The Roles of Serum and Carbon Dioxide in Capsule Formation by *Bacillus anthracis*

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SUMMARY

Capsule formation by virulent strains of *Bacillus anthracis* on nutrient agar is known to depend on incubation in air with added CO₂ as well as the addition of serum or bicarbonate to the medium. The minimum effective concentration of CO₂ varies with the pH of the medium in a way which shows that capsulation depends on a threshold concentration of bicarbonate in the medium. Serum is more effective than bicarbonate and appears to act by binding an agent which inhibits capsule formation since it is replaceable by activated charcoal. The inhibitor might be a fatty acid since certain acids prevented capsule formation. Capsules are formed on nutrient agar containing added bicarbonate only after the culture has become very dense which suggests that the organisms either inactivate the inhibitor or become resistant to its action as their growth rate falls on approaching the stationary phase.

INTRODUCTION

Virulent strains of *Bacillus anthracis* are invariably capsulated *in vivo* but form capsules *in vitro* only under special conditions. These include incubation in air with added CO₂ (Ivánovics, 1937) and the use of nutrient agar containing either serum (Sterne, 1937) or bicarbonate (see Thorne, 1956). Bicarbonate has displaced serum in recent investigations (see Housewright, 1962) but the naked eye appearance of colonies on serum agar and on bicarbonate agar incubated in the same atmosphere immediately suggests that serum agar gives far better capsulation, and that serum and bicarbonate are therefore not equivalent in capsule formation. This observation, which was repeatedly confirmed in studying the genetic control of capsulation (Meynell, 1963), and also the unusually high concentrations of serum and CO₂ said to be required, led to the present work. The results show that serum is replaceable by 0.2% (w/v) activated charcoal. Both are thought to act by binding an inhibitor which is present in the medium and in their presence 'physiological' concentrations of CO₂ suffice for capsule formation. Capsulation on bicarbonate agar is believed to occur only after the inhibitor has become ineffective. Microscopy confirmed this hypothesis for organisms growing on bicarbonate agar became capsulated many hours after those in comparable cultures on serum or charcoal agar.

METHODS

Organisms. The principal strain, 2160s, was a variant of *Bacillus anthracis* strain 2160, isolated after prolonged incubation of strain 2160 in broth containing 0.025 % (w/v) CaCl_2 (Renaux, 1952). It formed shorter chains, and therefore smoother colonies, than typical strains of *B. anthracis* (Nungester, 1929), did not form heat-resistant spores (McCloy, 1958), and was virtually avirulent for mice. However, it behaved like typical strains of this species in forming a capsule only when grown on serum or bicarbonate agar in CO_2 .

Five typical virulent strains were tested in a few experiments: two capsulated (C+) strains (1444, Hillsborough) from the authors' stocks, and C+ revertants from three strains (Vollum, A 69, A 77) received as C- mutants from Dr G. Ivánovics (Meynell, 1968).

Media. Only solid media were used. The usual formula was (% w/v): 'Lab-Lemco', 0.1; Tryptone (Oxoid), 0.2; Peptone (Oxoid), 1; NaCl, 0.7; Davis N.Z. agar, 1.5; pH 7.4. In some experiments, Oxoid Nutrient Broth No. 2, solidified with Oxoid Agar No. 3, 1.5 % (w/v) was used, either at pH 7.4 or 8.0, when it also contained 0.1 M 2-amino-2-hydroxymethylpropane-1,3-diol (tris), or at pH 6.2 or 6.8, when inorganic phosphate buffer was added to 0.1 M just before pouring plates. Other supplements were also added to molten agar at this time. Calculated amounts of NaHCO_3 were added from a M-solution sterilized by Seitz filtration; the calculations are explained in the legend to Fig. 1. Serum was added to 20 % (v/v). Bovine Serum Albumin Fraction V (batches AN 2070 and GC 1170; Armour Laboratories, Hampden Park, Eastbourne, Sussex) was added to 0.7 % (w/v) from a 7 % (w/v) solution in distilled water or in 0.1 M Sørensen buffer (pH 7.6) sterilized by Seitz filtration. Activated charcoal (Norit A or Hopkin and Williams decolorizing charcoal, code 2992) was added to 0.2 % (w/v) from a 10 % (w/v) suspension in distilled water previously sterilized by autoclaving at 121° for 15 min.

