Chemical Factors in the Germination of Spore-bearing Aerobes. The Effect of Yeast Extract on the Germination of *Bacillus anthracis* and its Replacement by Adenosine

By G. M. HILLS, *Microbiological Research Department, Porton, near Salisbury, Wilts.*

(Received 29 March 1949)

The rapidity of germination of bacterial spores when inoculated into a nutrient medium has been known for many years. Thus Fischöder (1909) showed that 98 % of anthrax spores became heat-sensitive in broth at 37° in 1 hr., but only 50 % became heat-sensitive in saline at the same temperature in 5 hr. Little work has been done, however, on the specific nutrients responsible for this type of effect, in spite of advances in the study of the requirements for bacterial growth (reviewed by Knight, 1945). Thus the review of Knaysi (1948) on the bacterial endospore adds little on the conditions for germination to that of Cook (1932), apart from the work of Knaysi (1945) himself on *Bacillus mycoides* (*B. cereus* var. *mycoides*). Using a microscopic technique, he concluded that the spore is adequately supplied with stored nitrogen, but requires a source of carbohydrate and maintenance of a neutral pH. On the other hand, Keilin & Hartree (1947), using a metabolic method, found that *B. subtilis* required a source of assimilable nitrogen and an unidentified dialysable factor present in yeast extract, peptone or tryptic digest of casein.

More recently, Wynne & Foster (1948*a, b, c*), and Foster & Wynne (1948), have studied the subject with special reference to *Clostridium botulinum*. Their findings showed a need for carbon dioxide (Wynne & Foster, 1948*c*), and inhibition by traces of unsaturated fatty acids, recalling inhibition of the growth of non-sporing bacteria, e.g. *Mycobacterium tuberculosis* (Dubos, 1945) and *Haemophilus pertussis* (Pollock, 1947). In spite of the widespread occurrence of carbon dioxide requirements and unsaturated fatty acid inhibitions in bacterial nutrition, no such effects were observed (Wynne & Foster, 1948*c*; Foster & Wynne, 1948) with the germination of the four aerobes studied, *B. brevis*, *B. megatherium*, *B. mesentericus* (*B. pumilus*) and *B. subtilis*.

In other fields of biology, there has been a general interest in problems of dormancy, and it has been recognized that the germination of many dormant cells is stimulated by essential metabolites produced and secreted by active tissues (see Brown, 1946), and in some instances the active principles have been either identified chemically or replaced by pure chemical compounds.

The germination of the mould, *Phycomyces blakesleeana*, was examined by Robbins and co-workers, who obtained two fractions from potato extract (Robbins & Hamner, 1940), both of which were essential for optimal activity. One of them could be replaced by guanine (Robbins & Kavanagh, 1942*a*) or by hypoxanthine (Robbins, Kavanagh & Kavanagh, 1942), and the latter was isolated from the extract by Robbins & Kavanagh (1942*b*). Other purines tested by Robbins (1943) were less active. The work of Hurni (1946) showed that the germination of *Rhizopus nodosus* was also stimulated by hypoxanthine, and that the early growth of four other Mucoraceae was similarly stimulated, though proof was not given that such stimulation was specifically connected with the phase of germination. With *Neurospora crassa*, on the other hand, the stimulating material in media containing autoclaved pentose was probably furfural (Emerson, 1948).

In a different field, the requirements for excystment of the ciliate protozoon, *Colpoda cucullus*, have been reported by Barker & Taylor (1933), Thimann & Barker (1934), Thimann & Haagen-Smit (1937), Haagen-Smit & Thimann (1938) and Prater & Haagen-Smit (1940). These authors found that, in addition to a source of soluble carbohydrate and various stimulating factors associated with carbohydrate metabolism, there was a need for one of two neutral compounds, $C_{13}H_{24}O_2$ or $C_{26}H_{44}O_2$, which were active at a concentration of 1 in $2.5-5.0 \times 10^7$. So far, it is not known if the nature of these compounds has been established, and their activity proved by the use of synthetic specimens.

It will be seen that in addition to unidentified factors, the compounds which have been implicated in the germination of dormant cells are of a very varied nature, no common pattern being yet discernible. The work now reported is concerned with the addition of results on the germination of spores of a single strain of *B. anthracis*. Results on other strains and species will be given in a later paper.

METHODS

The loss of resistance to heat, as a physiological criterion of germination shown by viable count, has been preferred in spite of the criticism (Cook, 1932;

Gardner, 1945) that this represents a pregerminative phase and does not correspond to true germination. The choice is justified, however, by the customary use of heat resistance in the diagnosis of spore formation and in freeing spore suspensions from vegetative cells. Support of this view has recently been elaborated by Wynne & Foster (1948*a*), and the evidence need not be repeated in detail. Briefly an organism, after loss of heat resistance, cannot be regarded as being in the spore state. A spore undergoing such a change must therefore be regarded as having germinated if still viable. The term 'germination' will therefore be used in this sense throughout.

Fig. 1. Effect of time at different temperatures on viable count of *Bacillus anthracis* (diagrammatic). *AB* at 0° in nutrient medium; *AC* at 35° in nutrient medium; *AD*, *BE* and *CFG* at 60° in inert diluent.

General principle. This is shown in Fig. 1. If spores were suspended in a suitable culture medium, and dilutions in a non-nutrient but harmless fluid were made at intervals for determination of the viable count, the changes in count were either small or insignificant during a period of about 30 min. whether the culture medium was maintained at 0° (*AB*) or 35° (*AC*). If the dilutions were heated at 60°, little further change was shown over a period up to 60 min., either with those made initially (*AD*) or with those made from culture medium which had been maintained at 0° (*BE*). With dilutions from culture medium which had been maintained at 35°, on the other hand, the count fell rapidly (*CFG*), usually reaching a comparatively stable lower level in less than 5 min. The count of spores remaining resistant to heat at 60° after any treatment will be referred to as the *S* count, while counts set up without heating at 60°, including both resistant spores and non-resistant vegetative forms, will be referred to as the *S* + *V* count. (These abbreviations are used instead of the terms 'spore count' and 'total count' to avoid confusion with the customary meaning of the latter in bacteriology.)

