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STUDIES ON FERMENTATIVE MECHANISMS IN
ZYMO-SARCINA VENTRICOLI.

T h e s i s
presented for the
Degree of Doctor of Philosophy
in the
University of Glasgow
by
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April, 1960.

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STUDIES ON FERMENTATIVE MECHANISMS

IN

ZYMOBACINA VENTRICULI.

"...sounds as of a fluid boiling or bubbling, and proceeding from the region of his stomach, were perceptible to himself and to those around him..."

Goodsir (1842)

"I cannot resist the temptation to emphasize the fact that we are dealing with a form of sugar fermentation which is essentially of the normal alcoholic type, but which is distinguished from it by the production of quite a considerable quantity of gaseous hydrogen."

Kluyver (1931)

P R E F A C E.

Zymosarcina ventriculi is a micro-organism with many unusual properties. It is one of the largest cocci known and can grow under extremely acid conditions, both in culture medium and in the human stomach. In addition, this organism is one of the few bacteria which produce large amounts of ethanol during the fermentation of glucose. These facts alone make Z. ventriculi of considerable interest to the microbiologist. In spite of this, the organism is so little known that it has been confused in the literature with entirely different bacteria. Z. ventriculi is not available in any of the national collections of cultures, which may explain why few studies on this organism have been made. In addition, when it has been isolated in pure culture, it has to be subcultured daily to be maintained.

Z. ventriculi has a long and interesting history dating back to 1842. Since that date, a number of well-known figures in medicine and microbiology, including Virchow, Suringar, Beijerinck, Kluyver and Van Niel, have been interested in this microbe. The history of Z. ventriculi has been extensively reviewed in the present work.

A general table of contents follows this preface. For ease of reference, detailed indices to the individual

sections are included immediately prior to each section.

I am grateful to Messrs. J. Smillie and W. Burns for technical assistance with some aspects of this work.

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Mr D.R.S. Cameron and Miss D. Allan photographed and copied the figures and plates in this thesis. The figures were drawn by Mr R. Callander. My wife translated certain papers and also typed this thesis.

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T.B.

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NOTE.

Sarcina ventriculi Goodair, the sarcina type species, is an anaerobic micro-organism. As it has been confused with aerobic sarcinae by many workers, Smit (1935) suggested that it might be included in a separate group Zymosarcina, together with the anaerobic saccharolytic sarcinae. This group is now recognized as a subgenus, Zymosarcina Smit (Bergey's Manual of Determinative Bacteriology, 1957). In the present work we have adopted this usage, and have referred to the organism as Zymosarcina ventriculi.

1. INTRODUCTION.

I. INTRODUCTION.

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1. HISTORICAL SURVEY.

The occurrence of a gastric fermentation caused by a sarcina was first recorded in 1842 by John Goodsir in Edinburgh. He proposed the name Sarcina ventriculi for this organism which he found in the gastric fluid of one of his patients. Goodsir's discovery was followed by considerable controversy concerning the relationship of the sarcina to the gastric fermentation. The symptoms of this apparently not uncommon complaint were dyspepsia accompanied by severe vomiting. Virchow (1847) states that "the sarcina has no relation whatever to the fermentation or to any pathological process." Hesse (1847) failed to cultivate this organism from the stomach in milk, flour-paste or sugar solutions, and doubted whether the sarcina was the only, or even the chief, cause of the "disease". He did emphasise however, that the clinical symptoms were reduced or disappeared with the disappearance of the sarcina, and that every form of treatment which reduced the fermentation also reduced the number of sarcina.

A similar case was reported by Hassal (1853). The frequent occurrence of this gastric fermentation may be surmised from the fact that by 1853 Hassal could say:

3.

"In the first drop of this (gastric fluid) placed under the microscope I detected the well known Sarcina ventriculi in great abundance."

The morphology of the organism was extensively studied by Suringar (1865, 1866). Like others, he was unable to cultivate the sarcina in artificial media but based his morphological investigations on material isolated from the stomach. It is noteworthy that he also claimed that the cell-wall contained cellulose. His coloured drawings clearly demonstrated that the sarcina belonged to the Coccaceae, differing from other members of the family in that the division takes place successively in three perpendicular planes.

In the early literature all organisms growing as sarcina tended to be included together, so that the oldest literature describes organisms simply as sarcina, and disregards their origin. (Common origins of various sarcina are the stomach, urine, beer and intestines of man or animal.) Later authors used the generic name Sarcina for many different species, which although morphologically resembling Goodsir's organism, were different in every other respect and should not have been placed in the same genus. Among these authors, Falkenheim (1885) in particular has caused confusion. He collected the

literature of sarcinae in his paper "Ueber Sarcina" and was therefore probably regarded as an authority on this subject. From a stomach containing a sarcina he apparently cultivated several strains of cocci which, according to Smit (1933), only form sarcina-like packets when grown in hay-infusion. The ready growth of these organisms and the absence of any fermentation, placed them distinctly apart from S. ventriculi. Nevertheless Falkenheim identified his organism with Goodsir's. This laid the foundation of a faulty conception which can be followed through the literature to the present day. Oppler (1894), Gruber (1895) and Coyon (1899) all perpetuated Falkenheim's mistaken views and apparently studied organisms which they named S. ventriculi, which had nothing to do with Goodsir's microbe. Stubenrath (1897) and Lehmann and Neumann (1897) actually denied the existence of such a microbe on the grounds that a number of sarcina may be isolated from the stomach, none of which corresponds to Goodsir's description. The error was later further perpetuated by Heissen (1921), by Bergey et al., (Manual of Determinative Bacteriology, 1st to 5th eds., 1923 to 1939) and to the present day by Topley and Wilson (1955).

In spite of this wide-spread confusion a number of workers have succeeded in finding the genuine Sarcina

ventriculi. De Bary (1887) described the organism correctly, as did Ehret (1898) and Latzel (1918). Ehret investigated the fermentation products in the diseased stomach but like De Bary and Latzel was unable to cultivate the organism. The first investigations of real importance were carried out by Beijerinck (1905, 1906). He succeeded in isolating the organism from garden soil and was the first worker to grow it in pure culture. He also demonstrated the anaerobic nature of the organism, and that to keep the organism "alive", it had to be subcultured while it was still actively fermenting. His first attempts to isolate an actively fermenting culture of the organism from gastric juice containing S. ventriculi were unsuccessful. Later (1911) he succeeded by syphoning gastric juice directly from the stomach into a flask containing acid wort at blood heat. Under these conditions fermentation proceeded just as when soil was used and the sarcina proved to be the same in both cases. In work of a later date, (Latzel (1918), Gorhard (1919), Heissen (1921)), attempts to cultivate the sarcina from gastric juice were unsuccessful.

In the late 1920's Sarcina ventriculi was extensively studied by Smit (1930). He suggested the name Zymosarcina to differentiate it from aerobic sarcinae. A carbon

balance for the fermentation of glucose by the organism indicated that the fermentation was essentially an alcoholic one. Kluyver (1931) also published a carbon balance for the fermentation which was in good agreement with that obtained by Smit.

No further work was published on the organism until 1948 when Koch described a method which he had developed for preserving cultures for longer periods than had previously been possible. Oehring (1953) has written a thesis on the sensitivity of S. ventriculi to antibiotics and chemotherapeutic agents.

In 1956 Van Niel, who had long been interested in this organism, but had been unable to find it in Californian soil, took advantage of his sabbatical visit to Europe to investigate the microbe. Milhaud, Aubert and Van Niel (1956) produced evidence that glucose is fermented by Embden-Meyerhof glycolysis.

More recently, Delaporte (1957) and Knöll and Niklowitz (1958) have carried out cytological studies on this organism. A defined medium for the growth of Sarcina ventriculi was recently described by Canale-Parola and Wolfe (1959). Further details of the carbohydrate metabolism of this organism have been reported by Bauchop and Daves (1959a,b) and by Arbuthnott, Bauchop and Daves (1960).

2. DESCRIPTION.

Singer (1914) has claimed that a diagram of Leewenhoek's (1684) represents "sarcina". This claim has been disputed by Dobell (1932) who pointed out that Leeuwenhoek intended the diagram to represent epidermal scales from the human skin, as seen under low magnification. In view of this, full credit must go to Goodsir (1842) as the discoverer of the first sarcina. The Sarcina ventriculi of Goodsir is in fact regarded as the type species. (Bergey's Manual, 1957). Goodsir originally described the genus as follows:

"Sarcina. Plants coriaceous, transparent, consisting of sixteen or sixty-four four-celled square frustules, arranged parallel to one another in a square transparent matrix.

Species 1. *Sarcina ventriculi*, mihi. Frustules 16; colour light brown; transparent matrix very perceptible between the frustules, less so around the edges; size 500 to 1000 inch., Hab. the human stomach."

Perhaps an even more vivid picture can be conjured up by the following description by Goodsir:

"These circumstances gave the whole organism the appearance of a wool-pack, or of a soft bundle bound with cord, crossing it four times at right angles, and at equal distances."

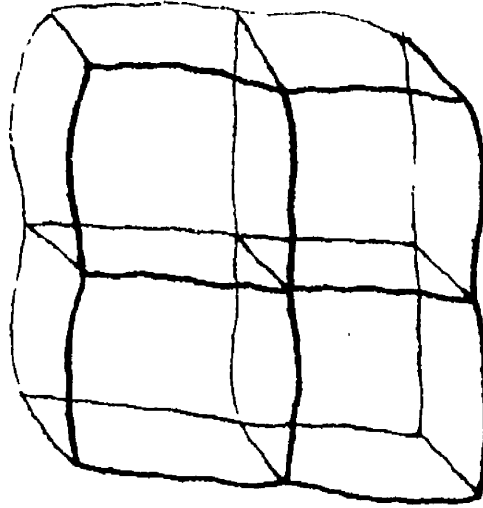
Sixteen "frustules", arranged in a square, would therefore have a side of four "frustules". Since each "frustule" contains four cells and is square, a side of four "frustules" would therefore consist of eight cells. Converting Goodsir's measurements into metric units, eight cells would measure 20.3 - 25.4 μ . The diameter of a single cell would therefore be 2.5 - 3.2 μ . However, since the cells in the packets are to some extent flattened against each other (Goodsir, 1842), a more realistic figure from the point of view of these measurements might be 3 - 4 μ for the diameter of a single cell. Goodsir's original drawing of sarcina is reproduced in Plate 1. The excellent drawings of Hassal (1853) are reproduced in Plate 2. Measurements of the "packets" represented here give a value of approximately 20 μ for an eight-celled side. This is in good agreement with Goodsir's results.

De Bary (1887) described sarcina which he found in gastric juice, the size of the cells being 3 - 4 μ in diameter. He also stated:

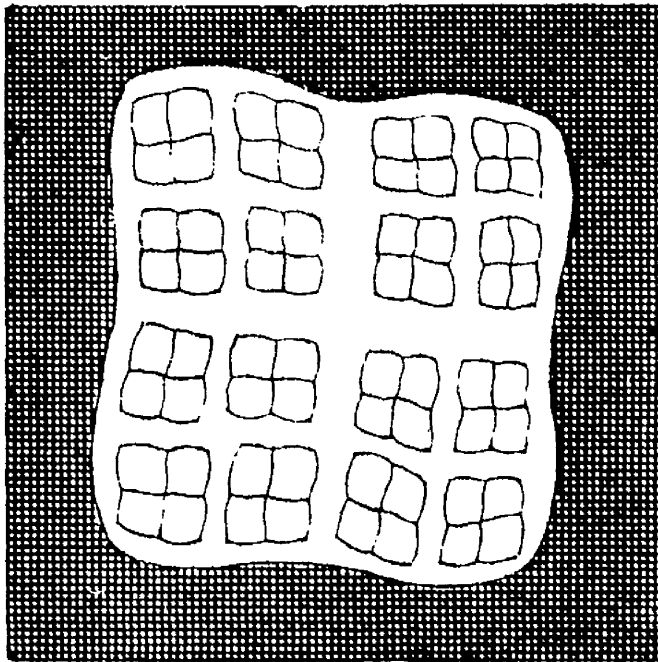
"...in large quantities of material from the human stomach I generally found two distinct forms side by side, a large celled one (described above) and another in which the cells are smaller (2 μ) and less clearly translucent. I can give no information respecting the genetic relations of

PLATE 1

Drawing of *Z. ventriculi*. (Goodsir, 1842).



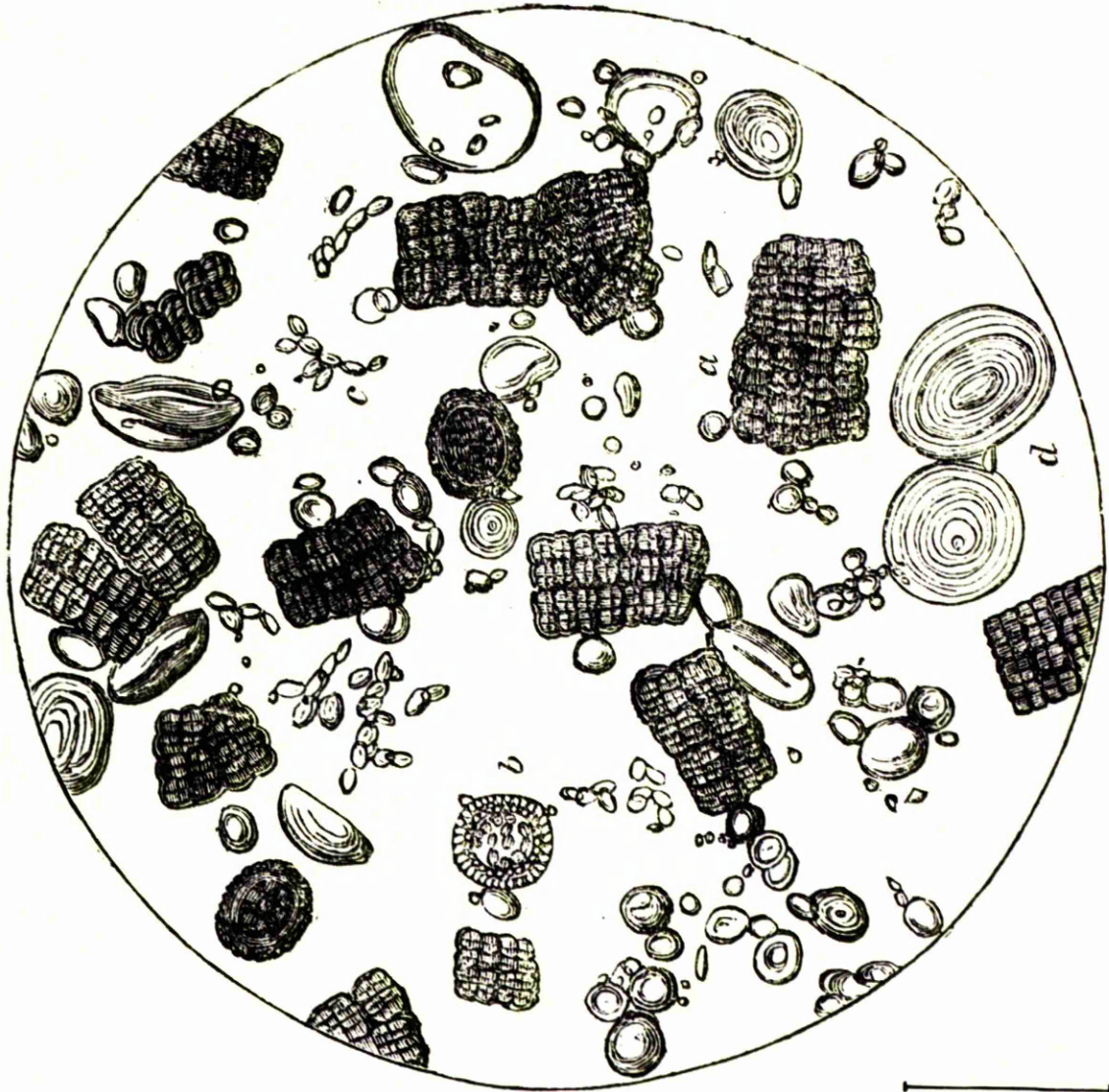
(a) "One of the four-celled frustules."



(b) "*Sarcina ventriculi* in a perfect state."

PLATE 2

Drawing of *Z. ventriculi*. (Hassal, 1853).



40 μ

the two forms."

This is certainly a strange observation particularly since Beijerinck (1905) apparently confirmed it:

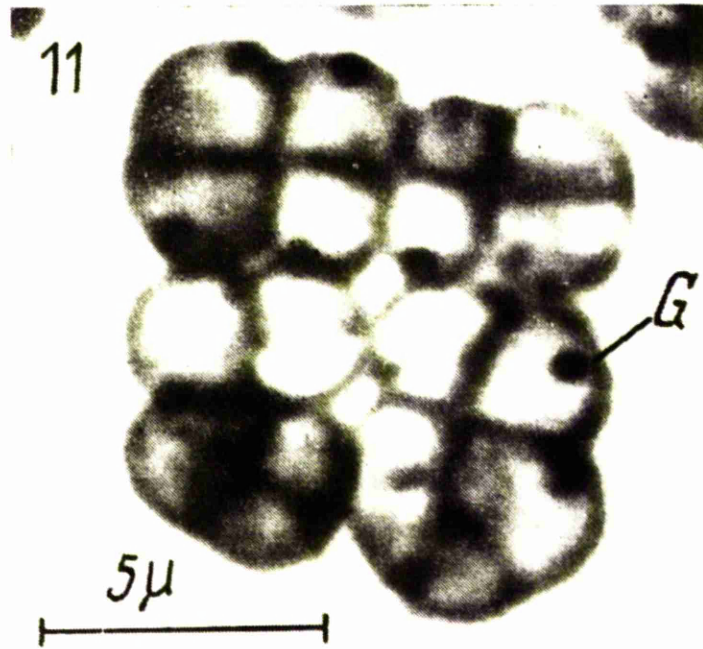
"...pure or almost pure culture of sarcina, of which the elementary cells measure for the greater part about 3 - 5 μ ... The cells are colourless and transparent and the packages present irregular sides. Here and there, but much less generally, a brownish intransparent form is seen, with more regularly cubical packages of which the cells measure 2 - 2.5 μ ."

Delaporte (1957) described the individual cocci as being 3 - 4 μ in diameter. She also made the surprising claim that under special conditions (not specified) giant cells, possibly 10 μ in diameter are sometimes found. Since a particular cytological study of these forms has been promised the publication of these results is awaited with great interest.

The work of Knoll and Miklowitz (1958) would seem to indicate the diameter of the individual cocci to be of the order of 2.5 μ . (Plate 3). It is interesting that Knaysi (1951) quoted the diameter of S. ventriculi as being 2.5 μ . Unfortunately he gave no reference to the source of his information. This considerable discrepancy in the figure quoted for the size of the individual cocci of Z. ventriculi

PLATE 3

Photomicrograph of *Z. ventriculi*. (Knöll and Niklowitz, 1958).



will be discussed later.

For the sake of completeness the description of the organism given in Bergey's Manual (1957) is appended. As an illustration of the error instituted by Falkenheim (1885) and perpetuated by many workers, the description given by Topley and Wilson is also included.

Description in Bergey's Manual of Determinative Bacteriology (1957).

Large Spheres, 3.5 to 4.0 microns in diameter, occurring in packets of 8, 16, 32 or more elements. Non-motile.

Gram-positive.

Growth occurs only in sugar media containing peptones.

Gelatin: No liquefaction.

Deep glucose agar colonies: Multilenticular, surrounded by a cloudy zone. Abundant gas.

Glucose agar slant: Round, whitish colonies, several millimetres in diameter.

Glucose broth: Abundant, flaky growth. Abundant gas.

Acid. No turbidity.

Plain peptone water: No growth.

Sugar peptone water: Abundant growth. Gas.

Milk: Slow growth. Acid and coagulation.

Indole not produced.

Acid and gas from glucose, fructose, sucrose, maltose,

lactose, and galactose. No acid from xylose, arabinose, raffinose, mannitol, dulcitol, salacin, starch, glycerol or inulin.

Cellulose reaction positive.

Neutral red broth changed to fluorescent yellow.

Utilizes peptones, wort and yeast water as sources of nitrogen. Cannot utilize amino acids or inorganic nitrogen.

Coagulated proteins not attacked.

Nitrites not produced from nitrates.

Principal products from metabolism are carbon dioxide and ethanol.

Microaerophilic to anaerobic.

Temperature relations: Optimum, 30°C. Minimum, 10°C.

Maximum, 45°C. Killed in ten minutes at 65°C.

Optimum pH, between 1.5 and 5.0; pH limits for growth, 0.9 to 9.8.

Non-pathogenic.

Source: Originally found by making a microscopic examination of vomit. Found in cases of duodenal ulcer, never in cases of stomach cancer (Smit).

Habitat: Found in the stomach, garden soil, dust, sand and mud.

Description in Popley and Wilson's Principles of
Bacteriology and Immunity (1955).

Morphology.- Spherical coccus, 0.8 - 1.0 μ in diameter, arranged in cubical packets and groups. In liquid media it occurs in pairs, small groups and packets. Non-motile.

Gram-positive, but decolorizes easily. Non-acid fast.

Agar Plate.- 48 hours, 37°C. Circular colonies, 1mm. in diameter, convex, amorphous, opaque, pale-yellow, with a smooth surface and entire edge; rather viscous in consistency, and easily emulsifiable.

Agar Slope.- 48 hours, 37°C. Moderate, confluent, raised, opaque, creamy-yellow growth with a smooth or contoured surface and an undulate edge.

Gelatin Stab.- Moderate filiform growth; no liquefaction.

Broth.- 48 hours, 37°C. Moderate uniform turbidity with a viscous deposit, disintegrating on shaking. No surface growth.

Metabolic and Biochemical Activities.- Aerobic, facultatively anaerobic. Optimum temperature for growth 22 - 30°C.

Pigment formed most readily at 22°C. No haemolysin formed.

Ferments no sugars. I.N. unchanged. M.R.-. V.P.-.

M.B. reduction -.

Pathogenicity.- Non-pathogenic.

3. ISOLATION.

"Already the older observers as Schlossberger (1847), Simon (1849) and Cremer (1858) have tried although in vain, by a kind of accumulation experiments, to cultivate the stomacal sarcina, wherefore they prepared, as nutrient liquid, artificial gastric juice with different additions. Remarkable, and illustrating the biological views of those days, is the fact, that for the infection they did not use the stomacal contents themselves, but beer yeast, supposing that the sarcina might originate from the yeast cells, which somewhat resemble it, and are always found in the stomach together with the sarcina itself." - Beijerinck (1905).

Beijerinck (1911) in fact appears to be the only worker who has succeeded in isolating a pure culture of Z. ventriculi from gastric juice. However, since his first isolation of the organism from garden soil in 1905, a number of other workers have succeeded in isolating the sarcina from this source using his methods. It is therefore pertinent to examine these methods. Beijerinck at this period was engaged in investigating soil organisms which could develop in a "sugar-containing culture fluid" under acid conditions with "imperfect oxidation". During the course of the investigations he was most surprised to

discover a fermenting sarcina developing under certain of these conditions. Essentially, Beijerinck's finding was, that if the growth medium (maltwort) was acidified to such a degree that it became unsuitable for the growth of other bacteria, inoculation with garden soil gave rise to a fermenting sarcina. He found that these conditions were obtained by the addition of 10 - 12 ml. N H_3PO_4 or 6 - 8 ml. N HCl to 100 ml. of wort. When 1 - 2 gm. of soil were placed in a stoppered bottle completely filled with this liquid and cultivated at 35° , a fermentation commenced after 12 - 16 hours and quickly increased, so that by 24 hours it was fermenting vigorously. The stopper was usually blown out and a thick scum covered the liquid. Microscopic examination of the soil in the flask showed that it contained a great number of very large sarcina-packets which resembled the sarcina from the stomach. By successive subculturing, every 24 hours, into fresh medium, it was possible after a few transfers, to obtain a pure culture of Z. ventriculi. Beijerinck emphasised the fact that these subcultures could only be successfully accomplished so long as the culture showed active fermentation. When fermentation ceased, 48 - 60 hours after commencement, further inoculations were unsuccessful. He also pointed out that where small inocula were being used, it was necessary to free the medium from air

by boiling it immediately before use and to employ a bottle completely filled with this medium so that on cooling no air redissolved in the medium.

As has already been stated, Beijerinck (1911) is the only worker who has succeeded in isolating a pure culture of Z. ventriculi from gastric juice. His early attempts to achieve this isolation failed. He was however fortunate in having had the experience of working with the organism isolated from soil. This undoubtedly gave him a considerable advantage over previous workers although it in no way detracts from his foresight and skill. As he said,

"My supposition that the cause of the failure might have been a too strong aeration of the infection material by which the anaerobic stomach sarcina had lost all its vegetative power, induced me to pay special attention to this point at a renewed experiment..."

In 1911 Beijerinck finally achieved the isolation of an actively growing culture by syphoning gastric contents directly from the stomach into a flask containing acid wort at blood heat. Under these conditions fermentation proceeded just as when soil was used, and the sarcina were found to be the same in both cases.

"It was proved that my supposition had been right: when transferring the contents of the stomach with the sarcina

to a fit culture liquid, so quickly that contact with the air might be considered as excluded, it was possible to make the growth and fermentation proceed vigorously." - Beijerinck (1911).

It would appear from this result that the failure of earlier workers to obtain a growing culture of Z. ventriculi from gastric contents may be largely explained in terms of the lack of anaerobic precautions taken in handling the material. As Beijerinck (1905) has stated,

"The "non-cultivability" (of Z. ventriculi) of De Bary (1857) may mean the same as anaerobiosis, for it is well known how difficult it is even at the present time, to cultivate anaerobics if the particulars of their life conditions are not exactly known."

Smit (1930, 1933) isolated the organism by similar methods. In his extensive investigations of Z. ventriculi he found that the pH of the medium which would yield positive results depended on the pH of the soil sample used. For some samples a pH of 1 - 2 was necessary but with other samples, the sarcina was only obtained when unacidified wort at pH 5.5 was used.

4. DISTRIBUTION.

4.1. The Stomach.

As has already been mentioned, many reports of finding Z. ventriculi in the human stomach have been made. Smit (1933) in fact claims that the normal habitat of Z. ventriculi is the diseased stomach of man. All attempts to find it in the normal stomach have so far failed. The organism apparently multiplies under conditions of pyloric ulceration and stenosis where food is held back abnormally long. In such cases of ulceration hyperacidity due to excessive secretion of HCl is common. In such strongly acid stomach contents, rich in carbohydrate, the sarcina finds a favourable environment where it can apparently multiply uninfluenced by the growth of other organisms.