Incubation of media. Plates were inoculated with a loop to give an area of confluent growth and streaks bearing isolated colonies. Cultures incubated in 5–40 % (v/v) CO_2 were held in anaerobic jars from which the necessary volume of air was evacuated and replaced by pure CO_2 ; thus, 20 % CO_2 implies 20 % (v/v) CO_2 + 80 % (v/v) air. In one of the experiments shown in Fig. 1 and Table 1, each jar contained 60 % air, the balance after adding the required amount of CO_2 being made up with N_2 . As this did not affect the results, jars simply contained different proportions of air in the later experiments. Cultures in 2.5 % CO_2 were set up in candle jars (Nye & Lamb, 1936).

Assessing the degree of capsulation. Plates were examined after incubation for 21–24 hr. at 37° . Capsulation is reflected in a mucoid appearance of the growth, and its degree was estimated from the naked-eye appearance of confluent growth and of colonies which were recorded separately as R, RS, SR, SR—more glistening, M+, M++, M+++ or M++++. SR broadly resembles a smooth salmonella colony, and M+++ resembles *Klebsiella pneumoniae*. The proportion of capsulated organisms was judged from films stained by M'Fadyean's method (1903) with polychrome methylene blue. For degrees of capsulation of M+ or greater, all the organisms appeared capsulated; between R and SR, the proportion of capsulated organisms increased from 10^{-7} to about 0.5.

RESULTS

The role of carbon dioxide

The concentrations of CO_2 customarily used to induce capsulation are exceedingly high (e.g. 20–30 %). This suggested that capsule formation depended not on the atmospheric concentration of CO_2 but on the concentration of bicarbonate ion (HCO_3^-) in the medium. This hypothesis was tested by growing cultures on agar of various pH in various concentrations of CO_2 , since the Henderson–Hasselbalch equation predicts that a given $[\text{HCO}_3^-]$ is produced by less and less CO_2 , the higher the pH. The Henderson–Hasselbalch equation can be written

$$\log [\text{HCO}_3^-] = \text{pH} - \text{pK} + \log [\text{CO}_2],$$

where the pH is that of the medium; pK is a constant related to the pK_1 of carbonic acid; and $[\text{CO}_2]$ is the molarity of dissolved CO_2 and equals $P\alpha(\text{CO}_2) 5.87 \times 10^{-7}$. P is the atmospheric pressure, α is the solubility of CO_2 , and (CO_2) is the % (v/v)

Table 1. *Degree of capsulation of Bacillus anthracis strain 2160s on various media of different pH incubated in various concentrations of carbon dioxide*

Medium	pH	% CO_2 in atmosphere									
		2.5		5		10		20		40	
		conf.	col.	conf.	col.	conf.	col.	conf.	col.	conf.	col.
Nutrient agar											
+ NaHCO_3	6.2	RS	R	RS	R	RS	R	RS	R	RS	R
	6.8	RS	R	RS	R	SR	RS	SR	SR	M++	M+
	7.4	RS	R	SR	RS	M+	M+	M++	M++	M++	M++
	8.0	RS	SR	M+	SR	M++	M++	M++	M++	M++	NG
+ NaHCO_3 + albumin or charcoal	6.2	RS	R	RS	R	SR	SR	M+	M+	M++	M++
	6.8	SR	M++	M++	M++	M++	M++	M++	M++	M++	M++
	7.4	SR	M++	M++	M++	M++	M++	M++	M++	M++	M++
	8.0	SR	M++	M++	M++	M++	M++	M++	M++	M++	M++
No supplement	6.2	RS	R	RS	R	RS	R	RS	R	SR	R
	6.8	RS	R	RS	R	SR	SR	SR	SR	SR	SR
	7.4	SR	R	SR	RS	SR	SR	SR	SR	SR	SR
	8.0	RS	RS	RS	RS	M+	M+	M+	M+	M+	M+
+ Charcoal	6.2	RS	R	RS	R	RS	RS	M+	M+	M++	M++
	6.8	RS	RS	M+	M++	M++	M++	M+++	M+++	M+++	M+++
	7.4	RS	M+	M+	M++	M+	M++	M++	M++	M++	M+++
	8.0	SR	M++	M++	M++	M++	M++	M+++	M+++	M+++	NG

