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

I. DEHYDROGENATION REACTIONS BY SUSPENSIONS OF *CL. SPOROGENES*

By WALTER KOCHOLATY and J. C. HOOGERHEIDE

From the Biochemical Research Foundation of the Franklin Institute, Philadelphia, Pa.

OUR knowledge of the metabolism of the spore-forming anaerobes has until now been rather restricted. This is due in part to the fact that from the biochemical point of view little attention has been given to this genus. This is regrettable, since this group of micro-organisms has the unique property of being able to live and multiply actively under anaerobic conditions with proteins as the only source of energy. A study of the metabolism of these organisms, therefore, may yield valuable information on protein metabolism in general.

Previous investigations consisted mainly in the detection of dissimilation products produced by members of this group of organisms. Later investigators, especially Fildes, Knight and Pappenheimer [1933–35] made useful contributions on a broader scale, dealing mainly with growth studies. The most fundamental discovery, however, was made by Stickland [1934; 1935] who showed that a cell suspension of *Cl. sporogenes* is able to activate some amino-acids as hydrogen acceptors and others as hydrogen donators, thus enabling an intermolecular reaction between two amino-acids to occur, one being oxidized, the other reduced. This study of coupled reactions between pairs of amino-acids, brought about by a suspension of *Cl. sporogenes*, was continued and enlarged by Woods [1936].

It is striking that among the many naturally occurring amino-acids there is such a limited number that can be used either as hydrogen donators or acceptors. It has been shown, for instance, that tryptophan belongs to the "essential" amino-acids for the growth of *Cl. sporogenes*; but, so far as can be deduced from previous work, this amino-acid plays no role as acceptor or donator. The question is whether or not the "Stickland reaction" is the only reaction which provides *Cl. sporogenes* with the necessary energy. It has also been shown that bacteria belonging to the facultative anaerobic group are able to activate as hydrogen donators many biochemically interesting compounds, such as alcohol, formic acid, succinic acid, glucose etc. The work of Stickland, on the other hand, suggests that in the case of *Cl. sporogenes* there are certain amino-acids that can be activated as hydrogen donators (or acceptors), while non-amino-acids do not seem to play any important role in this respect. Nevertheless, *Cl. sporogenes* actively ferments certain carbohydrates and, since a correlation between fermentability of sugars and their suitability to act as powerful hydrogen donators is always found, one wonders why glucose is not a better hydrogen donator.

This paper presents information on the conditions under which various substrates act as hydrogen donators, and the influence of different factors involved, in the metabolism of the anaerobes, particularly *Cl. sporogenes*. It

appeared likely that a cultivation period of 40–45 hr. as used by Stickland, was too long for an organism which so readily goes into the spore stage. That the age of the bacterial culture may have a very great influence upon the activity of the enzymes was pointed out recently for *B. coli* by Wooldridge *et al.* [1936], and by Wooldridge & Glass [1937]. The *p*H of the culture medium during the growth of the bacteria, a factor which was not previously considered to any extent, appeared also to bear an important relation to the activity of the enzymes. The most important factor, however, which indicates the power of the micro-organism to activate certain substrates as hydrogen donators was found to be the *p*H at which the dehydrogenation experiment was conducted. Previously little attention was given to this factor, a *p*H of about 7 being usually chosen.

There may be additional unknown factors, consideration of which is required in order to obtain a clear insight into the enzyme activity of these micro-organisms.

<div align="center">EXPERIMENTAL</div>

The experiments described in this paper were carried out with washed suspensions of *Cl. sporogenes*. After it had been established that different pure cultures behaved similarly, the main work was done with a culture obtained from the American Type Culture Collection and labelled No. 319. Tubes containing 10 ml. of medium (3 % neopeptone solution at *p*H 7·4) were inoculated from the stock culture in brain medium. After 24 hr. incubation these tubes were used for the inoculation of the culture bottles.

