LXXXV. THE PRODUCTION OF FREE SULPHUR FROM L-CYSTINE BY A SOIL BACTERIUM.

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(Received February 29th, 1936.)

IN the ordinary putrefaction of protein material the protein-sulphur is liberated mainly as hydrogen sulphide accompanied in a few cases by relatively small amounts of mercaptans. The present paper is an account of an organism which is capable of the aerobic degradation of L-cystine with the direct liberation of free sulphur.

In earlier work on the bacterial decomposition of cystine the medium used has always contained other sources of carbon such as carbohydrates or peptone, owing apparently to the difficulty of obtaining an organism capable of developing on cystine alone. Thus Sasaki and Otsaki [1912], who found that nineteen out of twenty-one different pure cultures formed hydrogen sulphide from cystine, employed Fränkel's medium containing asparagine, ammonium lactate and inorganic salts. Bürger [1914] also used this medium or a cystine medium which contained meat extract and lead acetate. He examined twenty-three different species of bacteria and observed hydrogen sulphide production in every case but never detected mercaptans.

The question of mercaptan formation from cystine is still not settled; Kondo [1923] in a review of the available evidence concludes that the mercaptan arises from the interaction of hydrogen sulphide with substances derived from carbohydrates or histidine, and that in the absence of these the sulphur is liberated as hydrogen sulphide.

During the course of our work a somewhat different mode of attack was described by Tarr [1933] who, using washed cells of *Proteus vulgaris*, quantitatively investigated the decomposition of cystine under anaerobic conditions. In this case too the sulphur appeared almost quantitatively as hydrogen sulphide, the cystine molecule yielding two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

In order to maintain the conditions of our own experiments as simple as possible an attempt was made to find some organism capable of utilising cystine as the sole source of sulphur, nitrogen and carbon. From garden soil a bacterium was isolated which was found to be able to attack cystine somewhat slowly in the absence of all other compounds of carbon, nitrogen or sulphur. On examination of the products it was found that free sulphur and free ammonia were produced in approximately equimolecular proportions and this sulphur appears to arise from the cystine without the intermediate formation of detectable amounts of hydrogen sulphide.

EXPERIMENTAL.

Isolation of the bacterium.

A modified Czapek medium containing cystine was inoculated with a suspension of soil obtained from the grounds surrounding the laboratory buildings.

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The medium had the composition: KNO_3 , 2.0 g.; K_2HPO_4 , 1.0 g.; MgCl_2 , $6\text{H}_2\text{O}$, 0.5 g.; KCl , 0.5 g.; FeCl_3 , 0.01 g.; water to 1 l. Several test-tubes each containing 10 ml. of the solution and approximately 0.1 g. cystine were inoculated with a few drops of the soil suspension. After three or four days one tube showed signs of bacterial activity. A loopful of this active liquid was transferred into a sterile tube of the medium and after a week from this into a third tube of the sterile cystine medium. On subsequent plating out on to nutrient agar the culture was found to be homogeneous and was transferred from the plates on to ordinary nutrient agar slopes.

Characteristics of the organism.

Morphology. Short bacillus. $2.0\text{--}2.5\mu$ by 0.5μ occurring singly, in pairs or in short chains.

Motile. The usual flagella stains and mordants failed however to reveal flagella at any stage of the cultivation.

Gram-negative.

Gelatin liquefied. Gelatin stab, napiform.

Agar colonies. Round; after 3 days at 20° diameter 0.75 mm.; after 7 days 2–3 mm.; after 40 days 5–6 mm.

Colonies, amorphous, homogeneous, homochromous. Surface smooth. Edges entire. Colour, grey-white by reflected, brown by transmitted light. Opalescent. Elevation between pulvinate and capitate.

Agar slant. Flat, dirty white with faint suggestion of a yellowish central streak.

Growth on agar completely inhibited at 37° .

Non-sporing. Suspensions in sterile water sterile after 20 min. at 80° .

Litmus milk. Alkaline, not coagulated.

Carbohydrate-peptone media. Lactose, sucrose, fructose, galactose, xylose and glucose, growth visible in one day, no gas, alkali production.

Potato slope. Smooth, raised, slightly yellowish orange streak becoming gradually browner.

Nitrate-peptone. Nitrate reduced to nitrite. Ammonia formed in traces only.