Apart from the apparent widespread occurrence of Z. ventriculi in the stomach of man under these conditions, the organism has also been found in the stomach of other animals. The sarcina has been seen in the stomach of a rabbit by Virchow (1847). Smit (1933) also found the organism in the partly digested stomach contents of a rabbit and a guinea-pig, when the gastric contents were cultured in acidified wort. In these cases the sarcina was not microscopically visible and the animals were in good health.

Smit believed therefore that the sarcina was only an accidental inhabitant of the stomach, probably having been introduced with the food.

According to Smit (1933) Z. ventriculi is also found in the intestines of patients who have the microbe in the stomach. It is not found however, in the normal faeces of man or animal.

No more recent reports of finding Z. ventriculi in gastric contents in cases of pyloric stenosis have been published.

4.2. Soil.

Z. ventriculi was first found in soil by Beijerinck (1905). Smit (1930, 1933) investigated the distribution of the organism in a wide variety of soil samples and found it in all surface soil samples which he tested. The sole exception was a sample which was taken from "some decimetres below the surface". Samples of sand from different places on the Dutch seashore as well as soil samples from the Dutch East Indies all gave positive results, which led Smit to state "that all samples of soil, sand or sludge from the surface of the earth, wherever taken, were positive; those taken below the surface were negative."

More recently Van Niel (1957) found Z. ventriculi in

soil samples in England, France, Holland and Israel. However, all attempts to isolate the organism from Californian soil had been unsuccessful. Nevertheless, Canale-Parola and Wolfe (1959) have apparently succeeded in isolating the organism from American soil.

These results reinforce Smit's belief that Z. ventriculi is widely distributed in soil.

One of the anomalies of the behaviour of Z. ventriculi is the fact that it exists widespread in surface layers of soil; yet when cultivated under laboratory conditions, it is extremely labile. Even soil which had dried and been kept in the laboratory for several months was still capable of producing active sarcina fermentations (Smit, 1953). All direct microscopic examinations of soil samples for Z. ventriculi proved to be unsuccessful. This led Smit to the belief that,

"Sarcinae exist in natural materials like soil and sand and retain their vitality, in a form until now unseen and unknown, being different from the known sarcina-packets and capable of surviving for a prolonged period."

This anomalous behaviour will be discussed later.

5. GROWTH AND NUTRITION.

The growth and nutrition of Z. ventriculi were first studied by Smit (1930, 1933). He found that the organism would not grow in sugar-free medium with or without oxygen. The hay-infusion recommended by Falkenheim (1885) is therefore an unsatisfactory medium. The fermentable sugars are glucose, fructose, galactose, maltose, sucrose and lactose. Raffinose, xylose, arabinose, starch, dextrans, glycerol, dulcitol, mannitol as well as organic acids and their salts are not utilized by Z. ventriculi.

Smit (1933) claimed that the source of nitrogen had to be in the form of peptonised protein. Until recently, the sarcina has been grown in complex media usually containing peptone and yeast extract together with a fermentable sugar. However, Canale-Parola and Wolfe (1959) have now described a defined medium for the growth of Z. ventriculi. The organism was found to exhibit an absolute requirement for biotin and nicotinic acid. In the presence of these two growth factors, a fermentable sugar and minerals, the microbe was found to require the following amino acids for growth: serine, histidine, isoleucine, leucine, tyrosine, methionine, tryptophan, phenylalanine, arginine, valine and glutamic acid.

Smit (1930) has investigated the effect of using different acids for the acidification of the medium (wort) to be used for the isolation of sarcina. Using a soil sample rich in sarcina, he found that the pH values shown in Table 1 were most suitable for the isolation. The acids used would seem to function in some manner other than by simply lowering the pH of the medium. Oxalic acid was found by Smit to be unsuccessful at any pH value. However it must be noted that these values are purely relative and will obviously depend on the nature of the soil sample used. Smit considered that it was therefore of interest to determine the minimum pH at which the fermentation of a pure culture could take place (Table 2). The effect of fermentation on the pH of the medium can be seen in Table 3. Where the initial pH was below 4.6, the pH of the medium was apparently unchanged by the fermentation. When higher pH values were used, Smit found that the pH of the medium was reduced to approximately 5.0.

The growth of the organism on solid medium was first accomplished by Beijerinck (1906). Plating Z. ventriculi on glucose-agar plates failed to produce any growth. However, by using actively fermenting cultures and inoculating into deep agar layers of malt-agar, colonies of Z. ventriculi were obtained. When these colonies were inoculated back

TABLE 1.

Optimum pH of wort, acidified with different acids,
for the isolation of *Z. ventriculi* from soil. (Smit, 1933).

Acid	pH
HCl	0.9
H ₂ SO ₄	1.5
HNO ₃	1.5
H ₃ PO ₄	2.0
Lactic	2.9
Acetic	4.7

TABLE 2.

Minimum pH of wort, acidified with different acids,
at which fermentation by *Z. ventriculi* could proceed.

(Smit, 1933).

Acid	pH
HCl	0.8
H ₂ SO ₄	1.1
HNO ₃	1.5
H ₃ PO ₄	1.35
Lactic	2.8
Pyruvic	3.0
Oxalic	3.4
Acetic	4.1

TABLE 3.

Effect of fermentation of glucose by *Z. ventriculi*
 on the pH of the medium. (Smit, 1933).

Initial pH	Final pH
1.37	1.4
1.61	1.65
2.40	2.41
2.84	2.90
3.65	3.74
4.62	4.21
5.48	4.06
7.32	5.08
8.30	5.06
8.77	4.84
9.16	4.77
9.60	5.15
9.80	4.48

into maltwort, vigorously fermenting cultures were once more obtained. Smit (1933) further pursued these studies and succeeded in simplifying the method. He found that by adding a few drops of impure culture to a tube of melted wort-agar at 50° and pouring the contents into a Petri dish, colonies of sarcina developed in this thin layer of nutrient agar. Smit also confirmed Beijerinck's finding that streaking the sarcina on the surface of an agar-plate failed to produce colonies. Smit found however, that when a large amount of active inoculum was plated on agar, a few sarcina colonies could be obtained. He believed that this effect was due to the cells protecting one another from the air, due to the large number of cells present. Smit found in addition, that when agar-plated cells were incubated in vacuo, growth was much better and surface colonies a few millimetres in diameter could be obtained.

One observation made by all workers who have used Z. ventriculi, is that cells grown in liquid culture lose their viability if they are not subcultured before fermentation ceases. Smit (1930) apparently succeeded in certain cases in prolonging viability for 4 - 5 weeks. This was accomplished by growing the organism in a tube of nutrient agar, and when the sarcina was actively fermenting, the tube was gassed with CO₂ and then sealed.

Koch (1948) was able to preserve viability up to at least three months, simply by incorporating 0.2% agar into his liquid medium. He used 100 ml. of hot trypsin-peptone solution mixed with 10 ml. of 2.5% trypsin-peptone-agar and 5 g. dextrose. 15 ml. of this solution were dispensed into standard tubes and sterilized. In place of a cotton-wool plug, a Kapsenberg (1940) culture stopper was used. Following sterilization, the tubes were cooled and inoculated with 0.2 to 0.5 ml. of an actively fermenting culture. The tubes were then incubated at 37^o, and a "net-like framework" of cells developed in the nutrient agar. Apparently the tubes were stored at 37^o, and up to three months later, inoculation into either trypsin-peptone solution or agar broth gave rise to actively fermenting cultures.

In these experiments, the optimum amount of agar was found to be 0.2%; both higher and lower concentrations of agar were found by Koch to be less effective in preserving viability.

6. METABOLISM AND FERMENTATION PRODUCTS.

Wilson, who carried out chemical analyses of gastric fermentation fluid for Goodsir (1842) arrived at the conclusion that the fluid contained hydrochloric, acetic and lactic acids. Hassel (1847) frequently mentioned "Essigbittersaure". Letheby (Hassel, 1853) claimed to have found hydrochloric and butyric acids in the gastric fluid. At a later date Thret (1898), investigating the fermentation products in the diseased stomach found ethanol, aldehyde, acetic and formic acid as well as CO_2 . "A small percentage of hydrogen was possibly formed." Beijerinck (1905) analysed the gas evolved and found it to consist of 75% CO_2 and 25% H_2 . He claimed to find lactic acid but no quantitative measurements appear to have been made. The acid was apparently identified by its smell.

The first quantitative measurements of fermentation products were performed by Smit (1930), (Table 4), who found that ethanol and CO_2 were the major products, together with smaller amounts of acetic acid and H_2 . On the basis of these results Smit was able to explain the changes observed in the pH of the culture medium during fermentation. The carbonic acid formed does not alter the pH when initially it is less than 5.0. However, "It easily changes the initial neutrality or

TABLE 4.

Fermentation of Glucose by *Z. ventriculi* (Smit, 1930).

Product	Percentage of Glucose Fermented.	Moles/Mole of Glucose.
CO ₂	41.7	1.71
Ethanol	40.3	1.58
H ₂	0.58	0.58
Acetic Acid	9.0	0.27
Acetyl Methyl Carbinol	trace	-
Formic Acid	1.08	0.04
Butyric Acid	-	-
Succinic Acid	-	-
Lactic Acid	3.05	0.06

alkalinity of the solution into the acidity of a saturated CO₂ solution (pH 5)." A carbon balance for the fermentation was also reported by Kluyver (1931), (Table 5). His results were in good agreement with those obtained by Smit.

No further studies were performed on this interesting fermentation until 1956, when Milhaud et al., using a washed cell suspension of Z. ventriculi at pH 5.0, obtained the carbon balance given in Table 6. As can be seen, there are considerable differences from the carbon balances obtained by earlier workers. All of these workers found approximately two moles of CO₂ per mole of glucose fermented. However, whereas Smit (Table 4) and Kluyver (Table 5) found ethanol to be the other major product, Milhaud et al. (Table 6) found equal amounts of ethanol and acetate together with almost two moles of H₂ per mole of glucose. Kluyver believed that his results substantiated what was essentially a yeast-type fermentation. On the other hand, the results of Milhaud et al. would seem to indicate a coliform-type fermentation.

As a result of fermentation experiments with (1-¹⁴C), (2-¹⁴C) and (6-¹⁴C) glucose, Milhaud et al. have presented evidence that Z. ventriculi ferments glucose by Embden-Meyerhof glycolysis (Table 7).

TABLE 5.

Fermentation of Glucose by *Z. ventriculi* (Kluyver, 1931).

Product	Percentage of Glucose Fermented.	Moles/Mole of Glucose.
CO ₂	47.7	1.94
Ethanol	43.7	1.71
H ₂	0.46	0.42
Acetic Acid	6.6	0.20
Acetyl Methyl Carbinol	1.7	0.04
Formic Acid	0.8	0.03

TABLE 6.

Fermentation of glucose by washed-cell suspensions of
Z. ventriculi at pH 5.0. (Milhaud et al., 1956).

	μ moles	moles/mole glucose
Glucose utilized	9.4	-
H ₂	15.9	1.7
CO ₂	17.8	1.9
Ethanol	7.5	0.8
Acetic Acid	8.9	0.9

TABLE 7.

Distribution of radioactivity¹ in each of the carbon atoms of the products of glucose fermentation by *Z. ventriculi* (Milhaud et al., 1956).

		(1- ¹⁴ C)Glucose (151,000)	(2- ¹⁴ C)Glucose (166,000)	(6- ¹⁴ C)Glucose (159,000)
Acetic Acid	CH ₃	67,900	0	68,000
	COOH	112	75,000	281
Ethanol	CH ₃	62,100	302	59,600
	CH ₂ OH	201	68,100	73
Carbon Dioxide	CO ₂	278	117	26

¹ counts/minute

7. CYTOLOGY.

The cytology of Z. ventriculi has recently been studied by Delaporte (1957) and by Knoll and Niklowitz (1958).

It can surely only be due to the fact that this organism is so little known that its cytology has not been previously studied. A coccus of 3 - 4 μ in diameter would seem to offer certain obvious advantages to the cytologist. If we represent the coccus by a sphere, and take the lower of these limits (3 μ) as the diameter, the volume of a single cell of Z. ventriculi works out at 14.1 μ^3 . If this is compared with the volume of some other bacteria some interesting facts would seem to emerge. A staphylococcus of 1.0 μ in diameter has a volume of 0.52 μ^3 . A coliform organism, represented by a cylinder with hemispherical ends, 1.0 μ in diameter and 3.0 μ in overall length, has a volume of 1.09 μ^3 . Both of these figures are fairly typical for cocci and bacilli respectively. The vast difference in size when compared with the figure for Z. ventriculi is obvious.

One property which would seem to be concomitant with an increased cell size is increased structural complexity within the cell. It would seem, therefore, that in the case of Z. ventriculi there is the possibility of a higher degree of structural complexity than is normally present in the

bacterial cell. The results of Delaporte (1957) and Knoll and Niklowitz (1958) indicate that to some extent at least, this may be true.

Knoll and Niklowitz (1958) studying ultra-thin sections with an electron microscope were able to determine details of the fine structure of the Z. ventriculi cell. They found that the cell packets as well as single cells were encased in a mucous layer (S), (Plate 4). Since each single cell is completely surrounded by a layer of mucus, it was suggested that this may act as a "cement" which holds the cells together in the cell-packet. Electron microscope studies of ultra-thin sections of other species of sarcina would obviously be of interest in this respect.

Knoll and Niklowitz have claimed that the cell wall (Zw), (Plate 4), consists of two membranes and is 30 μ thick. The inner membrane was believed to be the so-called plasma-membrane (Murray, 1957; Tomlin and May, 1955).

The cytoplasm (Cy) appears to be fairly uniform with no sign of structural complexity such as endoplasmic reticulum or cytoplasmic membranes as are found in the cytoplasm in animal cells. This is in keeping with findings with other bacteria (Bradfield, 1956). One important difference from other bacteria however, is the claim that Z. ventriculi may possess mitochondria (M), (Plate 5). So far, ultra-thin

PLATE 4.

Electron Micrograph of *Z. ventriculi*.

(Knöll and Niklowitz, 1958).

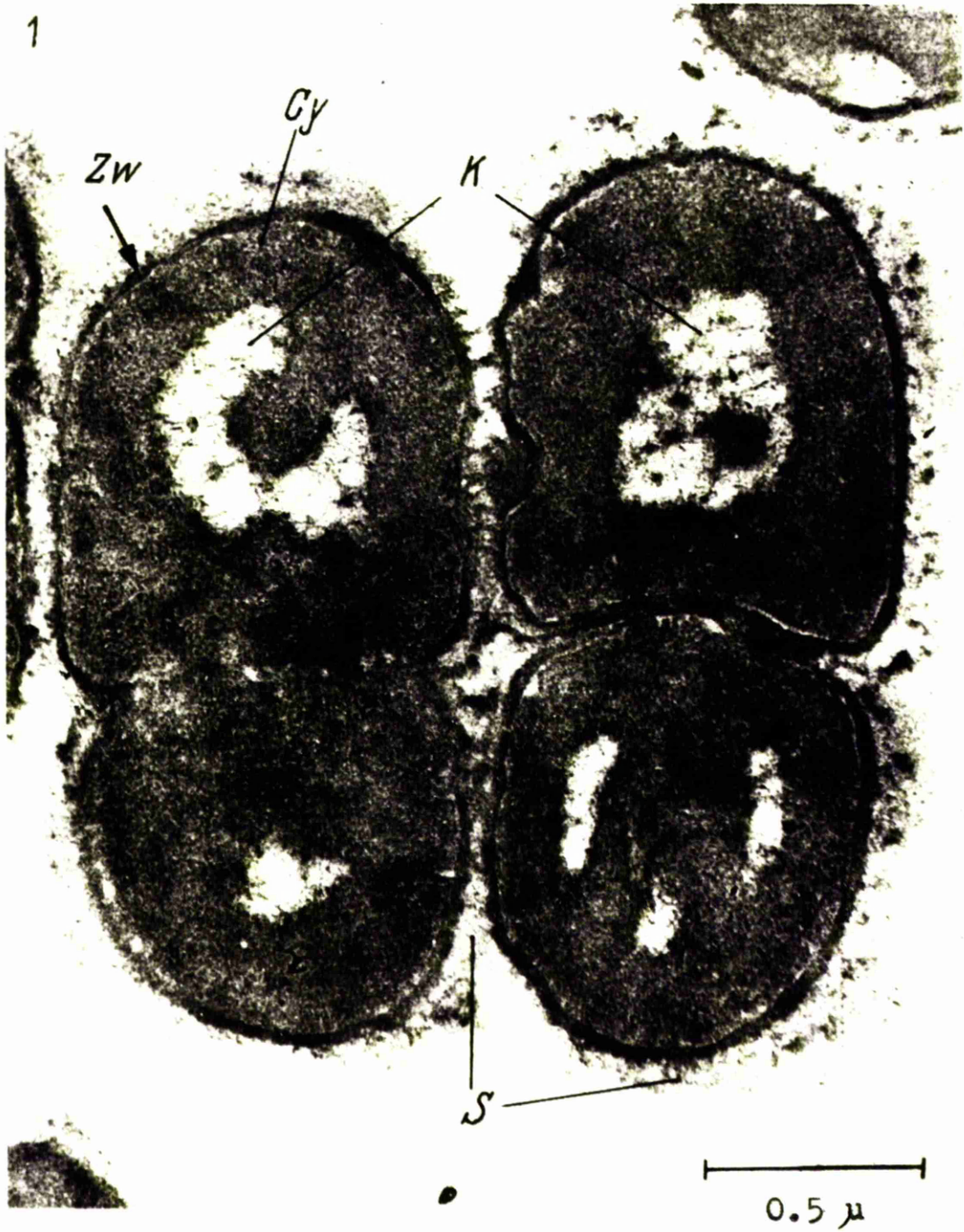


PLATE 5.

Electron Micrograph of a single cell of *Z. ventriculi*
(Knöll and Niklowitz, 1958).



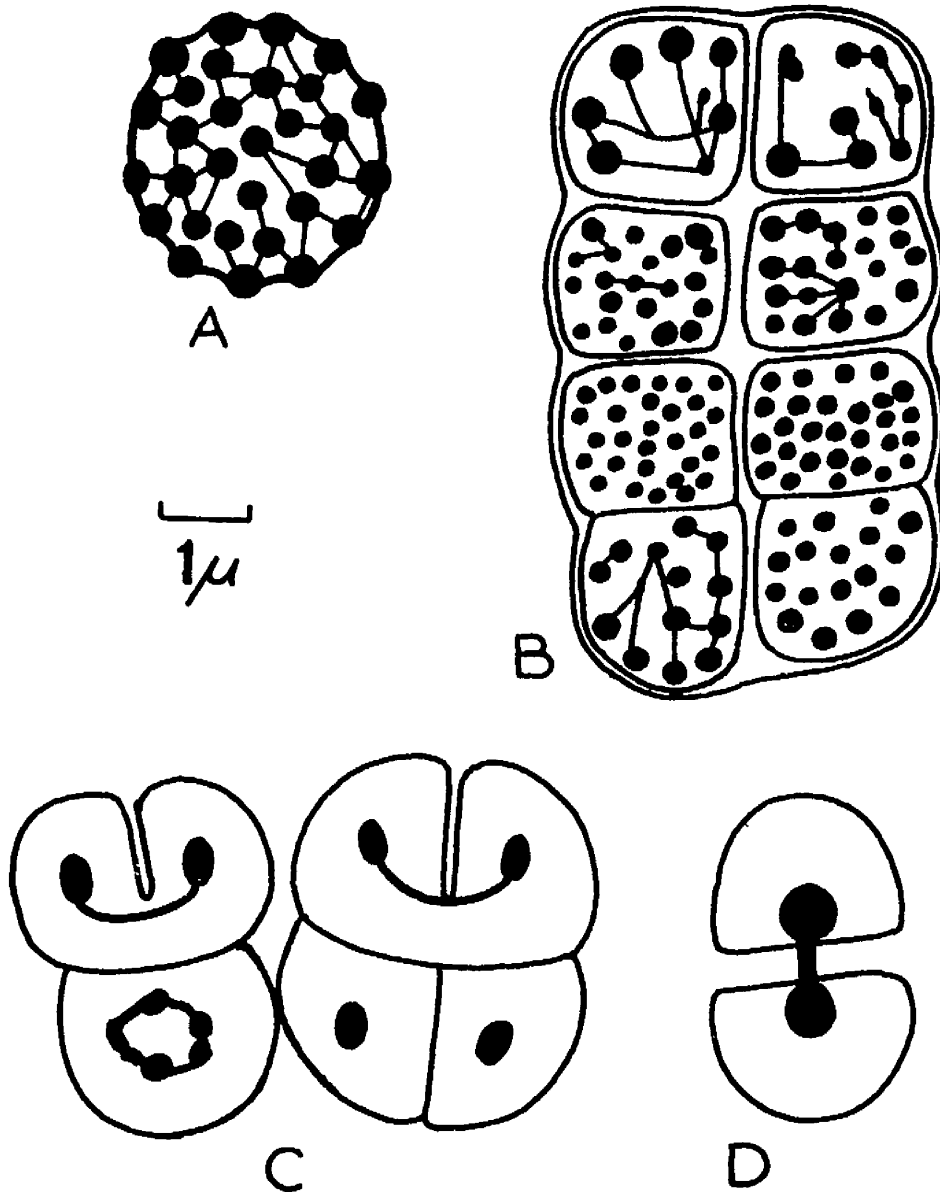
sections of bacteria of normal size do not show any structures resembling the mitochondria of other cells (Bradfield, 1956). Knoll and Miklowitz gave the size of this structure as being of the order of 0.25μ in diameter. This is comparable with the lower limits of sizes given by Palade (1956) for mitochondria ($0.2 - 0.5 \mu \times 0.3 - 5.0 \mu$). Since the size of mitochondria is comparable with the size of most bacteria ($0.2 - 2 \mu \times 0.3 - 5 \mu$; Knaysi, 1951), Mitchell (1959) has implied that it would therefore be unlikely to find mitochondria in bacteria. It will be interesting therefore, to see if the findings of Knoll and Miklowitz can be repeated independently.

Previous workers have found the nuclear material in cocci to be in the form of a dense central mass. (Delaporte, 1957). Although the formation of a dumb-bell form before cytoplasmic division occurs has also been described, no internal structure of the nuclear material has been discerned. Delaporte (1957) using histochemical techniques, described Z. ventriculi as possessing a dispersed nucleus with a very large number of chromatin threads - "a network-like nucleus with numerous chromocentres" (A, B, Fig. 1). Under certain conditions, e.g. the action of air, the network-like nucleus was found to condense into one small dense central homogeneous mass.

During cell division the nuclear material moves away from

FIG. 1.

Nuclear Material in *Z. ventriculi* (Delaporte, 1957).



the area where the transverse septum is about to form (C, Fig. 1). Delaporte observed no particular organisation of the nuclear material at this point. A bridge of nuclear material was found to join the two halves of the nucleus (D, Fig. 1) and progressively to shrink until the complete closure of the transverse septum occurred. The transverse septum was found to develop from the periphery to the centre of the cell, and the division of the cytoplasm appeared to be complete before the division of the nucleus was finished. Delaporte could detect no sign of chromosomes or of mitosis in Z. ventriculi. Knöll and Niklowitz (1958) also failed to find any evidence for chromosomes or for mitosis with their electron microscope studies of ultra-thin sections. However, they confirmed Delaporte's findings that the nuclear material (K) could alternate between a central compact arrangement (Plate 4) and an arrangement where it was diffused and divided over the whole cell (Plate 5). In agreement with the finding of Birch-Anderson et al. (1953) with Escherichia coli, Knöll and Niklowitz (1958) found with Z. ventriculi, that although the nuclear region appeared to be sharply defined, no well-defined membrane separating the nuclear region from the cytoplasm could be seen.

The results of cytological studies to date appear to indicate that the Z. ventriculi cell, in spite of its size,

is structurally only slightly more highly differentiated than other bacterial cells. However, the claim for the presence of mitochondria and the apparent structural complexity of the nuclear material would appear to be worthy of further study.

8. BIOCHEMICAL MECHANISMS.

Smit (1930), Kluyver (1931) and Milhaud et al. (1956) have shown that the products of glucose fermentation by Z. ventriculi consist predominantly of ethanol, acetate, CO₂ and H₂. Milhaud et al. (1956) have also obtained evidence that Z. ventriculi ferments glucose exclusively by Embden-Meyerhof glycolysis. In the present study we have therefore been mainly interested in the mechanisms of product formation below the "pyruvate level". At present two mechanisms are known which result in the formation of ethanol and CO₂. Two mechanisms which result in the formation of acetate, CO₂ and H₂ from pyruvate have also been demonstrated in micro-organisms.

8.1. Formation of Acetate, CO₂ and H₂.

The fission of pyruvate to acetate, CO₂ and H₂ was first demonstrated using extracts of Clostridium butylicum, by Keesell and Johnson (1942). A somewhat different fission of pyruvate was demonstrated in extracts of E. coli by Kalnitsky and Werkman (1943). Since both of these mechanisms have been described as "phosphoroclastic" reactions (Lipmann, 1946), we shall refer to them here as the "clostridial-type" and the "coliform-type" of phosphoroclastic fission respectively.

8.1.1. Coliform-type.

The "phosphoroclastic" fission of pyruvate in the Colon-Aerogenes group of organisms is believed to involve the intermediate formation of formate.



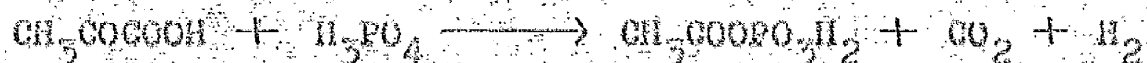
The formate produced is normally further metabolized to H_2 and CO_2 by the formic hydrogenlyase enzyme system.



The "elastic" reaction of pyruvate was first demonstrated in extracts of E. coli by Kalnitsky and Werkman (1943). The formation of acetyl phosphate from pyruvate was demonstrated by Utter and Werkman (1944). One of the main characteristics of this system is its ability to catalyse a rapid equilibration between labelled formate and the carboxyl group of added pyruvate (Utter et al., 1945). The formation of pyruvate from acetyl phosphate was demonstrated by Lipmann and Tuttle (1945). The co-factor requirements for the forward reaction and the formate exchange reaction were found to be inorganic phosphate, Coenzyme A (CoA) and diphosphothiamine (DPT) (Chantrenne and Lipmann, 1950; Kalnitsky and Werkman, 1943; Strecker et al., 1950; Strecker, 1951).

8.1.2. Clostridial-type.

The following reaction occurs in the saccharolytic clostridia:



Although CO_2 and H_2 superficially appear to be the equivalent of formate it would seem that the clostridial-type and the coliform-type of degradations involve different mechanisms. Formate is not usually metabolized by intact or cell-free preparations of the clostridia, and it has been shown that this compound is not an intermediate in pyruvate breakdown by Cl. butylicum (Koeppell and Johnson, 1942; Wilson et al., 1948). Recently Novelli (1955) produced evidence for the formate exchange reaction with extracts of Clostridium butyricum. More recently Hamilton and Wolfe (1959) have confirmed the inability of earlier workers (Wilson et al., 1948; Wolfe and O'Kane, 1955) to obtain extracts of Cl. butyricum which would effect a H^{14}COOH -pyruvate exchange.

