CXXVIII. METABOLISM OF THE STRICT ANAEROBES (GENUS: *CLOSTRIDIUM*)

II. REDUCTION OF AMINO-ACIDS WITH GASEOUS HYDROGEN BY SUSPENSIONS OF *CL. SPOROGENES*

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STUDIES were made of the conditions under which suspensions of *Cl. sporogenes* activate certain substrates as H-acceptors. Gaseous H_2 was used as the H-donator and the reactions carried out in Warburg manometers, in contrast to the methods used by other workers [Stickland, 1934; Woods, 1936]. It is believed that some of the objections to previous methods are eliminated by the method used in these studies. The method became possible only after it was found that *Cl. sporogenes*, under the proper conditions, actively utilizes gaseous H_2 .

EXPERIMENTAL

The strain of *Cl. sporogenes* used in these experiments was obtained from the American Type Culture Collection and was labelled No. 319. The organisms were cultured at pH 7.3 in 3% neopeptone solution, to which 1% glucose was added. Anaerobic conditions were provided in McIntosh and Fildes jars. Following incubation for 16 hr. at 37° the culture was centrifuged. The precipitated organisms were then washed with a volume equal to the original culture of 0.85% NaCl which had been previously boiled and cooled. After recentrifuging the organisms were resuspended in a previously boiled and cooled phosphate buffer solution of pH 7.2. Usually the organisms obtained from 250 ml. culture were suspended in 6 ml. phosphate buffer.

One ml. suspension of *Cl. sporogenes* in phosphate buffer was placed in each Warburg manometer vessel, the side arm of which contained 0.5 ml. 0.1M solution of the prospective H-acceptor. H_2 , purified by passing through a column of electrically heated copper particles, was directed through the apparatus for 10 min., after which the vessels were closed and placed in a water bath at 40°. After equilibrium had been reached (c. 5 min.) the substrate was added from the side arm and the change in gaseous pressure (in this case a decrease) measured. A control experiment in which the bacterial suspension and the substrate solution were not mixed together was carried out simultaneously.

In the first experiment glycine and hydroxyproline were used. Stickland [1934], employing another technique, had shown that these compounds acted as H-acceptors. As may be seen from Fig. 1 a very marked and uniform H_2 absorption with no decrease in rate even after 3-4 hr. was observed when either of these

compounds was added to the suspension of *Cl. sporogenes*. With glycine the absorption was slightly greater than with hydroxyproline. There was no appreciable H_2 absorption in the control.

The presence of glucose in the culture medium, while not entirely essential, was found to increase very markedly the ability of *Cl. sporogenes* to activate gaseous H_2 . In the absence of glucose some H_2 was usually absorbed but the amount was very small, a heavy bacterial suspension being required in order to detect the absorption. This may be the reason why Stephenson & Stickland [1931], who cultured *Cl. sporogenes* on a tryptic digest of caseinogen, failed to observe any H_2 absorption.

Fig. 1.

Fig. 2.

Fig. 1. Course of H_2 absorption of a washed suspension of *Cl. sporogenes* with glycine (0.2 m.-mol.) and hydroxyproline (0.1 m.-mol.) used as substrates. ●—● glycine, ○—○ hydroxyproline.

Fig. 2. Effect of pH on the rate of H_2 absorption. ○—○ hydroxyproline, ●—● tryptophan.

The addition of formate to the culture medium also increased the ability of the organisms to activate H_2 , although much less than did the addition of glucose. When formate was used it was necessary to decrease the peptone concentration in order to induce some formate utilization. The most active bacterial suspensions were obtained from a 16 hr. culture in 3% neopeptone + 1% glucose, or from a 36 hr. culture in a 1.5% neopeptone + 1% formate, the glucose-containing culture giving by far the best results.¹

As may be seen from Fig. 2 the optimum pH for H_2 absorption of a suspension of *Cl. sporogenes* obtained from a 16 hr. culture in 3% neopeptone + 1% glucose was found to be about 7.1.

Since the pH optima are the same for different substrates, we are probably dealing in each case with the activity of the same enzyme (hydrogenase) which is capable of activating H_2 . Substrate activation may shift this pH optimum slightly, particularly when the substrate activation is low at pH 7.1. This pH optimum of 7.1 differs considerably from that of the *Bact. coli* hydrogenase or from

¹ It was found necessary to exercise great care in eliminating traces of air from the anaerobic culture jars. Traces of air, which with heavy inocula apparently exert no influence on bacterial growth, very markedly diminished the H_2 absorption of bacteria obtained from such a culture. For the same reason it was found necessary to remove O_2 traces from buffer and substrate solutions by boiling them just before use.

that of the hydrogenase of sulphate-reducing bacteria with which Stephenson & Stickland [1931] found activity from pH 4 to 10 with the optimum at 6.3.

