CCXXII. STUDIES IN THE METABOLISM OF THE STRICT ANAEROBES (GENUS *CLOSTRIDIUM*)

VI. HYDROGEN PRODUCTION AND AMINO-ACID UTILIZATION BY *CLOSTRIDIUM TETANOMORPHUM*

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The organism employed in the present studies was a strain of *Cl. tetanomorphum* (National Collection of Type Cultures, No. 500) originally isolated by Robertson from a septic wound. It was described [McIntosh & Fildes, 1919] as producing a pink colour and gas in a meat medium with no digestion or blackening of the meat; gelatin was not liquified. Of the sugars tested only glucose and maltose were fermented. Similar results have been obtained by Kendall *et al.* [1922] with another strain of the organism; in addition, traces of gas were observed from glycerol and from nutrient broth. The metabolism of the organism does not appear to have been further investigated.

On cultivation of the organism anaerobically on a tryptic caseinogen digest broth a considerable quantity of gas was evolved. Analysis showed the gas to consist of hydrogen and carbon dioxide. Since the traditional source of hydrogen in bacterial fermentations is carbohydrate, it seemed to us of importance to trace the origin of the hydrogen formed from the tryptic broth, as the latter consists essentially of protein cleavage products and contains no detectable carbohydrate. Furthermore, *Bact. coli* and related organisms do not liberate hydrogen during growth on this medium.

In the present communication it will be shown that washed suspensions of *Cl. tetanomorphum* decompose a number of amino-acids with evolution of H_2 and CO_2. In addition a general survey of the metabolic activities of the organism has been made.

Experimental

Methods of estimation

(1) *Hydrogen.* Hydrogen production was measured in Warburg manometers. Conical cups with single or double side-bulbs and inner tubes, total volume 17–20 ml., were used. CO_2 was absorbed by the usual KOH-filter-paper absorbers in the inner tubes. The main cup usually contained the buffer and neutralized substrate and the bacterial suspension was tipped in from a side-bulb after equilibration. The manometers were filled with N_2 freed from traces of O_2 by passage over heated copper turnings.

(2) *Carbon dioxide.* CO_2 production was also estimated manometrically. The usual method employing bicarbonate buffer and a N_2-CO_2 gas mixture could not be applied since there was usually a concurrent production of H_2 and NH_3 and

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a change in the free acid concentration during the reactions. The following indirect method was therefore employed: (*a*) H_2 was estimated as above; (*b*) double side-bulb manometers were set up containing the bacterial suspension in the main cup, substrate in one side-bulb and 0·1 ml. 10% H_2SO_4 in the other. No CO_2 absorbent was employed in these cups. After the manometers were filled with N_2 and equilibrated, the initial bound CO_2 was determined in one series of manometers by tipping both acid and substrate into the main cup. In a second series of manometers the substrate only was tipped into the main cup and gas production followed. This gas consisted of H_2 and some of the CO_2 produced, the remainder being bound by the phosphate buffer. The mean value of H_2 evolution (as estimated in (*a*)) was expressed in terms of mm. pressure change in the manometers employed for the CO_2 determinations (*b*) and subtracted from the total pressure change. The remainder is equivalent to the change in pressure produced by free CO_2. The final bound CO_2 in the second series of manometers was then measured by tipping in the acid from the second side-bulb. The actual CO_2 production is therefore: free CO_2 + final bound CO_2 − initial bound CO_2. Similar control experiments in which water replaced the substrate were carried out.

(3) *Miscellaneous estimations.* Ammonia was determined by the method described by Stickland [1934] and Woods [1936]. Volatile acids were estimated according to Stickland [1935]. A photo-electric turbidimeter [Clifton *et al.* 1935], calibrated in terms of total cell count and dry weight per ml., was used to follow growth and to estimate the dry weight of the bacterial suspensions.

Growth experiments

The production of H_2 by *Cl. tetanomorphum* during growth on tryptic broth was followed manometrically, CO_2 being absorbed. Samples from a flask of tryptic broth, inoculated immediately after autoclaving and rapid cooling from a 24 hr. culture in Robertson's meat medium, were placed in a series of Warburg cups and the manometers at once filled with N_2 and shaken at 32°. Manometers were removed at 2 hr. intervals for total cell and dry weight determinations. Typical results are plotted in Fig. 1, together with Q_{H_2} values (μl. H_2 per hr. per mg. dry weight), calculated for each 2 hr. interval. Q_{H_2} serves as a measure of the activity of the organisms and changes with the age of the culture in a manner similar to that described by Clifton [1937] for the rates of O_2 consumption and CO_2 production by certain facultative anaerobes. A maximum Q_{H_2} was observed early in the logarithmic period of growth.

In large-scale experiments the gases produced were analysed in the Haldane apparatus. After preliminary CO_2 absorption, the volume of gas decreased on combustion with no concurrent formation of alkali-soluble products; the evidence therefore indicates that the gas is H_2. In a typical experiment the original gas in the culture flask consisted only of N_2, whilst after 48 hr. growth its composition was 28·6% H_2, 19·6% CO_2 and 51·8% N_2. Analysis of the gas collected in Durham tubes from broth cultures of *Cl. tetanomorphum* showed, in a typical case, 84·5% H_2 and 15·6% CO_2; a considerable amount of CO_2 is, of course, bound by the medium.