conf. = confluent growth; col. = isolated colonies; NG = no growth.

of atmospheric CO_2 (see Umbreit, Burris & Stauffer, 1957, chapter 2). It follows that $\log [\text{HCO}_3^-]$ plotted against $\log [\text{CO}_2]$ for a given pH gives a straight line and that the plots for different pH form a series of parallel straight lines (Fig. 1). Hence, as stated above, a given $[\text{HCO}_3^-]$ is produced by a variety of combinations of $[\text{CO}_2]$ and pH.

Cultures on nutrient agar plates, buffered at pH 6.2, 6.8, 7.4, or 8.0, and supplemented with appropriate concentrations of NaHCO_3 , were incubated overnight in different CO_2 concentrations and examined for capsulation. Figure 1*a* and Table 1 show that capsule production depended entirely on the concentration of HCO_3^- in the medium since it occurred with any combination of pH and CO_2 , provided that at least 0.056 M- HCO_3^- was present in the medium.

The role of serum

The early experiments on capsulation used nutrient agar containing a high concentration of horse serum (Sterne, 1937) and concentrations of 20% (v/v) or more have been used ever since. Our initial supposition was that serum acted as a buffer, increasing the concentration of HCO_3^- formed in the medium at a given CO_2 concentration in the same way as an increase in pH or addition of NaHCO_3 . Two observations, however, were against this explanation. First, the buffering power of the broth used for these experiments was hardly changed by adding 20% (v/v) horse serum and remained less than that of tryptic digest broth which gave less capsulation (Fig. 2). Secondly, overnight cultures on serum agar were far more mucoid than those on bicarbonate agar, the difference being most striking

Fig. 1. Relation of bicarbonate ion (HCO_3^-) concentration to atmospheric content of CO_2 at various values of pH. The lines show the relations predicted for pH 6.2, 6.8, 7.4 and 8.0 by the Henderson-Hasselbalch equation, with $\text{pK} = 6.32$; $P = 760 \text{ mm. Hg}$; and $\alpha = 0.56 \text{ ml./ml.}$ at 37° . The amounts of NaHCO_3 added to plates were also calculated from these values. The letters enclosed by circles indicate the degree of capsulation produced by strain 2160s after overnight incubation at 37° . Nutrient agar at pH 6.2 and 6.8 was buffered with 0.1 M-inorganic phosphate and at pH 7.4 and 8.0 with 0.1 M-tris. In addition, the calculated amount of NaHCO_3 was added to buffer H_2CO_3 formed in the medium by solution of CO_2 , leaving the phosphate or tris to maintain the initial pH during growth of the culture. All the media had certain points in common. Capsulation never occurred at any pH after incubation in air (0.03% CO_2). Colonial diameters were respectively half and a quarter those of the controls when the HCO_3^- concentration was in the ranges 0.01–0.07 M and 0.07–0.1 M. Only pin-point colonies were seen with HCO_3^- concentrations exceeding 0.1 M, and thickly inoculated areas of the plates showed only a watery smear of growth. (a) Plates with added NaHCO_3 only. (b) Plates with NaHCO_3 and either 0.7% (w/v) albumin or 0.2% (w/v) charcoal. The dashed lines show the threshold HCO_3^- concentration above which mucoid growth occurred. The threshold for bicarbonate agar is 0.056 M, about 18 times greater than for albumin or charcoal agar, where it is 0.0032 M.

with isolated colonies. In addition, there were quite marked differences in the capsule-promoting activity of different batches of serum; some were almost inactive. The sera of different animal species are known to differ markedly in this respect (Dr H. Smith, personal communication) and, in limited tests with single batches of human, sheep, and calf sera, we found that all were much more active than horse serum. Human serum showed the most activity, followed by calf and sheep serum.

