and mouse can deacetylate phenylacetyl cephalosporin, whereas man is unable to deacetylate cephalosporin C.

SUMMARY

1. Phenylacetyl cephalosporin was observed to give rise to a further microbiologically active material in the urine of mice receiving the compound subcutaneously. This also occurred with cephalosporin C and its *N*-dinitrophenyl derivative.

2. Many tissues, notably liver and kidney, are able to produce these compounds *in vitro*.

3. The metabolites have less microbiological activity than their corresponding parent compounds.

4. The metabolites are produced by deacetylation of the parent cephalosporins; different animal species have the ability to deacetylate different cephalosporins to different extents, e.g. man and monkey can deacetylate phenylacetyl cephalosporin but not cephalosporin C.

5. It is deduced that the clinical effectiveness of any cephalosporin will depend, not only on its microbiological activity *in vitro*, but also on the

rate at which it is deacetylated in the body and the activity of the resulting deacetyl compound.

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The Fermentation of Glucose by *Chlorella vulgaris*

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The anaerobic metabolism of algae has received little attention. Genevois (1927, 1929) showed that several different species produce acid but little carbon dioxide anaerobically. Gaffron (1939) and Michels (1940) showed that *Chlorella pyrenoidosa* and *Scenedesmus obliquus* form lactic acid from glucose anaerobically. Gaffron & Rubin (1943) found that strains of *Scenedesmus*, but not of *Chlorella*, produce hydrogen after a period of anaerobiosis. Barker (1935) studied, rather thoroughly, the anaerobic metabolism of the colourless alga, *Prototheca zopfii*, and found that lactic acid was formed from glucose anaerobically. He claimed that the conversion was quantitative, i.e. 1 mole of glucose gave 2 moles of lactic acid as in homolactic fermenting bacteria. Damasche (1957) has made a similar claim for *C. pyrenoidosa*, but neither Barker nor Damasche supplies experimental values that support this conclusion.

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Damasche also showed that the acid fermentation of glucose by *C. pyrenoidosa* is replaced, after some days of anaerobiosis, by a fermentation in which hydrogen is produced.

On the other hand, there are algae with a typical yeast type of alcoholic fermentation, e.g. *Ochromonas malhamensis* (Reazin, 1956). Weis & Mukerjee (1958) cite no quantitative results but recognize three types of algal fermentation. In the first, typified by *C. pyrenoidosa*, only lactic acid is produced from glucose; in the second, e.g. *C. ellipsoidea*, no acid is produced; and in the third type, represented by *C. vulgaris*, the products include both lactic acid and acetic acid.

We have worked only with *C. vulgaris* and have attempted to account quantitatively for the products of glucose fermentation. We find, in agreement with Weis & Mukerjee (1958), that both acetic acid and lactic acid are important products, but a considerable quantity of formic acid is also produced as well as small quantities of carbon

dioxide and hydrogen. In addition, about one-eighth of the added glucose is converted into polysaccharide.

EXPERIMENTAL

Growth of organism. *C. vulgaris* (Pearsall's strain) was grown in pure culture in a medium containing: KH_2PO_4 , 7.26 g.; K_2HPO_4 , 2.32 g.; NH_4NO_3 , 0.75 g.; MgSO_4 , 0.2 g.; sodium citrate monohydrate, 0.2 g.; saturated FeSO_4 soln., 0.08 ml.; 'A₄' trace-element soln. (Arnon, 1938), 1.0 ml.; glass-distilled water to 1 l. The pH, after autoclaving, was 6.1. Cultures were grown for 3 days at 25° with a light-intensity of 600 foot-candles and they were aerated with air containing 0.5% (v/v) of CO_2 . Cultures were starved before use by aeration for a further 18 hr. in darkness. The cells were harvested by centrifuging at 500g, washed and resuspended in 2 mm- MgSO_4 . The density of the cell suspension was measured at 640 μm and adjusted, by dilution, to a suitable value, generally about 16 mg. dry wt./ml.

Manometry. Gas exchange was measured by Warburg manometry at 25° in darkness. For some experiments, flasks of 200 ml. capacity containing 20–50 ml. of liquid were used. Flasks were filled with either N_2 (O_2 -free) or with 5% (v/v) CO_2 in N_2 . The CO_2 produced by the cells was absorbed by 10% (w/v) KOH, and H_2 with palladium-asbestos (Johnson Matthey and Co. Ltd.). Sulphuric acid (3N or 6N) was added at the end of some experiments either by tipping from a second side arm or by injection through the side-arm stopper.

