Chemical Factors in the Germination of Spore-bearing Aerobes. The Effects of Amino-acids on the Germination of *Bacillus anthracis*, with some Observations on the Relation of Optical Form to Biological Activity

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The first paper of this series (Hills, 1949) has shown that, in a basal medium in which essential amino-acids are supplied by a gelatin hydrolysate and tyrosine, the rate of germination of spores of *Bacillus anthracis* was greatly increased by boiled yeast extract and that the extract could be replaced by adenosine at a concentration of 10^{-6}M or about 0·25 mg./l. It thus became possible to study the effects of amino-acids on germination, since the optimum concentration expected for many of them, of the order of 100 mg./l. on the basis of the optimum concentration of gelatin hydrolysate (0·25 %), was much greater than that of the adenosine. Observations on amino-acid requirements were therefore not likely to be vitiated by trace impurities in the adenosine, which was, necessarily, a natural product.

Difficulty was encountered in the study of amino-acid requirements, however, since the synthetic medium for the growth of *B. anthracis* used by Gladstone (1939), and since modified by Brewer, McCullough, Mills, Roessler, Herbst & Howe (1946) supported a rate of germination, even in the presence of yeast extract or adenosine, too small for detection by viable counts, though some germination must have occurred since the strain used grew satisfactorily on the modification of the latter authors. Gladstone (1939) had shown, however, that growth in a modification of his medium containing fourteen amino-acids was inhibited by leucine, isoleucine and valine separately, but was stimulated when these three amino-acids were present in appropriate proportions. In a further simplified medium, containing a total of eleven amino-acids, these three were essential. It seemed possible, therefore, that the feeble germination in synthetic media, and the inhibition of germination which occurred with concentrations of gelatin hydrolysate above the optimal (Hills, 1949), might both be due to an unsuitable balance of amino-acids. Work was therefore begun by attempting to simulate the effect of excess gelatin hydrolysate by means of amino-acids, with the hope that this would reveal the cause of failure to germinate in the amino-acid medium.

METHODS

Technique. Experimental methods, based on the determination of viable counts by the surface plate technique of Miles & Misra (1938), were as described previously (Hills, 1949). It is again emphasized that all counts were viable counts and the abbreviation ($S + V$ count) is used for the sake of brevity to denote a count determined so as to include both spore and vegetative cells. The period of incubation was 30 min. at 35°, except where otherwise stated.

Materials. These were largely of commercial origin, but the adenosine and inorganic constituents of the media were purified by recrystallization thrice from water. One specimen of D-alanine was kindly presented by Roche Products Ltd. A second specimen, for which the author thanks Dr H. N. Rydon, was prepared by resolution of the benzoyl derivative of commercial synthetic DL-alanine with brucine, and had $[\alpha]_D^{22°} = -7·8°$ at *c*, 1·342 g. hydrochloride/100 ml. N-HCl, indicating 90 % optical purity. A specimen of L-alanine, also prepared by Dr Rydon by resolution of the benzoyl derivative with strychnine, was optically pure, having $[\alpha]_D^{21°} = +10·7$ at *c*, 1·684 g. hydrochloride/100 ml. N-HCl.

The author thanks Dr Rydon also for a specimen of synthetic DL-tyrosine.

Pyruvic acid was purified by vacuum distillation and stored in the cold as 5M-solution (Wendel, 1932). Lactate was purified by recrystallization of the Zn salt and Zn was removed by Na_2CO_3.

Concentrations of materials have been expressed as the molarity of a single enantiomorph; the total concentration of a DL-mixture was thus twice that given.

Statistical interpretation of results. Since the technique was shown in the previous paper to give counts conforming to Poisson distributions, it was possible to take each count as an estimate of its sampling variance, and the sampling variance of the difference between two counts at the same dilution was therefore equal to the sum of the counts. This simple technique sufficed for the assessment of the differences between counts in the same experiment by well-known methods as described, for example, by Fisher (1946) or Davies (1947).