Spore suspensions. Spores of a virulent strain of *B.*

anthracis, supplied by Dr R. L. Vollum, were dried in horse serum and stored *in vacuo*. Three different spore suspensions were prepared from this stock culture as follows: (*a*) Grown direct from the stock culture on the surface of CCY agar (Gladstone & Fildes, 1940) to about 50% sporulation in 2-3 days. (*b*) Grown from (*a*) to about 100% sporulation in 24 hr. in a vigorously aerated medium composed of 2% marmite, 1% cane molasses (decolorized with activated charcoal) and 0.5% $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$. The pH of this acid medium was adjusted to 7.3 with aseptic precautions after autoclaving. (*c*) Grown from (*a*) to about 90% sporulation in 48 hr. in the 'complete' synthetic medium of Gladstone (1939) with L-alanine in place of DL-alanine (Hills, 1949) and supplemented by 10^{-7} M-aneurin (Hagan, O'Kane & Young, 1943), 5×10^{-4} M- Ca^{++} (Brewer, McCullough, Mills, Roessler, Herbst & Howe, 1946), and 10^{-5} M-yeast adenylic acid. The inoculum was about 2000 spores/ml. (viable count), and the cultures were aerated by shaking at 120 oscillations/min. in 50 ml. conical flasks containing 10 ml. culture, the distance of travel being 5 cm. The strain was still virulent after growth on this synthetic medium (LD_{50} for mice, about 200 spores by the intraperitoneal route).

After reaping, all spore suspensions were heated 1 hr. at 60° to destroy remaining vegetative forms, washed thrice with distilled water and stored in the ice chest. Suspension (*a*) was used for the major part of the work, (*b*) was used only for a few preliminary experiments, and (*c*) was used after the discovery that a preparation of yeast adenylic acid promoted almost complete germination in a suitable synthetic medium. It was thus possible, by cultivation of spores in such a medium, to reduce transference with the inoculum of unknown biologically active metabolites. Without adenylic acid, the use of a synthetic medium involved cultures in which only a small proportion of the inoculum could have been shown to have germinated. Under these conditions, growth from those spores which germinated most easily may have produced a new population of spores with simpler requirements for germination than those of the original culture. Such suspensions were avoided.

Basal medium. One of the early difficulties was the design of a suitable deficient basal medium, since germination was too rapid for quantitative measurement in the usual laboratory media.

The medium finally adopted had the following composition: KH_2PO_4 (A.R., 4.5 g. in 450 ml. water); L-tyrosine (crude, see p. 357, 50 mg. in 25 ml. N-NaOH); HCl-hydrolysed gelatin (Nelson's photographic no. 1, neutralized, 12.5 ml. 20%). The constituents were made up together to 500 ml., brought to pH 7.4 with 1-2 ml. N-NaOH and autoclaved. This basal medium was double strength to allow for the addition of inoculum and various test preparations to a fixed total volume. Although not essential for germination, the following mixture, which was essential for good growth, was added aseptically just before use (0.5 ml./10 ml. double-strength medium): aneurin, 1 ml. 10^{-4} M; MgSO_4 , 40 mg.; $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$, 16 mg.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 110 mg.; glucose, 2.5 g.; in 5 ml. N-HCl with 20 ml. water; Seitz filtered. After this addition the medium was neutralized with 0.5M- NaHCO_3 (0.2 ml./10 ml. double-strength medium) and distributed aseptically in accurately measured quantities in $6 \times \frac{3}{4}$ in. test tubes which had been cleaned in acid bichromate and sterilized with gauze-covered plugs.

In some experiments this basal medium was replaced by one containing 33 mM-phosphate buffer (prepared from

KH_2PO_4 (A.R.) with CO_2 -free N-NaOH added to give pH 7.3).

Procedure. Before inoculation, the tubes of medium were warmed in a well-stirred water bath, thermostatically controlled at $35.0 \pm 0.1^\circ$. No attempt was made either to aerate or to secure anaerobic conditions. To avoid errors due to the continuation of germination during the sampling of a long series of tubes, experiments were set up by inoculating at timed intervals, usually 1 min. Sampling, by making tenfold dilutions in distilled water, also took place at corresponding intervals. Where initial counts were required, samples were taken just after inoculation, and the tubes of inoculated medium were returned to the water bath immediately after sampling. For $S + V$ counts, the tubes of diluent were cooled in ice water before sampling and were kept in the cooling bath after sampling until the contents were plated. For S counts, the tubes of diluent were heated to 60° before sampling and heating at this temperature was continued for 15 min. after sampling to destroy all germinated forms. The dilutions were then cooled and kept in ice water till the time of plating.

Except where stated otherwise, the inoculum was about 12,000 spores/ml. and the period of incubation was 30 min.