In Durham tube experiments the presence of glucose (M/10) in the broth medium stimulated both growth and gas production. It is impossible to determine from these experiments if the extra gas formed in the presence of glucose is due to fermentation of the sugar, or to the increased growth on the broth. It is clear that with this organism the results of the usual bacteriological

fermentation tests with sugars carried out by the Durham tube or fermentation tube technique must be accepted with caution. Formate ($M/10$) in the broth medium considerably inhibited both growth and gas formation.

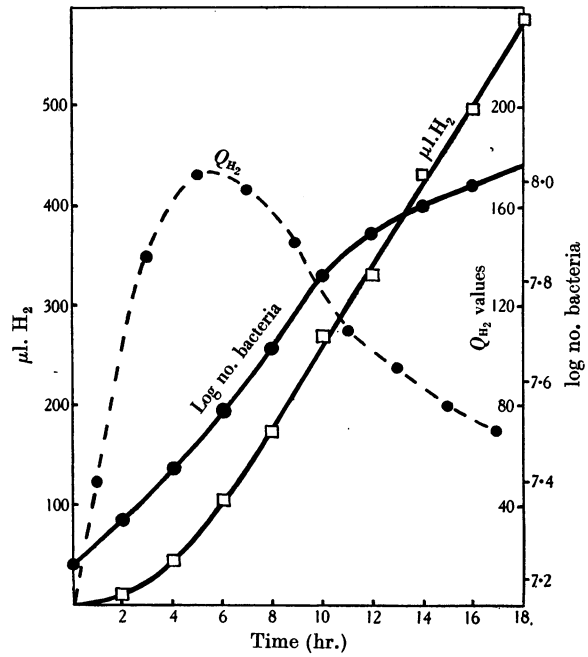

Fig. 1. Growth, H_2 production and Q_{H_2} on broth.

The electrode potentials developed during the growth of *Cl. tetanomorphum* on tryptic broth were determined as described by Clifton *et al.* [1934]. Growth and gas production became apparent when the potential of the broth was in the neighbourhood of $-0{\cdot}1$ v. at *p*H 7·2. A potential of $-0{\cdot}225$ v. was established in 24 hr. and was maintained at this level during the course of the experiment (5 days). That the potential is not influenced by the presence of H_2 is shown by the fact that the potential level was not appreciably altered when H_2 instead of N_2 was bubbled through the culture for several hours. Potentials of the same order are established during the growth of *Cl. tetani* and certain other anaerobes.

Experiments with washed suspensions

Preparation of suspensions. 900 ml. of tryptic caseinogen digest broth, containing a few pieces of meat from Robertson's meat medium, were autoclaved, rapidly cooled and at once inoculated with 1 ml. of the fluid from a 20 hr. culture of *Cl. tetanomorphum* on Robertson's meat medium. The culture was placed immediately in a McIntosh and Fildes anaerobic jar and incubated at 34° for 18–20 hr. At this time the culture usually contained approximately $1{\cdot}0 \times 10^8$ cells per ml. (0·1 mg. dry weight per ml.). Longer growth leads to less active suspensions and considerable spore formation. Irregular growth is obtained if the medium is allowed to stand exposed to air for even $\frac{1}{2}$ hr. before being rendered anaerobic.

The culture was centrifuged, the cells washed twice on the centrifuge with distilled water and finally suspended in 4–7 ml. distilled water or phosphate buffer *p*H 7·2. The suspension was at once thoroughly evacuated and the tube filled with N_2 and stored at 0° if the organisms were not used at once. It is essential that the spinning and washing processes be carried out as rapidly as possible and the final suspension kept out of contact with air; it will be shown (p. 1782) that the washed cells rapidly lose activity on exposure to oxygen.

Unless otherwise stated all experiments were carried out in phosphate buffer *p*H 7·2 and at 32°.

Production of hydrogen

As a first step in tracing the precursor of the hydrogen formed from broth the materials given in Table I were tested manometrically by the washed suspension technique. The washed suspension, like the growing organism, was highly active in liberating hydrogen from the tryptic digest broth. The high rate from broth compared with the very low rate from glucose alone shows clearly that traces of contaminating glucose cannot be responsible for the gas production from broth.

Table I

Substrate	Q_{H_2}
Tryptic digest broth (10 mg. N/ml.)	36·4
Acid hydrolysate caseinogen (17 mg. N/ml.)	32·8
Acid hydrolysate gelatin (29 mg. N/ml.)	20·8
Peptone (Difco) 2%	14·5
Glucose *M*/10	2·8
Formate *M*/10	0
Tryptic digest broth + glucose	31·7
Tryptic digest broth + formate	30·2

Substrate 0·7 ml., buffer *p*H 7·2 0·5 ml., bacterial suspension 0·4 ml. Water to 2·3 ml.

There remained a possibility that cell division might be necessary for hydrogen formation; it might be that the non-formation of gas from glucose and formate was due to the inability of the organism to grow on these substrates alone. This is disproved since glucose and formate slightly depress rather than increase H_2 production when mixed with broth (Table I).