In comparing the proportions germinating in separate experiments it is necessary to calculate the variance of the proportion. This is not given by the usual formula based on the binomial distribution, since the observations are not the numbers germinating and failing to germinate, but are estimates *n* of the spores subjected to a particular treatment

and s of those failing to germinate, both determined on equal portions. Since these vary independently according to Poisson distributions, the sampling variance of q ($=s/n$), the proportion of spores not germinating, is given by

$$V(q) = (\partial q/\partial s)^2 V(s) + (\partial q/\partial n)^2 V(n).$$

If n is the mean of k replicates and s that of l replicates

$$V(n) = n/k; \quad V(s) = s/l$$

and $$V(q) = (s/l + s^2/kn)/n^2 \quad \text{or} \quad (q/l + q^2/k)/n.$$

The second term of these expressions for $V(q)$ is usually small, contributing less than 10% to the standard error if $k = 5l$. This frequently arises, since the initial counts for a number of independent treatments rarely differ significantly, and may therefore be pooled to give the best estimate of n.

In studying the effects of amino-acids as inhibitors of germination (Table 8), uncontrollable variations in conditions led to variations in the degree of germination of the uninhibited control in the different experiments. It was then found advantageous to normalize the data by transformation of proportions to probits (see Finney, 1947). Since the probit x is related to the proportion q so that $dq/dx = z$, the ordinate of the normal curve corresponding to the proportion q, the sampling variance of x is given by

$$V(x) = (dx/dq)^2 V(q) = (q/lz^2 + q^2/kz^2)/n.$$

The sampling variance of the difference between two probits, based on the same estimate of n, is given by

$$\begin{aligned} V(x_1 - x_2) &= V(x_1) + V(x_2) - 2\,\mathrm{Cov}\, x_1 x_2 \\ &= V(q_1)/z_1^2 + V(q_2)/z_2^2 - 2\,\mathrm{Cov}\, q_1 q_2/z_1 z_2 \\ &= \{q_1/l_1 z_1^2 + q_2/l_2 z_2^2 + (q_1/z_1 - q_2/z_2)^2/k\}/n. \end{aligned}$$

If the reciprocal of this theoretical sampling variance was used to weight the probit differences derived from the data of Table 8, an χ^2 test showed that the variations between experiments were greater than expectation, but χ^2/ϕ, where ϕ is the number of degrees of freedom involved in determining the weighted mean of corresponding probit differences, showed no significant heterogeneity (Bartlett's test) due to the particular experiment involved ($P = 0{\cdot}7$), the amino-acid tested for inhibition ($P = 0{\cdot}9$), or its concentration ($P = 0{\cdot}7$). It was thus possible to use a pooled estimate of the variance, χ^2/ϕ, based on the data as a whole, and the larger number of degrees of freedom available from this estimate allowed greater precision in the application of the t test to the significance of the weighted means.

This advantage of uniformity of variance over a wide range justified the use of the probit transformation in spite of the disadvantage that, on approaching complete inhibition of germination, the probit tends to infinity with zero weight. The significance thus becomes indeterminate unless an estimate from the regression of probit on a function of concentration of inhibitor is available. This was not necessary for the present purpose since, in practice, the significance of such large inhibitions was not in doubt. By taking the minimum expected probit X as that observed at a lower concentration of inhibitor, it was possible to calculate a minimum value for the working probit, $x = X + (q - Q)/Z$ (as defined by Finney, 1947), where Q, Z are the proportions and ordinate of the normal curve corresponding to probit X, and q is the observed proportion not germinating at the higher concentration. The weight to be used with this working probit is that corresponding to Q and not to q. It will be observed that this technique allows appropriate weight to be given even to observations where errors of random sampling lead to an estimate of q greater than 1 (e.g. with D-alanine at 20 μM, Table 8, Exp. 3). It has been applied to this and three other observations in Table 8, since their omission leads to a lower estimate for variance for the data as a whole.

RESULTS

Replacement of hydrolysed gelatin in basal medium by a synthetic mixture of amino-acids. Preliminary experiments on the toxicity of amino-acids in the gelatin-tyrosine-yeast medium, showed that leucine, isoleucine and valine, which Gladstone (1939) had shown to be inhibitory to growth under certain conditions, did not inhibit germination under the conditions used, the concentrations being those which Gladstone had found to be effective. Of the eleven amino-acids present in the simplest form of his medium, however, DL-alanine was found to be strongly inhibitory, the activity being due to the D-component. The inhibition due to excess gelatin hydrolysate was probably not due to this cause, since that due to 10^{-4} M-D-alanine was reduced from 95 to 25% by increasing the hydrolysed gelatin concentration from 0·05 to 0·30%. It is probable that the inhibition due to high concentrations of hydrolysate was largely due to glycine; at 10 mM it reduced germination in 0·25% hydrolysate from 70 to 35% compared with a reduction to 50% when the concentration of hydrolysate was doubled. Taking 24% as the glycine content of gelatin (Block & Bolling, 1945), this corresponded to an increase of 8 mM in glycine concentration.