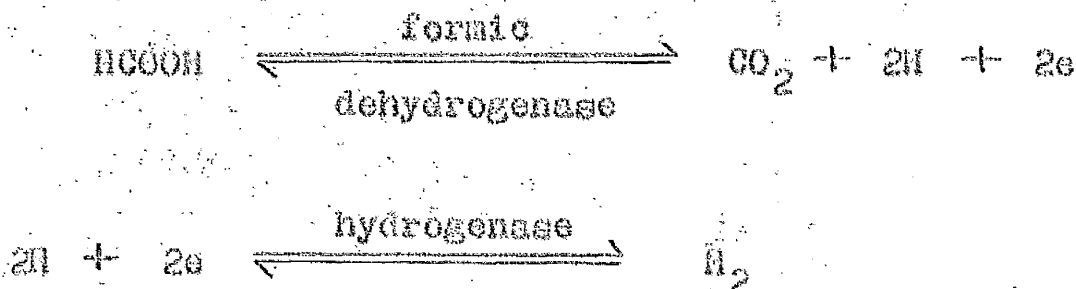
Reversibility of the clostridial "phosphorelastic" reaction was indicated by the incorporation of isotopic CO_2 into the carboxyl group of pyruvate (Wilson et al., 1948) and by the inhibiting effect of high hydrogen pressures on the rate of the forward reaction (Kubowitz, 1954; Mortenson and Wilson, 1951). On the other hand, attempts to demonstrate

an unequivocal incorporation of acetate or acetyl phosphate into pyruvate, in early work, were unsuccessful (Wilson *et al.*, 1948). Recently Mortlock and Wolfe (1959) claim to have obtained pyruvate formation from acetyl phosphate and CO_2 with extracts of *Cl. butyricum*.

The co-factor requirements of the clostridial clastic reaction were shown to be DPT, CoA and ferrous ions (Wolfe and O'Kane, 1953). The exchange reaction was found to require DPT, CoA and phosphate (or arsenate), (Wolfe and O'Kane, 1955). Later, it was found that the phosphate requirement for pyruvate cleavage or for CO_2 -pyruvate exchange could be replaced by CoA (Mortlock *et al.*, 1959).

8.1.3. Formic Hydrogenlyase.

It has already been mentioned that the formate produced in the coliform-type fission of pyruvate is normally further metabolized by the enzyme formic hydrogenlyase. Stephenson and Stickland (1951) suggested that "hydrogenlyase" was actually a two-enzyme system in which formic dehydrogenase and hydrogenase act in conjunction:



This postulated mechanism was soon discarded (Stephenson and Stickland, 1932; Stephenson, 1937). The theory was later revived and the question as to whether hydrogenlyase exists or whether its action is in reality a result of the simultaneous action of formic dehydrogenase and hydrogenase, has not yet been decided. In 1954 Wolf et al. produced a number of arguments attacking the "duplex" theory together with evidence for the fact that formic hydrogenlyase exists as a separate entity. Gest and Peck (1955) and Peck and Gest (1957a) have put forward further indirect evidence for the "duplex" theory of hydrogenlyase action.

8.1.4. Clostridial Hydrogenase.

Although Keepsell and Johnson (1942) found that extracts of Cl. butylicum did not display hydrogenase activity with methylene blue as acceptor, it was presumed that hydrogenase was an essential component of the enzyme complex (Gest, 1954). Later Wolfe and O'Kane (1953) also failed to detect hydrogenase in extracts of Cl. butyricum. The presence of hydrogenase has now been demonstrated by the use of the one-electron dyes, benzyl and methyl viologen (Peck and Gest, 1957b).

8.2. Ethanol Formation.

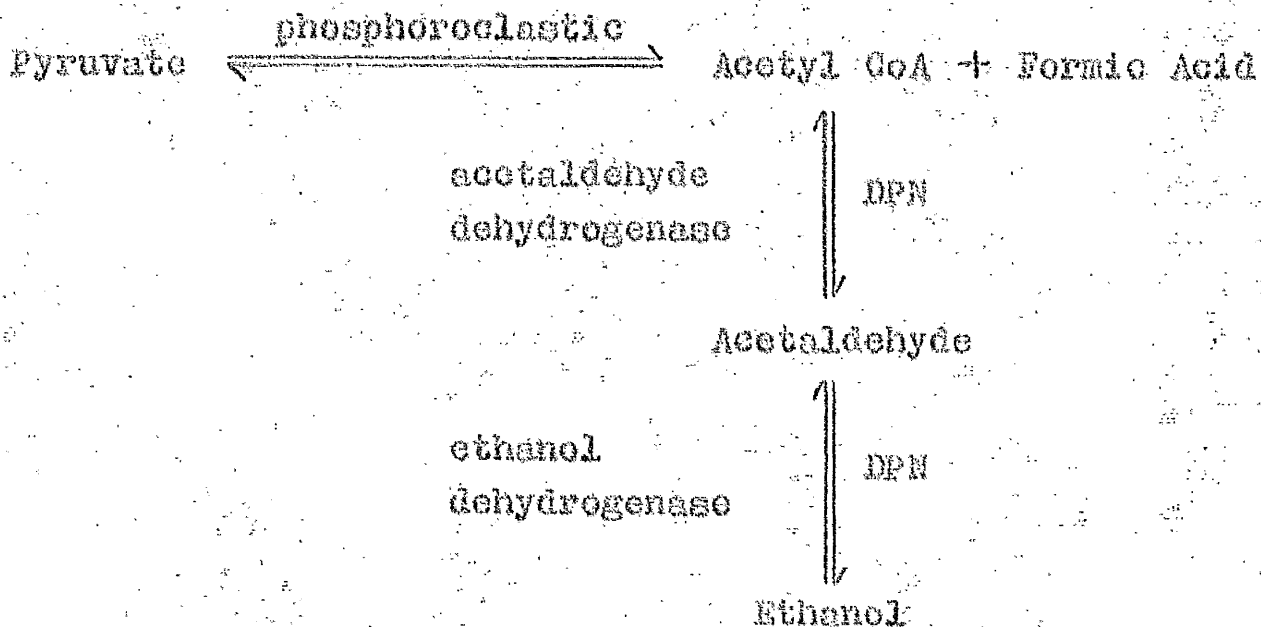
Relatively few micro-organisms possess the property of producing large amounts of ethanol from glucose. Despite this fact, the formation of ethanol and CO_2 from glucose by

49.

Saccharomyces cerevisiae, Leuconostoc mesenteroides and Pseudomonas lindneri occurs by three different pathways (Gunsalus, Horecker and Wood, 1955). However, the terminal mechanisms of ethanol formation do not appear to be as diverse as these facts might suggest.

The terminal mechanism of ethanol formation in yeast involves the direct cleavage of pyruvate to acetaldehyde and CO_2 followed by reduction of the acetaldehyde formed to ethanol. These two reactions are catalysed by the enzymes carboxylase and alcohol dehydrogenase respectively. Gibbs and DeMoss (1951) have shown that Ps. lindneri possesses a yeast type carboxylase. It seems possible therefore, that ethanol may be formed by the classical yeast-type mechanism in this organism.

In E. coli glucose is fermented by Embden-Meyerhof glycolysis and ethanol is a minor product of the fermentation. Ethanol appears to be formed by a different mechanism in this organism, and evidence for the following mechanism has been published by Dawes and Foster (1956).



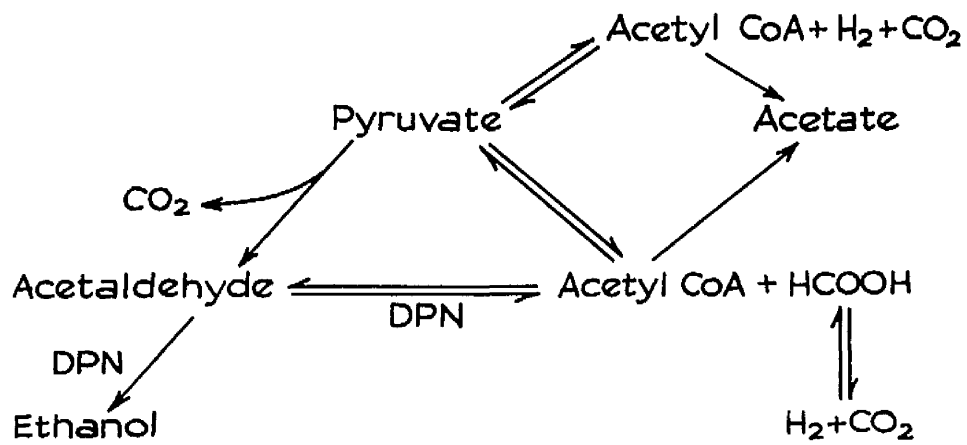
Gungalus et al. (1955) have stated that cells of L. mesenteroides reduce acetate to ethanol. It is possible therefore that this organism may possess a mechanism of ethanol formation similar to the one found in E. coli.

6.3. Summary.

The various anaerobic mechanisms in micro-organisms for the formation of ethanol, acetate, CO_2 and H_2 from pyruvate are shown in Fig. 2.

FIG. 2.

Anaerobic mechanisms for the formation of ethanol, acetate, $\text{CO}_2 + \text{H}_2$ from pyruvate in micro-organisms.



II. METHODS AND MATERIALS.

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1. BACTERIOLOGY.

1.1. Isolation.

The standard technique for the isolation of Z. ventriculi was as follows. Approximately 2 g. of soil were placed in a 250 ml. conical flask which was then filled with "acid" medium. The flask was plugged with cotton wool and incubated overnight at 37°. By 16 to 20 hours a vigorous fermentation was taking place and microscopic examination of the contents showed large numbers of sarcina to be present. The contents of the flask were stirred up and 10 ml. inoculated into a second flask in which the medium had been freshly boiled and quickly cooled. This procedure was repeated three or four times until a pure culture of Z. ventriculi was obtained. Inoculation from this culture into sterile medium (pH 7.0) gave a pure culture which could then be maintained.

In our experience, the only organism which is likely to interfere with the isolation of a pure culture of Z. ventriculi is yeast. Normally however, using the above technique, no trouble of this type was experienced. During the summer of 1959, which was a particularly dry and warm one, difficulties due to contamination by yeast were experienced. Due to the nature of Z. ventriculi it was found that none of the standard methods for isolating micro-organisms could effect a separation

of these two organisms. Obviously the commercial preparation Anti-Dione (Upjohn of England Ltd., Fleming Way, Crawley, Sussex), which selectively inhibits the growth of yeasts, would have been useful. Unfortunately, this preparation was not immediately available. However in this case it was found that the yeast could not utilize galactose. Therefore by substituting galactose for glucose in the "acid" medium it was possible to isolate a pure culture of Z. ventriculi.

1.2. Media.

Since we were essentially interested in biochemical studies with this organism, no attempt was made to devise a defined medium. The report of such a medium has recently appeared (Canale-Parola and Wolfe, 1959). The medium used when growing cells for all biochemical studies contained 2% (w/v) glucose, 1% (w/v) Dacto-peptone (Difco) and 1% (w/v) Yeast Extract (Difco). The pH of this medium, as made up, was 7.0; no adjustment of pH was made.

For the isolation of Z. ventriculi from soil an acid medium was used. The acid medium contained, in addition to the above components, 0.3% (v/v) conc. HCl. This addition lowered the pH of the medium to 3.5.

1.3. Growth Conditions and Harvesting of Cells.

400 ml. of medium were dispensed into a 500 ml. conical

flask which was plugged and sterilized for 15 minutes at 15 lbs./ sq. inch by standard techniques. If inoculation was to be carried out immediately, the flask was removed from the autoclave as soon as the pressure returned to zero, cooled quickly, and inoculated with a 5% (v/v) inoculum of actively fermenting cells. The flask was incubated overnight at 37°. If the flask of sterile medium had been stored some time before use, it was brought to the boil and cooled immediately before inoculation. The removal of air from the medium by this method was found to be a necessary precaution. Even when large inocula (10%, v/v) were used, growth often failed to occur if this procedure was not carried out.

In some experiments larger amounts of cells were required. These were obtained by scaling up the above procedures to 5 or 10 litres as required.

Harvesting of cells was considerably simplified by the fact that Z. ventriculi grows on the bottom of the culture vessel. It was possible therefore to pour off most of the growth medium and to harvest the cells by centrifugation from the small volume remaining. When large cultures (5 or 10 litres) were grown, the "supernatant" medium in the growth flask was removed by syphoning. The cells could then be obtained in a small volume and harvested by centrifugation.

1.4. Growth Cycle Experiment.

In one experiment the apparatus (B) of Dawes and Holms (1956) was used. This apparatus facilitated sampling and gassing with N_2 . 800 ml. of medium were sterilized in the flask. At zero time an inoculum of 10 ml. from an actively fermenting culture was added. Samples, 3 ml. and 80 ml., were withdrawn at various times for dry weight determinations and for the estimation of enzymic activities, respectively. Immediately before withdrawing samples, nitrogen was bubbled through the medium to ensure a uniformly distributed suspension. After 8.5 hours nitrogen was bubbled through the flask continuously until the experiment ended.

2. ANALYTICAL PROCEDURES.

2.1. General.

Bacterial densities were determined turbidimetrically, after suitable dilution, in a Hilger Spekker absorptiometer using Chance glass filters H508 and OB2. The instrument was calibrated against bacterial dry weight. Because of the rapid rate of sedimentation of this organism it was necessary to shake the suspension immediately prior to taking the reading.

Protein nitrogen was determined by the method of Stickland (1951).

2.2. Chemical Estimations.

Glucose was estimated by Nelson's method (1944).

Pyruvic acid was estimated by the method of Friedemann and Haugen (1943).

Lactic acid was estimated by the method of Hullin and Noble (1953).

Acetaldehyde was estimated by the Dawes and Foster (1956) modification of the Hullin and Noble (1953) method for lactic acid.

Acetyl phosphate was estimated by the method of Lipmann and Tuttle (1945) described by Stadtman (1957).

Acetyl methylcarbinol was estimated by the method of

Wosterfeld (1945).

Steam volatile acids were distilled in a Markham (1942) still, after acidification to approximately pH 2 with H_2SO_4 , and estimated by titration with 0.01 N NaOH using a Conway micro-burette. CO_2 -free air was bubbled through the liquid during titrations with phenol red as indicator, and the end-point was determined by comparison with a pH 7.6 standard, (Barker, 1957). When pyruvic acid was present in the reaction mixtures to be assayed the pH was adjusted to 3.5 (Lewis and Elsdon, 1955) to prevent pyruvic acid being distilled. Where samples had been previously acidified to precipitate protein, the pH of the reaction mixture was adjusted to approximately 5.0 using solid sodium bicarbonate.

Spectrophotometric measurements were made in a Hilger Uvispek instrument, fitted with a specially designed thermostatic cell holder using cuvettes of 1 cm. light path.

2.3. Special Materials, Chemicals and Enzymes Used.

Pyruvic acid was distilled in vacuo before use and neutralized with the calculated amount of sodium hydroxide.

Adenosine triphosphate (ATP) was supplied by the Sigma Chemical Co.

Triphosphopyridine nucleotide (TPN) was TPN 80 supplied by the Sigma Chemical Co.

59.

Diphosphopyridene nucleotide (DPN) was the "DPN reinst" of
Boehringer and Soehno.

Methyl viologen and benzyl viologen were obtained from
Jacobsen Van Den Berg and Co., 73, Cheapside, London, E.C.2.
(U-¹⁴C) Glucose, H¹⁴COOH and NaH¹⁴CO₃ were purchased from
The Radiochemical Centre, Amersham, England.

Glucose-6-phosphate Dehydrogenase was obtained from the
Sigma Chemical Co.

Suc Digestif, derived from the snail Helix pomatia, was
supplied by L'Industrie Biologique Francaise, 35 à 49,
Quai du Moulin de Cage, GENNEVILLIERS, (Seine).

Glass powder, purchased from Canadian Laboratory Supplies,
6655, Delmeade Road, Mount Royal Box 2090, Station "O", P.Q.,
was pyrex glass passed through a 200 mesh.

3. MANOMETRIC METHODS.

3.1. Washed-cell Suspensions.

Initially when washed-cell suspensions of Z. ventriculi were used in manometric experiments, no evolution of gas from glucose was detected, although the cells used had been gassing vigorously in the culture medium. It was found that certain precautions had to be taken in order to obtain active washed-cell suspensions.

Warburg flasks were prepared with all additions made except buffer and cell suspension. The side arms were stoppered and the manometer joints greased. Using an actively fermenting culture, Z. ventriculi was harvested as previously described. The growth medium was poured off and the cells in the volume remaining were made up to approximately 80 ml. with freshly boiled and cooled distilled water. This suspension was transferred quickly to a centrifuge tube and centrifuged for a few minutes. During centrifugation some of the buffer to be used was boiled and cooled, and the appropriate quantities pipetted into the Warburg flasks. By this time the centrifuge had stopped; the tube was removed, the supernatant poured off, and the cells taken up in 10 ml. buffer. The required amounts were pipetted into the Warburg flasks, the flasks attached to the

manometers and immediately gassed with nitrogen. From the time that the medium was decanted from the cells until the flasks were being gassed with nitrogen required approximately five minutes. Flasks were gassed with nitrogen for four minutes. It was found that by using this procedure, it was possible to obtain active washed-cell suspensions. Usually small amounts of glucose were carried over due to insufficient washing of the cells. The fermentation of this glucose was allowed to proceed to completion before substrates were added.

3.2. Acetaldehyde Formation.

In experiments where it was believed that acetaldehyde might be formed, the following procedure was used to prevent loss of acetaldehyde (b.p. 21°). At the end of the experiment the manometer and flask were taken from the water-bath without opening the stopcock. The flask was surrounded by crushed ice. Air was allowed to enter the manometer, when the manometer fluid rose to the top of the closed limb of the manometer, by quickly opening and closing the stopcock. This process was repeated several times until equilibration was achieved. The flask was then removed from the manometer and the reaction mixture decanted. In all subsequent procedures the reaction mixture was kept cold.

Apart from these special procedures, standard manometric techniques were followed. All manometric experiments were

carried out at 37.5° . The rate of shaking was 110 to 120 cycles / minute.

4. ENZYMIC PREPARATIONS.

4.1. Introduction.

One of the main problems in the investigation of glucose metabolism in Z. ventriculi has been the purely technical one of obtaining cell preparations or cell-free extracts which would utilize pyruvate. Washed-cell suspensions failed to metabolize pyruvate, and it was believed that this might be explained in terms of permeability phenomena. Therefore, besides attempting to obtain cell-free extracts, a number of methods which are thought to alter the permeability of bacterial cells, were employed.

The following methods were investigated:

I. Permeability Effects.

- (a) Slow vacuum drying at 0°. (Gunsalus, 1955).
- (b) Lyophilization (Gunsalus, 1955).
- (c) Acetone drying (Gunsalus, 1955).
- (d) Toluene treatment.
- (e) Freezing and thawing.
- (f) Storing in a "deep-freeze" cabinet (Koepsell and Johnson, 1942).

II. Mechanical Rupture.

- (g) Hughes' press (Hughes, 1951).
- (h) Ultrasonic oscillator (Mullard 50 watt Ultrasonic drill,

Type E 7680).

- (i) Nelco Homogenizer (Lemanna and Mallette, 1954).
- (j) Mickle tissue disintegrator (Mickle, 1948).
- (k) Alumina grinding (McIlwain, 1948).
- (l) Grinding with glass powder (Utter and Werkman, 1942).

III. Enzymic and Chemical Methods.

- (m) Penicillin treatment (Lederberg, 1957).
- (n) Lysozyme.
- (o) Suc digestif (Eddy and Williamson, 1957).

Methods (a), (b), (c), (e) and (f) failed to produce preparations which would metabolize pyruvate. Methods (g), (h), (j) and (k) failed to disrupt whole cells to any appreciable extent; method (j) was run for periods of up to twenty-four hours. Although cells were not disrupted by method (g), when treated in this way the cells were found to produce gas from pyruvate but at a slow rate. Methods (m) and (n) were investigated by observing the optical density of a cell suspension, and were found to be ineffective in disrupting cells.

Methods (d), (i), (l) and (o) were found to affect cells of Z. ventriculi.

4.2. Nelco Homogenizer.

The first and, until recently, the only method of obtaining cell-free extracts of Z. ventriculi was by the

method of Lamanna and Mallette (1954). Preliminary experiments demonstrated that the following method gave a high degree of disruption in a short period of time. Cells were suspended in 5.5 ml. buffer solution and transferred to the homogenizer vessel containing 8 ml. glass beads (Ballotini No. 12, Empire Glass Co., Leicester). The homogenizer was run for two minutes, with the vessel surrounded by crushed ice, stopped for two minutes to allow cooling of the motor, and run for a further two minutes. The homogenizer vessel was removed and the glass beads allowed to settle. The "supernatant" was decanted, the beads were washed three times with 2 ml. buffer, and the washings added to the original supernatant. Using cells grown overnight in 400 ml. medium, cell-free extracts with protein nitrogen concentrations of the order of 1.1 mg.N/ml. were obtained. These extracts were generally found to be inactive towards pyruvate but in some cases, pyruvate was metabolized at a slow rate. Various modifications of the above method, aimed at preserving the enzymic activity of the extracts, were found to be ineffective.

The following modifications of the above method were investigated:

1. Addition of one drop of octanol to the system to prevent foaming, which may cause denaturation of proteins.

2. Addition of glutathione or sodium hydrosulphite to prevent oxidation of sulphhydryl groups.
3. As the method used was a highly aerobic one, the apparatus was adapted so that it could be gassed continuously with nitrogen during the period of the disruption.

All of these modifications were ineffective for obtaining extracts with increased activity towards pyruvate. The preparations obtained by the original method were surveyed for a number of enzymic activities, and some active enzymes were found to be present.

4.3. Toluene Treatment.

In the preparation of purified hexokinase from yeast, the preliminary step involves treating the yeast with toluene (Kunitz and McDonald, 1946). This method was tried with Z. ventriculi and it was found that toluene-treated cells would metabolize pyruvate. The method used was as follows: 720 mg. (dry wt.) of cells were harvested as previously described and 3 ml. of toluene added to the wet cells. This mixture was incubated at 37° for one hour, stored overnight at 0°, made up to 25 ml. with distilled water and the toluene removed with a Pasteur pipette. One ml. quantities of this preparation were used in manometric experiments.

4.4. Suc Digestif.

Suc digestif, an enzymic preparation from Helix pomatia,

is highly effective in disrupting cells of Z. ventriculi. As suc digestif is itself an enzymic preparation, it was thought desirable first to produce protoplasts of Z. ventriculi, as Eddy and Williamson (1957) have done with yeast. If this could be achieved, the protoplasts could be washed free of suc digestif before being lysed to give cell-free extracts. Cells were suspended in 0.55 M rhamnose solution, with 0.1 ml. of suc digestif preparation added. No protoplast formation was observed although the cells were disrupted with the production of large cell fragments.

The possibilities of making use of such preparations have not been investigated.

4.5. Glass powder.

Grinding cells of Z. ventriculi with glass powder (Utter and Workman, 1942) in a mortar was highly effective in causing disruption of the cells. The cell-free extracts obtained appeared to possess a variety of enzymic activities. As a sample of glass powder was only recently obtained, these have not been fully investigated.

5. ISOTOPIC TECHNIQUES.

5.1. General Counting Procedure.

Radioactivity was determined by a thin end-window Geiger-Müller tube with a Panax scaling unit. Samples (except $^{14}\text{CO}_2$) were plated directly on to metal planchettes with an effective raised area of 1.5 cm^2 . Highly radioactive materials were diluted for counting and no corrections for coincidence were required. With the exception of $\text{Ba}^{14}\text{CO}_3$, all determinations were made at infinite thickness, and the absence of self-absorption was checked by plating decreasing amounts of the material.

Formate was plated directly from an aqueous solution (pH 7.0) and dried under an infra-red lamp.

$\text{NaH}^{14}\text{CO}_3$ was counted as $\text{Ba}^{14}\text{CO}_3$ using the methods described by Sakami (1955). The effective area of the precipitate was 1.5 cm^2 , so that the counts thus obtained were directly comparable with the counts obtained with other material plated directly on metal planchettes. Corrections for self-absorption and losses of material in the filtration apparatus were applied (Holms, 1957).

$^{14}\text{CO}_2$ was collected in NaOH and counted as $\text{Ba}^{14}\text{CO}_3$ as above, except in the manometric experiments using (U- ^{14}C)glucose, where $\text{Ba}^{14}\text{CO}_3$ was counted at infinite thickness.

Pyruvate was isolated and purified as the 2,4-dinitrophenylhydrazone. A few drops of a saturated solution of 2,4-dinitrophenylhydrazine in ethanol, containing 1% (v/v) HCl, were added to samples of reaction mixtures. The precipitated hydrazone was centrifuged down, washed once with water, twice with a 40 mM formate solution, and purified by the standard ethyl acetate - Na_2CO_3 extraction procedure (Friedemann and Haugen, 1943). The hydrazone was plated direct from ethyl acetate solution, and the amount plated was determined, after counting, by measuring the E_{450} value of the solution obtained by elution of the planchette with a 5% (w/v) sodium carbonate - 0.75 N sodium hydroxide solution.

In one experiment no precipitate of hydrazone was obtained. The reaction mixture was carried through the ethyl acetate - Na_2CO_3 extraction procedure, and the purified pyruvate 2,4-dinitrophenylhydrazone separated chromatographically. The hydrazone spot was cut from the chromatogram and eluted with 10% (w/v) Na_2CO_3 . The solution was acidified with conc. HCl, the hydrazone extracted into ethyl acetate and plated as above.

Pyruvate 2,4-dinitrophenylhydrazone was degraded by the method of Krebs and Johnson (1937).

Planchettes to be used for plating 2,4-dinitrophenyl-

hydrazone were treated by washing successively with 1.5 N NaOH, tap water and distilled water, and dried by blotting between filter papers.

5.2. Manometric Experiment with (U-¹⁴C)Glucose.

At the end of the experiment, alkali and filter papers were removed from the centre-wells of the Warburg flasks and transferred quantitatively to 10 ml. graduated measuring cylinders. 5 ml. of 0.2 mM Na₂CO₃ solution (CO₂-free) were added and the volume made to the mark with distilled water (CO₂-free). Portions (0.6 ml.) of this solution were taken for precipitation as Ba¹⁴CO₃ by the methods of Sakami (1955). To ascertain that counting was being carried out at infinite thickness larger amounts of the Na₂¹⁴CO₃ solution were used.