Unless otherwise stated, the culture medium consisted of 250 ml. of a 3 % solution of neopeptone (Difco standardized) in distilled water adjusted with NaOH to *p*H 7·4 before sterilization. No change in *p*H takes place during the growth period. The flasks were kept in a McIntosh and Fildes anaerobic jar and incubated at 37° for a period of 16–20 hr., sufficient to insure a heavy growth of the organisms. The culture was then centrifuged and washed with 250 ml. of freshly boiled (oxygen-free) saline solution, and finally suspended in 10–20 ml. of 0·85 % NaCl solution. Microscopic examinations showed that the bacteria were nearly all in the vegetative stage, practically no spores being observed. Smears on nutrient agar plates showed that no contamination was present. The enzymic activity of such a suspension was exceedingly high. However, since it decreased very rapidly on storage, even under anaerobic conditions, the experiments were carried out immediately after preparing the suspension. The method used for the investigation of the various substrates as hydrogen donators was the Thunberg technique as modified by Keilin. Thus in a hollow stopper of about 2 ml. capacity was placed 1 ml. of the bacterial suspension. The following constituents were placed in the tube: 1 ml. of $M/5$ phosphate buffer at *p*H 7·4 (if not otherwise stated), 1 ml. of $M/2000$ Brilliant Cresyl Blue and 2 ml. of the substrate solution (usually $M/10$) adjusted with NaOH to *p*H 7·4. The final volume was 5 ml. so that the substrate concentration was $M/50$, unless otherwise stated. After thorough evacuation by an oil pump, the bacterial suspension in the hollow stopper was combined with the reaction mixture, incubated in a water bath at 40° and the time necessary for the reduction of the dye noted.

The first experiments were designed to test the behaviour of such suspensions of *Cl. sporogenes* towards the naturally occurring amino-acids, some synthetic amino-acids and also towards many substances of biological interest.

As may be seen from Table I, among the compounds studied there are some amino-acids which under the conditions of this experiment act as outstanding hydrogen donators, namely *d*-alanine, *l*-leucine, *l-iso*leucine, *dl*-α-amino-*N*-valeric acid, *d*-valine. Among them *dl*-α-amino-*N*-valeric acid is a synthetic

Table I. *Relative velocities of dehydrogenation of various substrates by suspensions of Cl. sporogenes (16–20 hr. culture in 3°/₀ neopeptone at pH 7·4) in presence of Brilliant Cresyl Blue at pH 7·4*

The velocity with *d*-alanine is taken as standard = 100

100–50		10–1		0	
d-Alanine	100	Glycerol	9	Propionic acid	0
l-Leucine	100	*l*-Aspartic acid	9	Methyl alcohol	0
l-iso*Leucine*	100	*d*-Glutamic acid	9	Formaldehyde	0
dl-α-Amino-*N*-valeric acid	100	Malic acid	9	Glycine	0
d-Valine	76	Glycol	8	*l*-Proline	0
Pyruvic acid	51	Glyceric acid	8	*l*-Oxyproline	0
50–25		*l*-Dihydroxyphenylalanine	8	Maleic acid	0
Allyl alcohol	40	Histamine	8	Glutaric acid	0
l-Histidine	37	Laevulose	7	Glycollic acid	0
l-Phenylalanine	28	Fumaric acid	7	Glyoxal	0
25–10		Formic acid	7	β-Hydroxybutyric acid	0
Butylene glycol	24	*iso*Propyl alcohol	6	Acetic acid	0
Ethyl alcohol	20	*d*-Ornithine	6	*d*-Arginine	0
n-Butyl alcohol	20	Hydantoin	4	Acetamide	0
l-Tryptophan	17	*dl*-Tartaric acid	4	Acetylurea	0
l-Serine	16	Succinimide	4	Glycine anhydride	0
l-Tyrosine	16	Butyric acid	4	Oxaluric acid	0
l-Asparagine	13	Acetaldehyde	4	Urea	0
				Barbituric acid	0
d-Glucose	12	Succinic acid	3	Creatinine	0
				Creatine	0
1 : 3-Butylene glycol	12	Dialuric acid	3	Acetoine	0
				Formamide	0
n-Propyl alcohol	12	Citric acid	3	Alloxanthine	0
				Alloxan	0
Lactic acid	11	*l*-Lysine	3	Betaine	0
				Allantoin	0
Glyceric aldehyde	10	Dihydroxyacetone	2	Guanine	0
				Guanidine	0
Malonic acid	10	Oxalic acid	2		

acid and is not a constituent of proteins. However, a great variety of non-amino-acids are also able to act as hydrogen donators. Especially is this the case with pyruvic acid and different alcohols whereas glucose, lactic acid and glyceric aldehyde are fairly good donators.

Comparing our results with those of Stickland, who investigated only a very limited number of substrates, there is fairly good agreement, although our numbers for most of the substrates are somewhat higher. We believe that these differences are due to the fact that the vitality of our suspensions was higher than that of the suspensions used by Stickland, who cultivated the bacteria on a tryptic digest of caseinogen for 40–45 hr. It is possible also that the *p*H (not given in his paper) of Stickland's culture medium differed from ours. It has been our experience that very young bacterial cultures (16 hr.) are able to attack the compounds under study much more readily than older cultures, which often fail to activate some of the substrates as hydrogen donators. As has been pointed out, there are other factors that are of importance in influencing the dehydrogenase reactions which will be discussed in more detail.

