# XXVIII. HYDROGENASE.
## II. THE REDUCTION OF SULPHATE TO SULPHIDE BY MOLECULAR HYDROGEN.

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In a previous communication [Stephenson and Stickland, 1931] it was shown that many common species of bacteria possess an enzyme which activates molecular hydrogen; this enables them to reduce various hydrogen acceptors by means of hydrogen gas. It was there mentioned that the mixed culture, obtained from the River Ouse, in which this was first observed was able to reduce sulphate to sulphide by molecular hydrogen, and this reaction forms the subject of the present paper.

It was originally shown by Beijerinck [1895] that the hydrogen sulphide produced in mud arises anaerobically by the bacterial reduction of sulphates. Van Delden [1904] first obtained pure cultures of the organisms concerned (*V. desulphuricans* and *V. aestuarii*) in a medium in which sulphate was reduced to sulphide anaerobically at the expense of organic compounds (lactate and malate), the energy for development being derived from the reduction of the sulphate. Elion [1924] isolated a thermophilic organism (*V. thermodesulphuricans*) of the same type. Quite recently an exhaustive study of the metabolism of the sulphate-reducing bacteria has been made by Baars [1930]. Using several strains he investigated the products of oxidation of a large number of carbon compounds, and showed that the strains differed in the compounds which they were able to oxidise.

With regard to the reduction of sulphate by molecular hydrogen the only reference which we have been able to find is a statement by Niklewski [1914], who claims to have isolated an organism able to carry out this reaction, but gives no details.

### *Isolation and description of the organism.*

In order to isolate the organism from the mixed culture, the technique employed by Van Delden was used; this consists in plating on broth agar containing sulphate and iron salts, the concentrations used being 0·5 % sodium sulphate and 0·005 % potassium iron sulphate. The plates were incubated anaerobically, and the colonies responsible for the production of sulphide were

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distinguished by being jet black owing to the precipitation of iron sulphide. After several repetitions a culture was obtained which gave only black colonies; the pure culture was known as Strain 182.

Like the other sulphate-reducers described in the literature this organism can be grown anaerobically on an inorganic medium containing sulphate and lactate, thus proving that it derives its energy from the oxidation of the lactate by the sulphate:

$$2CH_3.CHOH.COOH + 3H_2SO_4 = 6CO_2 + 6H_2O + 3H_2S + 254 \text{ Kg. Cals.}$$

It also resembles *V. desulphuricans* in being a small comma-shaped organism, strictly anaerobic and non-spore-forming.

It has been shown by Baars that the different species of sulphate-reducers, while agreeing in their general morphological and physiological characteristics, differ among themselves in their abilities to use various carbon compounds for the reduction of sulphate. We therefore tried to establish the identity of our Strain 182 by sowing it into a number of media containing a variety of carbon compounds and sulphate. The medium [Baars, 1930] consisted of

| | % |
|---|---|
| $K_2HPO_4$ ... ... ... ... | 0·05 |
| $NH_4Cl$ ... ... ... ... | 0·1 |
| $CaSO_4$ ... ... ... ... | 0·1 |
| $MgSO_4.7H_2O$ ... ... ... | 0·2 |
| $FeSO_4$ ... ... ... ... | Trace |

with 30 millimols of carbon compound per litre, and the results were as follows:

| Carbon compound | *V. desulph.* | *V. rübentsch.* | Strain 182 |
|---|---|---|---|
| Glycerol | + | + | − |
| Mannitol | | + | − |
| Glucose | + | + | − |
| Fructose | + | + | + |
| Galactose | − | + | ? |
| Sucrose | − | + | − |
| Lactose | − | + | − |
| Maltose | − | + | − |
| Formic acid | + | + | + |
| Acetic ,, | − | + | − |
| Butyric ,, | − | + | − |
| Lactic ,, | + | + | + |
| Tartaric ,, | − | + | − |
| Succinic ,, | + | + | − |

We thus have to conclude that Strain 182 is not identical with any organism studied by Baars.

*Presence of hydrogenase in Strain 182.*

The bacteria were grown on 1200 cc. of a medium containing 25 % of tryptic broth, 0·5 % sodium formate, 0·5 % sodium sulphate and 50 % of inorganic medium [Stephenson, 1930]. After about 5 days' anaerobic incubation, the cells were separated from the medium by centrifuging, washed twice

with Ringer's solution and suspended in 50 cc. of Ringer's solution. By the method already described [Stephenson and Stickland, 1931] it was proved that this strain actively reduces methylene blue by molecular hydrogen, *i.e.* contains hydrogenase.

| | Reduction time (mins.) |
|---|---|
| Suspension (diluted 1 in 5) *in vacuo* | >180 |
| „ in hydrogen | 4 |
| Boiled suspension in hydrogen | >180 |

The ability to use hydrogen is not a peculiarity of our strain, as it was found by the same method that *V. rübentschickii* also contains hydrogenase.

| | Mins. |
|---|---|
| Reduction time *in vacuo* | >150 |
| „ in hydrogen | 17 |

*Reduction of sulphate by molecular hydrogen.*

(*a*) *Qualitative.* 1 cc. of a washed suspension of bacteria of Strain 182 was incubated at 37° for 48 hours with 1 cc. of 0·6 % sodium sulphate in phosphate buffer at $p_H$ 7·4, in hydrogen and *in vacuo*. In the tubes containing hydrogen a strong smell of hydrogen sulphide and a strong reaction with nitroprusside were observed, and in the controls both tests were negative. By the same method the reduction of sulphate by hydrogen in presence of *V. rübentschickii* was demonstrated.

