XCIX. THE ANAEROBIC DECOMPOSITION OF *l*-CYSTINE BY WASHED CELLS OF *PROTEUS VULGARIS*.

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ADEQUATE summaries of the literature dealing with the products resulting from the decomposition of amino-acids by various bacteria have been prepared by Stephenson [1930] and Buchanan and Fulmer [1930]. In the case of cystine a study of these reviews and of more recent literature makes it evident that previous investigators have concerned themselves with the formation of hydrogen sulphide, ethylmercaptan and diethyl sulphide from cystine, and not with the complete disintegration of the molecule. In the present paper the anaerobic decomposition of cystine by means of "washed cells" [Sandiford and Wooldridge, 1931] of *Proteus vulgaris* is fully described.

EXPERIMENTAL.

The strain of *Proteus vulgaris* employed was obtained from the Department of Pathology, Cambridge. The organism was inoculated in the form of a thin suspension on to the surface of caseinogen digest agar [Cole and Onslow, 1916] sloped in 1 litre Roux bottles. After 24 hours at 37° the organisms were washed from the surface with a sterile sulphate solution (Na_2SO_4, 0·9 %; K_2SO_4, 0·05 %), centrifuged under aseptic conditions and washed once with the same solution. Subsequently the bacterial cells were suspended in sterile sulphate solution to give a fairly thick suspension, the dry weight of bacteria being reported for each experiment. Such suspensions were stored at 1° under aseptic conditions and were used within 5 days of preparation. A sulphate solution was employed in the place of Ringer's solution since the presence of chloride interfered with subsequent volatile acid determinations.

The cystine employed gave the following analytical figures: N, 11·34; S, 26·24 %; theoretical N, 11·67; S, 26·69 %.

Fig. 1 shows the aeration train employed in the experimental work. The flasks employed had the following capacity: *A*, 2000 cc.; *B*, 500 cc. and *C*, 150 cc. The technique was as follows. Exactly 500 mg. of cystine were weighed out and placed in flask *A*, the rubber stopper and glass tubing inserted as illustrated, and the whole sterilised by autoclaving for 20 minutes at 15 lbs. pressure. The cystine was sterilised in the dry form since there are indications that there is a greater tendency for it to decompose when sterilised in an aqueous medium. 400 cc. of sterile *M*/40 phosphate buffer of p_H 7·8 were then added aseptically by the tube *X*, and the tube was closed by means of a screw-clip. Flask *B*, containing 300 cc. of 2 % zinc acetate solution slightly acidified by means of 1 cc. of 5 % acetic acid, was then connected as shown and a stream of hydrogen washed through 5 % $AgNO_3$, alkaline pyrogallol and 10 % H_2SO_4 bubbled rapidly

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through the aeration train for 2 hours in order to remove all the CO_2 present in the flasks. 100 cc. of a suspension of *P. vulgaris* were then added carefully by the tube *X*, and flask *C*, containing about 100 cc. of carbonate-free, approximate 0·1 *N* $Ba(OH)_2$, was connected to the end of the aeration train. The entire set-up was placed in an incubator at 37° and a rapid stream of washed hydrogen bubbled through the solutions for 24 hours. Experiments showed that, at the end of this time, all the cystine had dissolved and had completely broken down, the final p_H of the liquid being about 7·0 to 7·2. Nothing but typical *P. vulgaris* colonies developed on caseinogen digest agar plates prepared from suitable dilutions of the fluid in flask *A*, and smears stained by Gram's method showed nothing but negative cells which appeared to be identical with those found in the original suspensions.

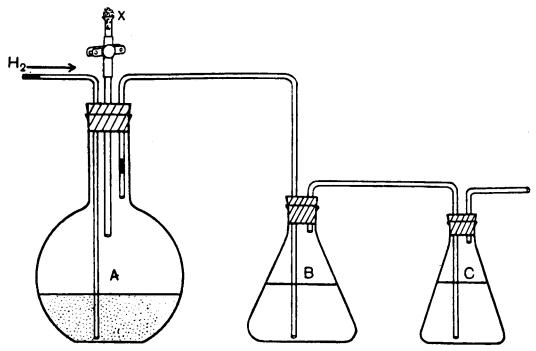

Fig. 1.