Fig. 2. Titration curves of different liquid media. The buffering capacity is inversely proportional to the slope of the titration curve. Key to curves: 1, Lemco broth + 0.7% (w/v) albumin dissolved in water; 2, Lemco broth + 20% (v/v) horse serum; 3, Lemco broth + 0.7% (w/v) albumin dissolved in 0.1 M-phosphate buffer; 4, Lemco broth alone; 5, Tryptic digest broth.

Massive capsulation resulted when horse serum was replaced by Bovine Serum Albumin Fraction V added to the medium in a concentration equal to that of the albumin provided by whole serum. The same order of capsulation was obtained whether the stock albumin solution was made in distilled water or in 0.1 M-phosphate buffer (pH 7.6). Capsulation occurred on albumin agar when the bacteria were separated from the medium by cellophan (a.p.d., 3 m μ) or by gradocol membranes (a.p.d., 5 m μ), regardless of whether they were inoculated on a membrane placed on the surface of the agar or across unsupplemented agar separated from albumin agar by a strip of cellophan. In the second case, the growth on nutrient agar next to the cellophan was more capsulated than that farther away.

These results suggested that albumin (and presumably serum) acted either by absorbing a low molecular weight inhibitor of capsulation from the medium or by

providing a dialysable factor which promoted capsule formation. Binding of an inhibitor was strongly supported by finding that activated charcoal (0.2%, w/v) was just as efficient as albumin in promoting capsulation. On either medium incubated in 10–20% CO₂, isolated colonies were so mucoid that they coalesced and the confluent growth sometimes poured into the lid of the plate. It seemed very improbable that charcoal contributed a stimulating factor, since either of two brands were active (Norit A or Hopkin and Williams), whether used as supplied or after successively refluxing with equal parts of chloroform and methanol for 6 hr., heating in 6 N-HCl for 30 min. at 121° followed by repeated washing with boiling distilled water, and heating to redness in nitrogen for 1 hr.

The effects of albumin and charcoal were examined in more detail by retesting the influence of CO₂ concentration and pH. Fig. 1*b* shows that, while the general relation found for bicarbonate agar also held for these media, the threshold HCO₃⁻ concentration required for capsulation was lowered about 18-fold. No capsulation occurred on either medium when incubated in air. The lowering of the threshold explains why capsulation was obtained on unbuffered medium containing albumin or charcoal despite some fall in pH on incubation in CO₂ (Table 1). It appears that capsulation can occur either with a low HCO₃⁻ threshold even when the pH falls (albumin or charcoal agar) or with a high threshold when the fall in pH is prevented (bicarbonate agar). As expected, the maximum amount of capsulation was produced when the pH was maintained and the HCO₃⁻ threshold was lowered (albumin or charcoal agar + bicarbonate buffer).

These observations also explain why capsulation does not occur on conventional nutrient agar at pH 7.4. On exposure to CO₂, sufficient carbonic acid forms in the medium to lower its pH and, consequently, to raise still further the concentration of CO₂ needed in the atmosphere to produce the threshold concentration of HCO₃⁻. However, capsulation occurs, as expected, when the agar is made exceptionally alkaline before incubation in CO₂, e.g. by raising the pH to 8.5 with an alkali like NaOH (Thorne, Gomez & Housewright, 1952) or by adding bicarbonate (Thorne, 1956).