Viable counts and their accuracy. The principle of the surface-plate technique of Miles & Misra (1938) was used with minor modifications in technical details. The composition of the medium for plating was 2% Evans peptone, 0.5% NaCl and 1.5% agar, pH 7.6. To ensure uniformity of successive batches of medium, it was made from a single batch of peptone, of which a sample had been shown to give satisfactory counts of the test organism, never significantly less, and, with some suspensions, many times greater than those on tryptic ox agar. Plates were poured without cooling the melted agar and were dried, inverted over the lids, for 90 min. at 60° . Dropping pipettes, delivering 42 drops/ml., were used to distribute 7 drops of appropriate dilutions over each of 3 plates. Plates were incubated either for 12 hr. at

37° or for 15 hr. at 30° , when the colonies were discrete and of uniform size, about 1 mm. in diameter, provided that the number on each plate did not exceed 250. In designing the experiments, control plates were inoculated so as to yield about 200 colonies from the spores which had failed to germinate after incubation in the deficient basal medium. With the usual inoculum, this was attained by plating, as described, 0.5 ml. of the tenfold dilution made in sampling. Samples showing a fall in count due to germination were plated at the same dilution.

Ideally, the chance of observing a particular value in a series of replicates should conform to a Poisson distribution, for which both the mean and the variance are estimated by the mean of the observations (Fisher, Thornton & Mackenzie, 1922). For a total control count of 500–700 colonies on the three plates, the standard error was thus about 25 colonies and the limits were of the order of $\pm 8\%$ with a probability, $P=0.95$. Ultimately, there were available for testing conformation to a Poisson series, 47 sets of quadruplicate controls and 21 sets of quintuplicate. With such a small series, it was necessary to group the data at both tails of the distribution, each amounting to 10% of the whole, to avoid expectations less than 5 in any group (Table 1). Owing to the presence of four aberrant values exceeding the theoretical 1% level, the χ^2 for deviations from the theoretical distribution could not therefore accurately summarize the whole of the available information. It indicated a larger probability, 0.5, for the occurrence of greater deviations by chance than 0.18 shown by the total χ^2 for variations within sets of replicates, the latter measure being very sensitive to the presence of a few excessively variable replicates, while giving little information on the general form of the distribution. Taking both criteria into consideration, it was considered justifiable to regard this sample of counts as conforming to a Poisson series, but in using the square root of a count as an estimate of its standard deviation, in order to assess the significance of differences, the precaution was

Table 1. *Conformation of control counts to Poisson series*

Probability of observing greater deviations by chance	No. of sets of replicates observed			No. of sets expected (N)	Difference (n - N)	$\frac{(n - N)^2}{N}$
	Sets of 4	Sets of 5	Total n			
1.00	0	0	0	6.8	+2.2	0.71
0.99	1	0	1			
0.98	2	2	4			
0.95	1	3	4			
0.90	5	2	7	6.8	+0.2	0.01
0.80	6	2	8	6.8	+1.2	0.21
0.70	6	5	11	13.6	-2.6	0.50
0.50	6	2	8	13.6	-5.6	2.30
0.30	8	3	11	6.8	+4.2	2.58
0.20	6	1	7	6.8	+0.2	0.01
0.10	1	0	1	6.8	+0.2	0.01
0.05	1	0	1			
0.02	1	0	1			
0.01	1	0	1			
0.00	3	1	4			
Total	47	21	68	68.0	0.0	6.33
Total χ^2 for deviations of counts from mean of set	175.4	69.3	244.7	—	—	—
Degrees of freedom (ϕ)	141	84	225	—	—	7
$\sqrt{2\chi^2 - \phi}$	1.97	1.15	0.93	—	—	—
Probability of observing greater deviations by chance	0.025	0.88	0.18	—	—	0.5

adopted of examining the data in a particular experiment for evidence of excessive variation.

Comparison of potencies of active preparations. The effects of preparations promoting the germination of spores in the presence of the basal medium were measured by the decrease in *S* count caused by the preparation in excess of the small decrease observed in the basal medium alone under the same conditions.

The control *S* count, after incubation in the basal medium, was used as an estimate of the number of spores which had been subject to the influence of the active preparations.

For comparison of potencies it was found that a convenient linear concentration relationship could be obtained by the probit-log concentration transformation (see Finney, 1947). Statistical justification of this procedure will be published elsewhere. Simple graphical methods sufficed, however, for general use.

RESULTS

Preliminary experiments

The rate of germination of a small inoculum, about 1000 spores/ml., in the usual laboratory media, was so rapid that a high proportion of spores lost their resistance to heat during the time required to make the inoculation and take a sample. On the other hand, the rate was so slow in the synthetic medium of Gladstone (1939), that it was not measurable by the technique adopted. The proportion of spores which became sensitive to heat in a complex medium during a convenient experimental period could be controlled, however, by increasing the size of the inoculum, or by diluting the medium. The latter method was chosen in the first place, in order to keep the inoculum size as small as possible, and so avoid transferring to fresh medium unknown essential metabolites present in the seed. It is recognized that this could be no more than a temporary expedient, since investigation of the effect of a particular nutrient might be of little value if concurrent deficiencies of other nutrients occurred.

In order to gain some idea of the types of material essential for germination, the CCY medium of Gladstone & Fildes (1940) was used since its composition, though complex, can be subjected to some degree of controlled variation. The bulk of the amino-acids were supplied by an acid hydrolysate of casein, while those destroyed by acid hydrolysis, especially tryptophan, were supplied by a tryptic digest of the same protein. Other growth factors were supplied by yeast extract. Using this medium at 10 % of the usual concentration (Table 2), it was shown that the tryptic digest was not essential, and that the small activity of the acid hydrolysate (Exp. 1), with or without tryptic digest, was much enhanced by yeast, although the latter, in the presence of buffer, was inactive (Exp. 2). The yeast extract was not replaceable by a mixture of known growth factors. Its fractionation was therefore undertaken with the object of identifying the active components.

Table 2. *The effect of constituents of CCY medium on germination*

(Basal medium, 0.05M-glycerophosphate, pH 7.3. The additions were prepared as described by Gladstone & Fildes (1940) and the final concentrations in the medium were acid-hydrolysed casein (*AC*), 0.07%; tryptic digest of casein (*TC*), 0.03%; yeast extract (*Y*) equivalent to 0.2% fresh yeast.)