(a) Influence of pH on dehydrogenation

Since it was impossible to investigate all of the compounds that may act as hydrogen donators, this work was restricted to four typical compounds: an amino-acid (*d*-alanine), a keto-acid (pyruvic acid), an alcohol (ethyl alcohol) and an aldehyde (acetaldehyde).

The buffer solutions used for these experiments were prepared from solutions of $M/5$ KH_2PO_4, $M/5$ Na_2HPO_4 and $M/5$ NaOH. Other buffer mixtures (phthalate, borate, citrate, veronal) were found unsatisfactory, partly because they inhibit dehydrogenase activity and partly because they do not cover the entire desired pH range. The buffering capacity of the phosphate buffer is almost zero in the more alkaline region but, owing to the fact that the theoretically possible amount of decomposition products is extremely small (equivalent to the amount of dye added), it was found that no change in the pH occurred during the experiment, even in this poorly buffered region. For each substrate the pH giving the most rapid reduction of the dye was taken as 100 ($= pH$ optimum). (Figs. 1–4, solid lines.)

As may be seen from these figures, the effect of pH on the rate of dehydrogenation is different for each of the four substrates investigated. The dehydrogenase action with d-alanine has its optimum at pH 9·3, with ethyl alcohol at pH 8·5, with acetaldehyde at pH 6·7 and with pyruvic acid at pH 6·0.

The course of the pH curves is in most cases a rather irregular one. This may be due to the fact that the dehydrogenation reaction is not a simple one, but occurs in different stages, the product of the first reaction being further converted by intermediate processes.

The sharp declines of the curves obtained, especially for pyruvic acid and acetaldehyde in the acid region, may be due to the fact that longer reduction times cause a marked destruction of the enzymes in this region. If this be true, the curves show (1) the resultants of pH-enzyme activity and pH-enzyme destruction, or (2) that the dye used at these pH values is thermodynamically insufficient with respect to the substrates or substrate-decomposition products.

These curves show clearly that it is not correct to conclude that an aminoacid, as for instance d-alanine, is a better hydrogen donator than, for example, pyruvic acid, as Table I suggests, since at another pH (6·0) the reverse order is shown. The degree of activity depends upon the pH.

The pH curve for the dehydrogenation of pyruvic acid is interesting from another point of view, namely it was found that *Cl. sporogenes* is able to ferment this compound with CO_2 formation under anaerobic conditions without the presence of any dye, also with an optimum at pH 6·0. (To be published later.)

In order to determine the influence of pH during the bacterial growth on dehydrogenation the experiments were repeated exactly as above mentioned, but instead of cultivating the bacteria at pH 7·4, it was done at pH 6·4, on the same medium. During the growth the pH increased slightly to pH 6·5 at the end of the culture period (20 hr.). The same pH curves for dehydrogenase activity could no longer be obtained. All curves showed a shift towards the acid range (Figs. 1–3, dotted lines), which was extremely pronounced in the case of d-alanine. Here the second optimum (in the former curve at pH 7·8) had shifted to pH 7·0 and became the main optimum, while the main optimum in the former curve (at pH 9·3) had shifted towards pH 8·2 and had become a secondary optimum. The result with pyruvic acid was similar, but the deviations for ethyl alcohol were not so great. This means that by simply cultivating the bacteria in a more acid range on the same medium, the pH-optima for the different substrates were shifted more or less. The explanation of this phenomenon, for which no analogy is found in the literature, is hypothetical at present, namely that the bacteria quickly adapt themselves to the more acid conditions in the culture medium by synthesizing slightly different enzymes which are more able to attack the various substrates at a lower pH.
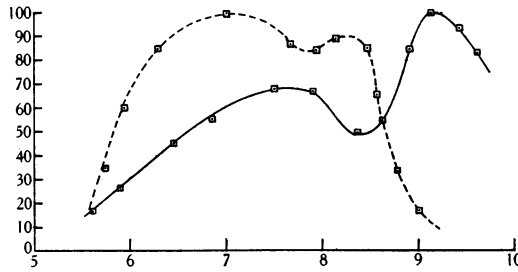
Fig. 1. *Cl. sporogenes.* pH-optimum for *d*-alanine dehydrogenase. pH of the culture medium 7·4 = ———; pH of the culture medium 6·4 = - - - -. Abscissa: pH. Ordinate: velocity.
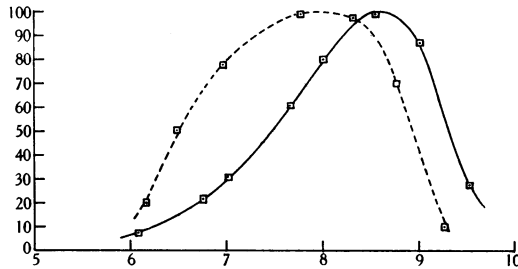

