¹⁴CO₂-Fixation by *Bacillus anthracis*

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Virulent encapsulated *Bacillus anthracis* incorporates ¹⁴C from ¹⁴CO₂. In the bacterial cell ¹⁴C is found preferentially in substances behaving like succinic acid, glutamic acid, aspartic acid and diaminopimelic acid. ¹⁴C is also very likely incorporated into peptides. Carbohydrates, at least the low-molecular ones, are not involved in ¹⁴CO₂-metabolism to any great extent.

The preliminary results of this study have been discussed earlier^{1,2}. The intention was to find the main products of CO₂-fixation in encapsulated *B. anthracis*.

EXPERIMENTAL

One colony from a mixture of highly-virulent capsule-forming *B. anthracis* strains was inoculated into each of twelve Warburg vessels containing 0.5 ml of a medium consisting of 2 parts meat juice and 3 parts serum at pH 7.4³ and incubated at + 37°C for about 60 h in an atmosphere of 20 % carbon dioxide containing 0.14 mC ¹⁴C. Controls were run in the same way without the inoculation of bacteria.

After incubation, when the microbes were encapsulated, the contents of the Warburg vessels were mixed in a glass tube and 6 ml distilled water and glass beads (diam. 0.2–0.4 mm) were added. After removal of air the tubes were shaken in a Microid apparatus for about 40 h at + 3°C. After this treatment only a few spores were left and these, in culture and pathogenicity tests, behaved like the original isolates. The suspension obtained was lyophilized and stored at –20°C. Before use, 6 ml of distilled water was added to the dry residue.

The crude suspension, S, containing high- and low-molecular substances was investigated by paper electrophoresis⁴. Ten μl S were placed on the paper and the electrophoresis was run in veronal buffer, μ = 0.125, pH 8.6, at a current of 0.5 mA/cm for 4 h. The strips were then dried for about one hour at + 60°C. For autoradiography they were covered with "Melinex 0" and placed on X-ray film (Kodirex). After 3–6 days exposure, the film was developed. The strips were examined under UV light, Mineralight 240–260 mμ, for the identification of nucleic acids and similar compounds. The strips were then stained with Amido black which reacts with proteins⁴, with fuchsin sulphurous acid to demonstrate carbohydrates and other substances with hydroxyl groups or hydroxyl and amino groups in the *cis*-position⁵, with Sudan black to make lipids visible⁶, and with methylene blue for the detection of metachromatic substances⁷.

The residue of solution S was then dialyzed continually against 2.5 l of distilled water during 2 days at + 3°C. The tube contents, the high-molecular fraction H, was lyophilized and stored at –20°C. Before use, 3 ml distilled water was added and 5 μl of the suspension was used for paper electrophoretic studies as described above.

The outer solution, 2.5 l, containing low-molecular substances, L, was evaporated in vacuum at $+37^\circ\text{C}$ to a volume of 1.0 ml. Of the solution obtained, 10 μl were used for paper electrophoretic separation and autoradiography as above. The strips were stained with aniline phthalate⁸ for the identification of carbohydrates and with ninhydrin⁹ for the detection of amino compounds.

Ten μl of solution L were used for preliminary paper chromatography in phenol-ammonia-cyanide⁹. After autoradiography the chromatograms were studied in UV and developed with aniline phthalate or ninhydrin. Ten μl of solution L were then investigated by paper chromatography in two dimensions with phenol-ammonia-cyanide and collidine-lutidine-diethylamine⁹. The chromatogram was used for autoradiography, studies in UV and development with ninhydrin. Ten μl of solution L were also used for two-dimensional paper chromatography in phenol-ammonia-cyanide and butanol-acetic acid-water, followed by autoradiography and development with aniline phthalate⁸. Some of the two-dimensional chromatograms were used as follows. The spots with high ^{14}C -activity were cut out. The substances were eluted and the solutions obtained were rechromatographed before and after hydrolysis in hydrochloric acid, in the phenol and collidine phases mentioned above¹⁰. The substances were again recognized by autoradiography and development with ninhydrin. From other chromatograms and electropherograms spots with high ^{14}C -activity were cut out, the eluted substances were re-chromatographed in ethanol-ammonia-water and propanol-eucalyptol-formic acid¹¹. The chromatograms obtained were reautoradiographed and acid substances on the chromatograms were identified with bromocresol green¹¹.