Exp. no.	Additions to basal medium	Spore count at			
		0 min.	15 min.	30 min.	60 min.
1	None	446	—	—	362
	<i>AC</i>	447	462	376	198
	<i>AC</i> + <i>TC</i>	509	478	382	208
	<i>AC</i> + <i>Y</i>	340	140	58	30
	<i>AC</i> + <i>TC</i> + <i>Y</i>	483	131	74	29
2	None	608	—	520	—
	<i>Y</i>	571	—	562	—
	<i>AC</i>	646	—	372	—
	<i>AC</i> + <i>Y</i>	615	—	48	—

The diluted casein hydrolysate did not, however, provide a suitable basal medium for the assay of yeast fractions, since the degree of germination, even in the absence of yeast extract, was too high except at dilutions which may well have been suboptimal in several essential metabolites. The synthetic medium of Gladstone (1939) was also unsuitable, since, as in buffer, the activity of yeast was not observed in this medium. It became necessary, therefore, to devise a suitable basal medium.

Development of a basal medium for assay of yeast fractions

A suitable basal medium should, in general: (i) contain optimal amounts of all essential nutrients except that to be assayed, and (ii) usually be as free from the material to be assayed as possible. Condition (i) is satisfied when the biological effect in the presence of an active supplement is independent of the concentration of the basal medium within suitable limits. Condition (ii) is satisfied when the effect in the basal medium alone is small.

After a number of trials, a hydrochloric acid hydrolysate of gelatin, supplemented by tyrosine and buffered by 33 mM-phosphate, pH 7.3, was found to allow a satisfactory response to yeast extract. In the presence of 0.28 mM-L-tyrosine, the optimum concentration of gelatin hydrolysate was 0.25 % (Fig. 2). Lower concentrations of gelatin were little less effective, but higher concentrations were inhibitory. No advantage in this respect was offered by a hydrolysate of lower salt content made with sulphuric acid and treated with barium hydroxide to remove sulphate ions. A fivefold increase in the tyrosine concentration, supplied either as the pure isomer or as twice the amount of synthetic DL-tyrosine, did not increase the extent of germination (Table 3). The initial counts showed that the presence

Fig. 2. Optimum concentration of hydrolysed gelatin for germination in presence of 0.005% tyrosine. *A*, basal medium with gelatin hydrolysed by 23% HCl, 0.75 hr. at 120°; *B*, basal medium with gelatin hydrolysed by 10% H₂SO₄, 3 hr. at 130°; *A* + yeast extract equivalent to 0.2% fresh yeast, $\square$; *B* + yeast extract equivalent to 0.2% fresh yeast, $\diamond$. To increase the range covered, the horizontal scale is proportional to the square root of the concentration.

of tyrosine in the dilutions during heating did not significantly affect the capacity of the spores to develop colonies on plating. For routine use, a

Table 3. *Effect of tyrosine on germination in presence of acid-hydrolysed gelatin and yeast extract*

(The basal medium contained HCl-hydrolysed gelatin, equivalent to 0.25% (w/v) gelatin, and yeast extract equivalent to 0.02% (w/v) fresh yeast in 33 mM-phosphate buffer, pH 7.3.)

Addition to basal medium	Concn. of each enantiomorph (mM)	Spore count at	
		0 min.	30 min.
None	—	375	283
L-Tyrosine	0.28	381	106
	1.4	369	89
DL-Tyrosine	0.28	384	99
	1.4	405	103

crude sample of L-tyrosine, obtained as a by-product from the preparation of tryptic digest of casein, was found to be as suitable as the pure preparations.

Table 4. *Effects of various metabolites on germination in acid-hydrolysed gelatin and tyrosine, with and without yeast extract*

(Basal medium, HCl-hydrolysed gelatin equivalent to 0.25% gelatin, 0.28 mM-L-tyrosine (crude) in 33 mM-phosphate buffer pH 7.3.)

Additions to basal medium	Concn. (M)		Concn. (M)
Cations		Tryptophan (L-)	0.5×10^{-3}
Ca ⁺⁺	1.0×10^{-3}		
Fe ⁺⁺	0.8×10^{-4}	Vitamins	
Mg ⁺⁺	0.5×10^{-3}	<i>p</i> -Aminobenzoate	0.8×10^{-5}
Glucose	1.3×10^{-2}	Aneurin	2.0×10^{-6}
Guanine and pyrimidines		Ascorbic acid	0.4×10^{-3}
Cytosine	4.5×10^{-5}	Choline	0.4×10^{-5}
Guanine	3.3×10^{-5}	Inositol	0.4×10^{-3}
Thymine	4.0×10^{-5}	Nicotinic acid	0.7×10^{-5}
Uracil	4.5×10^{-5}	Pantothenate	2.0×10^{-6}
Sulphur compounds		Pimelate	0.7×10^{-4}
Cystine (L-)	1.0×10^{-4}	Pyridoxine	0.8×10^{-5}
Glutathione	1.0×10^{-3}	Riboflavin	2.0×10^{-6}

(Since the technique gave control counts conforming to the Poisson distribution (Table 1), the standard error (*s*) of the difference between two counts (*D*) could be taken as the square root of the sum of the two counts. Ratios *D/s* exceeding 1.96 were considered to show significant differences (*P* = 0.95) and are given in heavy type.)