Fig. 2. *Cl. sporogenes.* pH-optimum for ethyl alcohol dehydrogenase. pH of the culture medium 7·4 = ———; pH of the culture medium 6·4 = - - - -. Abscissa: pH. Ordinate: velocity.
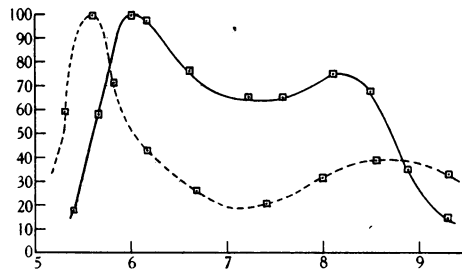

Fig. 3. *Cl. sporogenes.* pH-optimum for pyruvic acid dehydrogenase. pH of the culture medium 7·4 = ———; pH of the culture medium 6·4 = - - - -. Abscissa: pH. Ordinate: velocity.
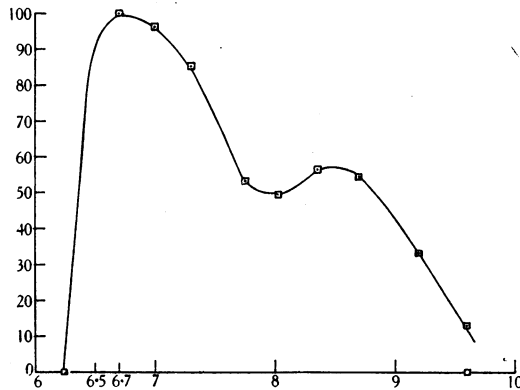

Fig. 4. *Cl. sporogenes.* pH-optimum for acetaldehyde dehydrogenase. pH of the culture medium 7·4. Abscissa: pH. Ordinate: velocity.

Whether this phenomenon is universal for the genus *Clostridium* is unknown at present, although similar results were obtained with another representative, *Cl. histolyticum*.

Table II. *Shift of the pH-optima for the dehydrogenation of d-alanine, pyruvic acid and ethyl alcohol by suspensions of* Cl. sporogenes *and* Cl. histolyticum *cultivated on the same medium (3°/₀ neopeptone), but at different pH*

pH-optimum for dehydrogenation of	Cl. sporogenes cultivated at		Cl. histolyticum cultivated at	
	pH 6·4	pH 7·4	pH 6·4	pH 7·4
d-Alanine	7·0	9·3	7·8	9·3
Pyruvic acid	5·6	6·0	5·4	6·0
Ethyl alcohol	8·0	8·5	8·3	8·3

(b) The influence of the medium

In order to see whether the composition of the medium on which *Cl. sporogenes* was grown had any influence upon the dehydrogenation activity, three different media were prepared: (1) the usual 3% neopeptone, (2) a tryptic digest of 3% gelatin, (3) a tryptic digest of 3% casein, all adjusted to pH 7·4. This pH did not alter during the cultivation time. The bacterial suspensions were made in the usual way and their activities compared against eight different substrates at pH 7·4. No significant difference was found, which justifies the conclusion (at least as far as the substrates studied are concerned) that the source of protein on which *Cl. sporogenes* is cultivated has little if any influence on the dehydrogenases produced. As the results obtained with the different media are practically identical, the figures for any one may be seen by referring to Table III, first column.

Table III. *Activity of suspensions of* Cl. sporogenes *(4·2 × 10⁹ cells per ml.) cultivated for 20, 45 and 70 hr. on 3°/₀ neopeptone (pH 7·4), measured on eight different substances at pH 7·4*

The velocity with *d*-alanine is taken as standard = 100. (Reduction time is given in min.)

	Age of the culture					
	20 hr.		45 hr.		70 hr.	
Substrates	Reduction time	Velocity	Reduction time	Velocity	Reduction time	Velocity
Glucose	25·5	12	51	10	—	—
Formic acid	43	7	—	—	—	—
l-Tryptophan	17·5	17	33	15	100	9
l-Asparagine	21·3	14	23	22	58	15·5
d-Alanine	3	100	5	100	9	100
d-Glutamic acid	30·3	9	48	10	115	8
Pyruvic acid	5·7	51	12	42	22·5	40
Ethyl alcohol	15	20	9	56	9	100

(c) Influence of the age of the culture

In order to investigate the influence of the age of the culture on dehydrogenase activity of the suspension obtained from such culture, a bottle with 750 ml. of 3% neopeptone was inoculated. During the incubation period of 70 hr. equal proportions were removed for study at 20, 45 and 70 hr. Cell suspensions were prepared and in all cases diluted until the suspension contained 4·2 × 10⁹ cells per ml.