It appeared most likely that the source of hydrogen in broth would be found among the amino-acids which are its main constituents. This view was supported by the experiments with the acid hydrolysates and peptone. The amino-acids available to us were therefore tested with washed suspensions for H_2 production (Table II). Most of the amino-acids were obtained from Hoffman-La Roche and were used in neutral solution. Cysteine was neutralized immediately before use;

Table II

Hydrogen produced	Hydrogen not produced
l(+)-Glutamic acid	Glycine
l(+)-Aspartic acid	*l*(−)-Alanine
dl-Serine	*l*(−)-Leucine
l(−)-Cysteine	*l*(+)-Valine
l(−)-Cystine*	*l*(−)-Proline
l(−)-Histidine	*l*(−)-Hydroxyproline
l(−)-Methionine	*l*(−)-Phenylalanine
l(−)-Tyrosine	*l*(−)-Tryptophan
	l(+)-Ornithine
	l(+)-Lysine
	l(+)-Arginine

Substrate *M*/30, organisms 12–16 mg. dry weight, *M*/15 phosphate buffer *p*H 7·2.

* Traces only.

cystine and tyrosine, being comparatively insoluble at *p*H 7, were used as neutral suspensions. With cystine and cysteine, filter-paper impregnated with 10% cadmium sulphate was placed in a second side-bulb as an additional means of removing the H_2S which is also formed. In experiments with methionine 10% zinc acetate absorbers were used, as a mercaptan appeared to be produced.

A number of the amino-acids liberating gas not absorbed by alkali (glutamic acid, aspartic acid, serine, cysteine, histidine) were tested on a larger scale without CO_2 absorption. The gaseous products on analysis proved to be H_2 and CO_2. No other gas was detected except hydrogen sulphide in the case of cysteine.

During the course of the work a number of carbohydrate and non-amino-substances, some of which were considered possible intermediates in amino-acid breakdown, were tested for H_2 production (see Table III). Analysis of the gaseous products of large-scale experiments with pyruvate, fumarate and malate confirmed the production of H_2 and CO_2.

Table III

Hydrogen formed	Hydrogen not formed	
Pyruvate	Formate	Tartrate
Fumarate	Galactose	Citrate
l(−)-Malate	Lactose	Oxalate
d-Glucose	Succinate	Acetoacetate
d-Maltose	Lactate	β-Hydroxybutyrate
Glycerol	Acetate	α-Ketoglutarate
	Propionate	Ethyl alcohol
	Butyrate	Acetaldehyde
		Aldol

Substrate *M*/40, organisms 12–16 mg. dry weight, *M*/15 phosphate buffer *p*H 7·2.

The absence of a formic hydrogenlyase enzyme is of particular interest since formate is usually regarded as the immediate precursor of hydrogen in fermentations brought about by facultative anaerobes. No data are available as to the formic hydrogenlyase activity of strict anaerobes which produce H_2 from carbohydrates. The possibility that the comparatively high concentration of formate used (*M*/30) was toxic to the organisms was checked by repeating the experiment with more dilute formate. The following results were obtained:

	μl. H_2 per hr.
Blank	27
Formate *M*/30	20
M/250	23
M/500	22

There is no indication of hydrogen production even with *M*/500 formate; furthermore the presence of formate slightly inhibits the blank H_2 evolution. It appears improbable that hydrogen is produced via formate in the case of *Cl. tetanomorphum*.

Relative rates of hydrogen production. The relative rates of hydrogen production from the different substrates varied somewhat with different batches of organisms. Of the various substrates pyruvate gave the most constant rate of H_2 evolution, Q_{H_2} values of 25–30 usually being obtained. Nine determinations with different batches of organisms yielded the following results:

	Q_{H_2} values
Range	24·7–30·0
Mean	27·2
Standard deviation	2·16

Compared with pyruvate, the other substrates varied considerably with different batches of bacteria both as to absolute rate of H_2 production and relative rate compared with one another. It was decided, therefore, to adopt the rate with pyruvate as a standard (=100) for any suspension and to express the rates with other substances as % of the rate with pyruvate. This value will be referred to as the activity index. Table IV summarizes the results of a large number of determinations with the various substrates and different suspensions arranged in order of mean activity index. The standard deviation gives some idea of the degree of variability experienced, in spite of growing the organisms and preparing the suspensions under, as far as was known, uniform conditions.

Table IV

Substrate	No. of exps.	Activity index		
		Range	Mean	Standard deviation
Pyruvate	.	.	100	.
l(+)-Glutamate	5	53–80	67·4	12·27
Fumarate	7	28–86	64·3	20·32
l(−)-Cysteine	5	30–64	45·0	13·56
dl-Serine	7	32–57	43·7	11·10
l(−)-Malate	8	14–57	40·1	14·56
l(+)-Aspartate	9	7–60	38·7	19·39
l(−)-Histidine	7	21–43	29·4	7·32
d-Glucose	6	7–37	20·7	11·20
l(−)-Tyrosine	3	15–24	20·0	.
Glycerol	2	7–18	12·5	.
l(−)-Methionine	3	8–12	10·0	.
d-Maltose	5	4–17	10·0	6·04
l(−)-Cystine	1	.	1·2	.

Substrate *M*/30, organisms 12–16 mg. dry weight, *M*/15 phosphate buffer *p*H 7·2.

The four-carbon compounds aspartate, fumarate and malate show the greatest variation; some suspensions were completely inactive towards these substrates although decomposing pyruvate and glutamate at about the usual rate. In the case of cysteine the rate of H_2 evolution fell off rapidly with time if comparatively large amounts (0·5 *M*/10) were used; this may be due to local poisoning by H_2S. The absorption of H_2S is, of course, always slightly in arrear of the formation of H_2S in the culture.