Since it now seemed likely that failure of a large proportion of spores to germinate in a synthetic amino-acid medium was due to DL-alanine, germination was tested in a medium containing eighteen amino-acids, including threonine, at the concentrations used by Gladstone (1939), but with DL-alanine replaced by the L-form. Other constituents of the medium were modified according to Brewer *et al.* (1946). Table 1 shows that this medium allowed a slow fall in spore count. In this medium the fall was much more rapid with the addition of a preparation of yeast adenylic acid, which was used at this stage before it was realized that its activity was probably due to its content of free adenosine. In buffer the fall was much slower and was not markedly stimulated by the adenylic acid. Although the combined count of vegetative cells and spores also fell slowly, the fall in spore count has been regarded as the best measure of germination, since at 0·5 hr. when no significant fall in $S + V$ count had occurred, the spore count in the best medium had fallen by over 90%. The fall in combined count on prolongation of incubation showed that a significant proportion of the heat-sensitive forms slowly became non-viable. This applied especially to those media which did not support growth (as shown by the count at 73 hr.) so that in these media the proportion

of spores remained high. Nevertheless, it would be misleading to take the fall in the proportion of spores as an index of germination, since this proportion may increase, as in the 'eighteen amino-acids' medium, with adenylic acid, due to a fall in the $S+V$ count while the number of spores actually remains practically constant.

medium of Exp. 2, containing aspartate and glutamate in addition, but the three excluding glycine were as effective as that basal medium.

If the concentrations of either alanine or tyrosine were increased above 0·5 mM, the germination increased but about 1 % spores did not germinate, even at 1–5 mM. At submaximal concentrations the

Table 1. *Germination and growth of* Bacillus anthracis *in a synthetic medium*

(The 'eighteen amino-acids' medium was that of Gladstone (1939), including salts and glucose, with replacement of DL-alanine by L-alanine ($5{\cdot}6\times10^{-4}$ M), and the addition of DL-threonine (3×10^{-5} M), aneurin (10^{-7} M), $CaCl_2$ (5×10^{-4} M), and $NaHCO_3$ (10^{-2} M). Yeast adenylic acid (y.a.a.), where added, was 10^{-5} M.

The inoculated media, 10 ml. in 6 in. × ¾ in. tubes, were incubated for the first 5 hr. in a water bath, at 35·0±0·1°, samples of 1·0 ml. being removed at intervals for counting. Subsequently the remainder of the culture was aerated by sloping on a rocker in the hot room in such a way that the tubes passed in a vertical plane through 35 oscillations/min. of amplitude about 5°, the minimum slope being as nearly horizontal as possible.

$S+V$ = viable count of spores and vegetative cells; S = spore count.)

	M/30 phosphate pH 7·3				'Eighteen amino-acids'			
	No y.a.a.		With y.a.a.		No y.a.a.		With y.a.a.	
	$(S+V)$	(S)	$(S+V)$	(S)	$(S+V)$	(S)	$(S+V)$	(S)
Time (hr.)	Counts ($\times10^3$)							
0·0	8·6	8·7	8·4	8·4	8·3	7·5	8·0	6·5
0·5	7·5	7·6	8·0	6·2	7·3	5·9	7·7	0·5
1·5	7·2	5·9	6·7	4·1	6·1	3·1	6·0	0·1
3·0	5·9	5·4	6·1	4·6	5·9	2·5	3·8	0·1
5·0	5·8	5·0	5·7	4·2	4·4	1·0	4·1	0·1
	Counts ($\times10^3$)				Counts ($\times10^7$)			
72	—	3·4	—	2·0	—	7·5	—	4·7