Gelatin shake. Small circular colonies on free surface, slowly eating into medium and producing complete liquefaction from above downwards.

Indole formed in 1% peptone solution.

Origin. Soil.

A culture has been deposited at the National Collection of Type Cultures.

On the Bergey system of nomenclature [Bergey, 1934] the organism would appear to be a species of the genus *Achromobacter* and differs in several characteristics from any described hitherto.

In consideration of the characteristic utilisation of cystine the name *Achromobacter cystinovorum* is suggested.

Method of experiment.

For the actual experiments the medium used for the isolation of the organism was further simplified by the elimination of sulphate, nitrate and magnesium, preliminary trials having shown that growth was not retarded by the absence of any or all of these. The medium finally employed consisted of K_2HPO_4 , 1.28 g.; KCl , 0.5 g.; hydrated FeCl_3 , 0.01 g.; water, 1 l., to which weighed amounts of cystine were added into each flask just prior to sterilisation. The weight of cystine usually employed was 0.5 g. per 100 ml. of the medium; this amount dissolved at 100° during sterilisation and partly crystallised out on cooling. The p_{H} of this medium was 7.0.

It will be seen that the medium contained no source of carbon, sulphur or nitrogen other than cystine.

The cystine used was prepared from human hair, was decolorised several times by charcoal in boiling hydrochloric acid solution, filtered, precipitated from the filtrate by sodium acetate and washed with water. A further purification was effected by treatment with warm ammonia solution (1 vol. sp. gr. 0.880 to 1 vol. water) filtering in order to remove traces of earthy phosphates, and precipitating the cystine from the filtrate by the addition of acetic acid. Finally the product was exhaustively extracted with hot water in order to remove residual tyrosine.

(Found S (Denis-Benedict method) and N (Kjeldahl): Sample 1: S, 27.1, 27.1; N, 11.4, 11.6%. Sample 2: S, 26.25, 26.7; N, 11.7, 11.6%. Cystine requires S, 26.7, N, 11.7%.)

1.0052 g. cystine in 100 ml. of 1.004 *N* hydrochloric acid gave $[\alpha]_{5461}^{19^\circ} - 260.2^\circ$ and $[\alpha]_D^{20^\circ} - 222^\circ$.

For preliminary qualitative work 1 l. flat-bottomed round flasks, each containing 500 ml. of the medium were used. Signs of activity usually appeared on the third day after inoculation and incubation at 20°. The medium became turbid and later the powdery cystine deposit gave place to a colourless sludge-like mass. On shaking the contents of the flask with ether and evaporating the washed ethereal extract crystals of sulphur were obtained. For the quantitative determination of the free sulphur, the total contents of the culture flask were acidified with hydrochloric acid and filtered through a weighed Gooch crucible, and the residue was washed and dried at 100° to constant weight. The crucible and contents were together extracted with carbon disulphide and again weighed, and the sulphur was calculated as the difference between the weights. This figure was in several cases checked by collecting the carbon disulphide extract in a weighed beaker and weighing the residue of pure crystalline sulphur. The sulphur values were also checked against uninoculated control flasks.

The absence of hydrogen sulphide and indeed of sulphydryl compounds generally was confirmed by frequent withdrawal of samples of the active medium and testing with ammonium sulphide and sodium nitroprusside. Sulphates too were entirely absent at every stage. Nitrogen determinations by Kjeldahl's method were carried out on several complete culture flasks at various stages of growth, and in every case, even where the superincumbent air had been continuously removed for carbon dioxide estimation, there was no loss of total nitrogen.

The ammonia production was determined either by formaldehyde titration of filtered samples periodically removed from the culture flask or by adding excess of sodium carbonate to the total culture and aspirating air through the liquid at 40° into standard acid. Separate control experiments showed that cystine itself gave no ammonia under these conditions.

Carbon dioxide formation was measured by aspirating a current of air over the surface of the culture into standard barium hydroxide solutions.

Estimation of sulphur and ammonia. 250 ml. Erlenmeyer flasks were used, each containing 100 ml. of the cystine medium. After the sulphur had been removed by filtration through a weighed Gooch crucible as previously described an aliquot part of the total acid filtrate was made alkaline by addition of sodium carbonate and the ammonia determined by aspiration into standard acid.