Hot-water extracts. Samples of cell suspension were heated rapidly to 100°. The cell debris was removed by centrifuging and extracted with portions of water at 80°. The combined extracts were stored at –15°.

Ethanol extracts. A known volume of cell suspension was pipetted into 4 vol. of boiling ethanol. The cell debris was removed by centrifuging and washed with ethanol.

Polysaccharide hydrolysis. The extracted cells (about 10 mg. dry wt.) were suspended in 1 ml. of $\text{N-H}_2\text{SO}_4$ and heated at 100° for 150 min. Saturated $\text{Ba}(\text{OH})_2$ soln. was added until the mixture was just alkaline. Excess of Ba^{2+} ion was removed by bubbling a stream of CO_2 . Then 1 drop of aq. ammonia solution (sp.gr. 0.88) was added and the precipitate removed by centrifuging. The supernatant and washings were taken for analysis and for chromatography. Hydrolysates of *Chlorella*, prepared in this way, contain chiefly mannose and glucose (Syrett, 1956). Paper chromatography of the hydrolysates in butan-1-ol-acetic acid-water (5:1:4, by vol.) showed that, after cells had fermented [^{14}C]glucose, ^{14}C incorporated into polysaccharide was present only as glucose polysaccharide. To measure accurately the changes in amount of glucose polysaccharide, it was essential to analyse the hydrolysates by a method specific for glucose; that described in the next section was suitable.

Glucose. This was determined in hot-water extracts and in the polysaccharide hydrolysates by converting it into glucose 6-phosphate which then reduced NADP in the presence of glucose 6-phosphate dehydrogenase; reduced NADP was determined by measuring its fluorescence. For this assay 1.3 ml. in a cuvette contained: 100 μmoles of potassium phosphate, pH 7.5; 10 μmoles of MgCl_2 ; 0.2 μmole of NADP; up to 0.06 μmole of glucose; 0.5 mg. of hexo-

kinase (L. Light and Co. Ltd.); 2.5 $\mu\text{g.}$ of glucose 6-phosphate dehydrogenase (C. F. Boehringer und Soehne, G.m.b.H.). The reaction was started by adding 0.5 μmole of ATP and was complete in 10–15 min. at room temperature. The quantity of NADP reduced was proportional to the quantity of glucose present and was measured fluorimetrically in a Locarte fluorimeter (primary filter LF/2; secondary filter LF 3/2).

Lactic acid. A 1 ml. sample of cell suspension was pipetted into 2 ml. of acid ZnSO_4 soln. (18 g. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ / l. of 0.16N- H_2SO_4), and the mixture was heated at 100° for 2 min., cooled and neutralized with 0.33N- NaOH ; 1 ml. of 20% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was added followed by 1 g. of solid $\text{Ca}(\text{OH})_2$. The mixture was centrifuged after standing for 30 min., and the supernatant and washings were made up to 10 ml. Lactic acid was determined on samples by a modified Barker-Summerson method (Hullin & Noble, 1953). With this method, complete recovery of known amounts of lactic acid, added to the cell extracts, was obtained.

Volatile reducing substances. These were determined by pipetting a 2 ml. sample of cell suspension into a Conway unit containing 0.5 ml. of 0.1N- $\text{K}_2\text{Cr}_2\text{O}_7$ in 10N- H_2SO_4 in the centre well; 1 ml. of 40% (w/v) KOH was added to the cell suspension. After standing overnight, 0.5 ml. of 50% (w/v) KI was added to the centre well and the liberated iodine titrated with 0.05N- $\text{Na}_2\text{S}_2\text{O}_3$ (Conway, 1947).

Volatile acids. These were determined after steam-distillation (Friedman, 1938). Samples (10 ml.) of cell suspension were added to 1 ml. of 10N- H_2SO_4 , and the mixture was steam-distilled after the addition of 3 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 ml. of 20% (w/v) sodium tungstate and 0.5 ml. of 40% (w/v) KOH. The distillate (125 ml.) was collected and titrated with 0.01N- NaOH , with phenol red as indicator, after vigorous aeration with a stream of CO_2 -free N_2 . Sometimes, after titration, the distillate was made alkaline with N- NaOH , concentrated to 30 ml., and redistilled from a flask containing 12 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g. of HgO and 0.5 ml. of 10N- H_2SO_4 . This second distillation removes any formic acid, lactic acid or pyruvic acid that may have been present in the steam-distillate. The recovery of known amounts of acetic acid after both distillations was about 95%. Alternatively, the neutralized steam-distillate was sometimes made alkaline and 'blown' to dryness with cool air for the determination of formic acid.