The amount of (U-¹⁴C)glucose used in the experiment (10 μmoles) together with 16.2 mg. carrier glucose was combusted by the Van Slyke-Folch oxidation procedure (Sakami, 1955). The ¹⁴CO₂ obtained was weighed to check that complete recovery was obtained, and transferred to a 10 ml. measuring cylinder. An amount of Na₂CO₃ to give a final concentration of 0.1 mM was added and the volume made to the mark with distilled water (CO₂-free). The quantities used for precipitation as Ba¹⁴CO₃ were as previously described.

In this experiment, samples were counted on stage 2 of the counting chamber.

6. CHROMATOGRAPHY.

Keto-acids were separated as their 2,4-dinitrophenyl-hydrazones by descending chromatography on Whatman No. 1 paper. The solvent system used was n - butanol, 80 ml.; ethanol, 22 ml.; ammonium carbonate buffer, 38 ml. (Cavallini et al., 1949).

III. RESULTS AND DISCUSSION.

III. RESULTS AND DISCUSSION.

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1. INTRODUCTION.

When Z. ventriculi was isolated from soil, a preliminary survey of possible fermentation products was carried out with glucose, pyruvate and formate as substrates. Initially, no gas production was obtained with glucose as substrate, although the cells used had been actively gassing in the culture vessel. Similar difficulties were experienced by Davies and Stephenson (1941) using washed cell suspensions of Clostridium acetobutylicum (Weizmann). Eventually, by pipetting cell suspensions from a growing culture directly into Warburg flasks under N_2 , it was found that H_2 and CO_2 were produced during growth. By taking the precautions previously mentioned (II, 3.1.), washed cells which would metabolize glucose were finally obtained.

At this point in the investigation neither pyruvate nor formate were found to be metabolized. The failure of washed cells to metabolize pyruvate might be explained in terms of permeability phenomena. The fact that formate was not metabolized was explained by postulating that, in this organism, H_2 was produced by the clostridial-type of pyruvate fission, rather than via the formic hydrogenlyase system. Shortly afterwards however, Z. ventriculi was found to possess an extremely active formic hydrogenlyase system

which was shown to be highly sensitive to atmospheric oxygen. This finding explained the earlier failure of washed-cell suspensions to metabolize formate.

In order to explain the distribution of isotope in the products of glucose fermentation, with glucose labelled in different positions, it was reasonable to assume that pyruvate is an intermediate. As the failure of Z. ventriculi to metabolize pyruvate had been tentatively explained in terms of permeability phenomena, efforts were made to surmount this difficulty by attempting to alter the cell permeability or by obtaining cell-free extracts.

Z. ventriculi proved to be refractory to most of the methods commonly used for disrupting cells. Although cells were effectively disrupted by the method of Lamanna and Mallette (1954), cell-free extracts which would metabolize pyruvate were not obtained by this method. Extracts prepared in this way were used, however, to carry out a survey, by spectrophotometric methods, of other enzymes which might possibly be present.

The treatment of yeast cells with toluene has been employed to obtain enzymic preparations (Kunitz and McDonald, 1946). The treatment of cells of Z. ventriculi in this manner allowed pyruvate to be metabolized. Subsequent work demonstrated that the activity could be explained in terms of

a yeast-type carboxylase. Although no evidence for a "phosphorelastic" fission of pyruvate was ever obtained with these preparations, the functioning of such a mechanism seemed likely, in view of the presence of a formic hydrogen-lyase system. Many further attempts made to detect such a metabolic activity using cell-free preparations were without success. However, during one such experiment, in a control flask, containing whole cells together with pyruvate, pyruvate was found to be metabolized. No adequate explanation of this unexpected result has been obtained, but it was shown to be reproducible and therefore presented a new approach to the problem of pyruvate metabolism.

Later, when a sample of glass powder (200 mesh) became available, cell-free extracts which would metabolize pyruvate were obtained by grinding cells in a chilled mortar with this glass powder.

The investigation of glucose fermentation in Z. ventriculi has followed a somewhat devious course, due for the most part to difficulties experienced in handling this highly "labile" organism. However, our understanding of the fermentation has gradually increased, and we are now in a position to propose a scheme which can adequately explain the diverse fermentation balances obtained by earlier workers.

2. GLUCOSE METABOLISM.

2.1. Washed Cell Suspensions.

As soon as washed cell suspensions capable of metabolizing glucose had been obtained, experiments were carried out to elucidate the differences in the fermentation carbon balances obtained by Smit (1930), Kluyver (1931) and Milhaud et al. (1956).

The fermentation of glucose at pH 5.0 by washed cell suspensions produced H_2 and CO_2 in the ratio of 1 : 1 (Fig. 3), in agreement with the results of Milhaud et al. (1956).

In microbial fermentations where different pathways exist, probably the best known factor controlling the pathways which operate, is the pH of the medium or reaction mixture (van der Lek, 1930; Tikka, 1935; Campbell and Gunsalus, 1944).

Since Milhaud et al. (1956) had produced evidence that glucose was fermented exclusively by Embden-Meyerhof glycolysis, it seemed possible that the reactions subsequent to the pyruvate stage of metabolism might be controlled by pH in this way. Some such mechanism could explain the divergent fermentation carbon balances obtained.

The pH optimum for the evolution of H_2 from glucose was found to be 6.0 (Fig. 4). The gases evolved over a pH range of 2 to 8 were investigated (Table 8).

FIG. 3.Fermentation of Glucose by *Z. ventriculi* at pH 5.0.

Protocol; 10 μ moles glucose (side arm); 65 μ moles K phosphate; 10 mg. (dry wt.) of cells; pH, 5.0; total volume, 3.8 ml.; 0.2 ml. 20% (w/v) KOH in the centre well where indicated. Atmosphere N_2 .

FIG. 3.

Fermentation of Glucose by *Z. ventriculi* at pH 5.0

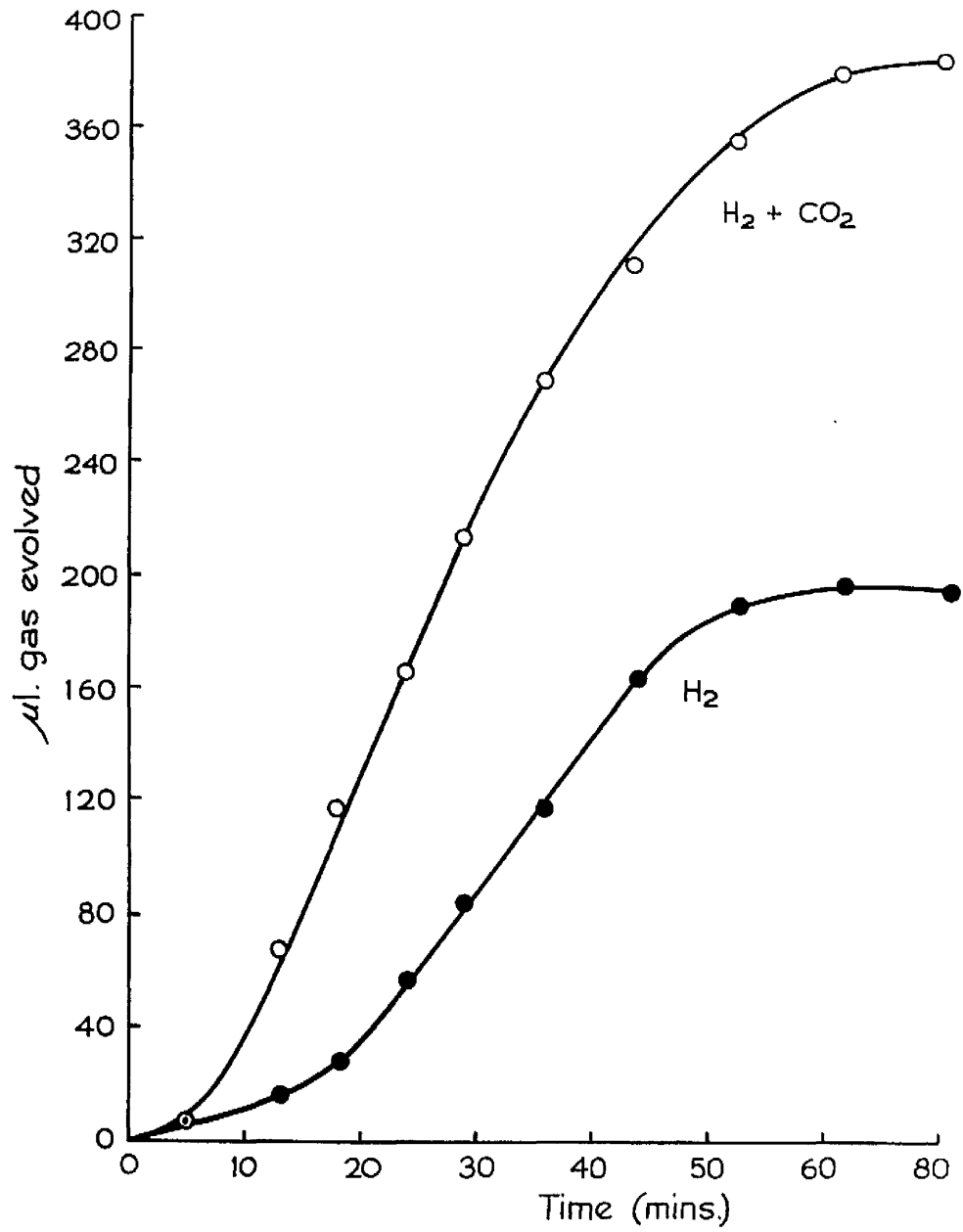


FIG. 4.pH Optimum of H_2 Evolution from Glucose by Z. ventriculi.

Protocol: 10 μ moles ($U-^{14}C$) glucose (1st side arm);
0.5 ml. 2 N H_2SO_4 (2nd side arm); K phosphate - citric
acid buffer (Gomori, 1955) at appropriate pH; 11 mg.
(dry wt.) of cells; total volume, 3.8 ml.; 0.2 ml.
20% (w/v) NaOH in the centre well. Atmosphere N_2 .

FIG. 4.

pH optimum of H₂ Evolution from Glucose
by *Z. ventriculi*.

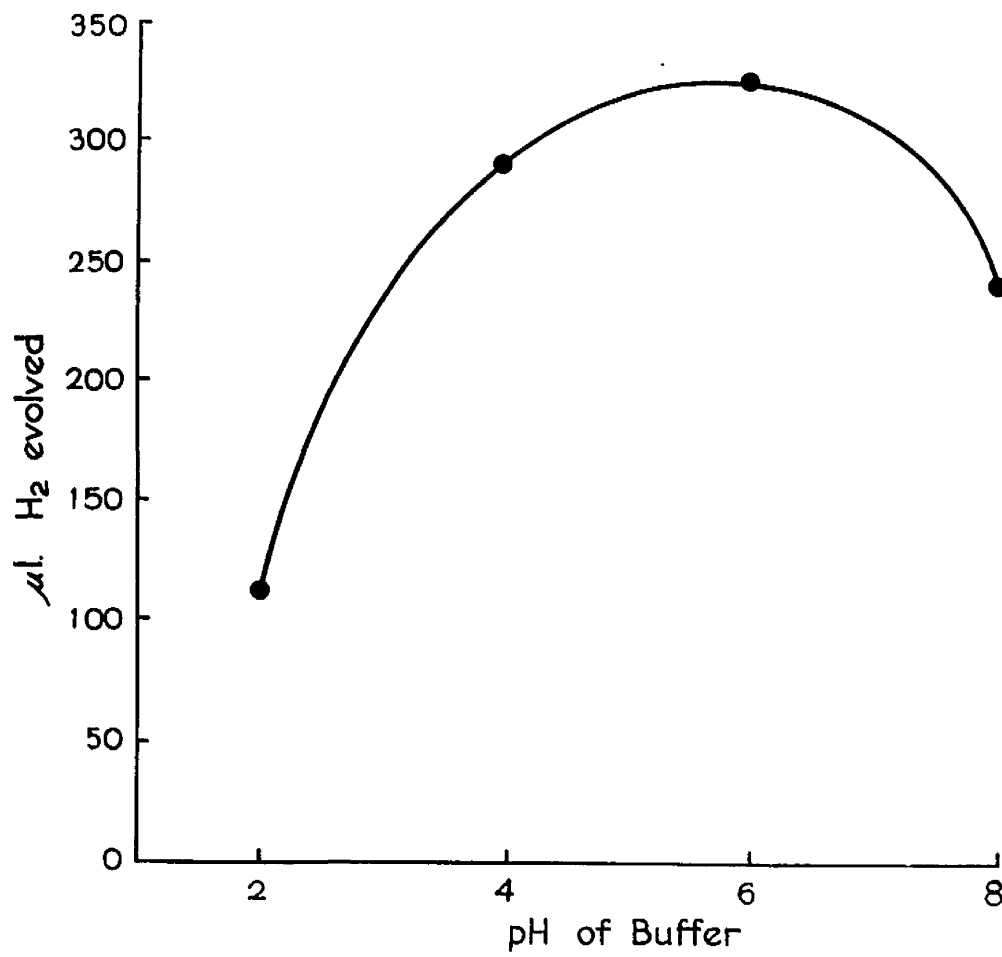


TABLE 8.

Gas Production from Glucose by *Z. ventriculi* at
different pH values.

Protocol: As for Fig. 4.

TABLE 8.

Gas Production from Glucose by *Z. ventriculi* at different pH values.

pH of Buffer	μmoles			Moles/Mole Glucose		Ratio CO ₂ /H ₂
	Glucose	CO ₂	H ₂	CO ₂	H ₂	
2	9.01	13.8	13.3	1.53	1.48	1.03
4	10.0	17.5	19.1	1.75	1.91	0.92
6	9.47	18.6	17.0	1.96	1.79	1.10
8	9.05	17.9	14.9	1.97	1.65	1.19

As can be seen from Table 8, the ratio CO_2 / H_2 is approximately unity at each pH value used. These results appear to indicate a fermentation of the type obtained by Milhaud et al. (1956) over the whole of this broad pH range. The results are certainly very different from the CO_2 / H_2 ratios obtained by Smit (1930) and Kluyver (1931), which were 3.3 and 4.6 respectively. At first sight then, it would seem that a pH controlling mechanism cannot explain the differences in the carbon balances obtained by earlier workers. However, there were some essential differences in the systems used by these investigators. Milhaud et al. (1956) used washed cell suspensions, whereas both Smit (1930) and Kluyver (1931) analysed the products of glucose fermentation in culture media. Unfortunately, neither Smit nor Kluyver gave full details of the medium used, and Milhaud et al. failed to mention the initial pH of their medium, although, by inference, it was probably 7.0. In view of these facts a more detailed investigation of cells grown at different pH values may be required. Although it would appear that pH does not influence the operation of the enzymic pathways of non-proliferating suspensions (Table 8), it is known that the pH of the culture medium can affect the enzymic constitution of the cell (Gale and Lipp, 1942). This may be the prime factor in this fermentation. Results

obtained more recently with Z. ventriculi (III, 4.3.) have shown that fermentative activities vary with the stage of the growth cycle. These results are detailed later and their implications discussed.

The elucidation of the controlling mechanism which determines the metabolic pathways to which the substrate (glucose) is directed is obviously an important aspect of the Z. ventriculi fermentation. In addition, the exciting possibility exists of using such a mechanism to produce a pure ethanolic fermentation. At any rate, it would not seem to be impossible, in view of the fermentation carbon balances obtained by Smit (1930) and Kluyver (1931).

2.1.1. Inhibitor Studies.

In general it is no longer valid to deduce Embden-Meyerhof glycolysis from positive results with the classical inhibitors, i.e. iodoacetate and fluoride, since only phosphohexokinase, aldolase and triosephosphate isomerase are unique to this pathway. However, in the case of a strict anaerobe, such findings, taken in conjunction with other evidence, yield useful confirmatory evidence.

The effect of different inhibitors on the production of H_2 from glucose is given in Table 9. Although iodoacetate is typically inhibitory at a concentration of 8 mM, KF had

TABLE 9.

Inhibition of Glucose Fermentation in *Z. ventriculi*.

Protocol: As for Fig. 3, except that inhibitors were present at the concentrations indicated. With iodoacetate and fluoride the experiment lasted 52 minutes. With sodium arsenite the duration of the experiment was 113 minutes.

TABLE 9.

Inhibition of Glucose Fermentation in *Z. ventriculi*.

Inhibitor	Concentration mM	H ₂ Produced μmoles	Inhibition of H ₂ Production per cent.
Iodoacetate	8.0	0	100
Potassium Fluoride	160.0	7.4	0
Sodium Arsenite	0.05	4.7	34
Sodium Arsenite	0.5	0.6	93

no effect on the evolution of H_2 from glucose. Daves and Holms (1958) found that KF did not affect glucose oxidation by Sarcina lutea. In other organisms enolase, the site of fluoride inhibition, has been shown to have a requirement for magnesium. Fluoride is believed to inhibit the action of this enzyme by forming a magnesium-fluorophosphate complex. The result obtained with Z. ventriculi may simply mean that enolase in this organism does not have a requirement for magnesium. Arsenite which inhibits the oxidative decarboxylation of pyruvate (Daves and Holms, 1958) inhibits H_2 production from glucose by Z. ventriculi. In Z. ventriculi the site of inhibition would appear to be a different one, since no pyruvate was found to accumulate in the presence of arsenite (Table 10). The fact that glucose disappearance was increasingly inhibited, and that the amount of pyruvate formed decreased with increased arsenite concentration, suggests that the site of action of the inhibitor is between glucose and pyruvate.

Although small amounts of pyruvate were found (Table 10), these were no greater than the amounts formed in the absence of inhibitor. Arsenite therefore does not cause pyruvate to accumulate in Z. ventriculi under these conditions. However, the observation that significant amounts of pyruvate were found indicates that pyruvate is an intermediate in the

TABLE 10.Effect of Arsenite on the Fermentation of Glucoseby *Z. ventriculi*.

Protocol: 10 μ moles glucose (side arm); 65 μ moles K phosphate; 11 mg. (dry wt.) of cells; inhibitors at concentrations indicated; total volume, 3.8 ml.; 0.2 ml. 20% (w/v) KOH in the centre well.

Atmosphere N_2 .

TABLE 10.

Effect of Arsenite on the Fermentation of Glucose
by *Z. ventriculi*.

Arsenite	Inhibition of H ₂ evolution from glucose ¹	Glucose utilized: ²	Pyruvate formed.
mm	per cent	μmoles	μmoles
0	-	8.8	0.48
0.05	34	8.9	0.48
0.5	93	2.9	0.08
5	100	0	0

¹ After 25 minutes.

² After 150 minutes.

Fermentation of glucose by Z. ventriculi.

Recently, Arbuthnott (1960) has carried out experiments where washed-cell suspensions of Z. ventriculi fermented glucose in the presence of sodium sulphite. These experiments (Table II) confirmed that acetaldehyde was also an intermediate in the fermentation of glucose. Experiments carried out earlier with pyruvate as substrate (III, 4.1.) had indicated that this result might be anticipated.

2.2. Cell-free Extracts.

The main object of obtaining cell-free extracts of Z. ventriculi has been concerned with securing preparations which would metabolize pyruvate. However, when cell-free preparations were obtained they were also tested for enzymes of glucose metabolism which might be present. The major difficulty encountered with all of the cell-free preparations so far obtained has been the presence of a highly active DPNH oxidase. A variety of methods has been used to try to remove this activity, namely dialysis, ammonium sulphate and pH fractionation, but without reproducible success. However, some of the enzymes concerned in the intermediary metabolism of glucose have been identified in these preparations.

2.2.1. Hexokinase.

Cell-free preparations obtained by the method of Lamanna

TABLE II.

Formation of Acetaldehyde from Glucose by *Z. ventriculi*
in the presence of sodium sulphite (Arbuthnott, 1960).

Protocol: 40 μ moles glucose (experiments 1 and 3),
80 μ moles glucose (experiment 2), (side arm); 126
 μ moles K phosphate; 20 mg. (dry wt.) of cells;
Na sulphite at concentration indicated; pH, 7.0;
total volume, 2.8 ml. Atmosphere N_2 .

TABLE 11.

Formation of acetaldehyde from glucose by *Z. ventriculi*
in the presence of sodium sulphite (Arbutnot, 1960).

Na_2SO_3 added.	Glucose added.	Glucose utilized.	Acetaldehyde found.
μmoles	μmoles	μmoles	μmoles
20.2	40	19.2	6.7
32.5	80	5.3	4.1
40.4	40	32.1	22.2

and Mallette (1954) contained hexokinase (Fig. 5).

2.2.2. Aldolase and Triosephosphate Dehydrogenase.

All attempts to demonstrate the presence of aldolase and triosephosphate dehydrogenase in extracts prepared by the method of Lamanna and Mallette (1954) failed, due to the presence of a powerful DPNH oxidase. Similar results were usually obtained using extracts prepared by grinding with glass powder. However on one occasion, the DPNH oxidase present in the preparation was not so active as usual and the presence of aldolase and triosephosphate dehydrogenase was demonstrated (Fig. 6).

Although DPNH has obviously been produced, indicating the presence of aldolase and triosephosphate dehydrogenase, the irregularities of the curve require comment. The results obtained in the early part of this experiment suggested that some DPNH oxidase might be present in the extract. At 70 minutes the cuvette was therefore quickly shaken and replaced in the spectrophotometer. As can be seen from the curve (Fig. 6) a sharp fall in the concentration of DPNH occurred, followed later by the further regeneration of DPNH. The irregular shape of this curve is explained therefore, in terms of an equilibrium between the aldolase-triosephosphate dehydrogenase system and the DPNH oxidase. A similar explanation might account for the lag period before DPNH

FIG. 5.Hexokinase of *Z. ventriculi*.

Protocol: 9 μ moles glucose; 40 μ moles NaHCO_3 ;
40 μ moles MgCl_2 ; 0.1 ml. glucose-6-phosphate
dehydrogenase (0.1%, w/v); 0.2 ml. extract (1.1 mg.
N / ml.); 0.2 μ moles TPN; 12.5 μ moles ATP (added last);
pH 7.1; total volume, 2.7 ml.; temperature, 37°.

FIG. 5.

Hexokinase of *Z. ventriculi*

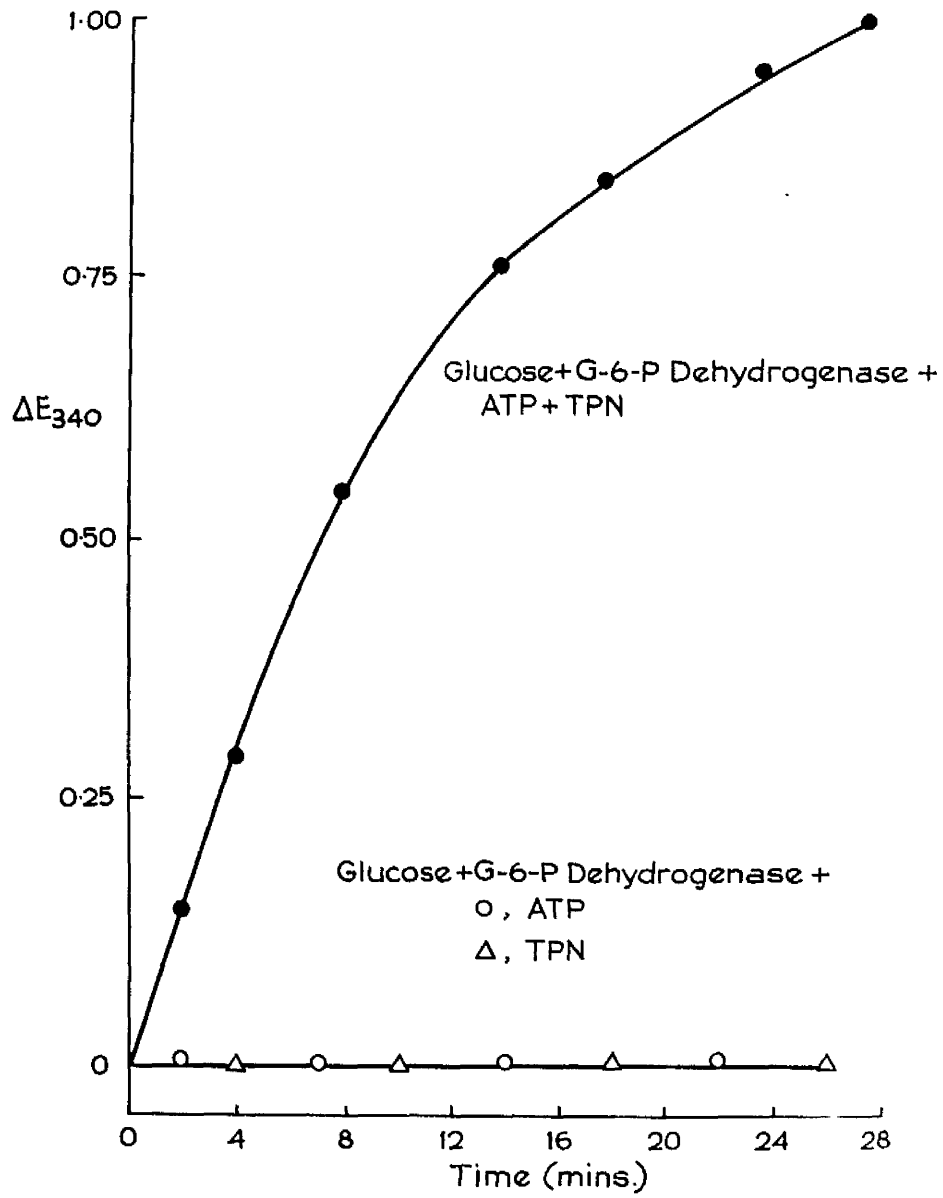


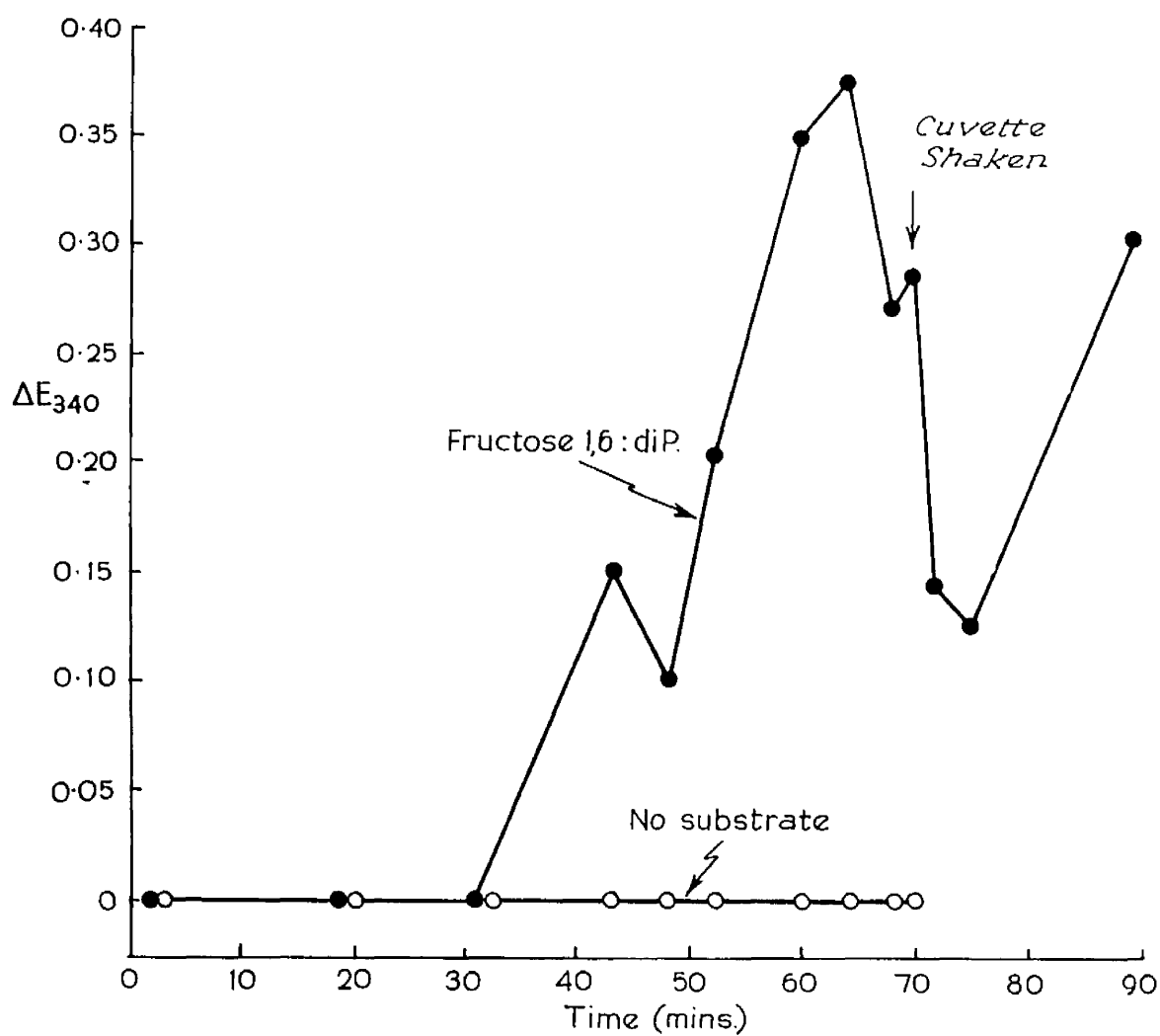
FIG. 6.