The data in Table I show the results obtained with a large number of biologically important compounds when used as substrates.

Table I. *Relative velocities of hydrogenation of various substrates by gaseous H₂ at pH 7.1 with a suspension of Cl. sporogenes, obtained from a 20 hr. culture in 3% neopeptone-1% glucose medium of pH 7.4*

The velocity with hydroxyproline is taken as standard (=100).

100-50			50-25		
Hydroxyproline	...	100	Acetylmethylcarbinol	...	50
Proline	...	100	Diacetyl	...	40
Glycine	...	100	Acrolein	...	47
Ornithine	...	100	Acetaldehyde	...	33
Arginine	...	80	Glyceric aldehyde	...	26
Tryptophan	...	67			
25-10			10-0		
Tyrosine	...	25	Acetic acid	...	5
Cysteine	...	22	Glutaric acid	...	5
Propionic acid	...	22	Glyoxal	...	5
Dihydroxyacetone	...	22	Oxalic acid	...	4
Glycerol	...	19	Aspartic acid	...	2
Glyceric acid	...	16	2:3-Butylene glycol	...	2
Butyric acid	...	16	Malonic acid	...	4
Cystine	...	16			
Methionine	...	15			
0					
Leucine	Maleic acid		Acetylurea		
Isoleucine	Acrylic acid		Oxaluric acid		
Valine	Fumaric acid		Uric acid		
Glutamic acid	Glycollic acid		Xanthine		
Histidine	Lactic acid		Hydantoin		
Alanine	Hydroxybutyric acid		Formamide		
Phenylalanine	Tartaric acid		Acetamide		
Lysine	Tartronic acid		Succinimide		
Asparagine	Malic acid		Betaine		
Serine	Glucose		Guanine		
Formic acid	Formaldehyde		Histamine		
Succinic acid	Ethanol		Creatine		
Citric acid	Urea				

As may be seen in Table I certain amino-acids (hydroxyproline, proline, glycine, ornithine, arginine and tryptophan) are the most active H-acceptors. A similar preference of *Cl. sporogenes* toward amino-acids was found in the detection of H-donators, as reported in a previous publication.

Hydroxyproline, proline and glycine were found by Stickland [1934], ornithine and arginine by Woods [1936] to act as H-acceptors. Their methods and ours give the same results so far as the most powerful H-acceptors are concerned. However, the H₂ absorption method used in these experiments adds many less powerful acceptors to the list because of its greater sensitivity.

Many substances other than amino-acids may act as H-acceptors, especially certain aldehydes (acetaldehyde, glyceric aldehyde, acrolein) and ketones (dihydroxyacetone, acetylmethylcarbinol, diacetyl) as well as glycerol, and propionic, butyric and glyceric acids.

It is worthy of note that many compounds which had been reported [Kocholaty & Hoogerheide, 1938] to act as H-donators, are found to act as H-acceptors too, i.e. ornithine, tryptophan, tyrosine, butyric and glyceric acids, acetaldehyde, glyceric aldehyde, glycerol, and certain others which have a

similar though lesser action. This fact suggests the possibility that an oxidation-reduction between 2 mol. of such a substrate occurs, 1 mol. being oxidized, the other being reduced. Evidence in support of this will be given later.

Cl. sporogenes causes fermentation of many substrates. Of the amino-acids tested, serine, arginine and leucine were fermented, as well as the sulphur-containing amino-acids cystine, cysteine and methionine. Of the substances other than amino-acids, glucose and pyruvic acid were strongly fermented. These fermentation reactions take place also in N_2 , and are independent of the presence of glucose in the culture medium. Some fermentable substrates act also as H-acceptors (arginine, cysteine, cystine, methionine, pyruvic acid) while others do not (leucine, serine, glucose). Although in some cases (cysteine, pyruvic acid) fermentation may be an intermolecular ("Stickland") reaction, in other cases (leucine, serine, glucose) it is highly probable that we are dealing with a true fermentation.