The organism is not a vigorous sugar fermenter. The rate of H_2 production from glucose is only $\frac{1}{5}$ of that from pyruvate and is considerably slower than that from most of the amino-acids. The activity of the glucose enzyme is somewhat variable. Maltose, the only other sugar fermented, is attacked very slowly.

It is hard to account for the variation in the relative rates at which the various substrates are attacked by different batches of organisms. With all strict anaerobes it is difficult to obtain identical growth even though the growth conditions are standardized; possibly enzymes attacking the various amino-acids are formed at different stages during growth and tend to disappear as their substrates become exhausted. It is hoped in future work to follow the relation between time of growth and the activity of the suspensions towards some of the substrates.

Total hydrogen formation. The absolute amount of H_2 evolved was determined for most of the substances attacked by the organism. The mean results of a large number of experiments, expressed as mol. H_2 formed per mol. of substrate, are given in Table V. In the case of substances attacked slowly, the organisms were tested for activity towards pyruvate at the end of the experiment in order to

Table V

Substrate	Mol. formed per mol. substrate: H_2	CO_2	NH_3
l(+)-Glutamate	0·27	0·85	0·92
dl-Serine	0·18* 0·14†	0·72* 0·53†	0·66* 0·47†
l(+)-Aspartate	0·90	1·41	0·87
l(−)-Histidine	0·14	0·67	2·17
l(−)-Cysteine	0·30	0·84	0·90
l(−)-Cystine	0·05	1·69	1·75
l(−)-Tyrosine	0·39	0·87	0·97
l(−)-Methionine	+	+	+
Glycine	0	0	0·05
l(−)-Alanine	0·01	0·02	0·04
l(−)-Leucine	0·02	0·06	0·10
l(+)-Valine	0	0·02	0·04
l(−)-Phenylalanine	0·01	0·02	0·06
l(−)-Proline	0·01	0	0·07
l(−)-Hydroxyproline	0·01	0	(−)
l(−)-Tryptophan	0·02	0	(−)
l(+)-Lysine	0·01	0·01	0·01
l(+)-Ornithin	0·01	0·01	0·03
l(+)-Arginine	0·01	0	0·04
Pyruvate	0·28	0·84	
Fumarate	1·04	1·51	
l(−)-Malate	0·98	1·50	
d-Glucose	1·95	+	
d-Maltose	+	+	
Glycerol	+	+	

* Experiment incomplete. † Value at which rate fell sharply.
+ = positive. (−) = not tested.

be sure that the cessation of H_2 production was not due to the organisms having become inactive. The amount of substrate taken was 0·05–0·3 ml. $M/10$ according to the rate of breakdown. Pyruvate solutions were standardized by the carboxylase method of Westerkamp [1933]. No attempt was made at quantitative determinations with substrates attacked very slowly, as the relatively large blank in long experiments renders such results unreliable. The amino-acids may be divided into two groups: (1) those giving large amounts of H_2, (2) those giving no H_2 or traces only; the latter is probably due to the presence of traces of the active amino-acids as impurities. Pyruvate, glutamate and cysteine yield approximately 0·3 mol. H_2 per mol. of substrate. With serine the results are a little complicated by the fact that as no *l*-serine was available the *dl*-compound was used. The curve for H_2 production from *dl*-serine (see Fig. 2) indicates that the *d*-isomeride is also attacked slowly by the organism, for after a rapid formation of H_2, the curves turn over sharply and H_2 formation then proceeds at a slow rate. With other substrates H_2 formation proceeds rapidly to completion and the cessation is abrupt. It will be shown later that the deamination of *dl*-serine proceeds in a similar way to H_2 formation (see Fig. 6), and further that the NH_3 formed during the period of rapid activity corresponds with the complete deamination of one isomeride of the *dl*-serine. The H_2 production at the break in the curve will therefore be assumed to indicate approximately the amount of H_2 formed from *l*-serine. Allowing for the fact that *dl*-serine was used it will be seen (Table V) that 1 mol. of *l*-serine also yields approximately 0·3 mol. H_2. Tyrosine gives rise to a rather larger amount of H_2 (0·39 mol.), whilst histidine gives only 0·14 mol. Only traces of hydrogen are evolved from cystine, possibly

owing to a reduction of a small part of this compound to cysteine by substances present in the organism.

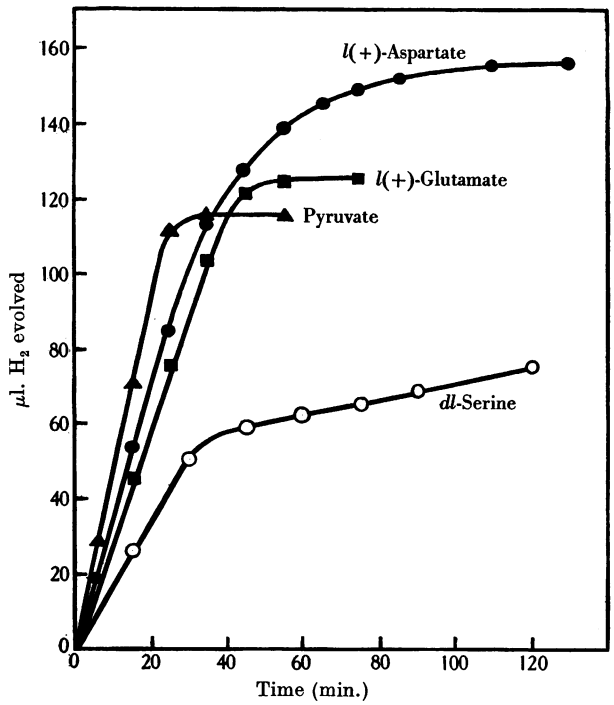

Fig. 2. Course of H_2 production. *l*(+) Glutamate 0·2 ml. *M*/10; pyruvate 0·2 ml. *M*/10; *l*(+) aspartate 0·3 ml. *M*/40; *dl*-serine 0·2 ml. *M*/10.