Aldolase and Triosephosphate Dehydrogenase of
X. ventriculi extract.

Protocol: 5 μ moles fructose 1:6 diphosphate;
24 μ moles glycylglycine; 12 μ moles 2-amino-2-
hydroxymethylpropane-1:3-diol (tris.); 6 μ moles
glutathione; 25 μ moles Na arsenate; 0.3 ml. extract
(0.5 mg. N / ml.); 0.2 μ moles DPN (added last); pH, 7.5;
total volume, 2.6 ml.; temperature, 37°.

FIG. 6.

Aldolase and Triosephosphate Dehydrogenase
of *Z. ventriculi* extract.



formation was observed.

2.2.3. Pyruvate Formation from 3-Phosphoglyceric Acid.

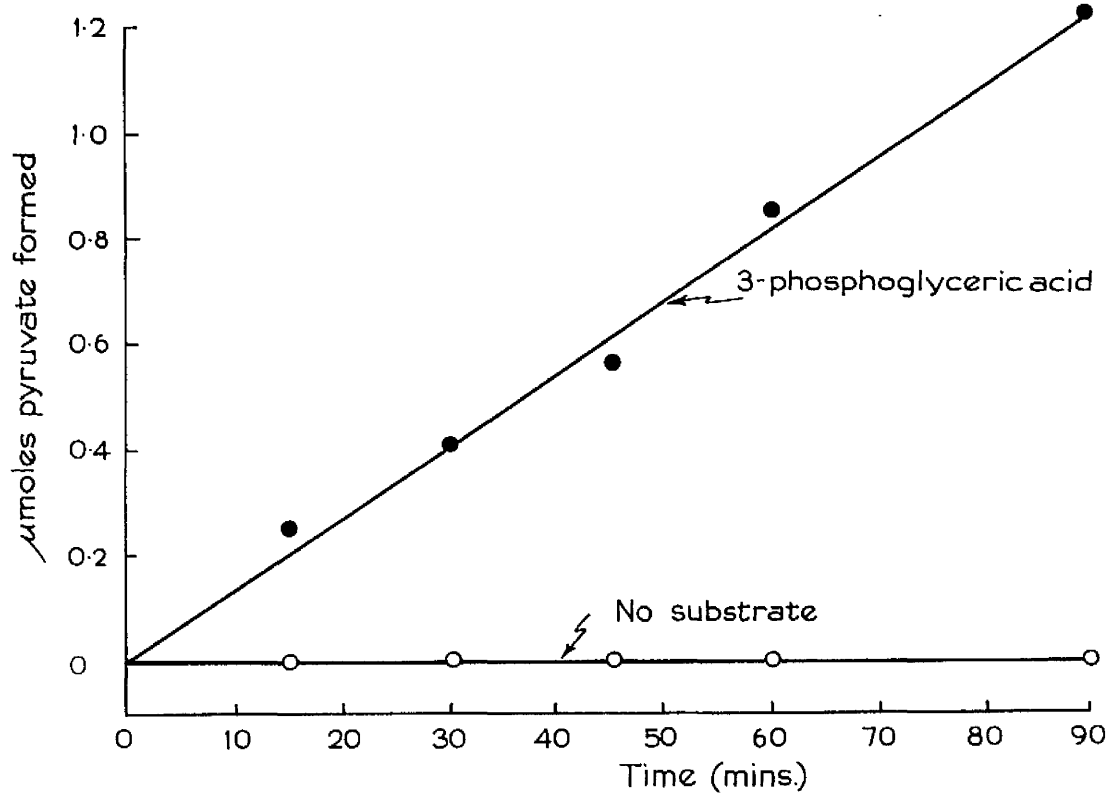
Pyruvate formation from 3-phosphoglyceric acid was demonstrated (Fig. 7) using extracts obtained by grinding cells of *Z. ventriculi* with glass powder.

FIG. 7.Formation of Pyruvate from 3-Phosphoglyceric acid
by an extract of *Z. ventriculi*.

Protocol: 20 μ moles 3-phosphoglycerate; 132 μ moles glycylglycine; 40 μ moles $MgCl_2$; 20 μ moles Na arsenite; 1.0 ml. extract (0.5 mg. N / ml.); 10 μ moles ADP; pH, 7.1; total volume, 7.1 ml.; temperature, 37 $^\circ$. Samples (1 ml.) were withdrawn at intervals and deproteinized with 0.2 ml. 40% (w/v) TCA. Pyruvate was assayed on the supernatants.

FIG. 7.

Formation of Pyruvate from 3-Phosphoglyceric Acid by an extract of *Z. ventriculi*.



3. FORMATE METABOLISM.

3.1. Formic Hydrogenlyase.

Early attempts to find a formic hydrogenlyase system with washed-cell suspensions of Z. ventriculi were unsuccessful. This led to the belief that this organism might possess a clostridial-type fission of pyruvate. Later however, formate was found to be metabolized, equimolar amounts of H_2 and CO_2 being produced (Fig. 8). Initially, the ratio of the gases evolved in the absence and presence of KOH was not typically 2 : 1. This effect was presumably due to the fact that the reaction was occurring too rapidly at this stage for the CO_2 evolved to be absorbed by the KOH in the centre well. Later, as can be seen, the ratio of CO_2/H_2 became typically 1 : 1.

The pH optimum for H_2 evolution from formate by washed-cell suspensions of Z. ventriculi was found to be 6.0 (Fig. 9). Although H_2 was produced from glucose over a much broader pH range, the pH optimum for this activity was also 6.0 (Fig. 4). Stephenson and Stickland (1952) found the pH optimum of formic hydrogenlyase in whole cells of Escherichia coli to be 7.0. The pH optimum for the evolution of H_2 from glucose in E. coli was found to be 6.2.

FIG. 8.Formic Hydrogeniyase of *Z. ventriculi*.

Protocol: 20 μ moles formate (side arm); 57.5 μ moles
K phosphate; 11 mg. (dry wt.) of cells; pH, 5.0;
total volume, 2.6 ml.; 0.2 ml. 20% (w/v) KOH in the
centre well as indicated. Atmosphere N_2 .

FIG. 8.

Formic Hydrogenlyase of *Z. ventriculi*.

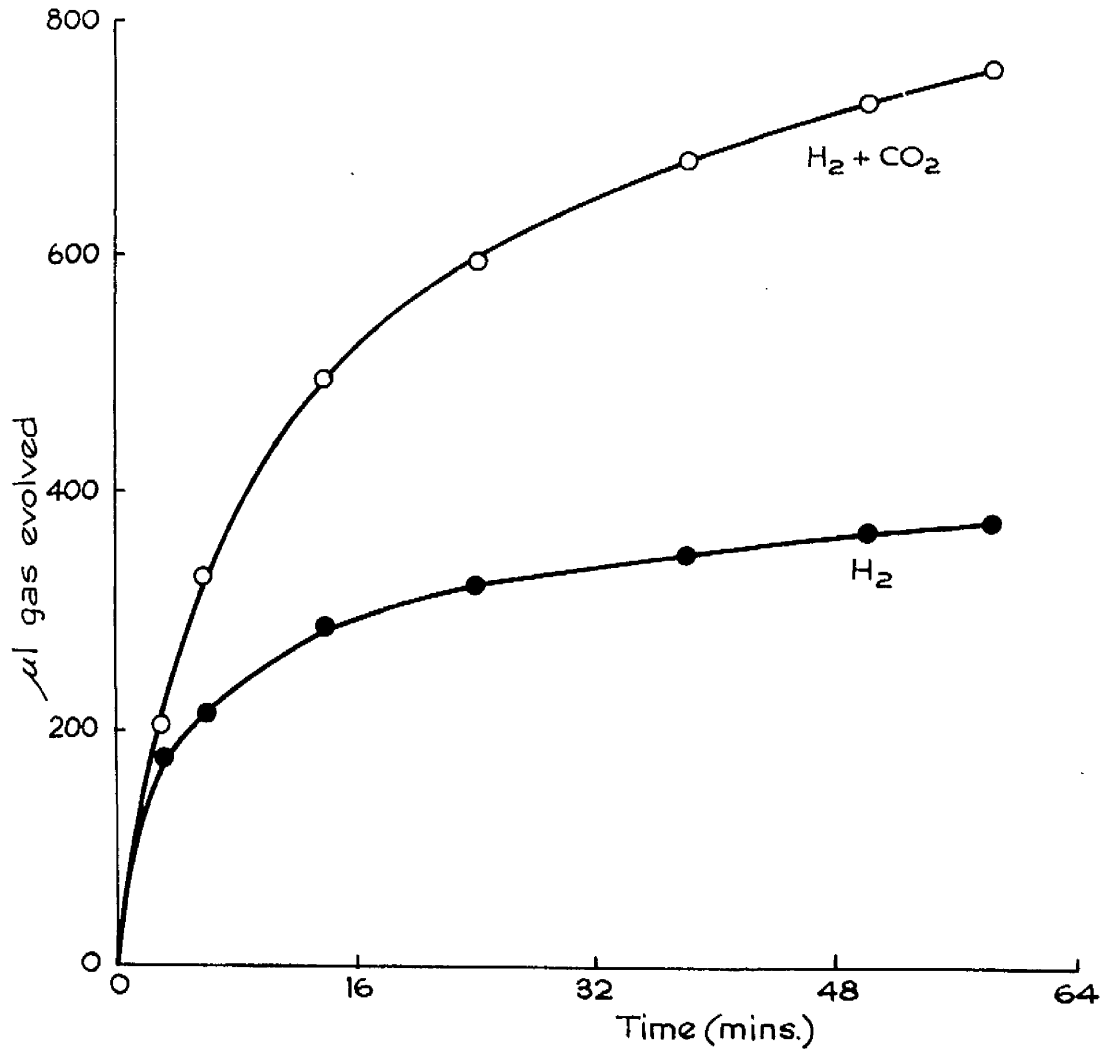
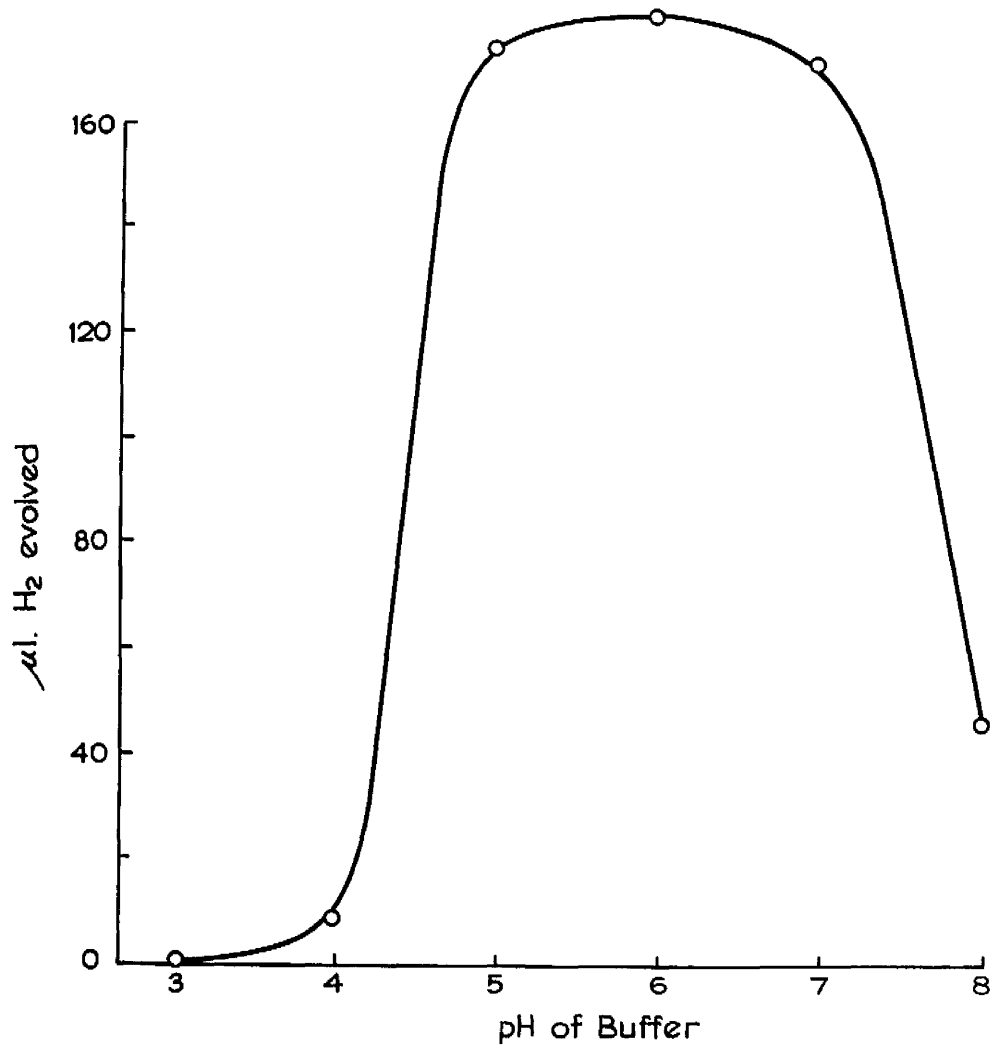


FIG. 9.pH Optimum of Formic Hydrogenlyase of *Z. ventriculi*.

Protocol: 109 μ moles formate (side arm); K phosphate-citric acid buffer (Gomori, 1955) at pH indicated; 11 mg. (dry wt.) of cells; total volume, 2.8 ml.; 0.2 ml. 20% (w/v) KOH in the centre well. Atmosphere N_2 . H_2 evolution at 50 min. is recorded, over which period the reaction was linear.

FIG. 9.

pH optimum of Formic Hydrogenlyase of
Z. ventriculi.



3.1.1. Inhibitor Studies.

The evolution of hydrogen from formate was sensitive to most of the inhibitors of the formic hydrogenlyase system in E. coli (Stephenson and Stickland, 1932), (Table 12). Nitrate and nitrite were typically inhibitory. It appears to be generally assumed (Pinsky and Stokes, 1952) that inhibition by nitrate is produced by its initial conversion to nitrite by a nitrate reductase. Certain results (III, 4.2.1.) suggest that Z. ventriculi does possess such a mechanism. The claim by Sait (1933) that Z. ventriculi does not possess a system for reducing nitrate therefore requires to be re-examined. Although such a mechanism may explain the inhibition by nitrate, the exact locus of action of nitrite itself on the preformed hydrogenlyase system is not known. The finding that cyanide inhibits the hydrogenlyase system at low concentrations agrees with the results obtained with the E. coli system by Stephenson and Stickland (1932). These workers found that 10^{-5} M cyanide produced 50% inhibition of H_2 evolution from formate. Inhibition by somewhat higher concentrations of formaldehyde as well as sensitivity to oxygen and carbon monoxide were also typical of the E. coli hydrogenlyase system.

One important difference between the Z. ventriculi system and the E. coli system was the effect of an atmosphere of

TABLE 12.Inhibition of Formic Hydrogenlyase of *Z. ventriculi*.

Protocol: 20 μ moles formate (side arm); inhibitors at concentrations indicated; 105 μ moles K phosphate; 11 mg. (dry wt.) of cells; pH, 5.0; total volume, 2.8 ml.; 0.2 ml. 20% KOH in the centre well. Atmosphere N_2 . For inhibition with H_2 the system used was as above except that the pH was 7.0. For inhibition with CO, Warburg flasks were first gassed with N_2 , then gassed with CO in a fume cupboard, for five minutes. The flasks were enclosed in opaque bags to prevent possible light dissociation of CO-Fe complexes.

TABLE 12.

Inhibition of Formic Hydrogenlyase of *Z. ventriculi*.

Inhibitor	Concentration	Inhibition of H ₂ production
	mm	per cent
NaNO ₃	1.0	63
NaNO ₂	1.0	76
KCN	0.005	50
KCN	0.05	100
HCHO	1.0	14
HCHO	80.0	88
H ₂	100%	5
O ₂	20%	100
CO	100%	95-100

hydrogen on hydrogen evolution from formate; Stephenson and Stickland (1932) obtained 50% inhibition under these conditions with E. coli. With Z. ventriculi only 5% inhibition of hydrogen evolution was obtained.

The large degree of inhibition of the formic hydrogen-lyase system in E. coli by an atmosphere of hydrogen suggested that the reaction might be reversible. This was confirmed by Woods (1936) who demonstrated that formate was formed from H_2 and CO_2 by washed-cell suspensions of E. coli. The finding that an atmosphere of hydrogen had little effect on the evolution of hydrogen from formate by Z. ventriculi appeared to constitute an essential difference between the two systems. An investigation of the reversibility of the formic hydrogenlyase system in this organism confirmed the previous result that an atmosphere of hydrogen had little effect on hydrogen evolution from formate (Fig. 10).

The results of Stephenson and Stickland (1932) and Woods (1936) on the action of H_2 on formic hydrogenlyase must have depended on the CO_2 present in the reaction mixtures. It seemed possible that the present results with Z. ventriculi might be explained by the fact that CO_2 was being removed from the buffer solution during the "boiling technique". In addition, Woods (1936) was able to obtain considerable uptake of H_2 when bicarbonate was added to the E. coli system.

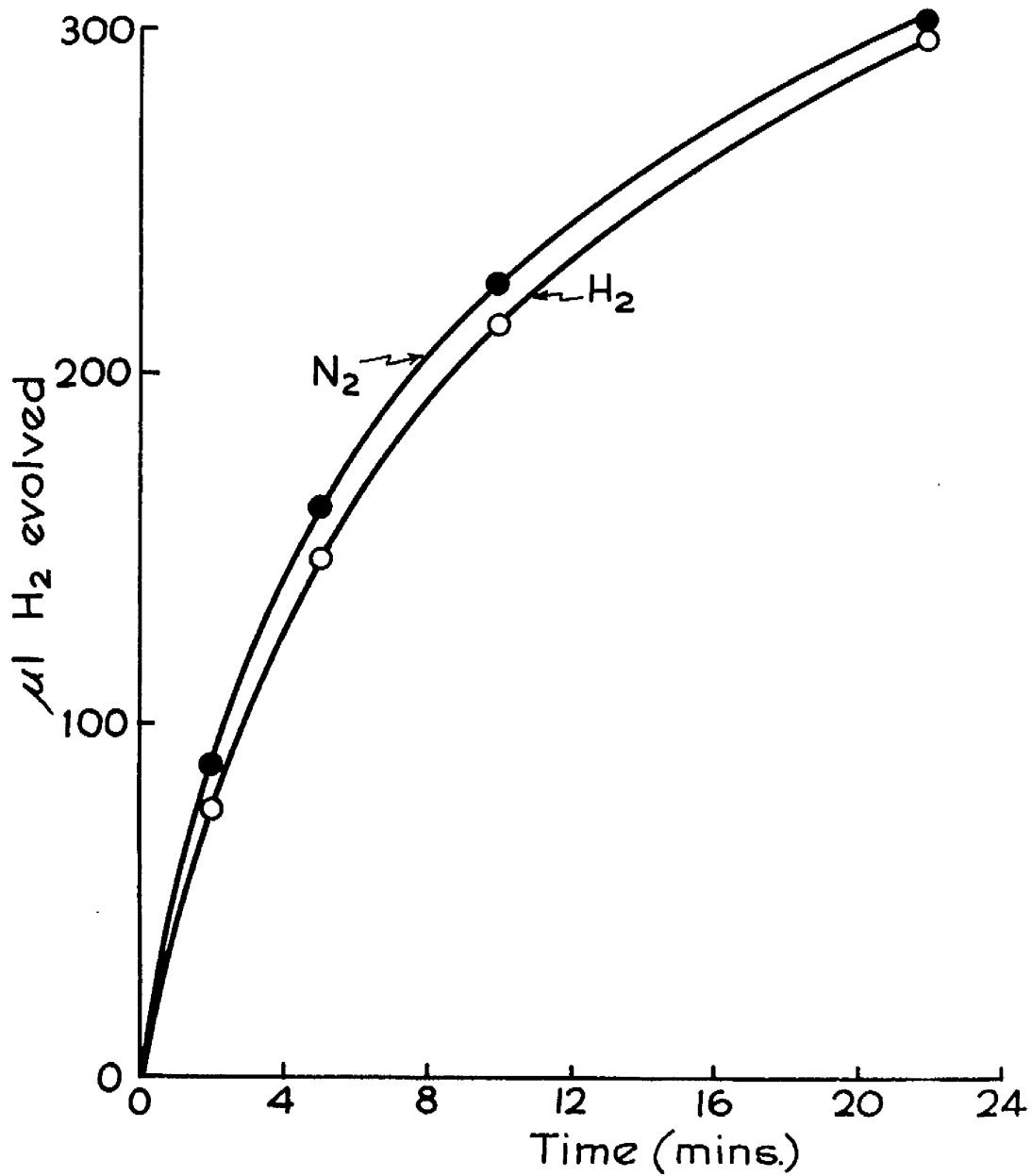
FIG. 10.

Effect of an atmosphere of H_2 on the evolution of H_2
from formate by *Z. ventriculi*.

Protocol: 20 μ moles formate (side arm); 57.5 μ moles
K phosphate; 10 mg. (dry wt.) of cells; pH, 7.0;
total volume, 2.8 ml.; 0.2 ml. 20% (w/v) KOH in the
centre well. Atmosphere N_2 or H_2 as indicated.

FIG. 10.

Effect of an atmosphere of H_2 on the evolution of H_2 from formate by *Z. ventriculi*.



When this was investigated with washed-cell suspensions of Z. ventriculi no hydrogen uptake was observed.

Despite these failures to detect reversibility of the hydrogenlyase system in Z. ventriculi, some results obtained more recently suggest that the complete pathway from pyruvate to H_2 and CO_2 may be reversible (III, 4.2.2.). In view of this finding, the failure to obtain reversibility of the formic hydrogenlyase system in Z. ventriculi must be re-examined.

5.2. Formic Dehydrogenase.

Washed-cell suspensions of Z. ventriculi have been shown to possess formic dehydrogenase activity with methylene blue as H acceptor (Fig. 11). Gest and Peck (1955) have shown that the one-electron dye benzyl viologen can also act as an H acceptor in this reaction. Although previous work with this dye has been carried out with cell-free extracts, whole cells of Z. ventriculi were tested for formic dehydrogenase (benzyl viologen). No activity was detected.

5.3. Hydrogenase.

Washed-cell suspensions of Z. ventriculi were found to possess hydrogenase using methylene blue as H acceptor (Fig. 12). Whole cells of Desulphovibrio desulphuricans have been shown to catalyse the hydrogenase "evolution assay" with reduced methyl

FIG. 11.Formic Dehydrogenase of *Z. ventriculi*.

Protocol: 20 μ moles formate (side arm); 57.5 μ moles
K phosphate; 20 μ moles methylene blue; 11 mg. (dry wt.)
of cells; pH, 5.0; total volume, 2.5 ml.; 0.2 ml. 20%
(w/v) KOH in the centre well as indicated. Atmosphere N_2 .

FIG. 11.