As may be seen from Table III, the activity of the bacterial suspensions towards many of the substrates decreases considerably with increasing age of the culture; the younger the culture, the greater the activity. A culture of 70 hr. gives a bacterial suspension which has a reduction time for d-alanine three times that of the 20 hr. culture. Similar values were obtained for l-asparagine and d-glutamic acid, while for pyruvic acid these numbers are four times as great and for l-tryptophan six times as great. Formic acid and glucose were not dehydrogenated at all by the 70 hr. culture. The behaviour towards ethyl alcohol was in decided contrast to that shown towards glucose. The bacteria from the 70 hr. culture dehydrogenated this substrate much more actively than those from the 20 hr. culture. With the organisms from the 70 hr. culture ethyl alcohol compares favourably with d-alanine as a hydrogen donator, while with the organisms from the 20 hr. culture it was only one-fifth as efficient.

(d) Influence of addition of glucose to culture medium

The experiments were carried out in the same manner as the preceding one, except that 1 % of glucose was added to the 3 % neopeptone medium. In order to avoid marked changes in the pH during the growth due to sugar fermentation, an initial pH of 6·1 was chosen for the experiment. This pH remained fairly constant during the whole incubation time, during which considerable gas was produced. Unfortunately, therefore, the results shown in Tables III and IV are not directly comparable. An attempt was made, without much success, to cultivate Cl. sporogenes on neopeptone at pH 6·1 in order to obtain comparable results. The pH of the solution gradually increased until after 1–2 days it had reached pH 7·0 (Table IV).

Table IV. *Activities of suspensions of* Cl. sporogenes *($4·5 \times 10^9$ cells per ml.) cultivated for 20, 45 and 70 hr. on $3°/_o$ neopeptone—$1°/_o$ glucose at pH 6·1, measured on 9 different substrates at pH 7·4*

| | Age of the culture | | | | | |
| | 20 hr. | | 45 hr. | | 70 hr. | |
Substrates	Reduction time	Velocity	Reduction time	Velocity	Reduction time	Velocity
Glucose	3·5	33	5	38	8	38
Formic acid	20·7	4·5	—	—	—	—
l-Tryptophan	10	9	22	6	52·5	6
l-Asparagine	7·3	14	20	7	27	11
d-Alanine	1·2	100	2	100	3	100
d-Glutamic acid	9·7	10	37	2	51·5	6
Pyruvic acid	2·2	55	3·5	56	4·7	63
Ethyl alcohol	0·7	168	3	67	15	20
Lactic acid	14·5	5·5	26·5	4	63	5

A glance at Table IV shows at once that glucose now acts as a powerful hydrogen donator. Even after 70 hr. cultivation its activity is exceedingly strong. The behaviours of formic acid, l-tryptophan, l-asparagine, d-alanine and d-glutamic acid are similar to those shown in Table III. Pyruvic acid dehydrogenation is increased gradually by longer incubation when glucose is present in the medium, while Table III shows a gradual decrease in activity when the medium does not contain glucose.

Most striking, however, is the behaviour of the alcohol dehydrogenation. After 20 hr. incubation time the activity of the alcohol dehydrogenase is extremely high and exceeds that of d-alanine, which has always been the most active

hydrogen donator, by even more than 50 %. The activity of alcohol dehydro-
genase decreases rapidly, however, as may be seen from the values obtained with
the cultures of 45 and 70 hr. This is in direct contrast to the increasing activity
during ageing when glucose is not added to the culture medium (Table III).

Any attempt to explain this phenomenon would be purely speculative. Our
knowledge of catabolism in anaerobes is too restricted at present to offer any
satisfactory explanations.