The ultimate fate of substrates acting as H-acceptors is a reduction, but it appears that in most cases the complete reaction is a rather complicated one. As would be expected, the less complicated cases are those in which the substrate acts exclusively as H-acceptor. In cases where the substrate acts as both donator and acceptor, there occur side reactions, reductions not involving the absorption of H_2 , but the simultaneous dehydrogenation of a second mol. Even in cases where the substrate acts only as H-acceptor, e.g. with proline, hydroxyproline and glycine, marked mutual differences are observed. Proline and hydroxyproline are reduced through the opening of their ring-bond, without deamination. Glycine, however, is reduced and simultaneously deaminized.

l-Proline. The reaction which takes place when *l*-proline acts as H-acceptor with a suspension of *Cl. sporogenes* and H_2 as donator is probably:

The H_2 absorption was found to be largely independent of the concentration of proline, showing no decrease until the concentration was diminished to 0.006 m.-mol. in 2.5 ml. of suspension. As may be seen from Fig. 3, the absorption becomes less and finally stops completely. As calculated from the amounts of H_2 and proline involved the reactants appear to participate in molecular proportions. No NH_3 was liberated.

NH_3 present at the end of the experiment was estimated by semi-micro steam distillation of the contents of the Warburg vessels after addition of 1 ml. 10% Na_2CO_3 . A blank determination was carried out on the same amount of substrate and bacterial suspension. The NH_3 was absorbed in 5 ml. $N/70 H_2SO_4$ and the excess acid titrated with $N/70 NaOH$.

The product of the reaction (probably δ -amino-*n*-valeric acid) has not been isolated or definitely identified, as there is not a satisfactory method for the identification of this substance in such small amounts. However, since Stickland [1935] showed that when proline acts as H-acceptor

Fig. 3. Course of H_2 absorption of a washed suspension of *Cl. sporogenes* with proline (conc. 0.100 m.-mol. (●—●) and 0.020 m.-mol. (○—○) respectively) as substrate.

with alanine as a donor deamination does not occur, the ring being opened and the proline reduced to δ -amino-*n*-valeric acid, it is probable that in this case a similar reaction occurs.

l-Hydroxyproline. The reaction which takes place when hydroxyproline acts as H-acceptor is similar to that occurring with proline. It was shown as before that 1 mol. hydroxyproline absorbs 1 mol. H_2 , and that no NH_3 is liberated.

Therefore the reaction taking place is probably:

Here again no suitable method was found for the isolation and identification of such small amounts of γ -hydroxy- δ -aminovaleric acid.

Glycine. As has already been mentioned glycine, unlike proline and hydroxyproline, is deaminated during reduction. One mol. glycine absorbs 1 mol. H_2 , liberating 1 mol. NH_3 .

The reaction which takes place is therefore:

A separate set of experiments showed that 1 mol. glycine yielded 1 mol. of a volatile acid, which was definitely identified as acetic acid.

The contents of the Warburg vessels, after the completion of the experiment, were transferred into a micro steam distillation apparatus and 2 ml. 4*N* H_2SO_4 were added. After distillation (c. 50 ml.) the amount of volatile acid was estimated by titration with *N*/70 NaOH, after removal of CO_2 by heating to 100°. Blank determinations with a known amount of acetate showed that the whole amount of acetic acid had thus been accounted for. For the identification of acetic acid, the Feigl test [1937] was used. We wish to express our thanks to the Microchemical Department Institute for the identification of this compound.

Fig. 4. Course of H_2 absorption of a washed suspension of *Cl. sporogenes* with glycine (conc. 0.100 (●—●) and 0.020 (○—○) m.-mol. respectively) as substrate.

As may be seen from Fig. 4, the H_2 absorption does not cease when all the glycine has disappeared but continues slowly, finally exceeding the theoretical

limit. This is due in some degree to the action of acetic acid as H-acceptor. The slope of the line of H_2 absorption for acetic acid is quite different from that for glycine, no difficulty being encountered in determining the end-point of the reaction.

There was further evidence that another side reaction occurred, but only to a very small extent. It was probably as indicated by the equation:

There was a slight development of CO_2 , and of NH_3 and acetic acid in the ratio of 4:3. It is doubtful if this reaction plays a very important role.

d-Ornithine. As was reported in our previous publication [Kocholaty & Hoogerheide, 1938], ornithine acts as a feeble H-donor. On the other hand it may be activated as a powerful H-acceptor (Table I). This is probably why the reaction with ornithine as the only substrate in H_2 is more complicated than that obtained by using the previously mentioned substrates. Of the two amino groups present in the ornithine molecule, only one is split off. This is in agreement with the results of Woods [1936], who found that when ornithine reacts with the donor alanine it accepts two H atoms and undergoes reductive deamination to δ -amino-*n*-valeric acid.