Formic Dehydrogenase of *Z. ventriculi*

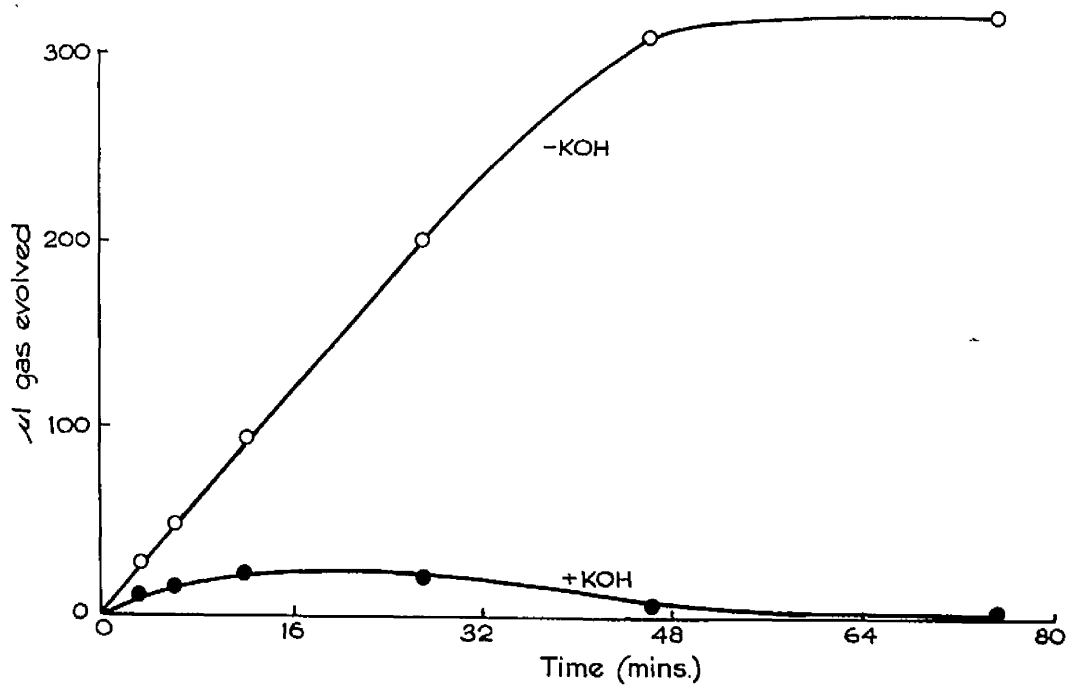


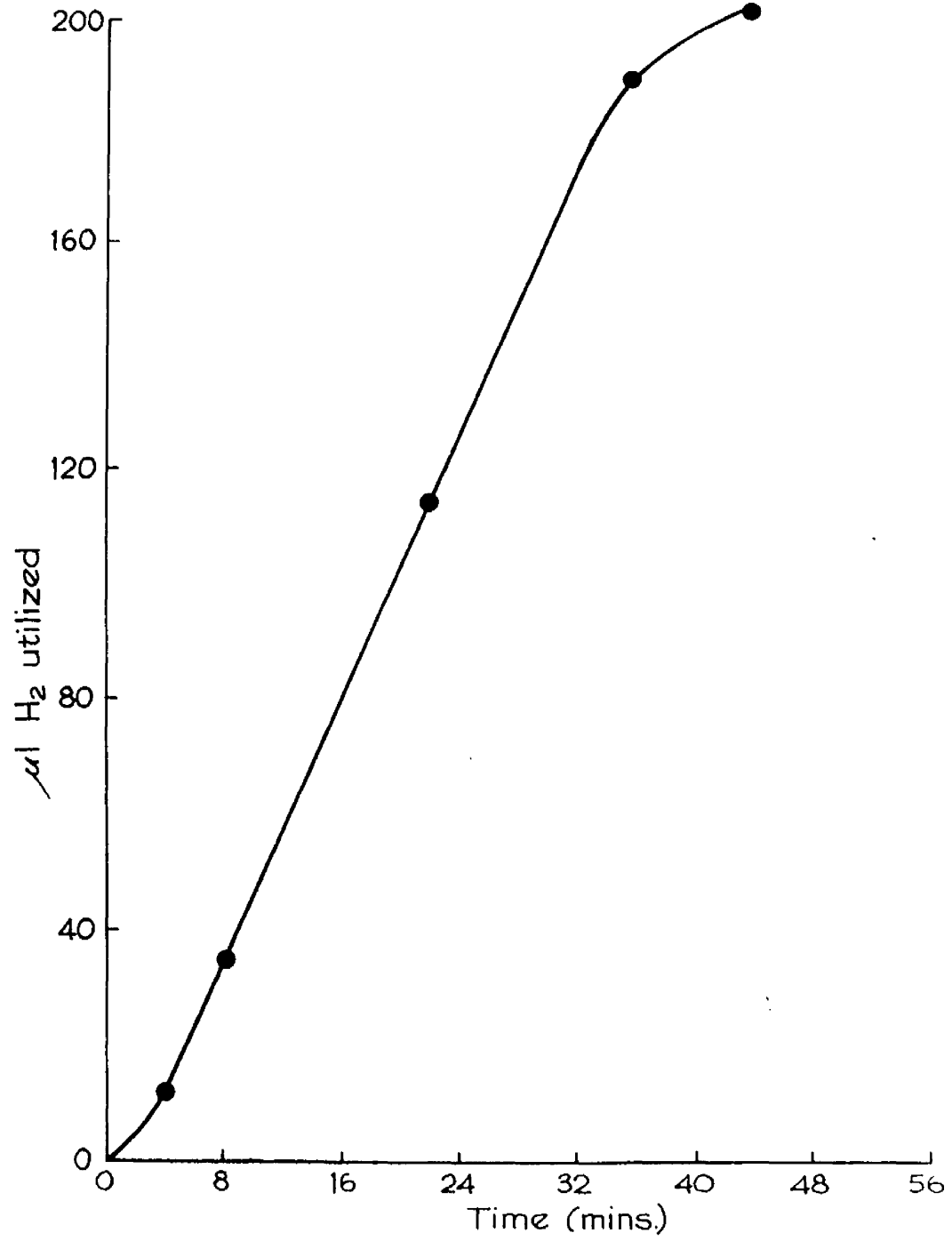
FIG. 12.

Hydrogenase of *Z. ventriculi*.

Protocol: 20 μ moles methylene blue (side arm);
57.7 μ moles K phosphate; 10 mg. (dry wt.) of cells;
pH, 5.0; total volume, 2.8 ml.; 0.2 ml. 80% (w/v) KOH
in the centre well. Atmosphere H_2 .

FIG. 12.

Hydrogenase of *Z. ventriculi*



viologen (Peck and Gest, 1956). Whole cells of Z. ventriculi did not appear to possess this activity.

4. PYRUVATE METABOLISM.

In the early stages of the investigation of the intermediary carbohydrate metabolism of Z. ventriculi, it was found that pyruvate was not metabolized by intact cells. It seemed likely that this result might be explained in terms of permeability phenomena but, whatever the explanation, this was a serious setback. The central role of pyruvate in intermediary carbohydrate metabolism is now well established. In addition, the results of the isotopic experiments of Milhaud et al. with Z. ventriculi suggested that the metabolic route from glucose to pyruvate was by the known reactions of Embden-Meyerhof glycolysis. The pathway or pathways of product formation from pyruvate were therefore of particular interest. Consequently some means had to be found of circumventing the apparent impermeability of pyruvate. Unfortunately Z. ventriculi proved refractory to most of the methods commonly used for obtaining cell-free extracts of bacteria. Most of the methods investigated which are believed to alter the permeability of bacterial cells were also unsatisfactory. These difficulties have been more fully discussed elsewhere (II, 4.).

Z. ventriculi has been shown to possess a formic hydrogen-lyase system (III, 3.1.). In E. coli this system has been

shown to be adaptive (Stephenson and Stickland, 1933). If this be the case with Z. ventriculi, for cells grown on glucose to possess the lyase system, formic acid would have to be an intermediate. The fact that previous workers have found small amounts of formic acid in their analyses of fermentation products also supports this argument (Smit, 1930; Milhaud et al., 1956). The coliform-type of phosphoroclastic fission of pyruvate is the only known reaction which yields formate from pyruvate. It seemed reasonable therefore, to believe that some such reaction must occur in Z. ventriculi. Early studies on pyruvate metabolism were, in consequence, spent in investigating a possible "phosphoroclastic" fission of pyruvate in Z. ventriculi.

4.1. Pyruvate Carboxylase.

Metabolism of pyruvate was first obtained with toluene-treated cells of Z. ventriculi. When the bicarbonate assay of Kalnitsky and Werkman (1943), described by Strecker (1955), was used to detect "phosphoroclastic" activity, no activity was found. Gas was evolved from pyruvate however, in the absence of bicarbonate, and was found to consist entirely of CO_2 (Fig. 13.).

The activity of toluene-treated cells towards glucose, pyruvate and formate was investigated. On one occasion formate

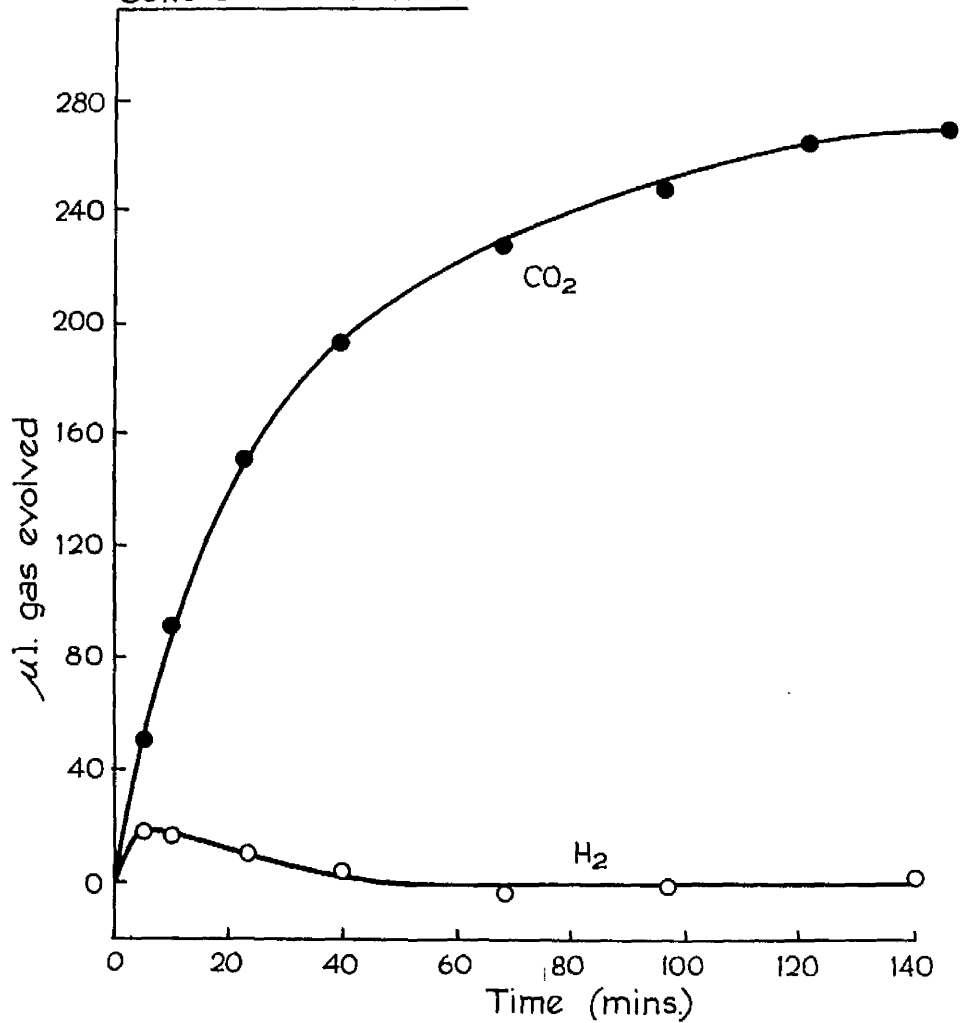
FIG. 13.

Fermentation of Pyruvic Acid by Toluene-treated
cells of *Z. ventriculi*.

Protocol: 20 μ moles pyruvate (side arm); 130 μ moles
Na citrate; 1.0 ml. of toluene-treated cells; pH, 6.0;
0.2 ml. 20% (w/v) KOH in the centre well as indicated.
Atmosphere N_2 .

FIG. 13.

Fermentation of Pyruvic Acid by Toluene-treated
Cells of *Z. ventriculi*.



was found to be metabolized by such a preparation (Fig. 14.). This was a particularly interesting result since even in this case, where the formic hydrogenlyase system is obviously present, no H_2 was produced from pyruvate. This suggested that if Z. ventriculi possessed a "phosphoroclastic" reaction, this system, like the hydrogenlyase system, must be an extremely labile one. All subsequent work has confirmed this impression. No gas production was obtained from glucose under these conditions, although CO_2 was actively produced from pyruvate. This finding suggested that there is at least one labile reaction in the chain of reactions between glucose and pyruvate.

A carbon balance for the fermentation of pyruvate by toluene-treated cells was obtained (Table 13). The results demonstrated that the evolution of CO_2 could be explained in terms of a predominant pyruvic carboxylase reaction. In the absence of KOH the major products were CO_2 and acetaldehyde, typical of a yeast-type carboxylase. Small amounts of acetyl-methylcarbinol (acetoin) were also formed. This appears to be characteristic of the pyruvic carboxylase reaction (Green et al., 1941; Gibbs and DeMoss, 1951; King and Cheldelin, 1954). Although Juni (1952) has failed to separate the acetoin-forming mechanism from carboxylase in a purified system from yeast, Gibbs and DeMoss (1951) obtained carboxylase free of the acetoin-forming system with preparations of

FIG. 14.Action of Toluene-treated Cells of *Z. ventriculi*
on Glucose, Pyruvate and Formate.

Protocol: 20 μ moles pyruvate, 20 μ moles formate or
5 μ moles glucose as indicated (side arm); 150 μ moles
Na citrate; 1.0 ml. of toluene-treated cells; pH, 6.0;
0.2 ml. of 20% (w/v) KOH in the centre well as indicated.
Atmosphere N_2 .

FIG. 14.

Action of Toluene-treated Cells of *Z. ventriculi*
on Glucose, Pyruvate and Formate.

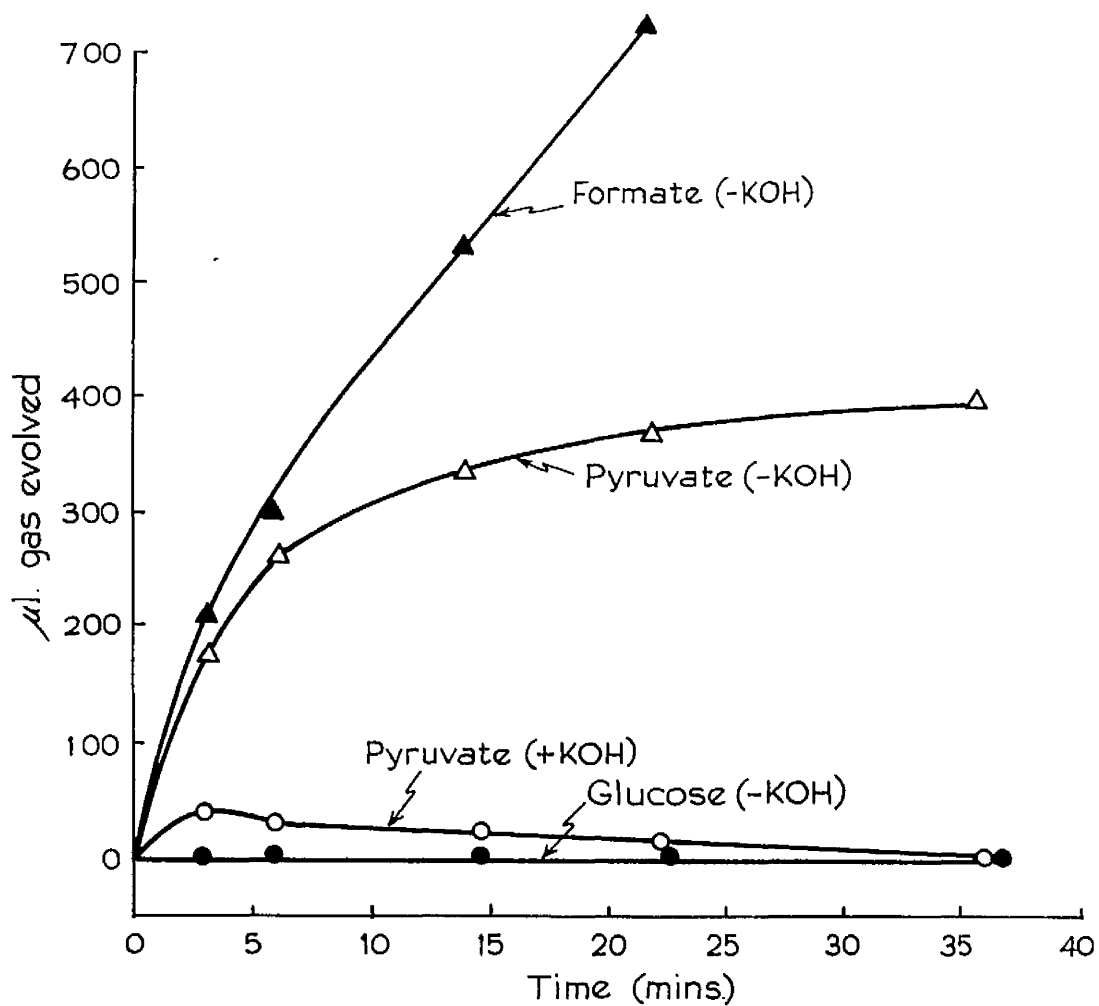


TABLE 13.

Fermentation of Pyruvic Acid by a Toluene-treated
preparation of *Z. ventriculi*.

Protocol: As for Fig. 13 except that 50 μ moles
pyruvate were used.

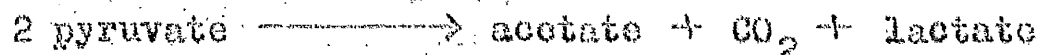
TABLE 13.

Fermentation of Pyruvic Acid by a Toluene-treated preparation of *Z. ventriculi*.

	KOH absent		KOH present	
	μ moles	moles/mole pyruvate	μ moles	moles/mole pyruvate
Pyruvate utilized	32.2	-	32.2	-
CO ₂	27.4	0.85	-	-
Acetaldehyde	21.5	0.66	4.3	0.13
Acetylmethylcarbinol	1.4	0.04	0.6	0.02
Volatile Acid	3.9	0.12	1.4	0.04
Lactic Acid	4.6	0.14	7.1	0.22

Ps. lindneri.

The finding of small equimolar amounts of acetate and lactate in the carbon balance suggests that the Krebs' dismutation reaction (Krebs, 1937) may operate in these preparations.



By subtracting the amount of CO₂ equivalent to the acetaldehyde and acetoin produced from the total CO₂ evolved, the resulting value for CO₂ was equimolar with the values for acetate and lactate found. This agrees with the above equation. Small equimolar amounts of lactate and acetate were also formed from pyruvate by cell-free extracts obtained by grinding with glass powder. (5.2 μmoles lactate and 6.0 μmoles acetate were produced from 36.0 μmoles pyruvate at pH 7.0).

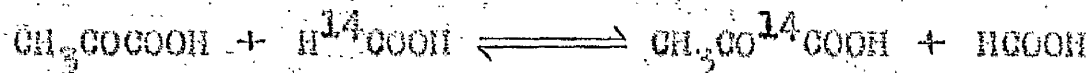
In the presence of KOH a complete carbon balance was not obtained. As previously found (Fig. 13.) no gas was produced. A material which was believed to be aldehyde resin, was formed on the filter paper in the centre well of the Warburg vessel. The recovery of acetaldehyde and other products was reduced under these conditions, which suggested that acetaldehyde was the precursor of these other compounds.

4.1.1. Inhibitor Studies.

The evolution of CO_2 from pyruvate was found to be sensitive to the same inhibitors (Table 14) as a yeast-type carboxylase (Green et al., 1941; King and Cheldelin, 1954). Heavy metals and inhibitors of sulphhydryl groups (p-chloro-mercuribenzoate) were typically inhibitory at low concentrations, as well as acetaldehyde and formaldehyde at somewhat higher concentrations.

4.2. The Pyruvate Elastic Reaction.

All attempts to demonstrate a elastic reaction of pyruvate in Z. ventriculi were unsuccessful using the bicarbonate assay technique of Kalnitsky and Werkman (1943), which depends on the production of two equivalents of acid from one equivalent of pyruvate. As indirect evidence suggested that such a mechanism might exist in Z. ventriculi, attempts were made to secure evidence of the "exchange" reaction, a characteristic property of the coliform-type of phosphoclastic fission.



It was believed that this property might constitute a more sensitive method for detecting the elastic reaction. The system used is given in the protocol to Table 17. All

TABLE 14.Inhibition of Pyruvic Carboxylase of *Z. ventriculi*.

Protocol: As for Table 13 except that inhibitors were added at the concentrations indicated. The duration of the experiment was 30 minutes, by which time 4.2 μ moles CO_2 were evolved from pyruvate in the absence of an inhibitor.

TABLE 14.

Inhibition of Pyruvic Carboxylase of *Z. ventriculi*.

Inhibitor	Concentration	Inhibition of CO ₂ production.
	mm	per cent
CuSO ₄	0.36	52
AgNO ₃	0.2	30
AgNO ₃	2.0	95
Hg(NO ₃) ₂	0.2	100
p-Chloromercuribenzoate	0.36	100
Acetaldehyde	20.0	48
Formaldehyde	80.0	100

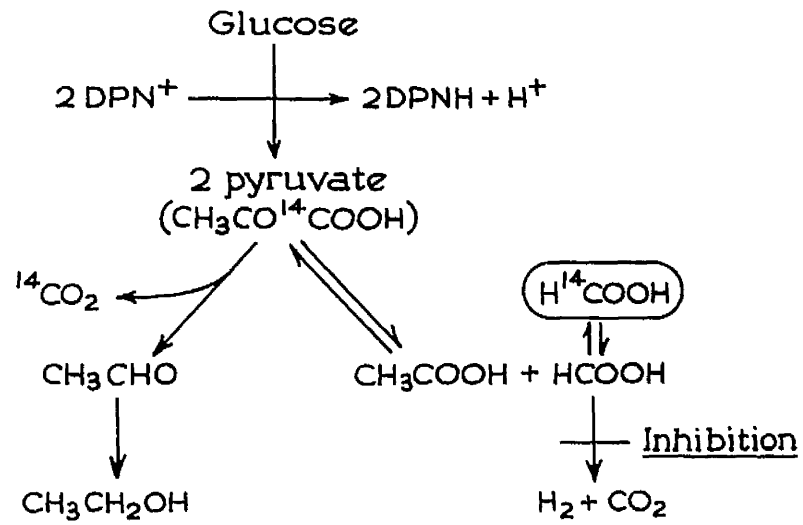
attempts to obtain evidence for the exchange reaction were unsuccessful using toluene-treated cells and cells treated in Hughes' press (Hughes, 1951).

4.2.1. Selective Inhibition.

The failure to obtain direct evidence for a clastic reaction of pyruvate led to the development of an indirect approach to the problem. Since Z. ventriculi had been shown to possess a pyruvate carboxylase, and was believed to possess a pyruvate clastic reaction, the following method seemed worth investigating. If formic hydrogenlyase could be completely inhibited, the fermentation of glucose in the presence of added H^{14}COOH might lead to $^{14}\text{CO}_2$ production via the "exchange" reaction and pyruvic carboxylase (Fig. 15). The production of $^{14}\text{CO}_2$ under conditions where formic hydrogenlyase was completely inhibited would thus appear to constitute clear evidence for pyruvate- H^{14}COOH exchange. Although in theory this seemed a feasible approach, in practice the conditions necessary for its execution could not be easily achieved. The main difficulties were that formic hydrogenlyase had to be inhibited completely without appreciable inhibition of any of the other reactions. As the reactions involved included the pathway from glucose to pyruvate together with the terminal reactions shown in Fig. 15, the complete system was a complex one.

FIG. 15.

Proposed method for obtaining evidence of pyruvate - $H^{14}COOH$ exchange in *Z. ventriculi* by inhibiting Formic Hydrogenlyase.



Experiments using nitrate as inhibitor of the hydrogenlyase system produced results which appeared to suggest that complete diversion of glucose fermentation to the carboxylase pathway had been achieved (Fig. 16.). Two moles of CO_2 were produced per mole of glucose fermented and no H_2 was evolved. However, when the experiment was repeated with H^{14}COOH , the CO_2 obtained from control flasks which contained H^{14}COOH but no glucose, was as highly labelled as the CO_2 obtained from the test flask containing glucose together with H^{14}COOH . Obviously therefore, complete inhibition of the hydrogenlyase system under these conditions had not been achieved. The result obtained with the fermentation of glucose in the presence of nitrate suggests that nitrate may be acting as a proton acceptor under these conditions. In other words, Z. ventriculi may possess a nitrate reductase system. Smit's claim (1930) that Z. ventriculi does not reduce nitrate therefore requires re-examination.

4.2.2. Washed-Cell Suspensions.

At this point in the investigation of pyruvate metabolism in Z. ventriculi an enzymic preparation "Suc digestif" from Helix pomatia, was obtained. This preparation had been used successfully for removing the cell wall of yeast (Eddy and Williamson, 1957) and Neurospora crassa (Bachmann and Bonner,

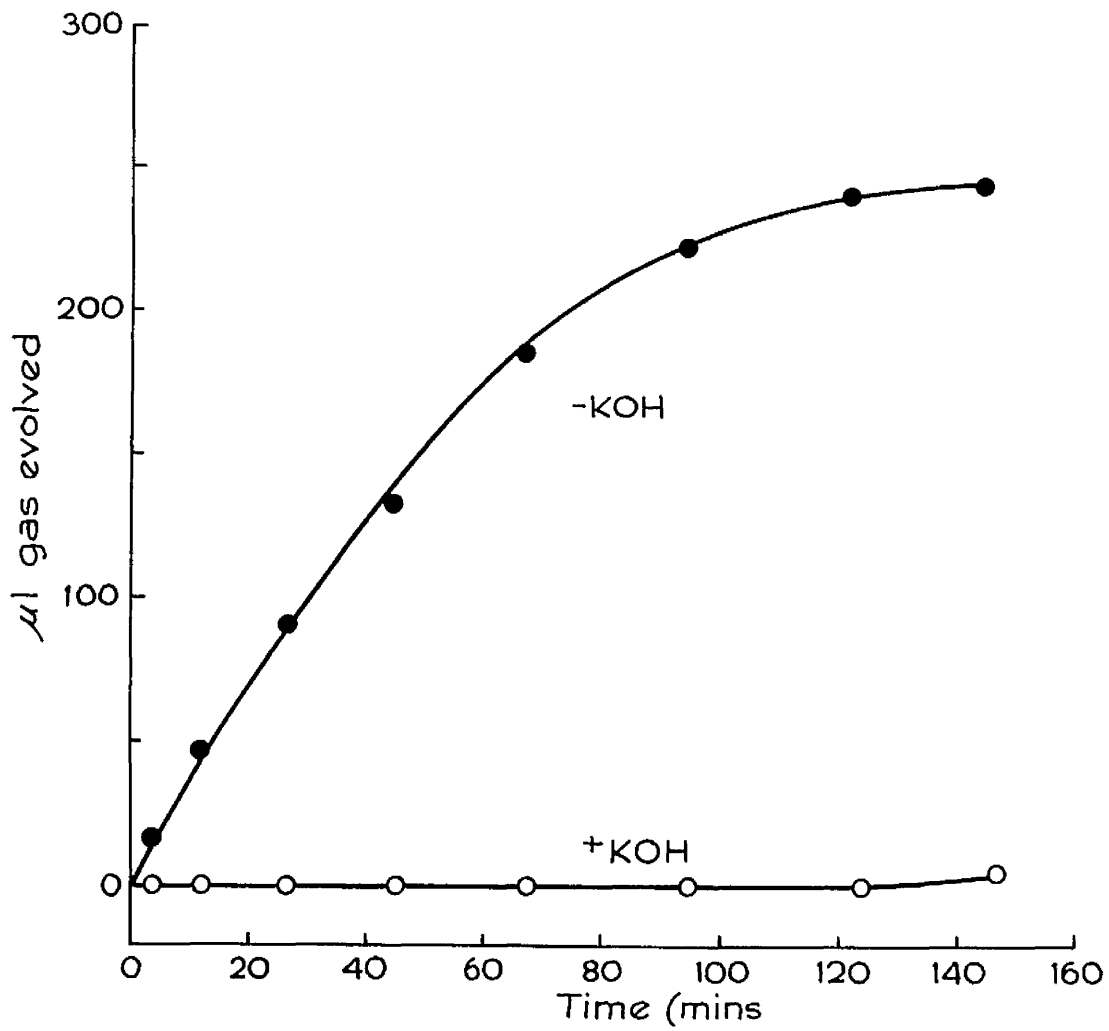
FIG. 16.