Determination of essential amino-acids. It was now comparatively simple to determine the amino-acids essential for germination by omission of groups of them in turn as shown in Table 2. In Exps. 1 and 2 the basal media were adequate, and the fall in spore count was not increased by any of the thirteen amino-acids added. The inhibition by D-alanine was shown in Exp. 1, and leucine, isoleucine and valine were together partially inhibitory in the basal medium of Exp. 2. Exp. 3 shows that the group containing glycine and L-alanine was effective with phenylalanine and tyrosine as the only amino-acids in the basal medium, but aspartate and glutamate were ineffective. This latter mixture was also ineffective in the presence of glycine and L-alanine (data not tabulated). The inhibitory effect of glycine was more marked in these mixtures containing few amino-acids, so that in this experiment where it was present in both effective mixtures little germination was observed in 0·5 hr. and it was necessary to prolong the experiment for 2 hr. before more than 50 % germination was observed. Exp. 4 shows that L-alanine and tyrosine together were effective, phenylalanine being stimulatory in their presence and glycine inhibitory. These effects were shown most clearly after 1 hr. incubation. The four amino-acids together were less effective than the basal

actual germination observed was somewhat variable, but a typical experiment (Table 3) showed that 100–150 μM was adequate for over 50 % germination. In another experiment with 300 μM-alanine, 300 μM-tyrosine and 1 μM-adenosine (now used in place of adenylic acid) the germination, 75 %, was practically as great as the 90 % observed in the gelatin-tyrosine medium with the addition of the same concentration of adenosine. A tenfold dilution of the CCY medium of Gladstone & Fildes (1940) was rather more effective, only 1 % of the spores remaining heat resistant as with higher concentrations of alanine and tyrosine. With tryptic digest of beef, on the other hand, even at a dilution tenfold greater than that usual in culture media, the germination of the small inocula used was so rapid that the spore count fell about 75 % during the 1–2 min. required for mixing the inoculum with medium at 35° and transferring a sample to the diluent at 60°. Since no significant fall occurred with the other media under these conditions, it must be concluded that the broth contains nutrients, so far unidentified, which can increase the rate of germination much beyond that attainable with those nutrients now known to be effective.

Inessential components of the medium. The omission of aneurin, glucose or bivalent cations from the

Table 2. *Amino-acid requirements for germination*

(All basal media contained salts and aneurin as in Table 1.)

Exp. no.	Time (hr.)	Basal medium: Constituents	Basal medium: Concn. (μM)	Additions: Substances	Additions: Concn. (μM)	Spores not germinated (%)	Spores not germinated (S.D.)
1	0·5	As in Table 1 with omission of cystine, methionine and tryptophan; with yeast adenylic acid	5	None		4·9	1·0
				L-Cystine	250	4·7	1·0
				DL-Methionine	200	10·9	1·5
				Two above		6·3	1·1
				Two above + DL-Serine + DL-Tryptophan	1000 + 1200	6·4	1·1
				Four above + D-alanine	280	88·9	3·1
2	0·5	Glycine	3300	None		25·3	1·7
		L-Alanine	560	DL-Leucine + DL-Isoleucine + DL-Valine	570 + 380 + 130	63·8	3·8
		DL-Aspartate	750				
		DL-Glutamate	300				
		DL-Phenylaline	300	L-Arginine + L-Histidine + DL-Lysine	500 + 250 + 270	37·0	2·9
		DL-Tyrosine	280				
		Yeast adenylic acid	2	DL-Threonine + DL-Proline + DL-Hydroxyproline	30 + 440 + 380	41·8	3·0
				Nine above		46·7	3·2
3	2·0	DL-Phenylalanine	300	None		68·7	3·5
		DL-Tyrosine	280	Glycine + L-Alanine	3300 + 560	34·0	2·5
		Yeast adenylic acid	2	DL-Aspartate + DL-Glutamate	750 + 300	73·8	3·7
				Four above		32·2	2·4
4	1·0	DL-Tyrosine	280	None		90·4	4·4
		Yeast adenylic acid	2	Glycine	3300	93·5	4·5
				L-Alanine	560	39·9	2·9
				DL-Phenylalanine	300	89·8	4·4
				Glycine + L-Alanine	3300 + 560	77·9	4·0
				Glycine + DL-Phenylalanine	3300 + 300	92·1	4·5
				L-Alanine + DL-Phenylalanine	560 + 300	10·7	1·5
				Three above		55·3	3·4
	1·0	As Exp. 2		None		12·9	1·6

Table 3. *Effect of concentrations of* L-*alanine and tyrosine on germination*

(The basal medium contained glucose, salts and aneurin as in Table 1, together with $1·5 \times 10^{-6}$ M-yeast adenylic acid.)