Formic acid. This was determined by the method of Perlin (1954) in which formic acid is oxidized to carbon dioxide by lead tetra-acetate; the gas produced is measured manometrically. The dried steam-distillate was dissolved in 5 ml. of 90% (v/v) acetic acid. A sample (2 ml.) of the solution was placed in a Warburg flask, the side arm of which contained 0.5 ml. of freshly prepared reagent [20 mg. of potassium acetate and 25 mg. of lead tetra-acetate (L. Light and Co. Ltd.)/ml. in 90% (v/v) acetic acid]. The reaction was carried out at 30° and was complete in 2 hr. Standard sodium formate solutions were treated in the same way as steam-distillates and determined at the same time.

Separation of extracts into cationic, anionic and neutral fractions. An aqueous extract (about 25 ml. from 40 mg. dry wt. of cells and adjusted to pH 3) was run slowly through a column (80 mm. $\times$ 8 mm.) of Zeo-Karb 215 (Cl^- form). The solution which came through was adjusted

to pH 9 and run slowly (1 ml./min.) through a column (130 mm. $\times$ 8 mm.) of Amberlite IRA-401 (CO_3^{2-} form). The fraction adsorbed on neither column was classified as neutral. Cations were eluted from Zeo-Karb columns with 25 ml. of aq. ammonia solution (sp.gr. 0.88), and anions were eluted from Amberlite columns with 45 ml. of $\text{m}-(\text{NH}_4)_2\text{CO}_3$.

Partition chromatography of organic acids on a silica-gel columns. The silica gel (British Drug Houses Ltd.) was dried by heating at 400° . The alkaline aqueous sample was evaporated almost to dryness on a water bath; it was then placed on ice and acidified with a few drops of $2\text{N-H}_2\text{SO}_4$. Dry silica gel was added immediately and the free-flowing powder transferred to the top of a column (200 mm. $\times$ 10 mm.) of silica gel in chloroform (equilibrated with $0.5\text{N-H}_2\text{SO}_4$). The column was eluted with mixtures of butan-1-ol in chloroform (Bulen, Varner & Burrell, 1952). Fractions (10 ml.) were collected and titrated, after the addition of water and phenol red, with 0.01N-NaOH in a stream of CO_2 -free N_2 .

Paper chromatography. Either Whatman no. 4 or 3MM paper was used. Amino acids and lactic acid were separated in butan-1-ol-acetic acid-water (4:1:5, by vol.). This solvent was also used for two-dimensional chromatograms after running one way in water-saturated phenol. Volatile acids in the steam-distillate were chromatographed, after the addition of morpholine, on no. 4 paper for 16 hr. in a mixture of butan-1-ol (30 ml.), cyclohexane (30 ml.), propan-1,2-diol (10 ml.), aq. ammonia solution (sp.gr. 0.88) (0.7 ml.), morpholine (0.07 ml.) and water (3.5 ml.) (Guillaume, Beerens & Osteux, 1956). After being dried at room temperature for 30 min., the chromatogram was sprayed with a mixture of 3 parts of cresol red solution (*o*-cresol red, 0.2 g.; N-NaOH , 0.3 ml.; water, 15 ml.; ethanol, 70 ml.) to 1 part of sodium diethylbarbiturate buffer (sodium diethylbarbiturate, 10 g.; water, 100 ml.; ethanol, 150 ml.). After the acidic spots had been detected, the paper was heavily sprayed with the buffer solution alone in order to retain the volatile acids on the paper for radioautography. These procedures gave good separation of formic acid, acetic acid and propionic acid. Kodirex No-Screen X-ray film was used for the preparation of radioautographs.

Estimation of ^{14}C . Samples of extracts were plated on ground-glass disks and dried to give infinitesimally thin samples for counting. Organic acid samples were made alkaline with N-NaOH before plating. The samples were counted with a scintillation head. In one experiment 0.05 ml. samples were mixed with 1 ml. of liquid scintillator (Nuclear Enterprises, N.E. 220) and counted in a liquid-scintillator counter. The activity of radioactive areas on paper was determined by counting with a thin end-window Geiger-Müller tube.