Effect of NaNO_2 on the evolution of gas from Glucose
by *Z. ventriculi*.

Protocol: 5 μ moles glucose (side arm); 105 μ moles
K phosphate; 12 mg. (dry wt.) of cells; 35 μ moles
Na nitrate; pH, 5.0; 0.2 ml. 20% (w/v) KOH in the
centre well as indicated. Atmosphere N_2 .

FIG. 16.

Effect of NaNO_3 on the evolution of gas from Glucose by *Z. ventriculi*.



1958). "Suc digestif" was equally successful in disrupting cells of Z. ventriculi. Although attempts were made to obtain protoplasts from Z. ventriculi using the system of Eddy and Williamson (1957), no protoplast formation was observed. In the course of an experiment with "suc digestif" where cells of Z. ventriculi were being disrupted in Warburg flasks, it was found that in a control flask which contained whole cells together with pyruvate, but without "suc digestif", pyruvate was fermented. This was an unexpected finding, particularly in view of the many attempts which had previously been made to obtain washed-cell suspensions capable of metabolizing pyruvate. Subsequent work demonstrated that this result was reproducible. Possible explanations are discussed later (III, 4.3.).

The fermentation of pyruvate at pH 7.0 by washed-cell suspensions of Z. ventriculi appears to be a complex one (Table 15); a complete carbon balance has not been obtained. However, the evolution of hydrogen together with the production of large amounts of volatile (acetic) acid suggests that the major pathway under these conditions is a clastic fission of pyruvate. The fact that no acetaldehyde was found suggests that carboxylase does not operate under these conditions. This belief is supported by the findings of Barron *et al.* (1951) and Steppani *et al.* (1959), who demonstrated

TABLE 15.

Fermentation of Pyruvate by Whole Cells of

Z. ventriculi at pH 7.0.

Protocol: 53 μ moles pyruvate (side arm); 105 μ moles
K phosphate; 10 mg. (dry wt.) of cells; pH, 7.0;
0.2 ml. of 20% (w/v) KOH in the centre well as
indicated; total volume, 2.8 ml. Atmosphere N₂.

TABLE 15.

Fermentation of Pyruvate by Whole Cells
of *Z. ventriculi* at pH 7.0.

	KOH present		KOH absent	
	μ moles	moles/mole pyruvate	μ moles	moles/mole pyruvate
Pyruvate utilized	53	-	38	-
Gas evolved	17.5	0.53	53	1.4
Volatile Acid produced	22	0.67	26	0.68
Lactate produced	0.3	0.01	0.3	0.01
Acetaldehyde produced	0	-	0	-

that pyruvate was not metabolized via carboxylase, under anaerobic conditions, by whole cells of yeast. The results of Barron et al. (1951) were explained essentially in terms of permeability phenomena. Since the ratio of gas evolved in the absence and presence of KOH approximates to 3, it seemed that the results might be explained in terms of combined elastic and dismutation reactions. However, the small amount of lactate found eliminated this possibility. At pH 7.0 therefore, pyruvate appears to be metabolized by washed-cell suspensions of Z. ventriculi via a elastic mechanism together with some unknown reaction.

By decreasing the pH of the reaction mixture to 4.9, the fermentation of pyruvate by washed-cell suspensions was found to be considerably simplified. The first results obtained at pH 4.9 are shown in Table 16.1. The fermentation of one mole of pyruvate yielded one mole each of acetate, CO₂ and H₂. This was the first clear demonstration of a elastic reaction of pyruvate in Z. ventriculi. The results can obviously be explained solely in terms of the metabolism of pyruvate by a phosphoclastic pathway. Since this organism has been shown to possess a formic hydrogenlyase system, the mechanism might reasonably be supposed to entail the elastic fission of pyruvate to acetate and formate, followed by the conversion of the latter to CO₂ and H₂ by

TABLE 16.

Fermentation of Pyruvate by Whole Cells of
Z. ventriculi at pH 4.9.

Protocol: As for Table 15 except that the pH was 4.9.

TABLE 16.

Fermentation of Pyruvate by Whole Cells of
Z. ventriculi at pH 4.9.

	KOH present		KOH absent	
	µmoles	moles/mole pyruvate	µmoles	moles/mole pyruvate
1. Pyruvate utilized	17.5	-	15.5	-
Gas evolved	16.0	0.92	30.5	1.97
Volatile Acid produced	18.7	1.07	15.8	1.02
2. Pyruvate utilized	21.0	-	7.0	-
Gas evolved	20.3	0.97	15.7	2.24
Volatile Acid produced	19.4	0.92	7.5	1.07

the hydrogenlyase system. However, a rigid demonstration of formate participation in this system is desirable. In the clostridial elastic fission of pyruvate to acetate, CO_2 and H_2 , formic acid is not an intermediate and the cells have no formic hydrogenlyase activity.

In this first experiment, equimolar amounts of pyruvate were utilized in the absence and presence of KOH, and H_2 and CO_2 were produced at approximately equal rates (Fig. 17). Subsequent work demonstrated that this was not always the case (Table 16.2.). Essentially, the fermentation pattern was similar, in that one mole of pyruvate produced one mole each of acetate, CO_2 and H_2 . However, one important difference emerged; in the absence of KOH much smaller amounts of pyruvate were fermented. Since other conditions were identical, it appeared that the removal of a reaction product, viz., CO_2 by the KOH, allowed the reaction to proceed further to completion. In addition, a complete fermentation balance was obtained in the absence of KOH. The results, therefore, could not be explained in terms of the accumulation of some intermediate. These facts strongly suggest that the complete elastic system, from pyruvate to CO_2 and H_2 , is reversible.

4.2.3. Isotopic Studies.

The successful demonstration of a elastic reaction

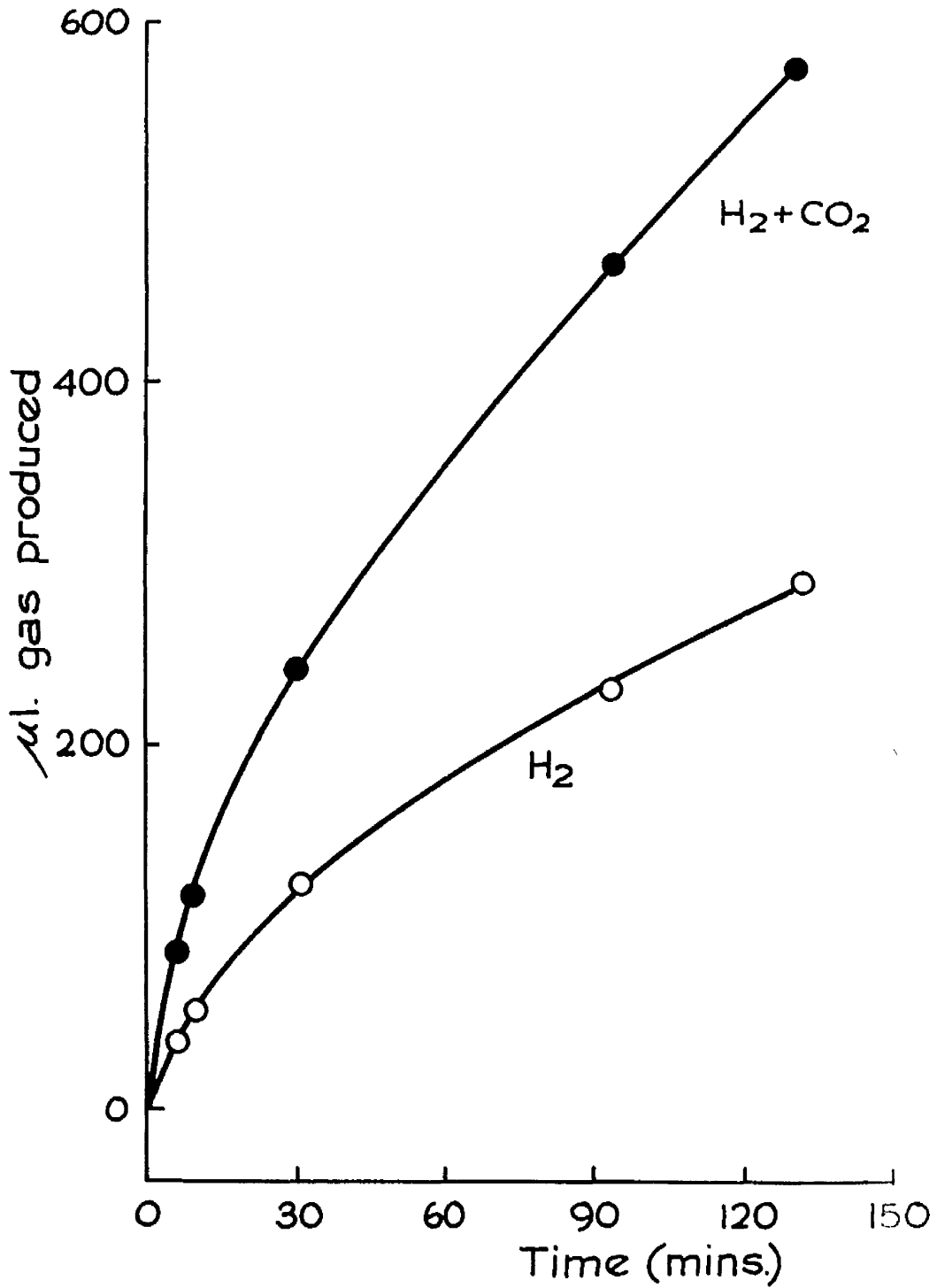
FIG. 17.

Fermentation of Pyruvate by Whole Cells of
Z. ventriculi at pH 4.9.

Protocol: As for Table 15 except that the pH was 4.9.

FIG. 17.

Fermentation of Pyruvate by
Z. ventriculi at pH 4.9.



operating as the sole pathway of pyruvate metabolism at pH 4.9 suggested the possibility of obtaining the pyruvate- $H^{14}COOH$ exchange reaction with whole cells. If this reaction could be demonstrated, it would constitute strong evidence for the functioning of the coliform-type of elastic fission. In order to demonstrate the exchange reaction under these conditions it was thought desirable to inhibit the formic hydrogenlyase system in some way. If this were not done, the results might be criticized on the grounds that $^{14}CO_2$, liberated from $H^{14}COOH$ by the hydrogenlyase system, might find its way into pyruvate by some other route, such as CO_2 -fixation reactions and the Wood-Werkman mechanism.

The first method used to inactivate the hydrogenlyase system was simply to wash cells of Z. ventriculi with phosphate buffer which had not been freed of atmospheric oxygen. This procedure succeeded in abolishing the hydrogenlyase activity of the cells. Unfortunately, the ability of the cells to metabolize pyruvate was also lost. This result demonstrated that the elastic system was extremely labile, which explained the failure to demonstrate its presence in all extracts and "treated" cells.

A number of objections can be raised against the use of the other inhibitors of the formic hydrogenlyase system in the demonstration of the exchange reaction under these

conditions. It seemed undesirable to use cyanide, since the cyanide ions might complex with pyruvate, thus preventing at least some of the pyruvate from participating in the exchange reaction. Carbon monoxide did not always produce the complete inhibition required. Nitrite, which seemed the most promising inhibitor for this purpose, was found to interfere with the formation of the 2,4-dinitrophenylhydrazone used for isolation of the pyruvate at the end of the reaction. Although this is a difficulty which can obviously be circumvented in some way it has not yet been further pursued.

In view of these difficulties it was decided, while appreciating the possibility of $^{14}\text{CO}_2$ incorporation into pyruvate, to see if any labelled carbon was in fact incorporated into pyruvate from H^{14}COOH .

The system used consisted of 50 μmoles pyruvate and 50 μmoles H^{14}COOH . Under these conditions 100% exchange would produce pyruvate with a specific activity (counts/minute/ μmole) one half of the specific activity of the added formate. In early experiments, where inhibitors of the hydrogenlyase system had been used, KOH was included in the centre well of the Warburg vessel. This enabled a check to be made for the presence of $^{14}\text{CO}_2$ evolution via formic hydrogenlyase. Obviously, under these conditions

the expression of results in the form of a percentage of theoretical exchange was meaningless since, as in fact happened, $^{14}\text{CO}_2$ was evolved, and this removed part of the carbon from the reaction. Even in these experiments however, the incorporation of small amounts of ^{14}C into pyruvate was obtained.

With whole cells of Z. ventriculi at pH 4.9, and without KOH in the system, 0.8% of the theoretical exchange was obtained with H^{14}COOH (Table 17). In an experiment carried out in the presence of cyanide, before the objections to cyanide were realized, all of the counts were found to be in the carboxyl group of recovered pyruvate (Table 18). Although the pH of the buffer used was 4.9, and any $^{14}\text{CO}_2$ would be evolved from the medium immediately under these conditions, this pH does not necessarily represent the internal pH of the cell. (The fact that Z. ventriculi ferments glucose at extremely low pH values suggests that this organism possesses a highly efficient internal pH regulating mechanism.) In view of this consideration, and the small amount of exchange obtained, it seemed possible that this might have occurred by some CO_2 -fixation mechanism within the cell, as formic hydrogenlyase was active in those preparations.

In order to test the possibility of $^{14}\text{CO}_2$ -pyruvate

TABLE 17.

Incorporation of Labelled Carbon into Pyruvate
from $H^{14}COONa$ and $NaH^{14}CO_3$ with whole cells
and extracts of *Z. ventriculi*.

Protocol: 50 μ moles pyruvate (1st side arm);
50 μ moles $H^{14}COONa$ or $NaH^{14}CO_3$ (2nd side arm);
140 μ moles K phosphate; 10 mg. (dry wt.) of cells
or 1.0 ml. extract (0.5 mg. μ /ml.); pH, 4.9 or 7.0;
total volume, 5.8 ml. Atmosphere N_2 . Pyruvate and
 $H^{14}COONa$, or $NaH^{14}CO_3$, were tipped together and the
reaction allowed to continue for 90 minutes.

TABLE 17.

Incorporation of labelled Carbon into Pyruvate
 from $\text{H}^{14}\text{COONa}$ and $\text{NaH}^{14}\text{CO}_3$ with whole cells
 and extracts of *Z. ventriculi*.

	$^{14}\text{CO}_2$ -Pyruvate	H^{14}COOH -Pyruvate		
	Whole Cells	Whole Cells		Extracts
	pH 7.0	pH 4.9	pH 7.0	pH 7.0
Specific Activity of recovered pyruvate (c.p.m. / μmole)	1618	17	156	0
$\text{H}^{14}\text{COONa}$ added (c.p.m. / μmole)	—	4250	4250	4250
$\text{NaH}^{14}\text{CO}_3$ added (c.p.m. / μmole)	4200	—	—	—
Per cent of theoretical exchange	67.4	0.8	7.3	0

TABLE 18.

Incorporation of Labelled Carbon into Pyruvate
from H¹⁴COONa with *Z. ventriculi* at pH 4.9.

Protocol: 50 μ moles pyruvate (1st side arm); 50 μ moles
H¹⁴COONa (2nd side arm); 105 μ moles K phosphate; 53.2
 μ moles K cyanide; pH, 4.9; total volume, 3.8 ml.; 0.2 ml.
20% (w/v) KOH in the centre well. Atmosphere N₂.
Pyruvate and formate were tipped together and the reaction
allowed to continue for 120 minutes.

TABLE 18.

Incorporation of Labelled Carbon into Pyruvate
from H^{14}COOH with Z. ventriculi at pH 4.9.

	cpm/ μmole
Specific Activity of recovered pyruvate.	70
Specific Activity of recovered pyruvate COOH.	65
H^{14}COOH added.	4290

exchange a similar experiment was carried out at pH 7.0 where $^{14}\text{CO}_2$ -pyruvate and H^{14}COOH -pyruvate exchange were compared (Table 17). The incorporation of H^{14}COOH into pyruvate was nine times greater at pH 7.0 than at pH 4.9. This result cast further doubts on whether the observed incorporation was a genuine H^{14}COOH -pyruvate exchange. It was possible however, that the results might be explained in terms of the pH optimum of the exchange reaction in Z. ventriculi. However, the large extent of $^{14}\text{CO}_2$ -pyruvate exchange obtained at pH 7.0 with whole cells (Table 17) greatly weakened any argument for a genuine H^{14}COOH -pyruvate exchange under these conditions. In addition, the high value obtained for the $^{14}\text{CO}_2$ -pyruvate exchange compared with the value obtained for the H^{14}COOH -pyruvate exchange precluded the possibility that $^{14}\text{CO}_2$ -pyruvate exchange was occurring via hydrogenlyase and the clastic system. It follows therefore, that Z. ventriculi must possess some carboxylation mechanism. Since the yeast-type carboxylase is not normally reversible (Ochoa, 1951), and as in any case carboxylase does not appear to operate in Z. ventriculi under these conditions, this carboxylation must be an additional mechanism of pyruvate metabolism possessed by this organism. Possible mechanisms to explain these results are discussed later (III, 6.1.2.).

Further support for the concept that the $^{14}\text{CO}_2$ -pyruvate

exchange reaction was a mechanism distinct from the hydrogen-lyase-clastic system was afforded by results obtained with extracts of Z. ventriculi, prepared by grinding with glass powder. With such preparations pyruvate isolated from the $^{14}\text{CO}_2$ -pyruvate system was found to be highly radioactive. Unfortunately, the amount of pyruvate remaining at the end of the experiment was so small that it could not be isolated by the normal techniques. Instead it was isolated chromatographically, eluted and then counted in the normal way. However, the amount obtained was so small that an accurate specific activity could not be obtained. In an experiment with a similar preparation, the pyruvate isolated from the H^{14}COOH -pyruvate system was found to be unlabelled (Table 17).

4.3. Growth Experiment.

Although the pyruvate clastic reaction in Z. ventriculi had been found to be extremely labile, both in whole cells, "treated cells" (II, 4.1.) and cell-free extracts, it was felt that this explanation of the earlier failures to detect the system in whole cells was not entirely satisfactory. In earlier experiments pyruvate was tested on various occasions at the same time as glucose, and although the cells actively metabolized glucose to produce H_2 and CO_2 , pyruvate was not utilized. One possible explanation of the results seemed to be that the fermentative activity might vary at different

stages of the growth cycle. An experiment to investigate this possibility was carried out using the growth apparatus of Dawes and Holms (1956). The method used in this experiment has been previously described (II, 1.3.) and the results are shown in Fig. 18 and Table 19.

As can be seen from Fig. 18, enzymic activity towards glucose, pyruvate and formate declined steadily throughout the growth cycle. The rapid production of hydrogen from pyruvate occurred only at an early stage in the growth cycle, although rapid production of CO_2 from pyruvate appeared to continue until a later stage. This effect can be seen most clearly in Table 19. Nevertheless, the production of total gas from pyruvate was always much lower than the production of hydrogen from glucose. This finding strongly suggested that the permeability of the cells towards pyruvate may be a controlling factor here. This concept is supported by the finding that, although under certain conditions whole cells of Z. ventriculi metabolize pyruvate, this does not appear to occur via carboxylase. Carboxylase has been shown to be present by treating cells with toluene, which suggests that there are permeability factors preventing the operation of carboxylase in whole cells with pyruvate as substrate. This argument is supported by the evidence obtained by Barron et al. (1951) and Stoppani et al. (1959) with yeast. It does not

FIG. 18 and TABLE 19.Enzymic Activities at Different Stages during
the Growth Cycle of *Z. ventriculi*.

Protocol: 35 μ moles pyruvate, 10 μ moles glucose or
20 μ moles formate (side arm); 105 μ moles K phosphate;
cells, dry wt. as shown; pH, 4.9; total volume, 2.8 ml.;
0.2 ml. 20% (w/v) KOH in the centre well as indicated.
Atmosphere N_2 .

FIG. 18.

Enzymic Activities at Different Stages during the Growth Cycle of *Z. ventriculi*.

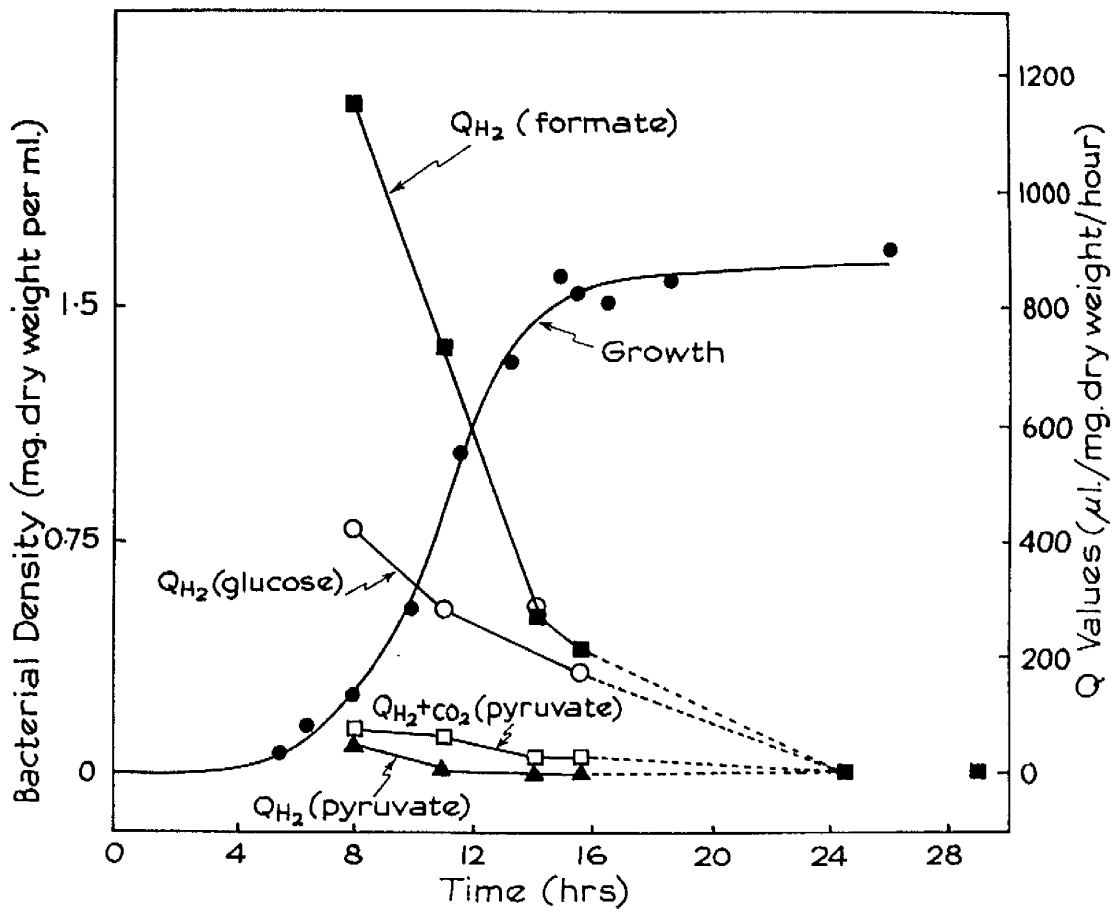


TABLE 19.

Enzymic Activities at Different Stages during
the Growth Cycle of *Z. ventriculi*.

Time of Growth (Hours)	Glucose	Formate	Pyruvate	
	Q_{H_2}	Q_{H_2}	$Q_{H_2 + CO_2}$	Q_{H_2}
8.0	420	1156	67	50
11.0	283	726	60	8
14.0	266	275	19	5
15.5	169	203	16	6
24.5	0	0	0	0
29.0	0	0	0	0

soon impossible then, that under conditions different from the above and where whole cells metabolize pyruvate via a clastic reaction, that permeability phenomena may also be a controlling factor in the operation of this clastic pathway. The operation of such a mechanism during the later stages of the growth cycle could explain the failure of whole cells to produce hydrogen from pyruvate except at an early phase of the growth cycle. This fact might also explain early failures to detect gas production from pyruvate. However, pyruvate is metabolized during a large part of the growth cycle, apparently by some unknown mechanism, which was previously mentioned (III, 4.2.2.). No metabolism of pyruvate was ever detected in the early metabolic studies on Z. ventriculi where whole cells were used. At present no adequate explanation of these early failures to detect metabolic activity towards pyruvate can be put forward. At this point it seems worth while to consider however, what these results may actually mean in terms of the normal growth of Z. ventriculi. It may be remembered that during this experiment the medium was efficiently mixed by bubbling N_2 through the growth vessel. There can be no doubt that this thorough mixing greatly accelerated the growth rate. The stationary phase was reached after approximately 16 to 20 hours. In the normal "bottom" fermentation of Z. ventriculi

growth continues for 2 to 3 days. The minimum adjustment in our assessment of these results therefore necessitates an alteration of the time scale. It seems possible however, that there may be more fundamental differences between the cells grown by these two methods. This information can only be obtained by further experiments. Nevertheless from this experiment the concept has emerged that the permeability factors operating in the metabolism of pyruvate may be complex. It appears that the elastic system as well as the carboxylase reaction may be controlled in this manner.

It must be emphasized that these mechanisms are not believed to be operative in controlling the normal fermentation of glucose where pyruvate could be produced as an endogenous metabolite, and the problem of its passage from the exterior to the interior of the cell does not arise.

5. BACTERIOLOGY.

As has already been mentioned, the present work has been primarily concerned with biochemical studies of Z. ventriculi. However a few observations have been made which seemed worthy of note but did not fall into this category. They have therefore been included in the present section under the above heading.