RESULTS

The results of the paper electrophoretic studies are shown in Fig. 1. In the crude suspension, S, ^{14}C is distributed in fractions 1a + b, 2 and 3; 1a + b and 2 have a strong ^{14}C -activity, and 3 a weaker. The slow migrating part of fraction 1, 1a, absorbs UV light, is stained by Amido black, Sudan black, fuchsin sulphurous acid and very weakly with methylene blue, properties which can be due to the presence of nucleic acids, proteins, lipids, carbohydrates and

Fig. 1. Paper electrophoretic separation of homogenates from ^{14}C -labelled *B. anthracis*. Symbols. C = radioactive zone, U = ultraviolet light absorbing zone, A = amido black stainable zone, Sb = Sudan black stainable zone, F = Fuchsin sulphurous acid stainable zone, M = Methylene blue stainable zone, N = Ninhydrin stainable zone, S = crude suspension, H = high-molecular fraction of suspension, L = low-molecular fraction of suspension, w = weak reaction, st = strong reaction.

Fig. 2. One dimensional paper chromatography (Phenol-NH₃-HCN) of the low-molecular fraction from *B. anthracis* homogenate. ¹⁴C-distribution.

¹⁴ C	<i>R_F</i>	Reference substances and their reactions:			
		UV240 – 260	Aniline phthalate	Ninhydrin	I.P.
0.00				
	0.20		Glucuronic acid Galacturonic acid	Aspartic acid	2.8
				Glutamic acid Diaminopimelic acid	3.2 5.5
	0.40			Serine	5.7
				Asparagine Glycine Threonine	5.4 6.0 5.6
0.60		Rhamnose Glucosamine	Glutamine α-alanine	5.7 6.0
			N-acetyl glucosamine Galactosamine	Hydroxyproline Methionine Leucines	5.8 5.7 6.0 – 6.1
0.80	Proline		Proline	6.3

glutamyl polypeptide. Fraction 1 b reacts as most proteins with Amido black. The second fraction absorbs UV light, a property characteristic for heterocyclic and aromatic compounds, and is stained by methylene blue like, *e.g.*, glutamyl polypeptide. The strongly negatively charged fraction 3 does not react with the developers used.

The high-molecular part, H, of the suspension behaves after electrophoresis, autoradiography and different development techniques like fraction 1a of suspension S.

The low-molecular part, L, of the suspension, is separated into 4 fractions. Fractions 1a + b have a low ¹⁴C-activity and 2 and 3a high ¹⁴C-activity. Fraction 1a absorbs UV light and gives as well as fraction 1 b a positive nin-

Fig. 3. Two dimensional paper chromatography (phenol- NH_3 -HCN and collidine-lutidine-diethylamine) of low-molecular fraction from *B. anthracis* homogenate. ^{14}C -distribution.

hydrin reaction. The slow migration rate of fraction 1a + b suggests the presence of amino acids with $\text{IP} = 5-7$, e.g. diaminopimelic acid, glycine, α -alanine, proline, hydroxyproline, serine, asparagine, threonine, glutamine, methionine, leucines. Fraction 2 contains substances which absorb UV light very weakly, and which are slightly metachromatic but which give a pronounced colour with ninhydrin. Amino acids and some peptides with acid IP, e.g. glutamic acid, aspartic acid, and polypeptides of glutamic acid, behave in this way. Fraction 3 behaves similar to fraction 3 of the crude suspension, S. It does not stain with ninhydrin. After elution, paper chromatography in 2-dimensions (ethanol- NH_3 /propanol-eucalyptol-formic acid), autoradiography and development of the chromatograms with bromocresol green one spot appears with properties corresponding to those of succinic acid.