After 30 min. at 35°						
Additions to basal medium	Initial spore count	<i>D/s</i>	No yeast		0.03% yeast	
			Spore count	<i>D/s</i>	Spore count	<i>D/s</i>
None	644	—	641	—	253	—
Cations	651	0.19	529	3.28	243	0.45
Glucose	638	0.17	593	1.37	296	1.84
Guanine and pyrimidines	684	1.10	648	0.19	348	3.88
Sulphur compounds	684	1.10	627	0.39	234	0.86
Tryptophan	642	0.06	584	1.63	247	0.27
Vitamins	665	0.59	605	1.02	286	1.42
All	679	0.96	638	0.08	303	2.13

In order to check the adequacy of the supply of essential factors in this basal medium, the effects of various known growth factors were tested, not only on the blank, but also on the germination with suboptimal amounts of yeast extract (Table 4). The materials tested included cations, glucose, guanine, pyrimidines, sources of organic sulphur, tryptophan and water-soluble vitamins. No pronounced effects were observed, but those which were statistically significant were the small inhibition of 15% by guanine plus pyrimidines in the presence of yeast, and the small promotion of germination of 14% by cations in the absence of yeast. These effects could have no influence on the assay of yeast fractions, since the concentrations, at levels similar to those found optimal for growth by Brewer *et al.* (1946), were at least 50-fold greater than those likely to be present in yeast extracts of much greater activity. The cations, however, together with aneurin and glucose, were included in the basal medium, since they were essential for good growth and therefore, presumably, for survival of germinated forms. Details for preparation of the medium are given in the section on Methods, p. 354.

Replacement of yeast factor by adenine derivatives

After the development of a suitable basal medium, the problem was pursued along two independent lines. Boiled yeast extracts, or boiled autolysates up to twenty times as active as such simple extracts,

Table 5. *Effect of nucleic acid derivatives on germination in basal medium*

(Ribose, where added, was 4×10^{-4} M. Other additions were 2.5×10^{-4} M, except yeast adenylic acid, 10^{-5} M, guanylic acid, 10^{-4} M and yeast extract equivalent to 375 μ g. fresh yeast/ml. (Exp. 1) or 500 μ g./ml. (Exp. 2).)

Exp. no.	Additions to basal medium	Spore count after 30 min. at 35°	
1	None	495	
	None	491	
	None	463	
	Adenine	492	
	Adenosine triphosphate	550	
	Adenylic acid	216	
	Guanine	490	
	Guanylic acid	302	
	Thymus nucleic acid	434	
	Yeast nucleic acid	265	
	Yeast extract	201	
2		Without ribose	With ribose
	None	724	766
	None	938	938
	Adenine	908	944
	Guanine	757	948
	Hypoxanthine	764	944
	Xanthine	892	826
	Adenylic acid	126	67
	Yeast extract	122	102

were fractionated to give preparations active at 2.5–5.0 μ g./ml. These experiments are not described in detail, since the testing of materials known to be present in yeast extract showed activity in preparations of many nucleic acid derivatives (Table 5).

A commercial sample of yeast adenylic acid was highly active, and a guanylic acid preparation also showed some activity, but adenine and guanine were inactive. Preparations of yeast and thymus nucleic acids also showed some activity. In a second experiment (Table 5) the purines were tested with and without ribose to see if the latter made the synthesis of an active compound possible. Significant effects were not observed. Fig. 3 shows that the maximum

Fig. 3. Effect of nucleotide preparations and yeast extract on germination. Adenylic acid, $\diamond$; guanylic acid, $\square$; yeast extract, $\triangle$.

effect was given by both adenylic acid and guanylic acid at a concentration of about 35 μ g./ml. or 10^{-4} M, but the latter showed only 40% germination, compared with over 90% for adenylic acid. The effect of adenylic acid, however, was qualitatively different from that of yeast, since twofold increase of yeast concentration increased germination from 45 to 70%, whereas fivefold increase in adenylic acid concentration was necessary to produce a similar change in germination.

It must be emphasized that the preparations used up to this stage had been commercial products which had not been specially purified. The activity may therefore have been due to impurities. This was also suggested by the properties of the material in yeast extract, in particular the failure to get a barium salt insoluble in 67% ethanol. Further, during the course of work on the amino-acids essential for germination (Hills, 1949), it was found that there was a five- to ten-fold increase in the activity of the sample of adenylic acid used (Table 6) in 5 mm.-aqueous solution at its own pH (3.1) during a period of about 6 months at room temperature. In view of the probability of hydrolysis under these conditions, a commercial sample of adenosine was tested at the same time and found to have practically the same

activity as the old solution of adenylic acid, i.e. five to seven times the activity of freshly made solutions of preparations of yeast adenylic acid from two different commercial sources. Adenine cytosine dinucleotide had the same activity as adenylic acid on a molecular basis, but a guanosine preparation showed much smaller activity.

Table 6. *Concentrations of preparations of nucleosides and nucleotides for 50 % germination under standard conditions*

(The conditions were 30 min. at 35° in the basal medium described on p. 354. The concentration for a final spore count 50 % of that in the basal medium was estimated from points in the neighbourhood of 50 % survivors on the assumption that the probit-log concentration regression line was parallel to that of the standard yeast preparation, which gave 50 % survivors at 131–151 µg./ml. in these experiments.)

Addition to basal medium	Concn. for 50 % germination (µM)
Adenosine	0.8
Yeast adenylic acid	
Sample A, fresh solution	4.0
Sample A, 6-month-old solution	0.5
Sample B, fresh solution	5.5
Adenine cytosine dinucleotide	6.1
Guanosine	80

In view of the importance of tyrosine in germination and the similarity in the stability of tyrosine codecarboxylase (Gale & Epps, 1944) to the germination factor from yeast, it seemed likely that this coenzyme might be a possible biologically active impurity in commercial preparations of nucleic acid derivatives. A sample, kindly supplied by Dr E. F. Gale, was, however, found to be inactive even at 1 mg./ml.