RESULTS

Acid production during glucose fermentation. Little gas was evolved when small quantities of glucose were incubated anaerobically with cells in phosphate buffer, but when bicarbonate buffer was used, at the same pH, much gas was produced. The addition of acid after fermentation had ceased showed that 90% of the gas liberated during the

fermentation in bicarbonate buffer was the result of acid production. Thus the fermentation of glucose by *C. vulgaris* is essentially an acid fermentation.

Since little gas was produced compared with the quantity of acid during the fermentation of small quantities of glucose, gas production was neglected in a number of experiments in which 1, 1.5 or $2\ \mu\text{moles}$ of glucose were added to cells suspended in bicarbonate buffer, and acid production was estimated from the volume of carbon dioxide released from the bicarbonate. About 2 equiv. of carbon dioxide was formed/mole of glucose metabolized (Table 1), but this is an overestimate of acid production since gas production by the cells has been neglected.

Gas production during glucose fermentation. Both carbon dioxide and hydrogen were formed during the fermentation of endogenous substrates, and the formation of both was increased by the addition of glucose. No other gas was evolved since Warburg flasks containing both potassium hydroxide and palladium-asbestos showed no pressure change. In our experiments, hydrogen production was observed from the beginning of the experiments and no period of dark adaptation was necessary. The rate of hydrogen production was approximately doubled by illumination of 30 foot-candles intensity; in this it resembles the hydrogen production of other anaerobic algae (Gaffron & Rubin, 1943; Frankel, 1952).

The additional gas production when glucose was added amounted to about 0.13 mole of hydrogen/mole of glucose metabolized; carbon dioxide production was of the same order (Table 2).

Lactic acid formation. The observation that about 2 acid equiv. was formed/mole of glucose metabolized suggested that *C. vulgaris* might convert glucose quantitatively into lactic acid, as suggested by Barker for *Prototheca zopfii*. Analysis of the contents of Warburg flasks after fermentation had ceased showed, however, that not more

Table 1. Carbon dioxide liberated during glucose fermentation by *Chlorella vulgaris* in bicarbonate buffer

Warburg flasks contained 30 mg. dry wt. of cells suspended in 2 ml. of 4 mM-KHCO_3 and in an atmosphere of 5% (v/v) CO_2 in N_2 ; the pH was 6.75. The metabolism of the largest quantity of glucose was complete in 120 min. Endogenous CO_2 production has been subtracted.

Glucose added (μmoles)	No. of determinations	CO_2 liberated	
		($\mu\text{l.}$)	(moles/mole of glucose metabolized)
1.0	14	44 ± 1.4	1.97 ± 0.06
1.5	14	64 ± 1.2	1.91 ± 0.04
2.0	12	84 ± 1.7	1.88 ± 0.04

Table 2. *Gas production during glucose fermentation by Chlorella vulgaris*

Each flask contained 30 mg. dry wt. of cells suspended in 0.033M-potassium phosphate buffer, pH 7.1. Glucose (12 μ moles) was added to one set of flasks; after 6 hr. analysis of the flask contents showed that all of the added glucose had disappeared. The H₂ production was estimated from flasks containing KOH; the CO₂ production from flasks containing palladium-asbestos, 3N-H₂SO₄ being added to liberate bound CO₂ at the end of the experiment. The total gas production was estimated from flasks containing neither Pd nor KOH; a mean flask constant equal to $(K_{H_2} + 2K_{CO_2})/3$ was used.

	H ₂ production	CO ₂ production	Total gas production
Control (i.e. no added glucose)	52 μ l.	97 μ l.	164 μ l.
Experimental (i.e. 12 μ moles of glucose added)	85 μ l.	146 μ l.	240 μ l.
Difference	33 μ l.	49 μ l.	76 μ l.
	1.5 μ moles	2.2 μ moles	3.4 μ moles
$\therefore$ Gas liberated (mole/mole of glucose metabolized)	0.13	0.18	0.29

than 30 % of the added glucose could be accounted for as lactate (Table 3). Moreover, when a larger culture was incubated anaerobically with glucose and sampled at 20 min. intervals, lactic acid production was directly proportional to the amount of glucose that had disappeared but it accounted for only one-quarter of this glucose.

These experiments were carried out at about pH 7. At higher pH values, lactic acid production increased so that, at pH 7.8, it accounted for one-half of the glucose metabolized (Fig. 1).

Formation of volatile acids. Since lactic acid alone did not account for the total acid production during fermentation, volatile acids were sought. Steam-distillation of the products of glucose fermentation showed that considerable amounts of volatile acids were produced. Acid production, measured manometrically, was accounted for completely by lactic acid and volatile acids (Table 4). In this experiment volatile reducing substances such as ethanol were also determined; their production was negligible.