Fig. 2 shows the results from the one-dimensional paper chromatograms. ^{14}C -Activity is high in spots with R_F -values between 0.00–0.11; 0.13–0.20 and 0.24. The other spots with R_F -values about 0.29; 0.37; 0.42; 0.56 and 0.78 have lower ^{14}C -activity.

Under UV light a slightly dark spot appears at $R_F = 0.07$ and slightly light spots at $R_F = 0.29$; 0.42; 0.78.

By development with aniline phthalate slightly positive reactions occur at $R_F = 0.05$; 0.15; 0.58 and 0.70.

With ninhydrin as developer, coloured spots are obtained at $R_F = 0.15$; 0.27; 0.37; 0.42; 0.56; 0.74–0.82.

By using 2-dimensional paper chromatography a better separation is obtained (Fig. 3). High ^{14}C -activity is found in an area of R_F (phenol): 0.00–0.30; R_F (collidine): 0.00–0.20 and another of R_F (phenol) 0.80 and R_F (collidine) 0.13.

Staining with ninhydrin gives positive spots in the area with high ^{14}C -activity, except for a small spot with R_F (phenol): 0.05 and R_F (collidine): 0.05. Among the other slightly ^{14}C -active spots several are ninhydrin positive.

After elution, hydrolysis with acid, re-chromatography, autoradiography and staining with ninhydrin the highly ^{14}C -active and ninhydrin positive areas in the 2-dimensional chromatograms show spots corresponding to those of glutamic, aspartic and diaminopimelic acids. The highly ^{14}C -active and almost ninhydrin negative spot gives 3 ^{14}C -active spots after elution, re-chromatography in ethanol- NH_3 /propanol-eucalyptol-formic acid and autoradiography. One has R_F (base): 0.4; R_F (acid): 0.14; a second R_F (base): 0.12 and R_F (acid):

0.16 and the third R_F (base): 0.19 and R_F (acid): 0.56. The last spot behaves similarly to succinic acid.

The slightly ^{14}C -active, ninhydrin-positive spots show close agreement with glycine, α -alanine, hydroxyproline and proline, although asparagine, serine, threonine, glutamine, methionine and leucines cannot be excluded.

In the 2-dimensional chromatograms with phenol and butanol, which were autoradiographed for 3 days, 3 strongly ^{14}C -active spots appear. One spot has R_F (phenol): 0.05 and R_F (butanol): 0.15; the other R_F (phenol): 0.15 and R_F (butanol): 0.35 and the third R_F (phenol): 0.25 and R_F (butanol): 0.08. One slightly ^{14}C -active spot has R_F (phenol): 0.85 and R_F (butanol): 0.13. None of these four spots gives a positive reaction with aniline phthalate.

COMMENT

The study shows that virulent, encapsulated *B. anthracis* incorporates ^{14}C from $^{14}\text{CO}_2$. ^{14}C is found particularly in substances behaving like succinic acid, glutamic acid, aspartic acid and diaminopimelic acid. ^{14}C is also very likely incorporated into peptides. The carbohydrates, at least the low-molecular ones, are not involved in the $^{14}\text{CO}_2$ -metabolism to any great extent.

Earlier studies by Ivanovics¹², Sterne¹³ and Nordberg³ have shown that virulent *B. anthracis* becomes encapsulated in a CO_2 -atmosphere. From their studies on the effects of CO_2 upon the synthesis of D-glutamyl polypeptide, a constituent of the capsule, Thorne *et al.*¹⁴ have concluded that "The difference in the CO_2 -requirements for peptide synthesis is one of the few differences we have found between the virulent and the avirulent strains".

Since *B. anthracis* contains racemase¹⁵ and in other organisms there are close interrelations between the labelled substances found, these substances conceivably act as a pool for the synthesis of peptide. However, the labelled acids can also take part in the metabolism in some other way to contribute to the qualitative behaviour of the encapsulated virulent *B. anthracis*.

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