The evidence suggests that a similar reaction occurs when gaseous H_2 acts as H-donor, the main reaction probably being as indicated by the equation:

the first reaction product being the same as is formed by the reduction of proline.

When a surplus of ornithine was present and the reaction was stopped at a point where there was still an active H_2 absorption, it was found that considerably more NH_3 had been formed than is indicated by the above equation. This was due to secondary reactions also observed by Woods [1936]. The nature of these secondary reactions is not known, but they are probably oxidation-reduction reactions in which one mol. ornithine is oxidized while another mol. is being reduced. This explanation agrees with the fact that ornithine may act either as H-acceptor or donor.

When ornithine was incubated in N_2 with a suspension of *Cl. sporogenes* there occurred a slight but uniform production of CO_2 with considerable amounts of NH_3 .

When a surplus of ornithine is present, the quantity of NH_3 formed from ornithine in H_2 equals that calculated from the H_2 absorption according to the above equation, plus the quantity split off in the same time in N_2 .

The reaction occurring in N_2 is not clearly understood. The ratio of CO_2 to NH_3 is not constant, but increases with increasing concentration of ornithine. With 0.015 m.-mol. ornithine the ratio was very nearly 1:5, but when 0.05 m.-mol. was used the ratio was almost 1:1. This indicates that we are not dealing with a single reaction.

In the later stages of the reaction when the ornithine has been completely deaminized, H_2 absorption continues, but at a much lower rate (Fig. 5). It is probable that one of the products of the oxidation-reduction reaction then acts as a substrate, being reduced without deamination.

l-Tryptophan. Tryptophan, found to be an active H-acceptor, is also an active H-donor. Since it may act either as H-acceptor or donator secondary reactions would be expected to occur even more than with ornithine.

The principal reaction which occurs with this compound as H-acceptor and gaseous H_2 as the donator is probably:

The presence of indolepropionic acid was demonstrated by extraction with ether and precipitation as a nitroso-compound.

Fig. 5.

Fig. 6.

Fig. 5. Course of H_2 absorption of a washed suspension of *Cl. sporogenes* with ornithine as substrate (conc. 0.040 (●—●) and 0.020 (○—○) m.-mol.).

Fig. 6. Effect of pH on anaerobic decomposition of pyruvic acid and tryptophan by washed suspensions of *Cl. sporogenes*, measured as CO_2 and NH_3 respectively. ●—● pyruvic acid, ○—○ tryptophan.

The absorption of H_2 by tryptophan usually ceases when about $\frac{1}{3}$ — $\frac{1}{2}$ of that calculated for the above equation has been absorbed. NH_3 estimation shows, however, that all of the tryptophan has been deaminized.

In N_2 tryptophan is readily decomposed, 1 mol. tryptophan yielding approximately 1 mol. NH_3 in addition to small amounts of CO_2 and considerable amounts of indolepropionic acid. The reaction is in general agreement with the equation:

Approximately 1 mol. indolepropionic acid was formed to 2 mol. NH_3 during anaerobic decomposition of tryptophan *in vacuo* or in N_2 . Indolepyruvic acid, although not definitely identified, is probably the source of the small amount of CO_2 formed during anaerobic decomposition.

Very little influence of the pH on the rate of anaerobic decomposition of tryptophan was found inasmuch as there exists a broad optimum from pH 6.7 to 9.0 (Fig. 6). The optimum for H_2 absorption, however, was pH 7.0, the activity decreasing considerably on each side of this value.

Fermentable substrates

No H_2 uptake occurred when serine was mixed with a suspension of *Cl. sporogenes* in an atmosphere of H_2 ; instead carbon dioxide and considerable amounts of ammonia were produced as a result of fermentation. This fermentation proceeds equally well in N_2 .

When the sulphur-containing amino-acids, cysteine, cystine or methionine were used, active fermentation occurred with H_2S as the chief gaseous product. While with cysteine and methionine fermentation begins immediately and continues at a uniform speed, gas development with cystine shows a considerable lag period. Contrary to the results with serine much H_2 is absorbed in an atmosphere of H_2 by these sulphur-containing amino-acids. This was true also for arginine. Although a good H-acceptor, fermentation occurs simultaneously with the production of CO_2 and NH_3 . The fermentation, however, decreases rapidly in a short time with little utilization of the substrate.

