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

I. THE CHEMICAL REACTIONS BY WHICH *CL. SPOROGENES* OBTAINS ITS ENERGY.

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OUR knowledge of the metabolism of the bacteria of the genus *Clostridium* (the strict anaerobes) is at present very scanty. It has been recently reviewed [Topley and Wilson, 1929; McLeod, 1930; Stephenson, 1930], so only a brief summary will be given here, including facts discovered since the above reviews were published. Special reference will be made to *Cl. sporogenes*, which has been used by many workers as a type species, and of which our knowledge is the fullest. The present position is as follows.

(1) These bacteria cannot grow in media containing an appreciable concentration of free oxygen. The degree of tolerance to low concentrations of oxygen varies from species to species. With regard to the reason for this lack of tolerance of oxygen no final decision has been reached. McLeod and Gordon [see McLeod, 1930] claim that it is due to production of hydrogen peroxide, while Quastel and Stephenson [1926] believe that oxygen acts by preventing the culture from reaching a sufficiently low oxidation-reduction potential.

(2) They are unable to grow except in media containing protein or amino-acids. Carbohydrates, if present in addition, are fermented, but are neither sufficient nor essential for growth.

(3) A compound containing the —SH group, or one from which the bacteria can produce an —SH group, is essential for growth (Quastel and Stephenson [1926] for *Cl. sporogenes*, and Burrows [1933] for *Cl. botulinum*).

(4) Tryptophan is an "essential" amino-acid for *Cl. sporogenes* [Fildes and Knight, 1933]. With other species opinions differ; for instance, with *Cl. botulinum* Fildes and Knight say that tryptophan is "essential," Burrows [1933] that it is not. The word "essential" is here used in the special sense that the bacteria require it because they are unable to synthesise it.

(5) In the case of *Cl. sporogenes* growth depends also on the presence in the medium of a trace of a vitamin-like substance [Knight and Fildes, 1933].

(6) Burrows [1933] showed that *Cl. botulinum* can grow on a synthetic medium containing no amino-acids except glycine, alanine, leucine, proline, lysine and cysteine.

(7) Wolf and Harris [1917; 1918–19] analysed the products of the action of *Cl. sporogenes* on various natural media, but could deduce little as to the reactions by which the products had been formed.

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It is evident that the quantitatively most important part of the chemical action of bacteria on the medium during growth must be the chemical reactions by which they obtain their energy. As the above summary shows, we know nothing so far of this aspect of the metabolism of the strict anaerobes, and it forms the subject of the present communication.

THE CHEMICAL REACTIONS BY WHICH *CL. SPOROGENES* OBTAINS ITS ENERGY.

The simplest medium on which *Cl. sporogenes* will grow consists of an acid hydrolysate of gelatin with the addition of traces of cysteine (or thiolacetic acid), tryptophan and vitamin [Knight and Fildes, 1933]. This medium contains practically nothing but amino-acids, so the source of energy must lie in anaerobic reactions involving only amino-acids. It is difficult to picture any reaction by which a single amino-acid molecule could break down anaerobically to yield energy, so the most likely type of reaction is one between two molecules of amino-acids, probably involving oxidation of one molecule and reduction of the other, as in the hypothetical equation

$$R^1.CHNH_2.COOH + R^2.CHNH_2.COOH + H_2O \rightarrow R^1.CO.COOH + R^2.CH_2.COOH + 2NH_3.$$

R^1 and R^2 may theoretically be the same group or two different groups.

A reaction of this type would be similar to certain anaerobic energy-yielding reactions of *Bact. coli*, *e.g.* sodium lactate and sodium fumarate reacting to give sodium pyruvate and sodium succinate [Quastel *et al.*, 1925]. If such a reaction between amino-acids were catalysed by *Cl. sporogenes*, it would be expected that some amino-acids would react as hydrogen donators (*i.e.* would reduce methylene blue in the presence of the bacteria) while others would act as hydrogen acceptors (*i.e.* would oxidise leucomethylene blue). These theoretical considerations have been tested by experiment, and found to provide a satisfactory explanation of the method by which *Cl. sporogenes* obtains its energy.

EXPERIMENTAL.

Preparation of bacterial suspensions.