(*b*) *Quantitative.* The apparatus (Fig. 1) consisted of a bolt-headed flask of

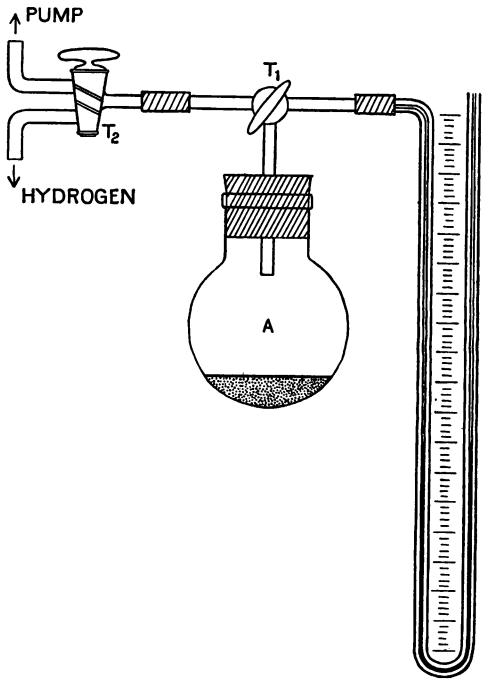


Fig. 1.

200 cc. capacity ($A$), fitted with a 3-way tap $T_1$; the latter was connected with a manometer. Into $A$ were put 10 cc. phosphate buffer, $p_H$ 7·2, 10 cc. of standard solution of sodium sulphate and 20 cc. of suspension of the organism. Before placing the stopper in the flask the tap $T_1$ was opened to the manometer, which was tipped till the mercury reached the tap; the latter was then closed by a turn through 90°. The rubber bung was then inserted in the flask, and its lower level marked on the glass. The distal tube of $T_1$ was then connected to a 3-way tap $T_2$, by which it could be joined either to a water-pump or to a supply of hydrogen. The flask was first evacuated, the liquid being shaken to expel dissolved air, and then filled with hydrogen, this process being repeated twice to ensure thorough removal of oxygen. $T_2$ was then closed, $T_1$ turned so as to connect the flask with the manometer and the flask disconnected. The whole was then placed in the incubator at 34°. When the temperature had become constant as shown by the manometer, the manometer and barometer readings were simultaneously taken, and these readings were taken at suitable intervals during the experiment until the absorption of hydrogen was complete (see Table I).

Table I.

| Date (August) | Time | Manometer diff. | Uptake | Correct. for change in bar. | Uptake (corrected) mm. Hg |
|---|---|---|---|---|---|
| 19 | 9.0 p.m. | +50·0 | 0·0 | 0·0 | 0·0 |
| 20 | 9.30 a.m. | + 1·0 | 49·0 | − 1·5 | 47·5 |
| 20 | 11.30 p.m. | −13·0 | 63·0 | − 1·5 | 61·5 |
| 21 | 10.0 a.m. | −20·5 | 70·5 | +14·5 | 85·0 |
| 21 | 10.30 p.m. | −40·0 | 90·0 | + 8·5 | 98·5 |
| 22 | 10.0 a.m. | −58·0 | 108·0 | + 1·5 | 109·5 |
| 22 | 10.30 p.m. | −63·5 | 113·5 | − 0·5 | 113·0 |
| 23 | 9.30 a.m. | −66·0 | 116·0 | + 6·5 | 122·5 |
| 24 | 10.0 „ | −73·0 | 123·0 | − 0·5 | 122·5 |
| 25 | 10.0 „ | −79·0 | 129·0 | − 6·5 | 122·5 |

The stopper was then removed and the contents of the flask made alkaline by the addition of 10 cc. of $N$ NaOH. Two 10 cc. samples were placed in 300 cc. flasks and acidified, and the hydrogen sulphide blown from them by a current of nitrogen into a series of three small absorption bottles, the first two each containing 10 cc. of $N/50$ iodine, the third about 10 cc. of toluene to absorb the iodine carried through by the current of gas. After 4 hours the iodine was titrated with $N/50$ thiosulphate, and the amount of hydrogen sulphide calculated. The residual sulphate, after removal of bacterial debris by filtration through kieselguhr, was estimated for us by a micro-method by Mr A. Colwell. The volume of the gas space in the flask and the tubes was measured.