DL-Tyrosine (μM)...	0	10	30	100	300
L-Alanine (μM)	Spore count (in 0·05 ml.) after 30 min. at 35°				
0	588	550	600	552	429
	607	—	—	—	—
50	565	600	581	148	94
150	483	478	386	91	39
500	469	472	248	66	16

medium had no significant effect in an experiment in which the total number of viable organisms, 98 ± 3 % spores ($P = 0·95$) initially, fell by 32 ± 4 % in media permitting a fall in spore count of 90 ± 2 % independently of the presence or absence of these constituents. In the absence of any one of the remaining constituents, adenosine, L-alanine or tyrosine, germination was much reduced, the $S + V$ count falling to $75·5 \pm 4$ % of the initial $S + V$ count and the spore count to 67 ± 4 %. This experiment confirms the cause in the fall in spore count as germination even in the simplest medium promoting such a fall. Replacement of the phosphate buffer by 25 mM-sodium bicarbonate in equilibrium with 5 % carbon dioxide in air, showed that phosphate was not essential but was probably stimulatory, giving 98% germination compared with 67 % in medium

buffered by sodium bicarbonate, though the corresponding blank figures for the buffers alone were 12 and 31 % respectively.

Replacement of tyrosine by analogues. Table 4 shows a typical experiment on the effect of replacing tyrosine by phenylalanine or dihydroxyphenylalanine. Tyrosine was active at 150 μM, and the other two inactive at this level, but active at 500 μM. In all cases the D-form present in the DL-mixture appeared to have no marked effect on the activity. Table 5 shows the effects of some other analogues of tyrosine, all showing some activity. The case of adrenaline, with activity of the same order as phenylalanine, calls for special comment since it showed that an unsubstituted α-amino group was not essential for activity. Controls showed that these compounds influenced only the spore count after incubation, variations in the final $S+V$ count and in the initial $S+V$ and spore counts being within the errors of random sampling.

Table 4. *Effects of concentration and optical form of aromatic amino-acids on germination in the presence of adenosine and* L-*alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 2 μM-adenosine and 500 μM-L-alanine. Dihydroxyphenylalanine was sterilized as 5 mM solution in 0·02M-HCl by Seitz filtration just before use.)

Concn. of added amino-acid (μM)	Spore count (in 0·05 ml.) after 30 min. at 35° with: Phenylalanine L-	Phenylalanine DL-	Tyrosine L-	Tyrosine DL-	Dihydroxyphenylalanine L-	Dihydroxyphenylalanine DL-
500	286	313	178	183	337	280
150	490	425	332	255	555	441
50	536	517	539	438	552	459
0	494; 468; 430					

Table 5. *Effects of analogues of tyrosine on germination in the presence of adenosine and* L-*alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 6 μM-adenosine and 500 μM-L-alanine. Just before use, the materials added were dissolved in 0·02N-HCl, Seitz filtered and added to the basal medium containing the amount of NaOH required for neutralization.)

Addition to basal medium (500 μM)	Viable counts (in 0·05 ml.) 0 min. $S+V$	0 min. Spore	30 min. $S+V$	30 min. Spore
None	596	546	494	441
Diiodotyrosine	596	501	460	341
Tyramine	579	525	480	295
Thyroxine	582	537	485	271
DL-Dihydroxyphenylalanine	579	531	497	231
Adrenaline	593	504	502	192
DL-Phenylalanine	603	527	490	151
DL-Tyrosine	586	512	486	96

Specificity of alanine. The stereochemical specificity of the enantiomorphs of alanine has already been indicated since it was necessary to replace DL-alanine by its L-component before germination was observed in a synthetic medium. Table 6 shows that L-alanine was not replaceable by β-alanine, nor by pyruvate or lactate, with or without ammonium chloride as a source of nitrogen. None of the last three was inhibitory in the presence of L-alanine, so that inhibition, as by D-alanine, did not occur with the corresponding form in DL-lactate. Reversal of D-alanine inhibition by L-alanine is shown in Table 7, a ratio of 10–30 molecules of promoter to one molecule of inhibitor giving about 50 % germination.