Other absorbents were also tested (Table 2). Coarse granular charcoal (British Drug Houses Ltd., for gas absorption; Holt, 1962) definitely promoted capsulation, although less efficiently than the finely particulate Norit and Hopkin and Williams charcoals, probably because the coarse granules sank to the bottom of the plate. Florisil was weakly active; its particles also tended to sink and its relative inefficiency might also have been due to poor dispersion. Alumina, silicic acid and cholesterol (Lwoff, 1947) produced a slight increase in the proportion of capsulated bacteria in the confluent growth as compared with the controls, but too inconstantly to prove their capsule-promoting activity. The anion-exchangers, DEAE and Amberlite CG-400, had definite activity, while the cation-exchangers, CEC and Amberlite CG-120, appeared to have none. Starch promoted capsulation, though not to the same extent as albumin or charcoal, and isolated colonies were usually more mucoid than the confluent growth and contained a higher proportion of capsulated organisms. The effect of starch might have been lessened owing to its hydrolysis by the amylase produced by the bacteria (Smith, Gordon & Clark, 1952; Knight & Proom, 1950). Alternatively, glucose formed by hydrolysis may have depressed capsule formation (see Discussion).

Table 2. *Action of absorbents in promoting capsulation of Bacillus anthracis strain 2160s*

Each absorbent was incorporated in nutrient agar pH 7.4 which was incubated in 20 % CO₂.

Absorbent and concentration (w/v)	Proportion of capsulated organisms in isolated colonies	Capsulogenic effect
Granular charcoal 2 %	0.05	Slight
Florisil* 1 %	0.01-0.05	Slight
Alumina† 1 %	10 ⁻⁸	None
Silicic acid ‡ 1 %	10 ⁻⁸	None
Lintner's soluble starch 0.6 %	0.1-M + +	Inconstant
Cholesterol§ 0.03 %	10 ⁻⁸	None
0.001 %	10 ⁻⁸	None
DEAE 2 %	0.8	Probable
CEC¶	10 ⁻⁵	None
Amberlite CG-120** 0.5 %	10 ⁻⁸	None
Amberlite CG-400†† 0.5 %	M + +	Probable

* 60-100 mesh magnesium silicate (L. Light and Co.).

† Grade 1 (M. Woelm).

‡ 100 mesh (Mallinckrodt).

§ Added from a 0.1 %, w/v, emulsion in water.

|| *N,N*-Diethylaminoethyl cellulose (Eastman Organic Chemicals).

¶ Cellex-CM. Carboxyl methyl cellulose (Bio-Rad laboratories).

** Cation-exchange resin (British Drug Houses Ltd).

†† Anion-exchange resin (British Drug Houses Ltd).

Relation of capsule formation on bicarbonate agar to bacterial concentration

If, in fact, albumin and charcoal removed an inhibitor of capsule formation from the medium, it remained to account for capsule formation on bicarbonate agar. Two possibilities were that the effect of the inhibitor was overcome by HCO₃⁻ concentrations permitting capsulation so that bicarbonate agar effectively resembled albumin or serum agar; or that the inhibitor ceased to affect the bacteria after their concentration had become fairly high. These alternatives could therefore be distinguished by seeing whether organisms growing on bicarbonate agar in appropriate concentrations of CO₂ were capsulated throughout growth like other species (Meynell, 1961), which is consistent with the first explanation; or whether capsules only appeared late in the growth of the culture after the inhibitor had become inactive. A corollary of the second explanation is that capsulation should appear far earlier in cultures on albumin or charcoal agar in which the inhibitor is assumed to be inactive.

Plates of bicarbonate, albumin, or charcoal agar at pH 7.4 were flooded with non-capsulated bacteria from an overnight culture grown in air, allowed to dry, and then incubated in CO₂ at 37°. Impressions were made every hour from blocks cut from each of three plates of each medium, stained with M'Fadyean's polychrome methylene blue, and examined. Initially, isolated organisms and short chains were seen. In later specimens, when the growth was too dense for impressions, either smears were made or a small amount of the growth was rubbed up in water.