From these observations it appears that the activities of nucleic acid derivatives might reasonably be accounted for by their content of adenosine, a preparation of which was the most active material tested.

Purification of adenosine

In order to complete the identification of adenosine as the active material, the changes in activity were examined (Table 7), following purification by recrystallizations from water without change in m.p. 224–226° (uncorr.). The final product, dried *in vacuo* over CaCl₂, gave the following analytical results (Weiler and Strauss): (Found, C, 44.4; H, 5.2; N, 25.6. Calc. for C₁₀H₁₃N₅O₄; C, 44.9; H, 4.9; N, 26.2 %). Levene & Bass (1931) gave m.p. 229°, with 1.5 mol. water but the conditions of drying were not stated. Fractions *D* and *E*, the mother liquor and crystals, respectively, from the third recrystallization, had closely similar activities at all four levels tested. The original commercial specimen *A* and the mother liquors *B* and *C*, from the first and second recrystallizations, respectively, were less active, except for the two lowest levels of preparation *C* in Exp. 1. The χ^2 test was therefore applied on the assumption that the best estimate for each of the levels tested was the mean of the corresponding values for *D* and *E*. Highly significant differences ($P < 0.001$) were observed for *A*, *B* and *C* in both experiments, but the deviations for *D* and *E* together were not significant ($P = 0.21$).

Sterilization of adenosine

In the experiments reported so far, the adenosine had been sterilized by autoclaving in aqueous solution (15 lb./in.²; 20 min.). The purest preparation *E*

Table 7. *Effect of recrystallization on activity of commercial sample of adenosine in promoting germination*

(The significance of differences was tested by χ^2 for the deviations of all points for each preparation from the means of corresponding points for preparations, *D* and *E*. The control counts, without addition of any preparation, were 668.4 and 597.8 in Exps. 1 and 2 respectively, each the mean of quintuplicates.)

		Spore count after 30 min. in basal medium + preparation				
		<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
		Mother liquors from				Solid phase from 3rd recrystallization
Exp. no.	Adenosine concn. (µg./ml.)	Original sample	1st recrystallization	2nd recrystallization	3rd recrystallization	
1	0.188	537	558	515	523	515
	0.470	425	426	334	370	330
	1.175	318	278	301	267	239
	2.94	163	191	193	151	160
2	0.188	528	608	637	393	397
	0.470	362	386	401	304	331
	1.175	243	290	175	132	164
	2.94	156	157	148	114	130
χ^2		153.8	303.7	296.6	10.76	
Probability of difference due to chance		0.001	0.001	0.001	0.21	

was tested after sterilization both by autoclaving and by Seitz filtration (Table 8). Exp. 1 showed no significant differences at any concentration, and although Exp. 2 suggested that the filtered solution was less active at each concentration tested, only at the highest concentration did the difference appear to be significant by a simple χ^2 test. Examination of replicate counts in this experiment showed greater

Effect of adenosine on vegetative and spore counts

Except for a few early experiments in complex media it has been assumed throughout this work that the fall in spore count on incubation in culture media was due to germination. Omission of controls on possible changes in the total count was justified in

Table 8. *Effect of purified adenosine on germination after sterilization by autoclaving or Seitz filtration*

(Thrice recrystallized adenosine was used (Preparation E, p. 359). Sterilization was carried out in 2 mm-solution; autoclaving for 20 min. at 15 lb./in.², and Seitz filtration through a Ford Sterimat S.B., 10 ml. through a pad 3.6 cm. in diameter. Control spore counts with no adenosine in the basal medium were 392.6 and 922.2 for Exps. 1 and 2 respectively (sum of three plates, each sum the mean of quintuplicate determinations). The period of incubation was 30 min. at 35°.)

Spore counts with adenosine									
Exp. no.	Concn. of adenosine (μ M)	Autoclaved		Filtered		χ^2 $\frac{(n_1 - n_2)^2}{n_1 + n_2}$	Degrees of freedom (ϕ)	Probability of greater difference due to chance (P)	
		Single plates	Sum (n_1)	Single plates	Sum (n_2)				
1	0.6	81	289	82	264	1.13	1	—	
		99		89					
		109		93					
	1.8	49	159	56	179	1.18	1	—	
		52		61					
		58		62					
	6.0	40	123	37	117	0.15	1	—	
		41		39					
		42		41					
	2	0.6	219	692	235	745	1.95	1	—
			231		241				
			242		269				
1.8		133	450	150	490	1.70	1	—	
		148		152					
		169		188					
6.0		60	211	65	258	4.71	1	0.03	
		74		89					
		77		104					
			1924		2053		10.82	6	0.09
						4.18	1	0.04	
Analysis of variance:									
	Treatments		ϕ	χ^2	F	P			
	Experiments		1	4.18	3.20	0.09			
	Concentrations		1	3.77	2.89	>0.1			
			2	2.35	0.90	>0.1			
	Interaction		2	0.52	0.20	>0.1			
				10.82					
	Plates		24	31.38					

variability than usual ($P=0.14$), especially for those counts showing the apparently significant difference between autoclaved and filtered solutions. Partition of χ^2 in the form of an analysis of variance and application of a variance ratio test showed that the difference was not, in fact, significant ($P=0.09$). The conclusion is that if any loss of activity occurred on autoclaving it must be equal to the loss by adsorption on Seitz filtration. There is no evidence that the activity of adenosine was due to breakdown on autoclaving.

the assay of preparations, since the observation of such controls would have doubled the experimental work, complicated the computations of activity and the errors of random sampling would have reduced the value of any adjustments which such changes might have made in the probit. Table 9 shows that the effect of adenosine was solely on the loss of heat resistance of spores; the 30% fall of the $V+S$ count on incubation was independent of the addition of adenosine to the basal medium, and whilst the proportion of heat-resistant organisms remained

practically 100 % after incubation in the basal medium, it fell to 11 % in the basal medium plus 2 μ M-adenosine. The remaining 89 %, which were no longer spores since they had become heat sensitive, must be regarded as having germinated since they were still viable, giving visible colonies on plates of a suitable nutrient medium. Adenosine alone was ineffective in the absence of the basal medium.