Allowing 0.001 g. for S and 0.002 g. for N in the controls the atomic ratios for S/ammonia-N shown in the right-hand column of Table I are obtained.

Simultaneous estimation of carbon dioxide, sulphur and ammonia. A train of bottles was set up in the following order. Potassium hydroxide solution 30%, dilute barium hydroxide solution, dilute sulphuric acid, the culture flask, dilute sulphuric acid, two Drechsel bottles each containing

Table I.

Wt. of cystine in 100 ml. medium, g.	Time from first sterili- sation days	Time after inoculation days	S produced g.	Ammonia-N produced g.	S/NH ₃ -N
Controls. Flasks merely subjected to discontinuous sterilisation.					
0.5000	6	—	0.0004	—	—
0.5000	14	—	0.0024	0.0018	—
0.5000	20	—	0.0013	0.0020	—
0.5006	7	—	- 0.0004	0.0006	—
0.4999	15	—	- 0.0004	0.0013	—
0.5000	21	—	0.0001	0.0016	—
0.4999	42	—	0.0008	0.0017	—
Inoculated flasks					
0.4999	13	7	0.0536	0.0248	1.01
0.4997	20	14	0.0830	0.0361	1.05
0.5008	27	21	0.0650	0.0321	0.94
				0.0313	
0.5009	15	8	0.0483	0.0219	0.95
0.4998	21	14	—	0.0294	—
0.4998	42	35	0.0801	0.0284	1.26
				0.0305	

measured volumes of standard barium hydroxide solution with phenolphthalein and finally a soda-lime tube connected to the aspirator. The air current was passed over the surface of the liquid culture medium by means of two tubes, a short inlet tube which passed just through the rubber stopper, and a longer outlet tube which reached almost to the surface of the medium. The evolved carbon dioxide was estimated by back-titration of the baryta bottles with standard barium hydroxide solution if they showed excess of carbon dioxide, or otherwise by allowing the barium carbonate to settle, pipetting off an aliquot part of the clear pink solution and titrating with standard HCl.

At the end of each experiment the sulphuric acid bottle was examined for ammonia, but as had been found in all previous experiments, none was present. Before disconnecting the culture flask its contents were cautiously acidified with hydrochloric acid and a rapid stream of air passed through the apparatus. The contents of the flask were then filtered through a tared Gooch crucible and sulphur and ammonia estimated in residue and filtrate respectively. After extraction of the residue with carbon disulphide for the removal of sulphur, the residue was in some cases examined for total nitrogen, *i.e.* synthesised bacterial protein-nitrogen. (See Table II.)

Table II.

Exp. no.	Cystine in medium g.	Time after inoculation days	Sulphur produced mg.	Ammonia-N produced mg.	CO ₂ produced mg.	Atomic ratio S/ammonia-N/CO ₂ -C
1	0.5010	17	50.8	20.6	123	1.1 : 1 : 1.9
2	0.5003	9	31.8	16.8	88	0.83 : 1 : 1.66
3	0.5005	13	37.6	14.9	127	1.11 : 1 : 2.73
4	0.4995	12	41.4	17.1	97	1.06 : 1 : 1.43
5	0.4996	8	11.6	10.3	92	0.49 : 1 : 2.9
6	0.5004	7	11.7	5.8	51	0.88 : 1 : 2.9
7	0.4998	7	11.2	6.5	66	0.76 : 1 : 3.3

In the last two experiments the S/N/CO₂ ratio was further checked (*a*) by a determination of bacterial protein-nitrogen in the solid residue after extraction with carbon disulphide and (*b*) by a determination of non-ammonia-nitrogen in the acid filtrate (Table III).

Table III.

Exp. no.	Insol. protein residue mg.	N in residue mg.	Non- ammonia-N in filtrate mg.	Total N found mg.	Cystine-N originally present mg.
6	12.4	1.5	51	$5.8 + 1.5 + 51 = 58.3$	57.5
7	11.4	1.6	50.5	$6.5 + 1.6 + 50.5 = 58.6$	59.7

SUMMARY.

A new soil organism is described which is capable of decomposing *l*-cystine in the absence of other sources of either carbon, nitrogen or sulphur.

The products obtained are free sulphur, ammonia and carbon dioxide.

The name *Achromobacter cystinovorum* is suggested.

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