Of compounds other than amino-acids, pyruvic acid and glucose are actively fermented under the conditions of these experiments. The optimum pH for the fermentation of pyruvic acid was found to be 6.2 (Fig. 6). This acid yields 1 mol. CO_2 per mol. of the acid, while among the degradation products approximately 0.5 mol. of a volatile acid was detected which was very probably acetic acid, since it gave a positive iodo-lanthanum reaction.

Glucose was the only substrate used with which much H_2 was produced.

Recently Woods & Clifton [1937] have shown that *Cl. tetanomorphum* decomposes many amino-acids with the liberation of H_2 . *Cl. sporogenes* behaves very differently in this respect inasmuch as none of the amino-acids tested but only glucose yielded H_2 .

DISCUSSION

Stephenson & Stickland [1931] suggest a correlation between the ability of certain species of micro-organisms to activate gaseous H_2 and their ability to ferment formic acid, only those species which ferment formic acid being able to activate H_2 .

Since *Cl. sporogenes* in general does not ferment formic acid it was considered to be unable to activate H_2 . This theory does not appear to be valid under all conditions in the case of *Cl. sporogenes* since formic acid is not fermented even by suspensions of the organisms which are highly active so far as H-activation is concerned. Although formate has usually been considered to be the precursor of H_2 produced by bacteria, evidence has been obtained recently which suggests that H_2 may arise by some mechanism other than by way of formate [Stephenson & Stickland, 1932; Stephenson, 1937; Woods & Clifton, 1937]. It seems to us, therefore, that there is more evidence of a correlation between the ability of certain bacterial species to produce H_2 gas, and the ability to activate H_2 gas, independently of its formation from formate or by some other mechanism. This appears more probable since the reversibility of the reaction $2H = H_2$ was clearly demonstrated with a suspension of *B. coli* by Green & Stickland [1934] and Woods [1936].

In many cases, certain specific bacterial enzymes are produced only when the organisms require them for the utilization of a particular substrate (adaptive enzymes). It is possible that hydrogenase is present in the resting *Cl. sporogenes* only when the organisms are cultured in a medium in which H_2 development occurs.

The production of H_2 from glucose and the utilization of H_2 with certain amino-acids by the same suspension is an example of a possible linking of carbohydrate and protein metabolism by this particular species.

The results obtained with the redox indicator method (used by Stickland for the detection of H-acceptors) and those obtained by using gaseous H_2 are comparable, since the latter method detects all the H-acceptors found by the indicator method but, in addition, it reveals many less potent H-acceptors, and thus is superior to the redox indicator method. It is applicable however only in a pH range in which H_2 is activated (pH 6–8).

A sharp distinction between H-donators and H-acceptors can no longer be maintained. Many substrates act as donators as well as acceptors, giving an intermolecular oxido-reduction, one molecule being oxidized, another reduced.

Fermentation reactions, Stickland reactions and these intermolecular oxido-reductions, appear to be the main sources of energy of *Cl. sporogenes*. The very decided preference of this organism for amino-acids is in accordance with its putrefying action on proteins.

This work indicates that certain amino-acids, such as *d*-lysine, glutamic acid and aspartic acid, are not utilized by *Cl. sporogenes* to any extent, since they are not fermented, and play no important role as H-acceptors or H-donators.

SUMMARY

1. Washed suspensions of *Cl. sporogenes* which had been grown on culture media containing glucose were found to be able to utilize gaseous H_2 .
2. This ability to utilize H_2 was used as the basis of a method for the detection of substrates able to act as H-acceptors for this organism.
3. Proline, hydroxyproline, glycine, ornithine, arginine, tryptophan, certain aldehydes and ketones and to a lesser extent many other substrates may act as H-acceptors with H_2 as donator.
4. The chemical reactions concerned in the reduction of certain of these acceptors have been studied. Evidence is given that each of the 6 amino-acids mentioned absorbs 1 mol. H_2 for each mol. amino-acid. Except for proline and hydroxyproline, which are reduced by the opening of the ring bonds, they all undergo reductive deamination.
5. Several substrates act as donators as well as acceptors, and hence give rise to oxido-reduction reactions in which one mol. is oxidized and another is reduced. These reactions are independent of the presence of H_2 , and occur also as side-reactions accompanying H_2 uptake.
6. Serine, cysteine, cystine, methionine, pyruvic acid, glucose and to a lesser extent arginine, are actively fermented by washed suspensions of *Cl. sporogenes*. Glucose was the only substrate from which H_2 was evolved.

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