Flask *A* was then placed in ice-water in order to avoid the possibility of further decomposition of any product in the solution, and, after a preliminary 15 minutes in order to allow the solution to cool, 2·5 cc. of *N* H_2SO_4 (3 cc. in control experiments) were added to bring the p_H to approximately 6·5, and hydrogen was bubbled through the solution for 2 hours in order to remove practically all remaining H_2S and CO_2. Control experiments, employing identical technique, but with no added cystine, were run under the same conditions with the same suspension in each case. The products arising from the decomposition were estimated by the following methods.

CO_2. The white $BaCO_3$ precipitate in flask *C* was filtered on a Büchner funnel and washed quickly with several successive amounts of distilled water. The CO_2 present in the precipitate was then estimated by the method described by Stephenson and Whetham [1922].

H_2S. The zinc acetate solution in flask *B* was diluted to 5000 cc., the white precipitate of zinc sulphide being thoroughly mixed in order to give a uniform suspension. 2 cc. of this suspension were taken for a colorimetric determination of H_2S by the method described by Almy [1925]. In control experiments the original zinc acetate solution was diluted to 500 cc. and 50 cc. of the resulting solution taken for the H_2S determination. The amount of H_2S formed in control experiments was negligible in comparison with that found in cystine experiments.

NH_3. The technique was based on that developed by Van Slyke [1911] for the estimation of ammonia, using the apparatus described by Stephenson and Whetham [1922] for the estimation of volatile acid. For the determinations the liquid in flask *A* was made up to exactly 500 cc. without removing the bacterial

cells. 50 cc. of the suspension were distilled with 2 g. of anhydrous Na_2CO_3 and the ammonia collected in standard H_2SO_4 under reduced pressure at a temperature not exceeding 50°.

Total volatile acid. The remaining 450 cc. of suspension from flask *A* were made alkaline to phenolphthalein with *N* NaOH, and then filtered through a layer of kieselguhr on a Büchner funnel with suction in order to remove most of the bacterial cells. The resulting clear solution was concentrated under reduced pressure to between 40 and 50 cc., and the number of cc. of volatile acid present determined by the method described by Stephenson and Whetham [1922]. The clear distillate containing the barium salts of the volatile acids was evaporated under reduced pressure and made up to exactly 100 cc. This solution, which represented 450 mg. of cystine, was employed for the estimation of formic and acetic acids.

Formic acid. The formic acid in 11·1 cc. of the above solution was estimated exactly by the technique described by Stickland [1929].

Acetic acid. The remaining 88·9 cc. of the above solution were made more strongly alkaline by the addition of 5 cc. of *N* NaOH, placed in a 150 cc. flask and heated to boiling on a wire gauze. Approximately *N*/10 $KMnO_4$ was then run into the hot solution until a purple colour resulted indicating an excess of permanganate, thus oxidising the formic acid present. The solution was cooled, and 1 % H_2O_2 added cautiously until the purple colour due to the excess permanganate disappeared. The precipitated MnO_2 was removed by filtering with suction, the clear filtrate made faintly acid with *N* H_2SO_4 and cold, saturated Ag_2SO_4 solution added in slight excess of that required to precipitate the chloride. The AgCl precipitate was removed by filtration, the solution made slightly alkaline by the addition of *N* NaOH and the resulting AgOH precipitate removed by filtration. The clear solution was evaporated under reduced pressure to between 40 and 50 cc. The amount of volatile acid present was then determined by the method already referred to with the single exception that the excess baryta in the receiving flask was titrated with standard H_2SO_4 in place of HCl. The barium sulphate was removed by centrifuging and the supernatant liquid evaporated in a large evaporating dish over a water-bath to between 25 and 30 cc. The solution was then filtered through a small filter-paper into a weighed crystallising dish. The clear solution was evaporated to dryness on a water-bath and then placed over concentrated H_2SO_4 in a vacuum desiccator for 24 hours. The dry residue was then weighed, the weight being compared with that calculated for $(C_2H_3O_2)_2Ba$, and the percentage of barium in the salt was then estimated as $BaSO_4$. A small quantity of the *para*toluidide was prepared from the mixed barium salts from two separate experiments according to the directions given by Mulliken [1904], and the melting-point of the resulting compound was determined alone and when mixed with a sample of acet*para*toluidide prepared from pure acetic acid.