Initially, on all three media, no sign of capsule was present and the bacteria were short, broad and vacuolated, the appearance associated with stationary phase cultures of this strain. After 2–3 hr. fairly long chains of thinner organisms formed, often lying side by side; and after 5–7 hr. a progressively more dense mass of tangled organisms was seen. On bicarbonate agar, about $1/10^6$ fully capsulated organisms or chains were seen from the 4th to the 9th hour but uniform capsulation never appeared before 11 hr., that is, several hours after dense confluent growth had formed. On albumin agar or charcoal agar, most of the bacteria showed capsules after 3 hr. incubation, first seen as a red flush around most of the organisms with sometimes a red granularity. All were definitely capsulated by 4–5 hr. The degree of capsulation after 3 hr. was equivalent to that seen on bicarbonate agar after about 11 hr. Moreover, after 10 hr., the growth on albumin or charcoal agar was glistening and easily suspended in saline, whereas the growth on bicarbonate agar had a dull rough surface and formed a more granular suspension.

The results were the same whether plates with 0.025 M- NaHCO_3 were compared with charcoal and albumin plates without NaHCO_3 incubated in 30% CO_2 or whether plates containing 0.03M added NaHCO_3 incubated in 10% CO_2 were compared with charcoal or albumin plates containing 0.015 M- NaHCO_3 incubated in 5% CO_2 , conditions which gave approximately equal capsulation after overnight incubation.

These results therefore support the view that the inhibitor removed from nutrient agar by charcoal or albumin becomes ineffective and ceases to prevent capsulation on bicarbonate agar once the bacteria have become sufficiently dense. Before that, the bacteria grew without capsules on bicarbonate agar. This conclusion was strengthened by the observation that on bicarbonate agar isolated colonies were always less mucoid than confluent growth, and that colonies which happened to be very near confluent growth were often markedly more mucoid than those on other parts of the plate. This was not generally seen on albumin or charcoal agar. Although these were the usual findings, the colonial and confluent growth could differ in three other ways.

(a) Where isolated colonies were more mucoid and contained a higher proportion of capsulated bacteria than did confluent growth. This was sometimes observed in the presence of albumin or charcoal with borderline concentrations of HCO_3^- (c. 0.004M), and also on certain other media, such as acid-hydrolysed casein + yeast extract, which gave poor growth. It seems likely that here it was not the inhibitor that limited capsule formation, but either HCO_3^- or a nutrient supplied by the medium.

(b) Where a mucoid rim formed round less mucoid, or non-mucoid, confluent growth. This was always seen when albumin or charcoal agar plates were inoculated by flooding and the growth formed after incubation in CO_2 was interrupted by bare areas which either had been left uninoculated or were produced by antibiotics (Table 3). Here again, some factor in the medium other than the inhibitor was probably limiting capsulation. A mucoid rim was also seen with some plates inoculated by loop: when they contained charcoal or albumin, where the isolated colonies were generally mucoid; and when they contained only bicarbonate, where the isolated colonies were rough and capsulation was evidently limited not only by a nutrient in the medium but also depended on there being a sufficient concentration of bacteria.

(c) Where mucoid growth only occurred in a few colonies situated very close to confluent growth. This was seen very occasionally on plates without absorbent with calculated concentrations of HCO_3^- of $1.8\text{--}3.2 \times 10^{-3}\text{M}$. It probably indicated a more extreme shortage of the necessary nutrient in the medium and removal of the inhibitor by confluent growth.

Table 3. *Effect of various agents on capsule formation by Bacillus anthracis strain 2160 s*

Nutrient agar plates at pH 7.4 containing Norit A charcoal were inoculated by flooding, and the test agents were placed in wells cut with a cork borer. The plates were incubated in 20 % CO_2 .