Table 9. *Effect of adenosine on vegetative and spore counts*

(Sample equivalent to 0.05 ml. undiluted culture.)

Medium	Count of sample			
	Before incubation		After 30 min. at 35°	
	Vegetative + spore	Spore	Vegetative + spore	Spore
Basal	688	680	498	492
Buffer + 2 μ M-adenosine	702	694	560	467
Basal + 2 μ M-adenosine	719	694	472	52

DISCUSSION

The importance of nucleic acid derivatives in bacterial nutrition was first shown by Richardson (1936), when he reported the need of *Staphylococcus aureus* for uracil during anaerobic growth, although the organism was capable of synthesizing this essential pyrimidine aerobically. Subsequent workers have established the need for nucleic acid derivatives by other organisms. In some cases, complex derivatives have been more effective than simple purines and pyrimidines, e.g. with *Neurospora* mutants (Loring & Pierce, 1944) or *Lactobacillus gayonii* (*L. fermenti*) (Hutchings, Sloane & Boggiano, 1946).

The mode of action of adenosine in germination, however, is at present obscure, since nothing is known either of the biochemical or biophysical changes which occur in the spore during germination, or of the metabolism of adenosine by the spore. Adenosine can be hydrolysed and deaminated by various non-spore-forming organisms (Lutwak-Mann, 1936) and can stimulate the deamination of adenine by *Escherichia coli* (Stephenson & Trim, 1938) or of serine by *Clostridium welchii* (Woods & Trim, 1942). It would be of interest to examine the extent to which spores may catalyse such reactions as well as those of the type, purine-riboside + phosphate $\rightleftharpoons$ purine + ribose-1-phosphate, described in animal tissues (Kalckar, 1945). Such processes involving ammonia and phosphate transfer might provide a link between the stimulation of anthrax spores by adenosine and that of *Phycomyces* spores by guanine or hypoxanthine (Robbins & Kavanagh, 1942a, b).

A distinction must be drawn between the requirements for germination and those for growth. The

synthetic ability of cells emerging from a dormant state may be less than those of an actively growing culture (cf. Brown, 1946). The requirements for germination would thus tend to be more exacting than those for growth, as shown by the stimulation of germination but not of growth, by adenosine in the case of *B. anthracis*. In germination, with changes in a single cell without multiplication, it may be more difficult to show a requirement for a particular nutrient, since the cell may carry a supply, though insufficient for optimal activity, and the amount available for each cell cannot be markedly reduced as during growth. This may be the case with pyridoxal phosphate, an important coenzyme in metabolism of amino-acids, including that of tyrosine (see Gale, 1946); however, although tyrosine was important in the germination of *B. anthracis* even high concentrations of the coenzyme had no influence. In growth, involving perhaps a million-fold increase in cell substance, provided that the cell is sufficiently equipped with essential metabolites for initiation of the process, the increased synthetic ability of the actively metabolizing culture may be capable of supplying essential materials which could not be synthesized by the germinating spore. In this way it is possible to explain the growth of *B. anthracis* in synthetic media not containing adenosine (Gladstone, 1939; Brewer *et al.* 1946). In such media growth is due to the germination of only a small proportion of the inoculum in the early stages, presumably under the influence of traces of adenosine in the cells. This proportion is frequently not detectable by the comparatively crude viable-count technique. Increased germination cannot be excluded after changes have been produced in the medium by the actively growing culture, but it is masked by formation of fresh spores.

Although adenosine was the most active material tested in a high degree of purity, commercial preparations of yeast nucleic acid and its derivatives showed some activity. These materials were not purified to show how far the activity was specific for adenosine and how far it was due to related compounds. Adenine, however, was inactive, so that the specificity was greater than that of the *Neurospora* mutant described by Pierce & Loring (1945), for which the maximum growth rate was largely dependent on the amount of adenyl radical available rather than its state of combination. In view of the high sensitivity of the germination of *B. anthracis* to adenosine, this process might be made the basis of a microbiological assay. Less than 0.5 μ g. could be detected and a three-point assay could be carried out on as little as 2 μ g. without modifying the experimental technique already used. Since only 1 % of the actual sample available was used in plating, the method could be made even more sensitive by the use of micropipettes. Of established

methods at present available, the guinea-pig auricle method of Drury, Lutwak-Mann & Solandt (1937) requires 1–2 $\mu\text{g.}$, while the use of a specific deaminase required about 1 mg. unless the reaction is followed by ultraviolet spectrometry (Kalckar, 1947) when the amount may be reduced to 0.1 $\mu\text{g.}$ The results given in Table 6 suggest an adenosine content of about 150 mg./100 g. fresh yeast for the best yeast extract used in this work. Ostern, Terszakowec & Hubl (1938), using the deaminase method, found 360 mg./100 g. dialysed acetone-dried yeast after 20 hr. autolysis, and this was equivalent to 60% of the adenine of the original cell nucleic acid. The amount found by the effect on germination therefore does not exceed the maximum expected from the work of Ostern *et al.* (1938), but a closer comparison is not possible on account of the effects of autolysis and other conditions.

SUMMARY

1. The metabolites essential for the rapid germination of spores of *Bacillus anthracis* have been

studied, using loss of resistance to heat as a measure of germination, determined by viable count.