*Calculation of results.*

(*a*) *Hydrogen used.* Change of pressure (corrected for change of barometer) = 445·5 mm. Hg.

Volume of gas space in flask = 134·0 cc., temperature, 34°.

Hydrogen used = $\frac{134 \times 445{\cdot}5 \times 273}{760 \times 307}$ = 69·9 cc. = 3·12 millimols.

(*b*) *Hydrogen sulphide produced.* Iodine used by total volume = 73·7 cc. $N/50$ = 36·85 cc. $M/50$ $H_2S$ = 0·74 millimols.

(*c*) *Sodium sulphate reduced.* The $Na_2SO_4$ added, 0·100 g. = 0·70 millimols, was completely reduced.

*Results.*

As four molecules of hydrogen are required to reduce one molecule of sulphate to sulphide, the figures for hydrogen in the table are divided by four in order to show the extent of the agreement:

$$H_2SO_4 + 4H_2 = H_2S + 4H_2O.$$

| Exp. | Sulphate reduced (millimols) | Hydrogen used / 4 (millimols) | Sulphide formed (millimols) |
|---|---|---|---|
| 1 | 0·70 | 0·78 | 0·74 |
| 2 | 0·40 | 0·38 | 0·35 |

*Control experiments.* (*a*) An exactly similar experiment to the above, without the addition of sulphate, showed no uptake of hydrogen. (*b*) A suspension of cells incubated with sulphate in nitrogen instead of hydrogen gave no hydrogen sulphide. (*c*) An identical experiment carried out with full sterile precautions, both in the centrifuging and washing of the organisms and in the setting up of the apparatus, gave similar results, and showed no contaminating colonies on plating at the end of the experiment.

*Reduction of other hydrogen acceptors.*

Baars showed that the various strains of *V. desulphuricans*, when sown into lactate synthetic medium containing sulphate, sulphite, hyposulphite, thiosulphate or sulphur, reduced them all to sulphide. We found that the washed suspension of cells of Strain 182 reduced sulphate, sulphite and thiosulphate to sulphide in the presence of hydrogen and not in the control experiments with hydrogen absent.

*Effect of hydrogen ion concentration.*

As hydrogenase was first studied in a group of organisms differing widely in their physiology from Strain 182, it seemed worth while to ascertain whether the enzyme showed any marked divergence of properties in association with this sulphate-reducing species. For this reason we compared the effect of hydrogen ion concentration on the enzyme of this organism with that on the enzyme in *Bact. coli* and Strain 111 previously recorded [Stephenson and Stickland, 1931]. The result is shown in Fig. 2; the explanation of the dotted lines and the lettering will be found in the earlier paper [1931, p. 210]. The curve is the same in its main character as those obtained with the other species, and it seems probable that we are dealing with the same enzyme.

The peak at $p_H$ 6·3 gives the optimum reaction for the activation of

hydrogen; we have at present no accurate method for determining the optimum $p_H$ for the activation of sulphate, so that the optimum for the complete reaction is unknown.

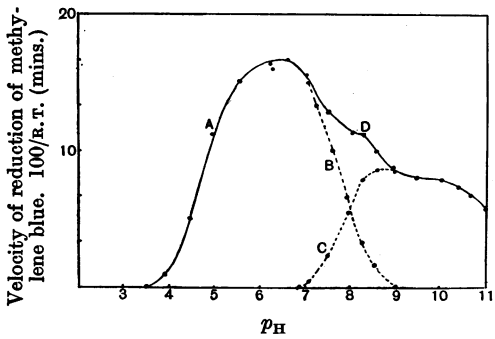


Fig. 2.

## SUMMARY.

1. The enzyme hydrogenase has been found in a typical sulphate-reducing organism from the River Ouse.

2. This organism is able to reduce sulphate quantitatively to sulphide by means of molecular hydrogen according to the equation

$$H_2SO_4 + 4H_2 = H_2S + 4H_2O.$$

3. Sulphite and thiosulphate are also reduced to sulphide.

We wish to record here our thanks to Prof. A. J. Kluyver of Delft for supplying us with strains of *V. desulphuricans* and *V. rübentschickii*.

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## REFERENCES.

Baars (1930). Over het sulphaat reductie door bacterie. (Dissertation, Delft.)
Beijerinck (1895). *Zent. Bakt. Par.* II, **1**, 1.
Van Delden (1904). *Zent. Bakt. Par.* II, **11**, 81, 113.
Elion (1924). *Zent. Bakt. Par.* II, **63**, 58.
Niklewski (1914). *Zent. Bakt. Par.* II, **40**, 430.
Stephenson (1930). Bacterial metabolism. (Longmans, London.)
Stephenson and Stickland (1931). *Biochem. J.* **25**, 205.