(e) Influence of substrate concentration

We investigated the behaviour of washed *Cl. sporogenes* suspensions towards
the substrates *d*-alanine, pyruvic acid and ethyl alcohol, in dilutions varying
from $M/5$ to $M/50,000$. The results given in Fig. 5 show the behaviour as related
to substrate concentration. The concentration of *d*-alanine within the limits of
$M/5-M/200$ is without influence upon the reduction time of the dye, indicating
a relatively high substrate affinity, while pyruvic acid and ethyl alcohol alike
show a quite different behaviour, being greatly influenced by the substrate
concentration. The experiments were carried out at the pH optima for the
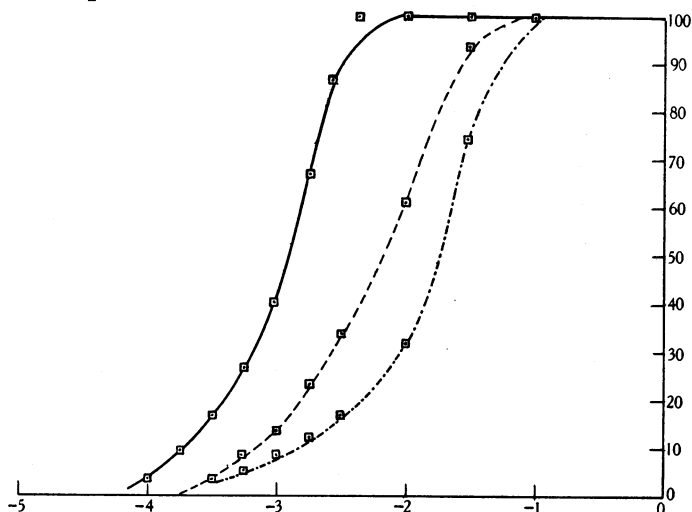investigated compounds.

Fig. 5. *Cl. sporogenes.* Influence of the substrate concentration. Substrates: *d*-alanine = ————;
pyruvic acid = — — — —; ethyl alcohol = —·—·—·—·. Abscissa: mol. substrate concentration
log $-1 ... -5$. Ordinate: velocity 0–100.

(f) Influence of addition of poisons on dehydrogenation

The influence of As_2O_3 and HCN in different concentrations on the dehydro-
genation of *d*-alanine, pyruvic acid and ethyl alcohol as substrates ($M/50$) was
studied.

The concentrations of HCN[1] and As_2O_3 used were $M/50-M/5000$ expressed
for the total volume.

[1] The investigations with the addition of the volatile HCN had to be carried out cautiously
to avoid as far as possible an escape of HCN during the evacuation of the Thunberg tubes. For
this purpose the HCN was placed in the hollow stopper at a more alkaline reaction and the buffer
used adjusted in such way that, after evacuating and combining the reaction mixtures in the
tubes, the required pH for the determination was obtained.

It may be seen from Fig. 6 that the behaviours of both As_2O_3 and HCN are similar toward one substrate, but that these behaviours may be completely different for different substrates. The dehydrogenation of ethyl alcohol is inhibited by increasing concentrations of HCN or As_2O_3, the dehydrogenation of d-alanine on the other hand is activated by addition of these compounds in concentrations up to $M/50$. This latter fact is not in agreement with the observations made by Stickland, who found that these compounds inhibited completely the dehydrogenation of d-alanine. By the addition of arsanilic acid we were also able to obtain an activation of the d-alanine dehydrogenation. The dehydrogenation of pyruvic acid is not affected by the addition of HCN, while arsenious acid, independently of the concentration, inhibits to the extent of 14 %.

Addition of $CuSO_4$ even in concentrations as low as $5 \times 10^{-4} M$ inhibits completely all dehydrogenation reactions studied on the same substrates, while on the other hand addition of NaF, even in concentrations as high as $M/5$, is without any influence upon the reduction of the dehydrogenases studied.
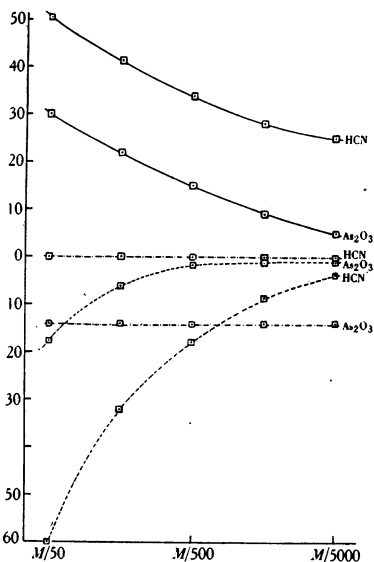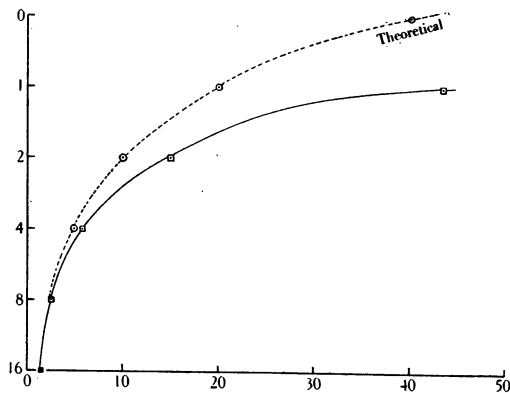

Fig. 6.   Fig. 7.