Glucose is broken down with the formation of 2 mol. of H_2; it would appear therefore that glucose cannot be disrupted via pyruvic acid since 2 mol. of the latter would give only 0·6 mol. H_2.

The four-carbon compounds aspartate, malate and fumarate were the only substances tested yielding 1 mol. H_2 per mol. of substrate. The H_2 evolution shows a tendency to fall off as the reaction nears completion (see aspartate, Fig. 2); the enzymes responsible for H_2 evolution seem to have a lower affinity for these substrates than is the case with pyruvate and glutamate.

The total H_2 formation with a given substrate appeared to vary somewhat with different batches of organisms, particularly in the case of substrates giving less than 1 mol. H_2. A large number of determinations have been made with pyruvate and glutamate as the reactions are rapid and the organisms blank is small in short period experiments. Table VI summarizes the results obtained

Table VI

	Pyruvate	Glutamate	Aspartate
No. of exps. ...	27	21	6
Mol. H_2 formed per mol.:			
Range	0·18–0·37	0·16–0·39	0·86–0·94
Mean	0·28	0·27	0·91
Standard deviation	0·0496	0·0728	0·0313

with different suspensions. All values are the means of duplicate estimations not varying by more than 3 %. Some figures for aspartate (which yields 1 mol. H_2) are included for comparison. In the case of pyruvate and glutamate these differences are well beyond the limits of experimental error as the extreme values represent a difference of over 100 %.

There remains a possibility that the blank H_2 production by the organisms alone does not occur in the presence of fermentable substrate and that the variation observed is due to differences in the blank subtracted rather than in the H_2 formed from the substrate. However the blank in these experiments was always less than 10 % of the H_2 evolution from e.g. pyruvate and there was no correlation between a high blank and low H_2 formation or vice versa (see Table VII). A similar variation in total H_2 is obtained if the organism blank is not subtracted.

Table VII

Mol. H_2 per mol. pyruvate		
Blank subtracted	Blank not subtracted	% blank of H_2 evolution
0·18	0·19	6
0·22	0·23	5
0·26	0·28	8
0·35	0·39	10
0·36	0·39	8

Substrates yielding 1 mol. H_2 give more constant results; with aspartate (Table VI) the maximum variation does not exceed 10 % and similar results are obtained with malate and fumarate. A possible explanation of the variable amounts of H_2 obtained from pyruvate and glutamate is that these substances, or some intermediate in their breakdown, are attacked in two or more ways by the organism, and that only one of these methods gives rise to H_2. The amount of H_2 produced would then depend on the relative strengths of the enzymes catalysing the different modes of breakdown. Such a theory of variability of the enzyme constitution of the cell is supported by the results already reported on the variability of the relative rates of H_2 production from the various substrates by different batches of organisms.

Effect of p*H on rate of* H_2 *formation.* Fig. 3 shows the influence of *p*H on the initial rate of H_2 formation from pyruvate and glutamate. The optimum is not sharp, the rate being at least 90 % of the maximum between *p*H 6·4 and 7·2 with pyruvate and between *p*H 6·7 and 7·4 with glutamate. The optimum in both cases is approximately *p*H 7. Fig. 4 illustrates an experiment with glutamate in which the rate of H_2 evolution at different *p*H values was followed for some time. It will be seen that the activity of the organisms falls off markedly in the acid range but remains more constant on the alkaline side of the optimum.

Optimum temperature. All the experiments described have been carried out at 32–33°. In short duration experiments faster rates were occasionally observed at 37° but the activity of the suspensions decreased rapidly at this temperature.

Sensitivity of the washed suspensions to oxygen. The necessity for keeping the washed suspensions of *Cl. tetanomorphum* anaerobic has already been emphasized. The results of a quantitative experiment designed to test the loss of activity of suspensions exposed to air in liberating H_2 from glutamate is shown in Fig. 5. A freshly prepared suspension of the organisms was divided into two parts; one part was at once thoroughly evacuated and stored at 0°. The second part was pipetted into the side-bulbs of manometer vessels and left exposed to the air

at room temperature. At intervals the manometers were made anaerobic and the initial rate of H_2 formation from glutamate measured. Similar determinations were made with the suspension stored *in vacuo* at 0°, the manometers being filled with N_2 in this case immediately after adding the organisms.

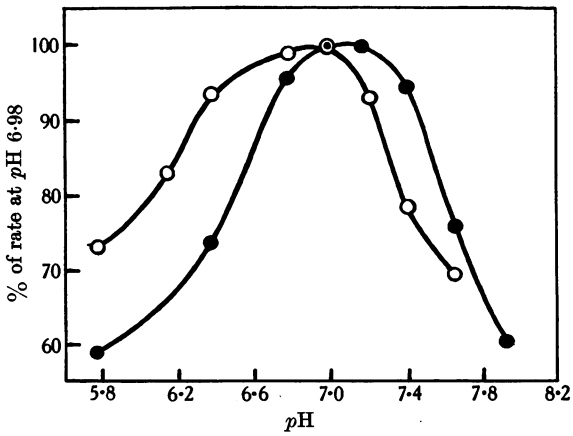

Fig. 3. Effect of *p*H on initial rate of H_2 formation.
• Glutamate. o Pyruvate.