5.1. Preservation of Cultures.

To preserve a culture of Z. ventriculi it is normally necessary to subculture while the organism is actively fermenting. However, some workers (Smit, 1930; Koch, 1948; Canale-Parola and Wolfe, 1959) have succeeded in preserving cultures of Z. ventriculi for longer periods of time than was normally possible. These methods have already been described (1, 5.). The method used by Koch (1948) appears to be most successful; unfortunately this work came to our notice only recently, and has not yet been investigated. Nevertheless a number of attempts were made to preserve cultures of Z. ventriculi. These were as follows:

Shake cultures of Z. ventriculi were prepared in 2% (w/v) agar, 2% (w/v) glucose, 1% (w/v) peptone and 1% (w/v) yeast extract with 0.1 ml. inoculum. Cotton-wool plugs were pushed down into the test-tubes, followed by a plug of

absorbent cotton wool. Five drops of 10% (w/v) pyrogallol and five drops of 15% (v/v) Na_2CO_3 were pipetted into each of the tubes and the tubes sealed with rubber bungs. The shake cultures were incubated overnight at 37° when growth occurred, and were then stored at 0° for varying lengths of time. After one week the contents of one of these tubes was inoculated into freshly boiled and cooled "acid" medium. No growth was ever obtained by these methods.

In the next experiment, instead of using a small inoculum in the inoculation of the nutrient agar, 1.0 ml. of concentrated cell suspension was pipetted into the tubes. This cell suspension was obtained by pouring off the medium from an actively fermenting culture. The cells remaining on the bottom of the flask were stirred to yield a thick suspension. The tubes were sealed with rubber bungs as before, but instead of incubating at 37° , they were immediately stored at 0° . By this technique active cultures were obtained up to fourteen days later. This is the longest period of time for which we have been able to preserve cultures of Z. ventriculi.

5.2. Clinical Investigation.

It was of considerable interest to ascertain whether Z. ventriculi could be found in the stomach of patients displaying the symptoms recorded by Goodsir (1942), Hassel

(1933) and Smit (1930). In addition, it seemed that it would be useful to compare biochemically an organism isolated from the stomach with the soil organism. To date, the gastric contents of five patients suffering from pyloric stenosis have been examined. In the fourth gastric sample examined microscopically Z. ventriculi was found in great abundance. Three samples in all were obtained from this patient, but all of them failed to produce an actively fermenting culture of Z. ventriculi when inoculated into appropriate media. Unfortunately, it was not possible to carry out Beijerinck's technique of syphoning gastric contents directly into the culture medium. Beijerinck therefore still remains the only worker who has succeeded in isolating an active culture of Z. ventriculi from gastric contents.

This particular patient, whose stomach contents were found to contain Z. ventriculi, was suffering from severe pyloric stenosis caused by a large carcinoma. The conditions for the finding of Z. ventriculi were classical in that food would be held back for abnormally long periods under these conditions. Smit (1933) had claimed that the organism was never found in the stomach in cases where the pyloric stenosis was caused by cancer. This observation has therefore been found to be incorrect.

The occurrence of Z. ventriculi in clinical conditions

appears to be less common to-day than formerly. This can be adequately explained in terms of the advancement of medical skill, both diagnostic and surgical. The occurrence of many cases of severe pyloric stenosis is presumably prevented by early diagnosis of the symptoms, and by prompt surgical action where necessary.

5.3. Morphology.

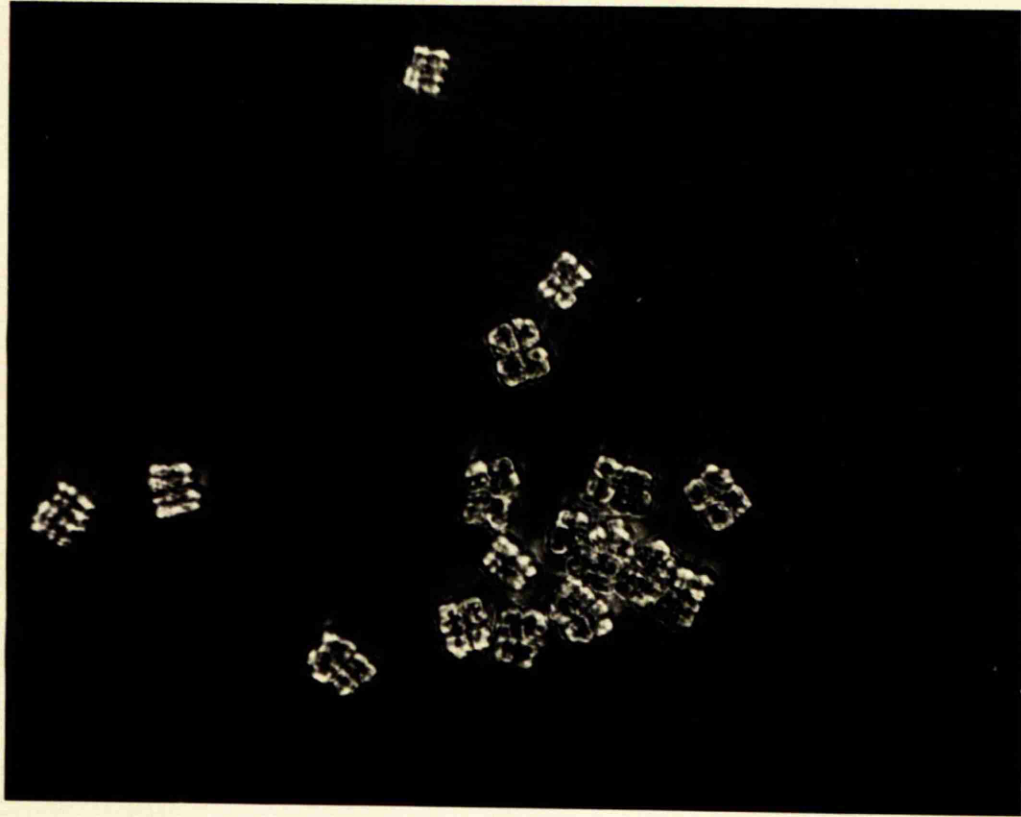
The observations of De Bary (1887) and Beijerinck (1905) on the existence of two types of cells of Z. ventriculi have been confirmed. Cells grown in complex medium at pH 7.0 consisted almost entirely of regular "packets" of opaque cells (Plate 6). Towards the end of the growth cycle however, a number of the "packets" were less regular and appeared to consist of slightly larger transparent cells. Cells grown at pH 3.5 were found to be almost entirely of the transparent type. In the acid conditions in which they found Z. ventriculi, De Bary (1887) and Beijerinck (1905) also observed that the transparent cells predominated. In the present work no comparative measurements of these two forms have yet been made. It may be premature to speculate, but it seems possible that the differences in the appearance of Z. ventriculi may depend on the conditions of growth.

Knoll and Niklowitz (1958) found that Z. ventriculi was surrounded by a layer of mucus. The presence or absence

PLATE 6.

Photomicrograph of *Z. ventriculi* from
an 18 hour culture (phase-contrast).

PLATE 6.



—| |—
40 μ

of such a layer, or the presence of differing amounts of this layer, may explain the varying appearance of the cells. The production of the mucous layer could be controlled by conditions of growth. The production of dextran by Leuconostoc mesenteroides is well known to depend on growth conditions.

5.4. Distribution in Soil.

We have extended the observations of Smit (1930) and Van Niel (1957) that Z. ventriculi occurs widely in soil. The organism has been found in various soil samples from Scotland, England, Newfoundland and Canada. Surprisingly, it was even found in sludge from the bed of Canadian freshwater lakes, although presumably under these conditions, good anaerobic conditions exist.

The problem of how Z. ventriculi can exist in the surface layers of the soil when it is so sensitive to atmospheric oxygen has not been investigated. (It has been found that soil kept under an atmosphere of oxygen could still initiate growth when incubated in the appropriate medium.) Smit (1935) believed that Z. ventriculi must exist in some unknown form in soil; this view has been reiterated by Koch (1948). However, such differences between natural and artificial environments are common, as for example with

the clostridia, and Smit's postulate does not now seem necessary. In spite of this fact, there is no satisfactory explanation of these anomalies. The concept of the "micro-environment" would seem to be equivalent to the "local pockets of anaerobiosis" of Thornton and Meiklejohn (1957). Although this concept seems feasible, it has not been a fruitful one, and would certainly be ruled out by logical positivists. This problem is indeed a difficult one. It may be that the microbiological equivalent of Heisenberg's "principle of indeterminacy" may apply here. This belief is supported by Smit's unsuccessful attempt to retain viability by mixing a pure culture of K. ventriculi with sterilized soil. The theory that strict anaerobes are unable to grow in the presence of atmospheric oxygen because of the production of lethal concentrations of hydrogen peroxide has been discussed by McClung (1956). This theory was advanced by Gordon, Holman and McLeod (1955), and McClung believes that it received much support from the work of Holman (1955). Although this may be true in certain cases, the theory does not explain the results obtained with K. ventriculi in the present work, or with Cl. acetobutylicum (Davies and Stephenson, 1941). In both of these cases, the metabolism of glucose by non-proliferating cells proved to be extremely sensitive to atmospheric oxygen. This fact

can also adequately explain the sensitivity of growing cells to atmospheric oxygen. It is believed that these examples illustrate a more general explanation of the reason for the failure of strict anaerobes to grow in the presence of atmospheric oxygen, namely, the sensitivity to oxygen of enzymic components such as sulphhydryl groups.

In view of the extreme sensitivity of vegetative cells of Z. ventriculi to atmospheric oxygen, it is difficult to imagine its role in its native environment. However, this is true of many micro-organisms, and the subject of microbial ecology is poorly understood (Gibson, 1957). Gibson (1957) has also stated that "it is rare in nature to meet anything even approaching a pure culture..." The occurrence of Z. ventriculi in gastric contents is one of these rarities. It is interesting to speculate that this may constitute another example of an organism finding some environmental "niche" (cf. the rumen) where it can proliferate without interference from other organisms. Due to advances in medical science, this would appear to possess only limited "survival" value.

6. CONCLUSION.

6.1. Embden-Meyerhof Glycolysis.

As Gunsalus et al. (1955) have pointed out, evidence for an Embden-Meyerhof pathway of glucose fermentation in micro-organisms has been based on:

- (a) production and utilization of postulated intermediates;
- (b) the presence of enzymes catalyzing reactions of the Embden-Meyerhof pathway;
- (c) sensitivity of these enzymes to inhibitors effective with muscle and yeast enzymes;
- (d) fermentation of ¹⁴C labelled glucose to products labelled as predicted by Embden-Meyerhof pattern.

With Z. ventriculi all of these criteria have been satisfied. Fructose-1:6-diphosphate and 3-phosphoglycerate have been shown to be metabolized by extracts of Z. ventriculi. The presence of aldolase and triosephosphate dehydrogenase has been demonstrated in extracts, as well as an enzymic pathway from 3-phosphoglycerate to pyruvate. The fermentation of glucose by whole cells of Z. ventriculi was shown to be sensitive to iodoacetate, one of the classical inhibitors of the muscle and yeast fermentation pathways. Milhaud et al. (1956), using glucose labelled in different positions, obtained products labelled as predicted by an Embden-Meyerhof

pattern. Their results also appear to exclude the possibility of any other pathway of glucose fermentation in Z. ventriculi.

The above evidence is conclusive proof that Embden-Meyerhof glycolysis operates in Z. ventriculi.

6.2. Pyruvate Reactions.

Elsden (1952) put forward the thesis that,

"...the primary mechanism found in muscle and yeast, the operation of which results in the formation of pyruvic acid, is found also in those bacteria which ferment carbohydrates, and that such differences in the end products as are observed are due to the ability of bacteria to synthesize a wide range of H acceptors from pyruvic acid."

Although this thesis is no longer generally acceptable, it would seem to apply to the Z. ventriculi fermentation.

Using toluene-treated cells, carboxylase has been demonstrated to be present in Z. ventriculi. The products obtained from the fermentation of pyruvate, together with the evidence from inhibitor studies, indicated that the carboxylase is the yeast-type (Green et al., 1941; Gibbs and Demoss, 1951; King and Cheldelin, 1954). Z. ventriculi is thus one of the few bacteria which possesses an enzyme system of this type.

(Pseudomonas lindneri and Acetobacter suboxydans are the other known cases.)

The presence of a clastic reaction of pyruvate has been demonstrated with washed-cell suspensions of Z. ventriculi. Although direct evidence has not been obtained, the presence of enzymes of formate metabolism, formic hydrogenlyase and formic dehydrogenase, strongly suggests that this reaction is the coliform-type, involving the intermediate formation of formate. Direct proof that the reaction is of the coliform-type depends on the demonstration of a pyruvate-formate reaction. Using radioactive formate, attempts to demonstrate an exchange reaction between pyruvate and formate have so far been unsuccessful. However, the fact that the clostridia do not possess enzymes of formate metabolism indicates that the pyruvate clastic reaction in Z. ventriculi differs from the pyruvate phosphoreclastic reaction in this group of organisms.

The formation of small amounts of lactate from glucose (Smit, 1930) and from pyruvate (III, 4.1.) suggests that Z. ventriculi possesses a lactic dehydrogenase reaction. The production of equimolar amounts of acetate, lactate and CO_2 from pyruvate further suggests that under certain conditions a dismutation reaction can also operate. A possible mechanism for this reaction will be discussed later.

Although no pyruvate- n^{14}COOH exchange reaction was detected, an active pyruvate- $\text{Mn}^{14}\text{CO}_2$ exchange reaction was

found to be present, both in whole-cell preparations and in extracts. It might be argued that these results could be explained in terms of a clostridial-type of pyruvate elastic reaction. In such a system pyruvate-NaH¹⁴CO₃ exchange would be expected, but not pyruvate-H¹⁴COOH exchange. It is believed however, that the presence of enzymes of formate metabolism is sufficient evidence to exclude this possibility. It follows therefore, that some other mechanism must exist for the incorporation of ¹⁴CO₂ into the carboxyl group of pyruvate. The reversibility of the carboxylase reaction would appear to be excluded (Ochoa, 1951). In addition, this system did not appear to operate in whole-cell preparations of Z. ventriculi. For the pyruvate-NaH¹⁴CO₃ exchange to occur by known reactions, there must be formation of some symmetrical compound such as fumarate, by the Good and Werkman reaction (1938, 1940). The occurrence of such a mechanism has not been investigated. In any case, there would appear to be some type of pyruvate-CO₂ reaction present in Z. ventriculi. It is obvious therefore, that a wide variety of reactions of pyruvate can operate in Z. ventriculi.

6.5. Formate Metabolism.

With washed-cell suspensions of Z. ventriculi, formic hydrogenlyase, formic dehydrogenase and hydrogenase were shown to be present. Evidence obtained with inhibitor

studies indicated that the formic hydrogenlyase was similar to the E. coli system (Stephenson and Stickland, 1952).

6.4. Fermentation of Glucose.

One of the intriguing features of the Z. ventriculi fermentation is the discrepancy in the carbon balances for the fermentation obtained by earlier workers (Tables 4, 5 and 6). The main features of these fermentation balances are compared in Table 20. As can be seen, Smit, Kluyver and Milhaud et al. all found approximately 2 moles of CO_2 per mole of glucose fermented. Whereas Smit and Kluyver found ethanol to be the other major product, Milhaud et al. found equal amounts of ethanol and acetate together with almost 2 moles of hydrogen per mole of glucose. Smit and Kluyver observed only minor amounts of acetate and hydrogen, and Kluyver (1931) stated:

"I cannot resist the temptation to emphasize the fact that we are dealing with a form of sugar fermentation which is essentially of the normal alcoholic type, but which is distinguished from it by the production of quite a considerable quantity of gaseous hydrogen."

The results of Milhaud et al., on the other hand, would appear to indicate a coliform-type of fermentation. At first sight these results may seem paradoxical; however, a closer examination reveals that there are two features common to all

TABLE 20.

Comparison of the main features of the fermentation balances found by different workers for the fermentation of glucose by *Z. ventriculi*.

	Smit	Klayver	Milhaud et al.
	moles	moles	moles
Glucose	1.0	1.0	1.0
CO ₂	1.71	1.94	1.9
Ethanol	1.58	1.71	0.8
H ₂	0.52	0.42	1.7
Acetic Acid	0.27	0.20	0.9

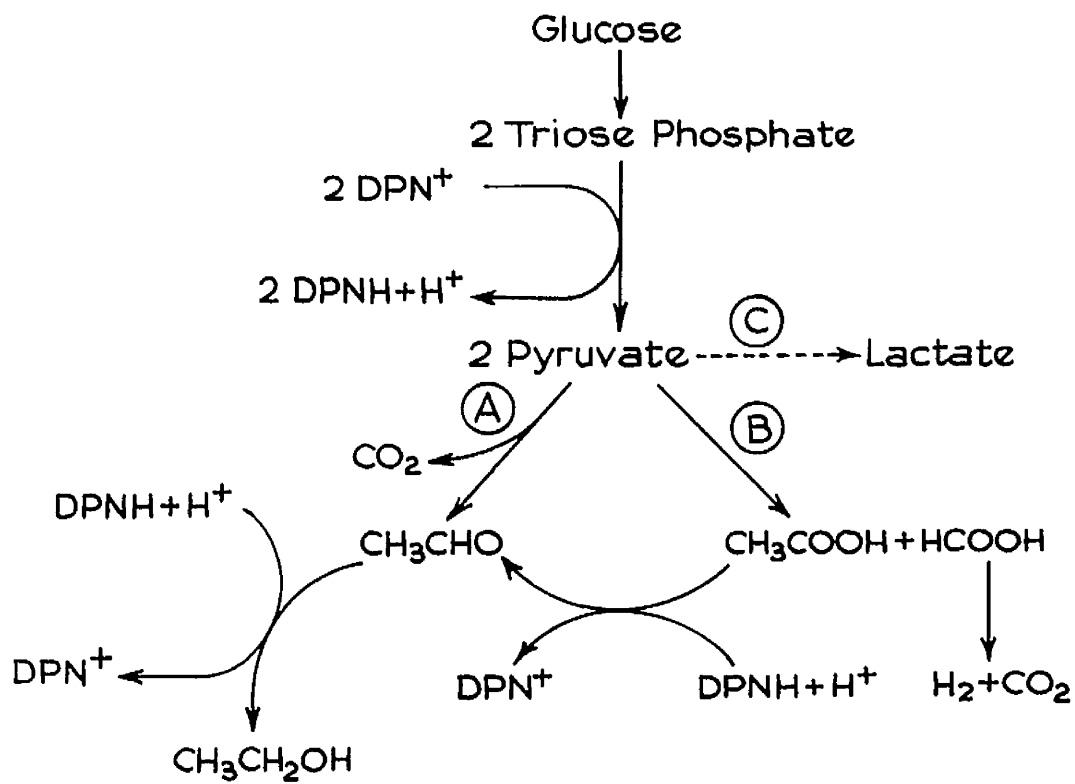
these fermentation balances. Two moles of CO_2 per mole of glucose were found in every case. In addition, the ratio of hydrogen to acetate was consistently found to be 2 : 1. It was realized at an early stage that these features were probably an important clue to the mechanisms involved in the Z. ventriculi fermentation.

On the basis of the results obtained in our studies, we can formulate a scheme which is able to explain all of these carbon balances obtained by the earlier workers (Fig. 19).

Glucose is fermented to pyruvate by Embden-Meyerhof glycolysis. Pathway A is the carboxylase pathway involving the decarboxylation of pyruvate to CO_2 and acetaldehyde, followed by the reduction of the latter to ethanol. Due to the presence of a DPNH oxidase in extracts of Z. ventriculi ethanol dehydrogenase has not yet been demonstrated. However, such a mechanism can reasonably be inferred. Pathway B involves the elastic fission of pyruvate to acetate and formate. The formate produced is metabolized to hydrogen and CO_2 by the formic hydrogenlyase system. As was previously mentioned, the fermentation balance obtained by Milhaud et al. could be explained in terms of a coliform-type of fermentation. The formation of ethanol in E. coli has been shown to involve the enzymic reduction of acetaldehyde by a coenzyme A

FIG. 19.

Proposed Scheme for the Fermentation of
Glucose by *Z. ventriculi*.



dependent acetaldehyde dehydrogenase (Dawes and Foster, 1956). It is proposed that Z. ventriculi also possesses this reaction.

If pathway A is ignored for the moment, it can be seen that the formation of ethanol via pathway B requires 2 moles of reduced DPK. Therefore, from the fermentation of one mole of glucose a maximum of one mole of ethanol can be formed by this pathway. The fermentation of one mole of glucose by this route would therefore result in the production of one mole each of ethanol and acetate, and two moles each of CO_2 and hydrogen. This pattern of products approximates to the fermentation balance obtained by Milhaud et al. (Table 20). It is worthy of note that the hydrogen/acetate ratio is always 2 : 1 whenever endogenous pyruvate is fermented by this pathway.

The results of Smit and Kluyver (Table 20) could be largely explained in terms of fermentation via pathway A, resulting in the formation of ethanol and CO_2 . Smit and Kluyver also found small amounts of hydrogen and acetate in the ratio of 2 : 1. This could be explained by the fermentation of small amounts of pyruvate by route B. It can also be seen that no matter which pathway or combination of pathways is utilized, the yield of CO_2 is always two moles per mole of glucose fermented.

Z. ventriculi has many unusual properties and this is apparently reflected at the biochemical level; this is the first example of an organism possessing both of these pathways of pyruvate metabolism. The operation of these two pathways in different ratios can therefore explain the fermentation balances obtained by Smit, Kluyver and Milhaud et al. The different ratios of pathways A and B which would require to be utilized, to explain the balances obtained by these workers, have been calculated (Table 21). The theoretical fermentation patterns are compared with the patterns of products obtained experimentally. As can be seen, there is good agreement in every case.

6.4.1. The Dismutation Reaction.

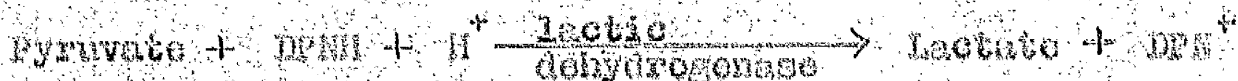
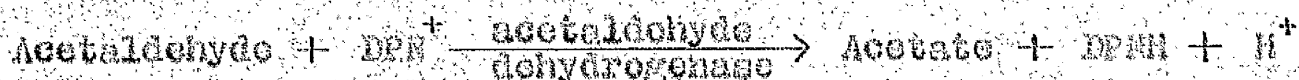
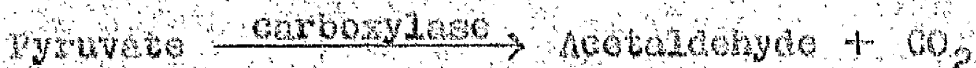
As lactic dehydrogenase has not been demonstrated directly, the formation of lactate from pyruvate in the fermentation scheme (Fig. 19.) is indicated by a dotted line. In any case, lactate is only a minor product of the fermentation (Smit, 1930). Nevertheless, we have obtained results which suggest that a dismutation reaction may occur under certain conditions (III, 4.1.). Such a reaction could be explained in terms of mechanisms included in the scheme for glucose fermentation, shown in Fig. 19.

TABLE 21.

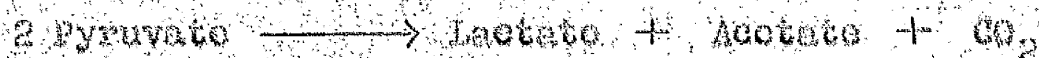
Comparison of products of glucose fermentation by
Z. ventriculi, according to theory¹ and experiment.

	Theory			Experiment	
	Pathway utilized		Total Products	Products found	Reference
	A	B	(A+B)	found	
moles	moles	moles	moles		
Pyruvate	0	2.0	-	-	Milhaud <i>et al.</i> (1956)
CO ₂	-	2.0	2.0	1.9	
Ethanol	-	1.0	1.0	0.8	
H ₂	-	2.0	2.0	1.7	
Acetate	-	1.0	1.0	0.9	
Pyruvate	1.46	0.54	-	-	Smit (1930)
CO ₂	1.46	0.54	2.0	1.71	
Ethanol	1.46	0.27	1.73	1.58	
H ₂	-	0.54	0.54	0.52	
Acetate	-	0.27	0.27	0.27	
Pyruvate	1.6	0.4	-	-	Kluyver (1931)
CO ₂	1.6	0.4	2.0	1.94	
Ethanol	1.6	0.2	1.8	1.71	
H ₂	-	0.4	0.4	0.42	
Acetate	-	0.2	0.2	0.20	

¹ One mole of glucose is fermented to two moles of pyruvate.



The overall sum of these reactions would produce the dismutation reaction pattern of products.



6.5. The Controlling Mechanism.

Although the fermentation scheme and the calculations presented in Fig. 19 and Table 21 respectively, can explain the carbon balances obtained for the Z. ventriculi fermentation, the mechanism which controls the ratio of pathways A and B is not fully understood.

The pH of the reaction mixture does not appear to affect the ratio of the pathways operating in the preformed enzymic system (III, 2.1.). The possibility that the pH of the growth medium may control the relative concentrations of the enzymes of the different pathways synthesised, has already been discussed (III, 2.1.). The results obtained in the growth experiment (III, 4.3.) would seem to offer further support for this concept. During the growth of Z. ventriculi

the pH of the medium changed from 7.0 to 4.8. At the same time the activity of the hydrogen-evolving system decreased steadily with glucose, pyruvate and formate as substrates. It is tempting to believe that there may be a direct correlation between these two facts. Although this has not been demonstrated, these findings are in keeping with the concept that the patterns of products in the Z. ventriculi fermentation may be explained in terms of pH effects at some level of enzyme biosynthesis.

SUMMARY

SUMMARY.

1. The enzymes hexokinase, aldolase and triosephosphate dehydrogenase have been demonstrated in crude extracts of Z. ventriculi.
2. Pyruvate formation from 3-phosphoglyceric acid has also been obtained with crude extracts.
3. The fermentation of glucose by whole cells of Z. ventriculi has been shown to be inhibited by iodoacetate but not by fluoride.
4. With toluene-treated cells of Z. ventriculi pyruvate is fermented mainly to CO₂ and acetaldehyde, with the formation of smaller amounts of acetoin, lactate and acetate.
5. The evolution of CO₂ from pyruvate by toluene-treated cells has been shown to be sensitive to the inhibitors of a yeast-type carboxylase.
6. At pH 4.9 whole cells of Z. ventriculi ferment pyruvate quantitatively to equimolar amounts of acetate, H₂ and CO₂.
7. Formic hydrogenlyase, formic dehydrogenase and hydrogenase have been demonstrated with washed-cell suspensions of Z. ventriculi.

8. The formic hydrogenlyase system has been shown to be sensitive to the inhibitors of the formic hydrogenlyase system in E. coli.
9. On the basis of these findings, a scheme for the fermentation of glucose by Z. ventriculi has been proposed which accounts for the fermentation balances obtained by earlier workers.
10. The results indicate that Z. ventriculi is unique in being the only organism which possesses both a yeast-type carboxylase and a clastic reaction for the metabolism of pyruvate.

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