Agent and concentration (w/v) in wells*	% charcoal in agar (w/v)	Width of growth inhibition zone (mm.)	Rough zone		
			Width (mm.)	Appearance	% capsulated organisms†
Agar extract‡	0.2	0	10	RS	—
	0.04	0	7	RS	b
Oleic acid 5 %	0.2	3	5	RS	a
	0.08	3	8	R	a
Palmitic acid 10 %	0.2	0	5	SR	b
	0.08	0	5	RS	a
Linolenic acid 12 %	0.2	10	5	R	a
	0.08	10	5	R	a
Linoleic acid 12 %	0.2	6	0	Rim§	—
	0.08	6	0	Rim	—
Stearic acid 10 %	0.2	0	10	R	a
	0.08	0	10	R	a
Sodium deoxycholate 10 %	0.2	7	3	R	a
	0.04	7	5	R	a
Proflavine 1 %	0.2	7	0	—	—
	0.04	7	0	—	—
Streptomycin 0.1 %	0.2	15	0	—	—
	0.04	15	0	—	—
Chloramphenicol 0.1 %	0.2–0.04	5–20	0	Rim	—
Vancomycin 0.1 %					
Erythromycin 0.1 %					
Kanamycin 0.1 %					
Furadantin 0.1 %					

* Each well contained 0.06–0.08 ml. The pH of the solutions was c. 7.2.

† Percentage of capsulated organisms in the rough zone. a, < 5 %; b, 10–50 %. Controls invariably had 100 %. — = not tested.

‡ Agar extract: 15 g. Davis agar was refluxed with equal parts of chloroform and methanol for 6 hr. The extract was evaporated to dryness and the lipoidal residue warmed in dilute NaOH. Part of the residue did not dissolve. The turbid suspension was neutralized and made up to 10 ml.

§ Rim: raised mucoid rim formed round the inhibition zone.

The nature of the inhibitor

The need for albumin or charcoal immediately suggested that the inhibitor might be a fatty acid present in the medium (see Pollock, 1949; Nieman, 1954). Against this is the fact that although these acids frequently inhibit bacterial growth, they have never been reported to inhibit specifically a function, like

capsulation, unconnected with growth. Various fatty acids and other agents were tested by placing them in wells cut in a plate of inoculated charcoal agar that was then incubated in CO_2 (Table 3). Lipoidal material isolated from agar by refluxing with chloroform + methanol inhibited capsule formation without detectably inhibiting growth, as did the fatty acids, palmitic and stearic. It does not follow, of course, that either acid is the postulated inhibitor, a conclusion which would require analysis of the medium before and during growth of the culture. Moreover, the same effect was produced by sodium deoxycholate which was not likely to be present in the media. Other fatty acids, e.g. linolenic acid, inhibited growth as well as capsule formation; this appears to be a specific effect, since all the chemotherapeutic agents tested inhibited growth alone. A mucoid rim sometimes surrounded the inhibition zone, as already mentioned in the preceding section.

Tests with typical virulent strains

Five typical strains of *Bacillus anthracis* (1444, Hillsborough, Vollum, A69, A77) were grown on the different media to show that strain 2160s was not exceptional. The results were much the same with all six strains, though there may have been some quantitative differences in their requirements. All five typical strains were heavily capsulated (M + + + +) on media containing 0.03 M- NaHCO_3 and either charcoal (0.2%, w/v) or albumin (0.7%, w/v) incubated in 10% CO_2 . They also produced capsules on bicarbonate agar and on albumin or charcoal agar, but their growth, particularly that of strain A77, was not nearly so mucoid as when absorbent and bicarbonate were both present.