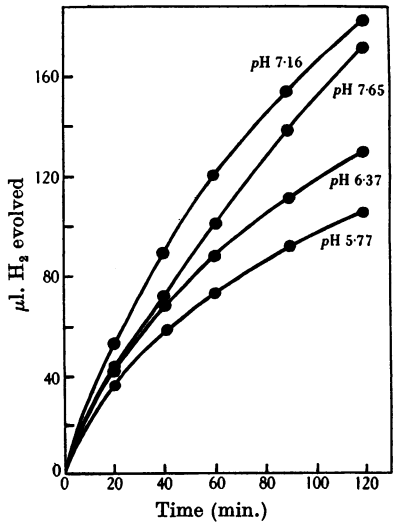

Fig. 4. Effect of *p*H on rate of H_2 production from glutamate.

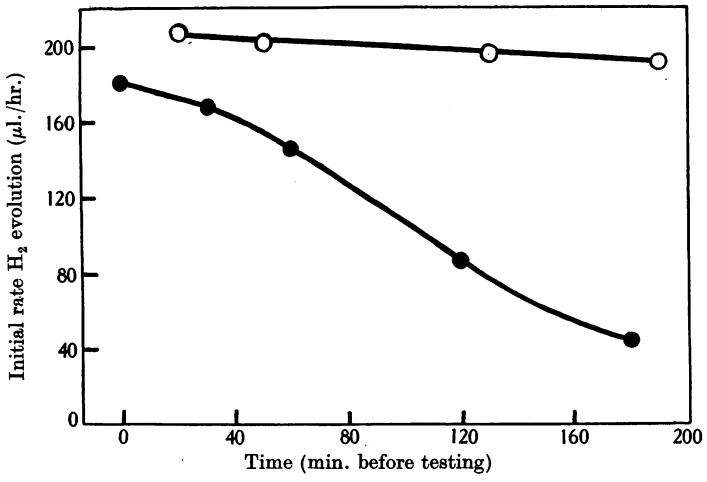

Fig. 5. Sensitivity of suspensions to oxygen.
• Organisms exposed to air at room temperature. o Organisms stored *in vacuo* at 0°.

It will be seen that there is a serious loss of activity with the organisms exposed to air, amounting to 20% in the first hour. It is obvious that in comparative experiments with this organism stringent precautions must be taken to avoid exposing the suspensions to O_2 while setting up the experiments. The practice adopted throughout the present work has been to evacuate the

freshly made suspension thoroughly, fill the tube with N_2 and keep at 0°. Experimental vessels were made anaerobic immediately after transferring the organisms from the storage tube.

The suspensions are comparatively stable when kept *in vacuo* at 0° (Fig. 5). Organisms stored in this way still retained 50 % of their original activity after 24 hr. and 15 % after 48 hr.

Production of carbon dioxide

Production of carbon dioxide was tested manometrically with all substrates giving rise to hydrogen. The amino-acids not producing hydrogen were also tested in order to determine whether decarboxylation occurred without H_2 formation. In the cases of cysteine and cystine the technique described earlier (p. 1774) had to be modified as the method estimates as CO_2 the H_2S which is also formed from these substances. In these cases therefore the manometers used for the H_2+CO_2 estimations contained 0·3 ml. acid 10 % cadmium sulphate (with filter-paper) in the centre tubes as H_2S absorbent. In the hydrogen estimations the KOH absorber for CO_2 also takes up H_2S; as an additional precaution $CdSO_4$ absorbers were placed in a second side-bulb. In the methionine experiments zinc acetate absorbers were used in both H_2 and H_2+CO_2 determinations to remove any mercaptan formed.

It will be seen (Table V) that all substrates attacked with the production of H_2 also yield CO_2. Quantitatively the substances giving approximately 0·3 mol. H_2 gave about 1 mol. CO_2. *dl*-Serine gives a greater amount of CO_2 than would be

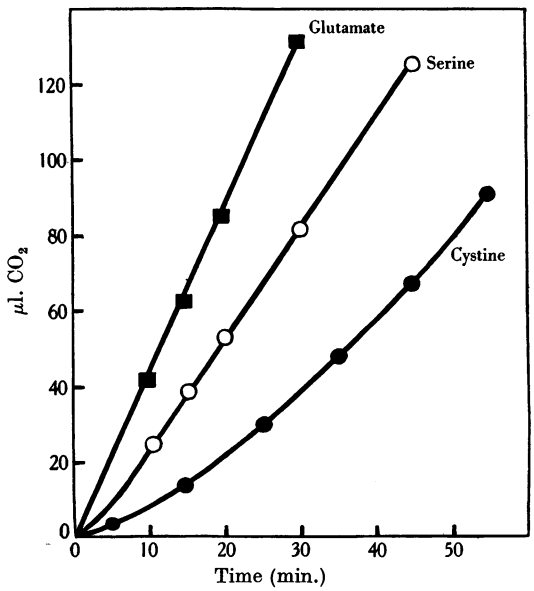

Fig. 6. Rate of CO_2 formation.

expected on the assumption that 1 mol. of CO_2 is formed and that only the natural isomeride is attacked; there is additional evidence from H_2 and deamination figures that the other isomeride is also attacked slowly. The four-carbon compounds aspartate, malate and fumarate (which give 1 mol. H_2) appear to evolve 1·5 mol. CO_2.