Table 6. *Specificity of* L-*alanine in promoting germination in the presence of adenosine and tyrosine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 1·5 μM-adenosine and 500 μM-tyrosine. The concentrations of the additions to the basal medium were NH_4Cl and pyruvate, 5 mM; DL-lactate and β-alanine, 2·5 mM; L-alanine, 0·5 mM.)

Additions to basal medium	Spore counts (in 0·05 ml.) 0 min.	30 min.
None	664	547
NH_4Cl	647	589
Lactate	653	596
Lactate + NH_4Cl	637	572
Pyruvate	668	589
Pyruvate + NH_4Cl	633	577
β-Alanine	661	480
L-Alanine	626	62
L-Alanine + NH_4Cl	641	34
L-Alanine + lactate	659	35
L-Alanine + pyruvate	645	166

Table 7. *Inhibition of germination by* D-*alanine*

(The basal medium contained 33 mM-phosphate, pH 7·3; 5 μM-adenosine and 500 μM-DL-tyrosine.)

Alanine (μM) L-	Alanine (μM) D-	Other additions	Spore count (in 0·05 ml.) 0 min.	30 min.
0	0	None	974	818
0	15	None	—	898
0	50	None	—	908
0	150	None	914	916
500	0	None	—	136
500	0	Brucine (20 μg./ml.)	845	248
500	0	Strychnine (20 μg./ml.)	897	231
500	15	None	—	403
500	50	None	—	655
500	150	None	—	836
1500	0	None	—	89
1500	15	None	—	142
1500	50	None	—	227
1500	150	None	—	461
5000	0	None	926	84
5000	15	None	—	73
5000	50	None	—	111
5000	150	None	910	385

Controls showed that traces of the alkaloids used in resolution could not be responsible for the inhibition, since in the presence of 500 μM-L-alanine, 15 μM-D-alanine (1·35 μg./ml.) reduced germination by more than twice as much as 20 μg./ml. brucine or strychnine.

Inhibition by other amino-acids. Indications of inhibition by glycine and by leucine, isoleucine and valine together had already been seen in determining the constituents responsible for germination in the original synthetic medium containing nineteen amino-acids. Observations were therefore made (Table 8) with a number of DL- and (where available) D-amino-acids, in a search for further examples of inhibition correlated with stereochemical form. Highly significant inhibitions ($P < 0·02$ for equal or greater effect in the same direction due to chance) were observed both at 1·5 and 5 mM only with glycine and DL-methionine. DL-Cysteine and D-valine were significantly inhibitory at 5 mM, but not at 1·5 mM, while with D-glutamate and DL-proline the inhibition at 5 mM was barely significant ($P \approx 0·05$). At this concentration D-leucine, DL-lysine, D-phenylalanine and DL-threonine showed no significant effect. Since D-alanine was significantly inhibitory ($P = 0·01$) even at 5 μM, the problem of the weaker inhibitions and their relation to stereochemical form has not been pursued further.

Table 8. *Inhibition of germination by amino-acids*

(The basal medium contained 33 mM-phosphate, pH 7·3; 2 μM-adenosine; 300 μM-L-alanine and 300 μM-DL-tyrosine.)

Exp. no.		1	2	3	4	5	6	7
Initial spores (in 0·05 ml.)		1	12	3	17	10	12	10
No. replicates	Mean count	1017	650	816	516	570	493	402