Nature of the volatile acids. After concentration, the neutralized steam-distillate was subjected to a second distillation with the addition of magnesium sulphate and mercuric oxide, as recommended by Friedman (1938). This distillation removes any pyruvic acid or lactic acid that distils over during the first distillation, and destroys formic acid. Under our conditions, less than 4 % of lactic acid present distilled over during the first distillation. Nevertheless, a considerable loss of acid occurred during the second Friedman distillation and the loss was always greater with samples from cell suspensions that had fermented glucose (Table 6). The loss suggested that formic acid might be a product of fermentation.

The volatile acid that distilled over during the second Friedman distillation accounted for about one-half of the total volatile acid. This acid reacted with lanthanum nitrate (Feigl, 1956), a reaction that is specific for acetic acid and propionic acid.

Table 3. *Lactic acid formation during glucose fermentation by Chlorella vulgaris*

Each Warburg flask contained 30 mg. dry wt. of cells suspended in 2 ml. of 4 mM-KHCO₃ in an atmosphere of 5% (v/v) CO₂ in N₂; the pH was 6.75. The flasks were sampled after 120 min. when the metabolism of the largest quantity of glucose was complete. Mean values from three experiments are given.

Glucose added (μ moles)	Lactic acid found (μ moles)	Glucose recovered as lactic acid (%)
0	0.02	—
1.0	0.40	19.0
1.5	0.91	29.6
2.0	1.22	30.0

Fig. 1. Effect of pH on lactic acid production by *Chlorella vulgaris* cells fermenting glucose in bicarbonate buffer. The pH was altered by varying the concentration of KHCO₃ in an atmosphere of 5% (v/v) CO₂ in N₂. Each flask contained 30 mg. dry wt. of cells and received 2 μ moles of glucose. Fermentation was complete in 120 min. and the flasks were then sampled. The results of two separate experiments (●, ▲) are shown; each point is the mean value derived from duplicate flasks.

When the distillate was made alkaline, evaporated to dryness and transferred to a silica-gel column, the acid, unlike propionic acid, was not readily eluted with 1% (v/v) butan-1-ol in chloroform; but it was eluted with 5% (v/v) butan-1-ol in chloroform with a single peak corresponding to acetic acid, and all the acid in the distillate was recovered in this peak. Added ^{14}C -labelled acetate was co-eluted with the acid peak. Thus the acid that distilled over during the second Friedman distillation was identified as acetic acid.

By paper chromatography in the alkaline butan-1-ol-cyclohexane-propan-1,2-diol solvent of Guillaume *et al.* (1956), it was found that acetic acid and formic acid were the only labelled volatile acids formed when uniformly labelled ^{14}C glucose was fermented. Four acidic spots could be detected on chromatograms, two slow-running with R_f values of 0.06 and 0.10 and two faster-running spots, one with R_f 0.30 corresponding to formic acid and one with R_f 0.36 corresponding to acetic acid. The two slow-running spots were not radioactive and they appeared to be present in about the same amount whether glucose was fermented or not; thus they could not be considered to be fermentation products. After fermentation of uniformly labelled ^{14}C glucose, both acetic acid and formic acid were radioactive, and the amount of ^{14}C in acetic acid was about twice that in formic acid, suggesting that the two acids were formed in approximately equimolar proportions. When the volatile acids resulting from ^{14}C glucose fermentation were subjected to the second Friedman distillation, only two-thirds of the ^{14}C was recovered. Again, this is as was expected, if labelled acetic acid and formic acid were present in equimolar proportions.

Formic acid was measured directly in dried alkaline steam-distillates by the method of Perlin

(1954). Formic acid and acetic acid accounted quantitatively for the volatile acid formed during glucose fermentation (Table 6).

Fermentation of ^{14}C glucose. When uniformly labelled ^{14}C glucose was fermented by *C. vulgaris*, about 85% of the ^{14}C could be recovered in ethanol-soluble compounds; 2.4% was recovered as carbon dioxide and an appreciable proportion (about 11%) was recovered as polysaccharide (Table 5). There was no incorporation into the insoluble residue which contained protein.

When the initial extraction was made with hot water and the residue extracted with ethanol, 99% of the soluble ^{14}C was present in the water extract. Thus one can conclude that lipids do not become labelled during the fermentation.