DISCUSSION

The results shown in Fig. 1 largely account for the high concentrations of CO_2 and of serum customarily used to induce capsule formation *in vitro*. The CO_2 concentration depends on the pH of the medium and on the amount of HCO_3^- required. Serum probably inactivates an inhibitor of capsular synthesis, and on serum or albumin agar at pH 6.8–7.4 excellent capsulation is produced by about 5% CO_2 , a concentration of the order found in the mammalian body. The action of purified charcoal makes it extremely unlikely that the function of serum or albumin is to contribute an essential nutrient to the medium. The differing characteristics of growth on bicarbonate agar appear only on examining either young cultures by microscopy or fully grown overnight cultures on solid medium where the confluent growth can be compared with isolated colonies. Many investigations seem to depend solely on chemical estimations of the capsular material, poly-D-glutamic acid, made on confluent growth formed after many hours or days incubation. On bicarbonate agar, capsules only appear after the culture has become very dense, which suggests that the organisms either inactivate the inhibitor (as, for example, other species bind fatty acid or Cu^{2+} (Davis, 1948; von Hofsten, 1962)) or that they become resistant to its action as their growth rate falls. It is not surprising therefore that the bacteria on bicarbonate agar are grossly heterogeneous in respect of capsulation in the early phases of growth, a point clearly brought out by stained films where the majority of organisms are non-capsulated when about $1/10^6$ are fully capsulated, presumably because they come from areas of denser growth. On albumin or charcoal

agar, the organisms are far more uniform, since capsulation begins throughout the population after 3–4 hr. incubation (see Mikhailov, Rozhkov & Tamarin, 1960).

It is evident from Fig. 1 that a higher HCO_3^- concentration is needed on bicarbonate agar, where the inhibitor is assumed to be present initially, than on albumin or charcoal agar. It may be that the inhibitor is competitively antagonized by HCO_3^- . The initial concentration of inhibitor in bicarbonate agar may, however, be so great that its effects are not overcome by any feasible HCO_3^- concentration, but, if it is progressively inactivated by the organisms, the higher concentrations of HCO_3^- used in these experiments succeed in inducing capsulation. Another explanation might be that by the time the inhibitor has become inactive, the culture has become dense and the physiology of the organisms has altered in such a way that capsular synthesis requires more HCO_3^- than with a rapidly growing culture. These alternatives could be distinguished by using steady-state cultures.

The postulated inhibitor has not been identified, though a fatty acid is clearly a possibility (Table 3); nor is it known whether it occurs naturally in the culture media or is produced by the organisms themselves (see Pollock, 1949; Nieman, 1954). The latter seems less likely, for isolated organisms growing on bicarbonate agar would then be producing sufficient inhibitor to prevent their own capsulation.

The action of the inhibitor is evidently linked to the part played by HCO_3^- in capsule formation. In general, CO_2 assimilated by heterotrophs appears to enter the tricarboxylic acid cycle (see Wood & Stjernholm, 1962) and, indeed, *Bacillus anthracis* growing on bicarbonate agar in the presence of $^{14}\text{CO}_2$ has been shown to form ^{14}C -labelled aspartate, succinate, etc. (Eastin & Thorne, 1968). Assimilation of CO_2 is known to depend on the action of biotin, which is replaceable by oleic acid. If the inhibitor is indeed a fatty acid, it may therefore act by interfering specifically with this assimilatory pathway; and, judging from studies of the effects of these acids on bacterial growth (e.g. Davis & Dubos, 1947), it would not be surprising if a given acid was stimulatory or inhibitory according to its concentration. The CO_2 requirement of some species is largely removed by adding tricarboxylic acid cycle intermediates to the medium (Lwoff & Monod, 1947; Ajl & Werkman, 1949) but, nevertheless, capsulation did not occur in cultures of strain 2160s incubated in air on either nutrient or charcoal agar containing 0.1 M-aspartate, succinate, glutamate or oxaloacetate at pH 7.4. A related observation was that glucose completely suppressed capsulation. At first, this was presumed to be due to the medium becoming acid and so lowering its content of HCO_3^- (Fig. 1). However, neutral red ($\text{pK} = 6.85$) did not indicate acidity, and, in any case, excellent capsules were formed even at pH 6.2 on charcoal or albumin agar incubated in 20–40% CO_2 (Fig. 1b). Glucose may therefore specifically repress capsular synthesis in strain 2160s; this point is still under examination.

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