2. A suitable deficient medium for the assay of promoters of germination contained 0.25% acid-hydrolysed gelatin and 0.005% L-tyrosine. Higher concentrations of hydrolysed gelatin were inhibitory.

3. The slow germination in the basal medium was accelerated significantly by yeast extract equivalent to 0.01% fresh yeast.

4. The yeast extract was replaceable by a purified preparation of adenosine which gave a significant effect at a concentration of 0.2 $\mu\text{g./ml.}$

I have great pleasure in thanking Dr D. W. Henderson and Dr D. D. Woods for careful criticism of this paper. The experimental work was carried out, with the technical assistance of Cpl. W. Bailey, R.A.M.C., while the author was a member of the Scientific Staff, Medical Research Council. Permission to publish has been granted by the Chief Scientist, Ministry of Supply.

REFERENCES

- Barker, H. A. & Taylor, C. V. (1933). *Physiol. Zool.* **6**, 127.
- Brewer, C. R., McCullough, W. G., Mills, R. C., Roessler, W. G., Herbst, E. J. & Howe, A. F. (1946). *Arch. Biochem.* **10**, 65.
- Brown, R. (1946). *Nature, Lond.*, **157**, 64.
- Cook, R. P. (1932). *Biol. Rev.* **7**, 1.
- Drury, A. N., Lutwak-Mann, C. & Solandt, O. M. (1937). *Quart. J. exp. Physiol.* **27**, 215.
- Dubos, R. J. (1945). *Proc. Soc. exp. Biol., N.Y.*, **58**, 361.
- Emerson, M. E. (1948). *J. Bact.* **55**, 327.
- Finney, D. J. (1947). *Probit Analysis*. Cambridge University Press.
- Fisher, R. A., Thornton, H. G. & Mackenzie, W. A. (1922). *Ann. appl. Biol.* **9**, 35.
- Fishoeder, F. (1909). *Zbl. Bakt.* (1. Abt. Orig.), **51**, 320.
- Foster, J. W. & Wynne, E. S. (1948). *J. Bact.* **55**, 495.
- Gale, E. F. (1946). *Advanc. Enzymol.* **6**, 1.
- Gale, E. F. & Epps, H. M. R. (1944). *Biochem. J.* **38**, 250.
- Gardner, A. D. (1945). *Lancet*, **i**, 658.
- Gladstone, G. P. (1939). *Brit. J. exp. Path.* **20**, 189.
- Gladstone, G. P. & Fildes, P. (1940). *Brit. J. exp. Path.* **21**, 161.
- Haagen-Smit, A. J. & Thimann, K. V. (1938). *J. cell. comp. Physiol.* **11**, 389.
- Hagan, W., O'Kane, D. J. & Young, G. A. (1943). Private communication.
- Hills, G. M. (1949). *Biochem. J.* **45**, 363.
- Hurni, H. (1946). *Experientia*, **2**, 66.
- Hutchings, B. L., Sloane, N. H. & Boggiano, E. (1946). *J. biol. Chem.* **162**, 737.
- Kalckar, H. M. (1945). *J. biol. Chem.* **158**, 723.
- Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 445.
- Keilin, D. & Hartree, E. F. (1947). *Antonie van Leeuwenhoek Ned. Tijdschr. Hyg.* **12**, 115.
- Knaysi, G. (1945). *J. Bact.* **49**, 617.
- Knaysi, G. (1948). *Bact. Rev.* **12**, 19.
- Knight, B. C. J. G. (1945). *Vitamins and Hormones*, **3**, 108.
- Levene, P. A. & Bass, L. W. (1931). *Nucleic Acids*. New York: Chemical Catalog Co.
- Loring, H. S. & Pierce, J. G. (1944). *J. biol. Chem.* **153**, 61.
- Lutwak-Mann, C. (1936). *Biochem. J.* **30**, 1405.
- Miles, A. A. & Misra, S. S. (1938). *J. Hyg., Camb.*, **38**, 732.
- Ostern, P., Terszakowec, J. & Hubl, S. (1938). *Hoppe-Seyl. Z.* **255**, 104.
- Pierce, J. G. & Loring, H. S. (1945). *J. biol. Chem.* **160**, 409.
- Pollock, M. R. (1947). *Brit. J. exp. Path.* **27**, 295.
- Prater, A. N. & Haagen-Smit, A. J. (1940). *J. cell. comp. Physiol.* **15**, 95.
- Richardson, G. M. (1936). *Biochem. J.* **30**, 2184.
- Robbins, W. J. (1943). *Proc. nat. Acad. Sci., Wash.*, **29**, 201.
- Robbins, W. J. & Hamner, K. C. (1940). *Bot. Gaz.* **101**, 912.
- Robbins, W. J. & Kavanagh, F. (1942a). *Proc. nat. Acad. Sci., Wash.*, **28**, 4.
- Robbins, W. J. & Kavanagh, F. (1942b). *Proc. nat. Acad. Sci., Wash.*, **28**, 65.
- Robbins, W. J., Kavanagh, V. W. & Kavanagh, F. (1942). *Bot. Gaz.* **104**, 224.
- Stephenson, M. & Trim, A. R. (1938). *Biochem. J.* **32**, 1740.
- Thimann, K. V. & Barker, H. A. (1934). *J. exp. Zool.* **69**, 37.
- Thimann, K. V. & Haagen-Smit, A. J. (1937). *Nature, Lond.*, **140**, 645.
- Woods, D. D. & Trim, A. R. (1942). *Biochem. J.* **36**, 501.
- Wynne, E. S. & Foster, J. W. (1948a). *J. Bact.* **55**, 61.
- Wynne, E. S. & Foster, J. W. (1948b). *J. Bact.* **55**, 69.
- Wynne, E. S. & Foster, J. W. (1948c). *J. Bact.* **55**, 331.