The ethanol-soluble compounds were separated by resin columns into cationic, anionic and neutral fractions. Most of the radioactivity was, as expected, in the anionic fraction, but some was in cationic and neutral compounds. Paper chromatography showed that the ^{14}C in the cationic fraction was confined to two ninhydrin-positive compounds that were identified by co-chromatography as alanine and aspartic acid. These were presumably labelled by exchange during fermentation as the amount of ^{14}C in them was proportional to the quantity of cells analysed rather than to the amount of glucose fermented. The radioactivity in the neutral fraction was confined to one unidentified compound with R_f about 0.5 in butan-1-ol-acetic acid-water (5:1:4, by vol.) and R_f about 0.7 in water-saturated phenol. This compound was not unfermented glucose.

Recovery of glucose carbon after fermentation. In Table 6 are summarized the results of an experiment in which 300 μmoles of glucose were metabolized by about 400 mg. dry wt. of *Chlorella* and the fermentation products analysed. The chief

Table 4. Acid production during glucose fermentation by *Chlorella vulgaris*

C. vulgaris (210 mg. dry wt.) was suspended in 18 ml. of 0.01M-KHCO₃ in an atmosphere of 5% (v/v) CO₂ in N₂. Then 60 μmoles of glucose were added to the experimental flasks. After 5 hr. 3N-H₂SO₄ was added to liberate residual bicarbonate as CO₂, and samples of the flask contents were removed for analysis. The amount of glucose remaining unmetabolized was 2.9 μmoles . Control values have been subtracted.

	Acid production			(μequiv./ μmole of glucose metabolized)
	(μl.)	(μmoles)	(μequiv.)	
Gas production	527	22.5	—	—
Acid production (by manometry)	2035	—	90.9	1.59
Volatile reducing substance as 'ethanol'	—	3.7	—	—
Lactic acid	—	33.0	33.0	0.58
Volatile acid	—	—	55.9	0.97
$\frac{\text{Lactic acid} + \text{volatile acid}}{\text{Acid production by manometry}} = 0.98$				

products, acetic acid, formic acid and lactic acid, were formed in approximately equimolar proportions. The other products recovered were carbon dioxide and polysaccharide. Together, these compounds accounted for 71 % of the glucose carbon that had disappeared. Polysaccharide disappeared during endogenous fermentation and is presumably a substrate for this as it is for endogenous aerobic respiration (Syrett, 1951).

In this experiment, 15 % of the glucose metabolized was assimilated into polysaccharide. This amount should be subtracted before the yield of

Table 5. Fermentation of [¹⁴C]glucose by *Chlorella vulgaris*

C. vulgaris (48 mg. dry wt.) was suspended in 3 ml. of 0.022M-potassium phosphate buffer, pH 7.15, in an atmosphere of N₂, and 15 μmoles (3 μC) of uniformly labelled [¹⁴C]glucose were added. Metabolism was complete after 4 hr. Then 6N-H₂SO₄ was added to release bound CO₂ and the flask contents were sampled. Similar flasks received glucose and acid together at the beginning of the experiment; these were analysed by the same procedure as a check on radiochemical contamination. All flasks were in duplicate.

	10 ⁻³ × Recovery of ¹⁴ C (counts/min.)				
	CO ₂	Ethanol-soluble	Poly-saccharide	Insoluble	Total
Initial	0	1445	20	5	1470
After incubation	34	1259	171	2	1466
(% of initial total)	(24)	(85.8)	(11.4)	(0.1)	(99.7)
10 ⁻³ × Distribution of ethanol-soluble ¹⁴ C, after fermentation, between cationic, anionic and neutral compounds (counts/min.)					
Cationic fraction	165				
Anionic fraction	715				
Neutral fraction	86				
Total	966*				

* This represents a 78 % recovery of the total ¹⁴C in the ethanol-soluble fraction.

Table 6. Recovery of glucose carbon after fermentation by *Chlorella vulgaris*

C. vulgaris (400 mg. dry wt.) was suspended in 48 ml. of 0.03M-potassium phosphate buffer, pH 7.3, under an atmosphere of N₂. The flasks contained palladium-asbestos to absorb H₂. The experimental flask received 300 μmoles of glucose and the experiment was terminated after 7 hr. Then 2 ml. of 6N-H₂SO₄ was added to liberate bound CO₂; it was also added to an initial flask. Samples (in triplicate) of cell suspension were removed from each flask for analysis and the mean values are given below. The initial values have been subtracted. Volatile acid collected after the second Friedman distillation is given as acetic acid. Formic acid was determined on dried samples of the first steam-distillate by the method of Perlin (1954).