Fig. 6. *Cl. sporogenes.* Influence of HCN, As_2O_3 upon d-alanine, pyruvic acid, ethyl alcohol dehydrogenases. d-alanine = ————; pyruvic acid = – – – – –; ethyl alcohol = –·—·—·– (to the lines has to be added HCN, resp. As_2O_3). Abscissa: log. mol. concentration of HCN resp. As_2O_3. $M/50$–$M/5000$. The substrate concentration is constant = $M/50$. Ordinate: 0–50 = % activation; 0–60 = % inhibition.

Fig. 7. *Cl. sporogenes.* Influence of the bacterial concentration. Substrate d-alanine ($M/50$). Abscissa: reduction time in min. 1–50. Ordinate: ml. bacteria suspension 16–1.

(g) Influence of variation in enzyme concentration

The effect of decreasing concentrations of washed suspensions of *Cl. sporogenes* used for dehydrogenation reactions was studied on the substrate d-alanine. A thick suspension of bacteria in saline was prepared in the usual way and diluted in geometric progression. Substrate and dye concentrations were in all cases the same, saline being added where necessary to make a total volume of 5 ml.

It was found that there was by no means a constant ratio between reduction time and bacterial concentration in the entire range of the dilutions. An approximately constant ratio exists only when rather high concentrations of bacteria are employed. The lower the bacterial concentration, the more pronounced the deviation from the hyperbolic function (Fig. 7).

Probably this is due to the fact that during the manipulations in which a limited contact with air is difficult to avoid, a small part of the activity is lost. As a matter of fact, smaller amounts of the suspensions or dilute suspensions are less protected against oxygen and a loss in activity is the consequence.

DISCUSSION

These investigations show that there are a great many factors which influence very markedly the results of dehydrogenation reactions with washed suspensions of *Cl. sporogenes* when reversibly reducible dyes are used as hydrogen acceptors. The following are important factors.

(1) The age of the bacterial culture. Usually the activity of the bacterial culture decreases with increasing incubation time. This is by no means always the case. Some substrates, such as ethyl alcohol, are more rapidly dehydrogenated by older cultures, when cultivated on neopeptone at pH 7·4.

(2) The pH of the medium during the growth of the bacteria. Marked changes in the pH curves for the different substrates were observed, when the bacteria were cultivated in a more acid medium. In general, these curves show that the substrates are attacked more readily in the acid ranges.

(3) The composition of the culture medium. Addition of glucose to the culture medium not only increased the ability of the organisms to use glucose as a hydrogen donator but also ethyl alcohol to a marked degree.

These factors, related to the conditions under which the bacteria have been cultivated, indicate the ease with which the organisms adapt themselves to changes in their environment. Organisms grown in broth only are not identical with those grown in broth to which glucose has been added, as the latter have developed to a high degree the enzyme mechanism necessary to utilize glucose, while this mechanism is rudimentary in organisms grown in broth alone. Repeated cultivation in a glucose-containing medium gradually develops the glucose-utilizing mechanism.

When cultivated in media of low pH the organisms adapt their enzyme production mechanism so that the substrates are more readily attacked at such pH values. It was impossible to demonstrate this for higher pH values. Conditions in old and young cultures differ. The changes in activity observed in this case are probably related to changes in the medium. The complexity of such medium makes it impossible to understand the reason for the different activities.

It is highly probable that this phenomenon of adaptability is not restricted to *Cl. sporogenes* but will be found in the entire microflora. Wooldridge recently described the influence on dehydrogenation reactions of ageing of a culture of *Bact coli*. It may be recalled that some lactic acid bacteria and yeasts are able to ferment certain carbohydrates only when they have been cultivated in the presence of these carbohydrates.

The present study shows that, in addition to the factors related to the conditions under which the bacteria are cultivated, there are also factors of importance in connexion with the preparation and investigation of the suspensions.

"Repeated washings" have been generally advocated in the preparation of bacterial suspensions for dehydrogenation experiments. It seems that with such procedure there is danger that the sensitive dehydrogenases, especially, may be partially inactivated or destroyed before the experiment is begun. When the culture is centrifuged in conical beakers at high speed a sharp separation of sediment and medium is obtained, and in this case one washing is sufficient to obtain a suspension with a very high activity and a very low blank value.

To locate definitely the dehydrogenating properties of the organisms, it was attempted to prepare an "enzyme solution" (after finding that the dehydrogenases are not secreted in the culture medium during the growth) by a careful destruction of the cell structure. However, this was not successful. The extreme lability of the dehydrogenases of *Cl. sporogenes*, even under anaerobic conditions at 0°, is remarkable and in striking contrast to that of *Bact. coli*, a suspension of which may be kept for a considerable time in the ice-box without loss of activity.