The results of two typical experiments are recorded in Tables I and II. It is evident from the data obtained that cystine breaks down under the conditions of the experiments to yield two molecules each of hydrogen sulphide, ammonia, acetic and formic acids, the last named presumably partially decomposing to form H_2 and CO_2:

$$\begin{aligned} &COOH.CH(NH_2).CH_2S{-}SCH_2.CH(NH_2).COOH + H_2 + 4H_2O \rightarrow \\ &\qquad 2H_2S + 2NH_3 + 2CH_3COOH + 2HCOOH \\ &\qquad\qquad \downarrow \\ &\qquad\qquad 2H_2 + 2CO_2 \end{aligned}$$

Table I. *Recovery of the various substances resulting from the anaerobic decomposition of 500 mg. of* l-*cystine.*

Exp. 1. 0·95 g. dry weight of bacteria used.
Exp. 2. 0·72 g. dry weight of bacteria used.

Experiment No.	NH_3 mg.	H_2S mg.	CO_2 mg.	Total* volatile acid in cc. of 0·1 *N*	Acetic† acid mg.	Formic acid mg.	Total‡ formic acid mg.
1							
Cystine added	74·0	139·0	114·0	60·4	250	73·5	
Control	3·4	0·2	30·8	3·1	0	0	
Difference	70·6	138·8	83·2	57·3	250	73·5	160·5
2							
Cystine added	68·0	132·0	146·0	56·9	236	80·5	
Control	3·4	0·2	43·0	3·0	0	0	
Difference	64·6	131·8	103·0	53·9	236	80·5	188·0

* The volatile acid arising in the control experiments has not been identified, and, since it represents only about 5 % of the total volatile acid formed in the presence of cystine, has been neglected.

† Barium calculated for $(C_2H_3O_2)_2Ba$ ... ... 53·79 %
Barium found, Exp. 1 ... ... 52·6 %
Barium found, Exp. 2 ... ... 52·6 %
M.P. of acet*para*toluidide, from pure acetic acid 146–7°
M.P. of acet*para*toluidide, prepared from mixed barium acetate of Exps. 1 and 2 ... ... 146–7°

No change was found in the M.P. when some of the above toluidide was mixed with *para*-toluidide prepared from pure acetic acid.

‡ The total formic acid is the sum of the formic acid found *plus* that calculated for the CO_2 recovered, assuming that formic acid breaks down according to the equation given.

Table II. *A comparison of the experimental and theoretical values based on the decomposition of cystine with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.*

	Recovery from 500 mg. cystine					
	Exp. 1		Exp. 2		Theoretical	
Compound	mg.	%	mg.	%	mg.	%
NH_3	70·6	100	64·6	92	70·2	100
H_2S	138·8	99	131·8	94	140·5	100
CH_3COOH	250·0	100	236·0	95	248·0	100
HCOOH (Total)	160·5	85	188·0	99	190·0	100

As yet all attempts to demonstrate the presence of lactic or pyruvic acid as a possible intermediary product of the reaction have proved unsuccessful. It is, therefore, impossible to state at present which of the several possible courses of anaerobic breakdown occurs.

Summary.

l-Cystine has been shown to undergo anaerobic decomposition in the presence of washed cells of *P. vulgaris* with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

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