	μmoles					
Glucose added	300.0					
Glucose remaining at end of experiment	4.4					
Glucose metabolized	295.6					
Glucose fermented (that metabolized minus that converted into polysaccharide)	250.8					
	Differences from initial values					
	Volatile acid ($\mu\text{equiv.}$)	Acetic acid (μmoles)	Formic acid (μmoles)	Lactic acid (μmoles)	CO_2 (μmoles)	Glucose in polysaccharide (μmoles)
Control (i.e. no glucose added)	25.0	23.4	11.0	8.8	5.0	- 4.5
Experimental (i.e. glucose added)	324.6	173.0	164.6	177.7	34.0	+ 40.3
Experimental - control	299.6	149.6	153.6	168.9	29.0	+ 44.8
Product (mole/mole of glucose metabolized)		0.51	0.52	0.57	0.10	0.15
Product (mole/mole of glucose fermented)		0.60	0.61	0.68	0.12	—
Glucose carbon recovered in product (%) [*]		16.9	8.7	28.6	1.6	15.2

* The total represents 71 % recovery of the glucose carbon metabolized.

fermentation products is calculated. When this is done, it can be seen that about 0.6 mole of acetic acid, 0.6 mole of formic acid and 0.7 mole of lactic acid were formed/mole of glucose fermented.

Comparison of the rate of glucose fermentation with the rate of aerobic oxidation. Many plants break down carbohydrates more quickly anaerobically than aerobically (Turner, 1951). This does not appear to be true of *C. vulgaris*. Cells (16 mg. dry wt.) were suspended in either phosphate buffer, pH 7.15, in aerobic Warburg flasks, or 0.01 M-potassium hydrogen carbonate, in flasks filled with 5% (v/v) carbon dioxide in nitrogen, final pH 7.15; 20 μ moles of glucose were added to each. The rate of aerobic breakdown of glucose was calculated from the rate of oxygen uptake assuming that 6 moles of oxygen are necessary to oxidize completely 1 mole of glucose; the r.q. is known to be unity (Syrett, 1951). The anaerobic rate of breakdown was estimated from the rate of pressure change in the flasks containing cells in bicarbonate, assuming that 2 molar equiv. of acid plus gas are released/mole of glucose metabolized (Table 1). The aerobic rate was found to be 0.153 μ mole of glucose/mg. dry wt., and the anaerobic rate 0.063 μ -mole/mg./hr. Thus, even allowing for uncertainties in this method of estimation, glucose was broken down twice as quickly aerobically as anaerobically, and a Pasteur effect did not appear to operate.

2,4-Dinitrophenol, which markedly stimulates the aerobic endogenous respiration, had no effect on the rate of endogenous fermentation (Table 7). Neither did 2,4-dinitrophenol, added to aerobic cells in bicarbonate buffer, induce any aerobic acid fermentation.

Table 7. *Effect of 2,4-dinitrophenol concentration on endogenous respiration (oxygen uptake) and on endogenous fermentation (acid production) by Chlorella vulgaris*

Cells (25 mg. dry wt.) were suspended in 0.06 M-potassium phosphate for aerobic measurements, or in 0.01 M-KHCO₃ in an atmosphere of 5% CO₂ (v/v) in N₂ for anaerobic measurements; the pH was 7.15 in all flasks. The cells were nitrogen-starved for 16 hr. before use to increase their endogenous content of carbohydrates (Syrett, 1953).

Concn. of 2,4-dinitrophenol (mM)	Rate of O ₂ uptake in aerobic conditions (μ l./mg. dry wt./hr.)	Rate of acid production in an- aerobic conditions (μ l. of CO ₂ /mg. dry wt./hr.)
0	2.41	2.00
0.5	5.22	2.08
0.75	6.84	1.92
1.0	8.00	2.08
1.5	8.04	2.00
2.0	5.41	2.06

DISCUSSION

The fermentation of glucose by *C. vulgaris* is largely an acid fermentation. Very little, if any, ethanol is formed, and gas production amounts to only 0.3–0.4 mole/mole of glucose metabolized (Tables 2 and 4). On the other hand, 1.5–2.0 equiv. of acid is formed/mole of glucose (Tables 1 and 4). Together lactic acid and volatile acids account, quantitatively, for the total acid production as measured manometrically (Table 4). The volatile acids consist of only acetic acid and formic acid in almost equimolar proportions (Table 6).