Added to medium: Amino-acid	Concn. (μM)	Spore count after 30 min. at 35°							Probit final spores: Weighted mean	S.E.	Probability of greater difference in one direction due to chance	Spores corresponding to probits (%): Mean	Difference
None	—	216	250	307	206	75	174	45	4·40	0·14	—	27	± 7
		204	237	287	221	95	154	33					
		198	—	—	208	—	—	—					
									Weighted mean difference				
D-Alanine	1·5	—	542	—	330	—	156	—	+0·39	0·32	0·12	42	+14
	5	—	576	728	406	—	252	—	+0·85	0·35	0·010	60	+33
	20	—	—	869	416	—	—	—	>1·57	0·38	<0·001	83	+56
	60	944	—	—	—	—	—	—	+2·29	1·33	0·026	96	+69
	200	904	—	—	—	—	—	—	+2·05	0·98	0·024	95	+68
Glycine	1500	630	—	—	329	—	—	—	+0·94	0·32	0·003	63	+36
	5000	1017	—	—	427	—	—	—	>1·95	0·36	<0·001	91	+64
DL-Methionine	1500	—	—	574	—	186	—	—	+0·71	0·32	0·017	54	+27
	5000	—	—	783	—	352	—	—	>1·49	0·36	<0·001	81	+54
D-Valine	1500	283	257	—	—	—	—	—	+0·18	0·24	0·23	33	+ 6
	5000	463	646	—	—	—	—	—	>1·07	0·25	<0·001	68	+41
DL-Cysteine	1500	—	—	165	—	96	—	—	−0·31	0·24	0·11	18	− 9
	5000	—	—	606	—	480	—	—	+1·28	0·46	0·004	75	+48
D-Glutamate	1500	93	307	—	—	—	174	55	−0·08	0·21	0·35	24	− 3
	5000	217	341	—	—	—	154	36	+0·38	0·22	0·047	41	+14
DL-Proline	1500	—	—	386	159	—	254	49	+0·16	0·22	0·24	33	+ 6
	5000	—	—	695	176	—	412	113	+0·46	0·29	0·062	44	+17
D-Leucine	5000	195	194	—	—	—	—	—	−0·10	0·24	0·34	24	− 3
DL-Lysine	5000	—	—	216	122	—	343	44	−0·13	0·22	0·28	23	− 4
D-Phenylalanine	5000	272	165	—	—	—	—	—	−0·01	0·23	0·49	27	0
DL-Threonine	5000	—	—	—	98	262	314	—	+0·17	0·25	0·26	33	+ 6

DISCUSSION

The most striking feature of this work is the high specificity of L-alanine in promoting germination and the inhibition by its enantiomorph. No such specificity was shown by tyrosine which was replaceable in some degree by related compounds, the carboxyl and unsubstituted amino groups not being essential for activity. The activity of the L-form was

not changed significantly when supplied by the synthetic DL-compound.

The small molecular ratio of D-alanine required to inhibit the action of the L-form was especially remarkable since inhibition of microbiological processes by structural analogues usually requires many molecules of inhibitor to one of promoter, e.g. 5000 for sulphonamides and *p*-aminobenzoate (Woods, 1940). In the metabolism of amino-acids by animal tissues, there are well-known examples of the occurrence of inhibitions by enantiomorphs at concentrations comparable to that of the substrate, e.g. synthesis of glutamine (Krebs, 1935); deamination of histidine (Edlbacher, Baur & Becker, 1940). In bacterial chemistry, on the other hand, the use of synthetic DL-amino-acids as standards in microbiological assay (for review see Snell, 1945*a*), to avoid active impurities of biological origin, has shown that, in general, only the L-forms have been metabolized, the antipodes being inert rather than inhibitory. In some cases the D-form may act as a supplement for the L-form, but this usually occurs only to a limited extent and requires the presence of sufficient L-form to initiate growth (for review see Rydon, 1948). The only species of micro-organisms which have so far been shown to be inhibited by D-amino-acids are *Lactobacillus arabinosus* (*Lb. plantarum*) (Fling & Fox, 1945) and *Escherichia coli* (Kobayashi, Fling & Fox, 1948). Acid production by the former, growing in a synthetic medium, was inhibited by D-leucine and D-valine at concentrations of the order 200-fold that of the L-forms essential for growth. The latter, growing in nutrient broth, was inhibited 80–90 % by 50 mM-D-leucine, 80 mM-D-valine, 120 mM-glycine or 140 mM-D-alanine. *Lb. arabinosus* was also inhibited by glycine at such high concentrations. The present work on germination shows that these amino-acids, except D-leucine, were inhibitory at 5 mM, as were DL-cysteine and DL-methionine also, but D-alanine was outstanding by inhibiting even at 5 μM. The inhibition of growth of *B. anthracis* by unbalanced concentrations of leucine, isoleucine and valine was not due to the D-forms (Gladstone, 1939), but with similar inhibitions due to norleucine, serine or threonine the effect of antipodes was not investigated, nor was this done with the inhibition of growth of *Streptococcus lactis* investigated by Snell & Guirard (1943), who also found glycine and β-alanine to be inhibitory. With this last-named organism, and with *Lb. casei* (Snell, 1945*b*), D-alanine not only failed to inhibit, but at a sufficient concentration, eliminated the need for pyridoxine in growth and in reversing the inhibitions by other amino-acids above mentioned.