Cystine, which gives only traces of H_2, is decomposed with evolution of twice as much CO_2 per molecule as is yielded by cysteine; this would be expected if each cysteine moiety of the cystine molecule were attacked in a manner similar to cysteine.

The rate of evolution of CO_2 from cystine is of some interest (Fig. 6). The reaction appears to be autocatalytic in the early stages, the rate of CO_2 formation increasing with time. Eventually the evolution becomes linear. With all other amino-acids the rate is linear from the moment of tipping in the substrate; serine and glutamate are given for comparison in Fig. 6. It was thought that the initiation of the decomposition of cystine might depend on the presence of a trace of cysteine, H_2 evolved or potentially formed from the latter reducing some cystine to cysteine, which would then be decomposed and at the same time reduce more cystine and so on. This would explain both the autocatalytic effect and the absence of H_2 formation with cystine. Experimentally, however, the addition of 1/20 the amount of cysteine to the cystine only reduced the autocatalytic period from 60 min. to 50 min.—an effect not sufficiently marked to support the above hypothesis.

The amino-acids forming only traces or no H_2 gave negative results for CO_2 formation. Except with cystine there was no decarboxylation without concurrent H_2 formation.

Deamination of amino-acids

The amino-acids tested above for H_2 and CO_2 formation were also tested for ammonia production when incubated anaerobically with washed suspensions of *Cl. tetanomorphum*. The usual procedure was to analyse the contents of Warburg pots for ammonia at the end of a quantitative experiment on H_2 or CO_2 production. The results are given in column 3 of Table V. All amino-acids yielding H_2 and/or CO_2 were deaminated. The other amino-acids (giving traces of, or no, H_2 and CO_2) showed only traces of NH_3. Glutamate, aspartate, cysteine, cystine, tyrosine and methionine were almost completely deaminated since they all produced between 0·88 and 0·97 mol. NH_3 per amino-group of the original amino-acid.

Since histidine contains only one amino-group in its side chain and since more than 1 mol. of NH_3 is produced, it follows that disruption of the iminazole ring must have occurred. Edlbacher and co-workers [1930; 1934] have obtained evidence with animal tissues of the breakdown of histidine via glutamic acid; a similar breakdown would explain the origin of H_2 from histidine with *Cl. tetanomorphum* since it has been shown that glutamic acid gives rise to H_2.

In the case of *dl*-serine deamination proceeds rapidly and parallel with H_2 formation until 50% deamination has occurred, corresponding with complete deamination of one isomeride of the serine (see Fig. 7). At this point NH_3 formation suddenly decreases sharply and proceeds at a slow rate. The rate of deamination with glutamate, which proceeds almost linearly and ceases abruptly, is given for comparison. The deamination results support the view, deduced from the course of H_2 evolution, that the *d*-isomeride of serine is also attacked, though much more slowly, by the organisms.

"*Stickland reaction.*" Working with *Cl. sporogenes*, Stickland [1934] demonstrated that linked reactions occurred between pairs of amino-acids, one of which was oxidized and the other reduced. Two typical reactions are those between alanine and glycine and between alanine and proline. In each case alanine is oxidized and deaminated; glycine is reduced and deaminated and proline reduced without deamination. Since none of these amino-acids is

appreciably deaminated alone by *Cl. sporogenes*, the production of NH_3 (beyond the controls) from a mixture of two amino-acids is an indication that a reaction

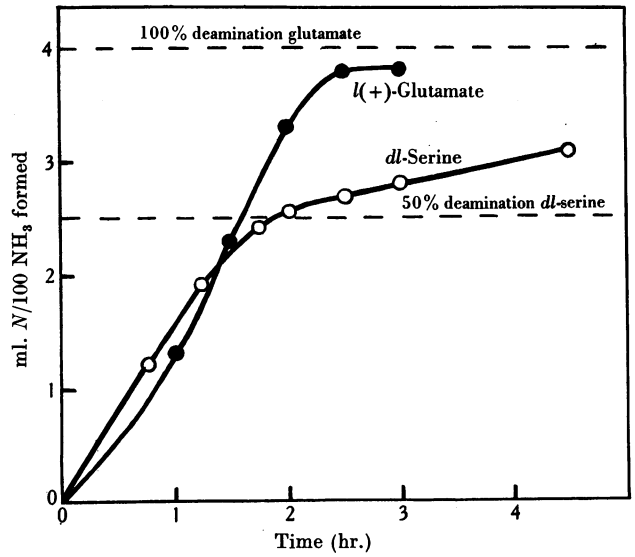

Fig. 7. Course of deamination 0·4 ml. *M*/10 *l*(+)-glutamate; 0·5 ml. *M*/10 *dl*-serine.

is taking place. The possible occurrence of such coupled reactions with *Cl. tetanomorphum* was tested with the alanine-proline and alanine-glycine systems and washed suspensions of the organism (Table VIII). There is no increase above the controls with either pair of amino-acids.