In some respects, the fermentation resembles that of *Escherichia coli* in which a mixture of acids, including lactic acid, formic acid and acetic acid, is formed from glucose (Stephenson, 1949). The proportions of the acids formed by *E. coli* are not constant but vary with pH (Tikka, 1935). The *Chlorella* fermentation is similar in that the proportion of lactic acid alters at higher pH values (Fig. 1). Again, like *E. coli*, *C. vulgaris* produces both hydrogen and carbon dioxide, and, like *E. coli*, the two gases are formed in about equal quantities (Table 2). In the *E. coli* fermentation, these gases are known to arise from the decomposition of formic acid. They may have a similar origin in the *Chlorella* fermentation, but the addition of formate to anaerobic *Chlorella* cells does not stimulate gas production (cf. Gaffron & Rubin, 1943).

Syrett (1958) has shown that the distribution of ¹⁴C in acetic acid and lactic acids produced during the fermentation of specifically labelled glucose is consistent with the formation of these acids via the Embden–Meyerhof–Parnas glycolysis reactions. The formation of acetic acid and formic acid in approximately equimolar amounts suggests that these acids may arise from the cleavage of pyruvate into 1 mol.prop. of each as in *E. coli*. If this is so, formic acid should be derived from C-3 and C-4 of glucose; this has not yet been confirmed experimentally.

We have attempted to draw up a carbon balance sheet for the fermentation of glucose (Table 6), but there is clearly a considerable discrepancy since only 71% of the added carbon was recovered. One point is the not inconsiderable proportion (about 15%) of the added glucose which was incorporated into polysaccharide. Algae, like higher plants, do not readily carry out synthetic reactions under aerobic conditions. The synthesis of polysaccharide presumably indicates that glucose fermentation synthesizes ATP. The discrepancy in the carbon balance sheet may arise because of other synthetic reactions, although neither protein nor lipid becomes labelled when [¹⁴C]glucose is fermented. Alternatively, other unidentified fermentation products may exist, for example, the radioactive neutral com-

pound that was formed during the fermentation of [^{14}C]glucose.

There is also a discrepancy in the hydrogen balance. Acetic acid and formic acid can be thought of as being derived from pyruvate by a hydroclastic reaction, not a reductive one; they are not highly reduced fermentation products. Thus the reduced pyridine nucleotide that is generated during glycolysis must be reoxidized in some other way. Decomposition to give gaseous hydrogen is a possibility, but the quantity of hydrogen produced is insufficient and, in any case, is offset by the formation of carbon dioxide. The explanation of the discrepancy in the hydrogen balance is probably that intracellular substances in the *Chlorella* cells are available for reduction. In comparison with the bacterial fermentations, the fermentation of glucose by *Chlorella* is a very slow process (cf. Gibbs, 1962). In the experiments reported above, about 8 mg. of glucose is fermented by 100 mg. dry wt. of cells in 6 hr. Thus the quantity of cellular carbon is large compared with the amount of glucose fermented, and reduction of intracellular substances could have occurred.

One further point is that glucose is broken down more quickly aerobically than anaerobically, and a Pasteur effect is absent. Gibbs (1962) has suggested that the principal reason for the slowness of the algal fermentations is the low activity of enzymes that catalyse the oxidation of reduced pyridine nucleotide under anaerobic conditions. The absence of a Pasteur effect and the failure of 2,4-dinitrophenol to stimulate the endogenous fermentation rate, in contrast with its pronounced effect on aerobic endogenous respiration, are consistent with this explanation.

SUMMARY

1. The fermentation products of *Chlorella vulgaris* are principally acids; at pH 7 the fermentation of 1 mole of glucose yields about 0.6 mole of formic acid, 0.6 mole of acetic acid, 0.7 mole of lactic acid, 0.3 mole of gas and little, if any, ethanol. The proportion of lactic acid increases at higher pH values.

2. The gas produced during the fermentation is a mixture of hydrogen and carbon dioxide in approximately equimolar proportions.

3. During glucose fermentation, 10–15 % of the added glucose is converted into polysaccharide. This, together with the known fermentation products, accounts for 71 % of the glucose carbon that disappears.

4. Glucose is broken down more quickly aerobically than anaerobically, and a Pasteur effect is absent. 2,4-Dinitrophenol, though stimulating aerobic endogenous respiration, does not affect the rate of endogenous fermentation, nor does it induce aerobic fermentation.

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