield of *viable* spores decreased from 100 % of the total yield on inactive medium to 20 % on the most active medium; (c) from the most active medium, 10^3 spores give about 7 counts/min. and, therefore, using an M 6 tube with background count 12 counts/min., the probable error on a 20 min. count is less than 5 %; (d) the proportion of ^{32}P found in the suspension decreased with increased activity of medium. The decrease appears to be due to the smaller number of spores formed.

Properties of the suspensions

If measurements of radioactivity were to be used as a reliable quantitative index of the presence of labelled spores, it was evidently necessary to be sure that substantially all the ^{32}P in the suspensions was firmly bound to the spores. Experimental results given below show that there was always some radioactivity in the suspending fluid and that the amount increased on standing. However, this amount of activity may frequently be neglected, particularly when the suspension is washed just before use.

Table 4. *Supernatant activity as percentage of total activity of suspension after final wash*

No. of washings	Suspension no.			
	17	19	34	37
1	390	360	—	290
2	46	39	26	21
3	11.3	8.1	5.1	4.6
4	5.4	5.2	5.7	2.0
5	3.1	3.0	6.8	—
6	3.1	2.8	5.4	1.5
7	5.1	1.4	3.7	—
8	4.5	—	3.4	—
9	2.0	—	—	—

Water soluble radioactivity. When the freshly harvested suspensions were repeatedly centrifuged with fresh lots of water, successive washings showed progressively less activity (Table 4). When suspensions were allowed to stand in the refrigerator the amount of activity in the supernatant increased. Repeated centrifuging and resuspension in the same fluid gave with one suspension a supernatant having 5 % of the total suspension activity after 1 day, rising to 22 % after 10 days. It seems possible that this increase was caused by slow diffusion out of dead spores and debris.

Phosphorus exchange. Inactive *B. subtilis* spores suspended in $\text{Na}_2\text{H}^{32}\text{PO}_4$ solution of activity 0.03 mC./ml. for 41 hr. at 4° did not retain significant activity. Successive washings showed very rapid decrease in the activity of the suspension, which was at each stage approximately equal to the activity of the supernatant fluid after centrifuging. This suggests that the ^{32}P in the labelled suspensions is actually incorporated in the cell substance. This was

confirmed by experiments indicating that ^{32}P did not exchange with ^{31}P when the cells were suspended in phosphate buffer, and also by the similarity in uptake of ^{32}P and of total phosphorus during growth. Surface active agents are known to increase cell surface permeability in certain circumstances. Experiments showed that a surface active agent did not significantly affect the loss of ^{32}P from labelled spores. Radioactive spores were suspended in water and in 0.7 % 'Tergitol' (a branched long chain sodium sulphonate; General Metallurgical and Chemical Corporation, Holborn, London), and stored at 4° for 46 hr. The activity of the whole suspension and of the supernatant after centrifuging were then measured. In one experiment the supernatant activity was 37 % of the whole suspension activity in the water suspension, and 56 % in the Tergitol suspension; a repeat experiment gave 42 % in water and 41 % in Tergitol. It was concluded that the Tergitol had no significant effect. This is in agreement with other observations which show that heat stable spores are usually indifferent to substances which attack or modify the vegetative cell wall.

Applications

These highly radioactive suspensions are particularly useful in studying the respiratory retention of bacterial aerosols. It is easy to detect, and to assess with sufficient accuracy, numbers of the order of 10^3 – 10^4 spores, so that experimental animals do not have to be exposed to very concentrated aerosols for long periods. We have published a preliminary account of such work (Buckland, Harper & Morton, 1950); similar work has been reported by Goldberg & Leif (1950), who used avirulent *Pasteurella pestis* labelled with ^{32}P .

In a previous communication (Buckland *et al.*, 1950) reference was made to the loss of viable organisms frequently observed when dilute suspensions of normal spores are bubbled or shaken, or even allowed to stand at room temperature or in the refrigerator. This loss has usually been ascribed to aggregation or to actual loss of viability; aggregation has been directly observed. However, we have found corresponding decreases of radioactive count, and found the spores, irrespective of aggregation, were adsorbed on the wall of the vessel. This is a factor of considerable importance in any investigation of chemical and physical influences on viability, and may cause doubt as to the validity of some reported results. The use of labelled organisms greatly facilitates the study of apparent losses of viable organisms due to adsorption on vessel walls.

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