Table VIII

(All values less organism blank)

Substrate	ml. *N*/100 NH_3 formed
Alanine	0·1
Proline	0·2
Glycine	0·2
Alanine + proline	0·15
Alanine + glycine	0·15

Quantities used: 0·4 ml. *M*/10 substrate, 1 ml. phosphate buffer *p*H 7·2, 0·4 ml. bacterial suspension, water to 2·4 ml. Incubated 4 hr. 32°.

Other reaction products

It is proposed to deal in a future paper with the more detailed chemistry of the decomposition of pyruvate and glutamate. A few points of a general nature may be mentioned here. All the substrates yielding H_2 which have been tested in large-scale experiments for the formation of volatile fatty acids have given positive results (Table IX); quantitative data are given only when the results of at least three determinations are available.

Evidence will be presented in a following paper that in the cases of pyruvate and glutamate the volatile acid is a mixture of acetic and butyric acids. With pyruvate the quantities found are in general agreement with the equation:

$$3CH_3.CO.COOH + H_2O \rightarrow CH_3.COOH + CH_3.CH_2.CH_2.COOH + 3CO_2 + H\ .$$

Table IX

(All values less appropriate control)

Substrate	Equivalents volatile acid formed per mol. substrate
Pyruvate	0·63
l(+)-Glutamate	1·46
dl-Serine	+
l(−)-Histidine	+
Fumarate	+
l(−)-Malate	+

A small amount of lactic acid (about 5%) is also formed from pyruvate, but not from glutamate.

Discussion

The liberation of molecular hydrogen during the decomposition of a number of amino-acids by *Cl. tetanomorphum* constitutes, as far as the authors are aware, a mode of breakdown of amino-acids not so far described for any type of cell. For bacteria in particular the process is of interest in that *Cl. tetanomorphum* appears to contain no formic hydrogenlyase enzyme; formate is usually considered to be the precursor of H_2 produced by bacteria. All the available evidence in this case is against the view that the H_2 arises through formate as an intermediary. Formate, even in very low concentration, gives no hydrogen when incubated with the organism. Since formate slightly depresses rather than increases the rate of H_2 formation from broth and from the organisms without added substrate, it seems unlikely that it is decomposed with formation of H_2 by interaction with some other intermediary. Additional evidence against this view is provided by the following experiment in which the absolute amount of H_2 from pyruvate was measured both in the presence and absence of formate:

	μl. H_2 formed	Q_{H_2}
0·2 ml. *M*/10 formate	0	0
0·2 ml. *M*/10 pyruvate	102	27·2
0·2 ml. *M*/10 formate + 0·2 ml. *M*/10 pyruvate	100	26·8

Formate increases neither the rate nor the total H_2 formed. The present evidence suggests therefore that some immediate precursor of H_2 other than formate must be sought. Discussion of the possible mode of origin of the H_2 is best deferred until the complete breakdown products of some of the substrates yielding H_2 have been quantitatively established. Stephenson & Stickland [1932] (see also Stephenson [1937]) obtained some evidence that with *Bact. coli* and glucose H_2 may arise by some other mechanism than via formate.

Recently Brown *et al.* [1937] have shown that pyruvic acid is dissimilated by suspensions of *Cl. butylicum* (Beyerinck) Donker (an organism of the butyric acid-butyl alcohol group characterized by the formation of isopropyl alcohol) in a manner very similar to that described for *Cl. tetanomorphum* in the present paper. At *p*H values above 6·3 the products obtained from pyruvate are H_2, CO_2, acetic acid and butyric acid. Two experiments quoted show mean values for H_2 and CO_2 production of 0·25 and 1·01 mol. per mol. of pyruvate utilized. Furthermore this organism does not attack formate.

As far as could be ascertained by the methods of investigation employed—H_2, CO_2 and NH_3 formation—only 8 of the commoner amino-acids (glutamic acid, aspartic acid, serine, cysteine, cystine, methionine, tyrosine and histidine)

are attacked by *Cl. tetanomorphum* to any appreciable extent. It is difficult to imagine any anaerobic method of decomposition of amino-acids in which neither NH_3 nor CO_2 would be produced. Since the organism grows vigorously on tryptic digest broth, which is essentially a mixture of amino-acids, it would appear that the decomposition of amino-acids is probably an important energy-yielding reaction of the cell.

It is noteworthy that, with the exception of cystine, all the amino-acids shown to be attacked in any way are broken down with evolution of H_2.

The decomposition of amino-acids with evolution of insoluble gas might play an important part in the setting up of gas gangrene in wounds infected by this organism. Although *Cl. tetanomorphum* itself has but little proteolytic activity, other anaerobic organisms also found in infected wounds, such as *Cl. histolyticum* and *Cl. putrificum*, have powerful proteolytic enzymes and would readily provide the amino-acid substrates necessary for H_2 formation by *Cl. tetanomorphum*.

Summary

1. Washed suspensions of *Cl. tetanomorphum* decompose a number of amino-acids with production of hydrogen. With the exception of cystine all amino-acids attacked by this organism in any way yield hydrogen; in each case carbon dioxide and ammonia are also formed.

2. The amino-acids decomposed in this way are: *l*(+)-glutamic acid, *dl*-serine, *l*(+)-aspartic acid, *l*(−)-histidine, *l*(−)-cysteine, *l*(−)-cystine, *l*(−)-tyrosine and *l*(−)-methionine. The other common amino-acids are not appreciably attacked.

3. Pyruvate, fumarate, *l*(−)-malate, *d*-glucose, *d*-maltose and glycerol are also decomposed with evolution of hydrogen and carbon dioxide.

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