The irregularity of the pH curves in Figs. 1–4 make them appear questionable. That the irregularities are real was demonstrated by repeating the experiments several times. This fact may be ascribed to the presence of other products, which arise from the original substrate. In the case of pyruvic acid for example, it was found that, when this substrate is incubated without dye under anaerobic conditions with a suspension of *Cl. sporogenes*, a rapid fermentation takes place as a result of which 1 mol. of CO_2 is formed per mol. pyruvic acid, indicating the formation of acetaldehyde. There are further indications that the acetaldehyde formed is converted into ethyl alcohol and acetic acid. This means that, when Brilliant Cresyl Blue also is present, not only pyruvic acid may be dehydrogenated but also acetaldehyde and ethyl alcohol, the fermentation products of pyruvic acid. These different substrates are perhaps decomposed by different enzymes at different pH-optima, the pH curve representing a resultant of all these processes. Similar phenomena may occur in the breakdown of *d*-alanine and other more complicated compounds.

Whether there is one enzyme (an "oxidoreductase") responsible for the dehydrogenation of the various substrates or whether there are produced dehydrogenases specific for each substrate or groups of substrates composed of similar compounds is not known. The different behaviours towards HCN and As_2O_3 suggest the latter possibility.

Finally, one important point should be stressed. The experiments show that *Cl. sporogenes* under certain conditions can activate not only amino-acids, as has been found hitherto but also many other compounds. When the organisms are grown on glucose broth, ethyl alcohol is an even better hydrogen donator than *d*-alanine. This suggests the possibility that a "Stickland reaction" may occur for instance between an amino-acid and alcohol.

As will be shown in a later publication there exist also non-amino-acids which act as hydrogen acceptors, suggesting the possibility of a reaction between *d*-alanine as donator and, as an example, acetylmethylcarbinol as acceptor. Even reactions between non-amino-acids should be possible with suspensions of *Cl. sporogenes*. The "Stickland reaction" would then have a more general significance if these reactions were possible.

We hope to continue investigations in this direction.

SUMMARY

1. A great number of amino-acids and biologically interesting compounds have been investigated as to their ability to act as hydrogen donators with washed suspensions of *Cl. sporogenes* under the stated conditions. It has been found

that many of these compounds could be dehydrogenated, although usually certain amino-acids are better hydrogen donators.

2. A study was made of the conditions which may influence the results: the age of the culture; the pH of the medium; the composition of the medium; and the pH at which the dehydrogenations were performed.

3. Usually the activity towards different substrates decreases during continued incubation. Some substrates like ethyl alcohol, however, are more rapidly dehydrogenated by older cultures when cultivated on neopeptone.

4. The pH curves for different substrates are completely different and the pH optima for dehydrogenation may vary greatly. Cultivated on neopeptone at pH 7·4 the pH-optimum for d-alanine was found to lie at 9·3, for ethyl alcohol at 8·5, for acetaldehyde at 6·7, and for pyruvic acid at 6·0. This indicates that comparison of dehydrogenating potencies must be made with due regard to the pH at which they have been investigated.

5. Marked changes in the pH curves for the different substrates were observed when the bacteria employed were cultivated at a more acid reaction than pH 7·4. In general these curves showed a shifting towards the acid side, indicating an adaptation of the organism, so that these substrates could be attacked more easily at low pH.

6. Addition of glucose to the culture medium not only increased the ability of the organism to use glucose as a hydrogen donator, but also, more markedly, that of ethyl alcohol.

7. The different behaviours of the dehydrogenases acting on d-alanine, pyruvic acid or ethyl alcohol towards poisons such as HCN, As_2O_3, NaF, $CuSO_4$ are described.

The authors wish to express their thanks to Dr Ellice McDonald, Director, for his interest and support throughout this work.

REFERENCES

Fildes (1935). *Brit. J. exp. Path.* **16**, 309.
—— & Knight (1933). *Brit. J. exp. Path.* **14**, 343.
—— & Richardson (1935). *Brit. J. exp. Path.* **16**, 326.
Knight & Fildes (1933). *Brit. J. exp. Path.* **14**, 112.
Pappenheimer (1935). *Biochem. J.* **29**, 2057.
Stickland (1934). *Biochem. J.* **28**, 1746.
—— (1935). *Biochem. J.* **29**, 288, 889.
Woods (1936). *Biochem. J.* **30**, 1934.
Wooldridge & Glass (1937). *Biochem. J.* **31**, 526.
—— Knox & Glass (1936). *Biochem. J.* **30**, 926.

ADDENDUM

While this paper was in the press a review, by Yudkin, appeared in *Biological Reviews*, **13**, 1 [1938], critically summarizing all the known cases of "adaptability" or "training" of micro-organisms. This will be discussed in a subsequent paper.