Even in the case of *B. anthracis*, the inhibition by D-alanine was confined to the germination of spores, and growth was not inhibited significantly. Gladstone (1939) grew six strains satisfactorily in an amino-acid medium containing DL-alanine, and although the strain used in the present work required supplementation of Gladstone's medium with aneurin, growth was then obtained in the presence of DL-alanine, even though the proportion of spores which germinated was not detected by viable counts.

It may be concluded that inhibition by stereoisomers of amino-acids is rare in bacteriological chemistry and that elucidation of the reason for this strong inhibition by D-alanine, the simplest amino-acid capable of showing optical isomerism, might have some bearing on the design of antibacterial agents of therapeutic value (cf. Work, 1948). In this connexion, it may be recalled that only the D-form of penicillinamine hydrochloride gave rise to active penicillins on condensation with appropriate oxazolones (quoted by Du Vigneaud, Carpenter, Holley, Livermore & Rachele, 1946). At present no reason can be offered for the importance of the two forms of alanine in promoting and inhibiting germination since existing knowledge of the metabolism of spores (Ruehle, 1923; Cook, 1931; Tarr, 1933; Virtanen & Pulkki, 1933; Keilin & Hartree, 1947) does not include their action on amino-acids.

SUMMARY

1. The germination of spores of a virulent strain of *Bacillus anthracis* has been studied by means of viable counts, and the amino-acid requirement has been determined.

2. Slow germination occurred in phosphate buffer at pH 7·3, but was greatly stimulated by 500 μM-L-alanine, 500 μM-L-tyrosine and 2 μM-adenosine together.

3. L-Alanine was not replaceable by related compounds and its action was strongly inhibited by D-alanine at a concentration 0·03 times that of its antipode. The inhibition was reversed by increasing the proportion of the L-form.

4. L-Tyrosine was replaceable by related compounds with little decrease in efficiency, none of the groups—phenolic hydroxyl, amino and carboxyl—being essential for activity. Its activity was not suppressed when supplied as DL-tyrosine.

5. Of seventeen other amino-acids tested, none showed significant stimulation of germination. Glycine, DL-methionine, DL-cysteine and DL-valine showed inhibition, but only at concentrations 1000 times that at which D-alanine inhibited.

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Studies in Vitamin A

14. THE ALLEGED MOBILIZATION OF VITAMIN A BY ADRENALINE

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In 1940 Young & Wald reported that the injection of adrenaline into a rabbit was followed by a very marked increase in the blood vitamin A level, which reached a maximum approximately 30 min. after injection. In view of its possible importance in helping to elucidate the mode of action of vitamin A and the possible clinical implications, the effect has been reinvestigated using both rats and rabbits as experimental animals.

EXPERIMENTAL

Animals. Rabbits were kept in a constant environment and given an unvarying diet of Lever's cubes and outer leaves of cabbage; rats were maintained similarly except that they were not given cabbage.

Dosing. The required dose of adrenaline was injected intraperitoneally into rats and intravenously into a marginal ear vein of rabbits.

Collection and examination of blood. The samples of rat blood were withdrawn by cardiac puncture using a method previously described (Glover, Goodwin & Morton, 1946). The samples of rabbit blood (approx. 10 ml.) were withdrawn without anaesthesia from a marginal vein of the ear not used for the injection of adrenaline.

In most experiments the plasma was separated from the red cells and examined for vitamin A using the method developed in this laboratory (Glover *et al.* 1946; Goodwin & Gregory, 1948). In some experiments whole blood was used and extracted according to the method of Young & Wald (1940), but the extract was examined for vitamin A using our method.

RESULTS

Rabbits. The results of the rabbit experiments are collected in Table 1. It will be seen that adrenaline has no appreciable effect on the vitamin A plasma levels of rabbit, and an analysis of the data using the *t* test indicated that the differences are not significant.

Young & Wald (1940) used whole blood for their vitamin A tests, but as it is now well established that under normal circumstances no vitamin A exists in the red cells, most of our experiments were carried out on blood plasma. There was, however, a possibility that adrenaline mobilized vitamin A into the red cells and that this would account for Young & Wald's results. To check this, two experiments were carried out in which whole blood instead of plasma was examined for vitamin A; again no mobilization was apparent.

Rats. When rats were used the experimental approach had to be altered slightly. The volume of