All the experiments described in this paper were done with washed suspensions of *Cl. sporogenes*. The culture was obtained from the National Collection of Type Cultures (No. 533, *B. sporogenes* Bellette), and was maintained on a meat medium. To prepare the washed suspensions, 800 ml. of tryptic digest of caseinogen in a one litre flask were inoculated directly with a loopful of the meat culture, and incubated for 40–45 hours in a McIntosh and Fildes anaerobic jar. The culture was then centrifuged, and the bacteria were washed twice by centrifuging with Ringer's solution and finally suspended in 10–20 ml. of Ringer's solution. The enzymic activity of such suspensions was very unstable, and experiments had to be carried out within a few hours of the washing of the bacteria; within 24 hours the activity had completely disappeared, even if the suspension were kept anaerobically in the ice-chest. Microscopic examination of many of the suspensions prepared in this way showed that they almost always contained nothing but vegetative cells, though rarely a few spores were visible.

Detection of hydrogen donators.

The usual method was employed. 1 ml. of phosphate buffer at p_H 7·5, 1 ml. of methylene blue or brilliant cresyl blue $M/2000$, 1 ml. of water and 1 ml. of the substances under investigation in neutralised $M/10$ solutions were added to a series of Thunberg tubes. After the addition to each in turn of 1 ml. of a

suitably diluted bacterial suspension, the tubes were evacuated and incubated in a water-bath at 40°, and the rate of reduction of the dye was observed. The averages of the results of a large number of such experiments are given in Table I. It will be noted first that those substances which are the most active

Table I. *Relative velocities of oxidation by dyes of various substrates. Velocity with alanine is taken as standard (=100).*

Substrate	Rate of oxidation	Substrate	Rate of oxidation
Sodium formate	0	*l*-Leucine	100
Sodium acetate	0	*l*-Phenylalanine	10
Sodium propionate	0	*l*-Aspartic acid	5
Sodium lactate	0	*d*-Glutamic acid	2
Sodium succinate	0	*d*-Arginine	0
Sodium pyruvate	40	*d*-Lysine	0
Glucose	0	*l*-Histidine	<2
		l-Proline	0
Glycine	0	*l*-Hydroxyproline	0
d-Alanine	100	*dl*-Serine	0
l-Alanine	0	*l*-Tyrosine	<2
d-Valine	60	*l*-Tryptophan	<2

hydrogen donators in the case of facultative anaerobes and aerobes (*e.g.* glucose, formate, lactate and succinate) were not oxidised at all by *Cl. sporogenes*. On the other hand, among the amino-acids were several very active hydrogen donators, especially the simple aliphatic monoamino-acids, *d*-alanine, *d*-valine and *l*-leucine. The only compound among those tried, apart from the amino-acids, which could be oxidised at all was sodium pyruvate.

The oxidation of alanine.

Before proceeding further, a few experiments on the reaction between alanine and methylene blue or cresyl blue must be described.

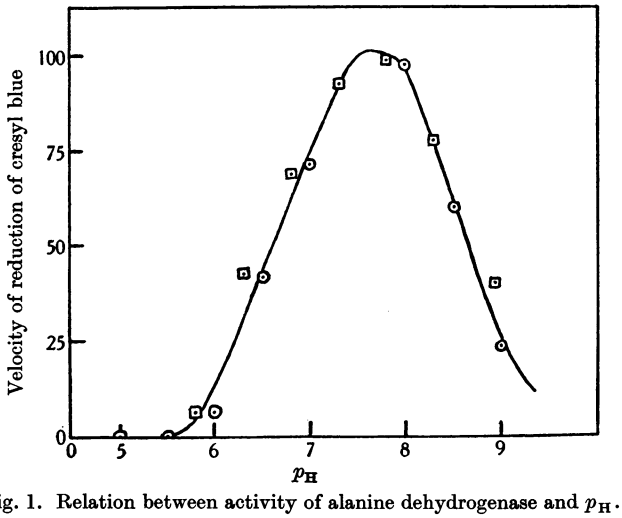

Fig. 1. Relation between activity of alanine dehydrogenase and p_H.

(*a*) *The relation between rate of oxidation and* p_H. A series of Thunberg tubes containing 1 ml. *M*/10 *d*-alanine, 1 ml. *M*/2000 cresyl blue, 1 ml. water, 1 ml. suspension of bacteria and 1 ml. of buffer (phthalate, phosphate or borate

according to the p_H) at different p_H values was incubated at 40° and the reduction time of the dye noted. The optimum p_H was found to lie at about 7·6 (see Fig. 1). A similar result was obtained with *l*-leucine.

(*b*) *The relation between rate of oxidation and concentration of alanine.* A similar series of tubes, all at p_H 7·5, with different concentrations of *d*-alanine was incubated at 40° and the reduction time of the dye observed. It was found that the affinity of alanine dehydrogenase for *d*-alanine was rather low ($K = 10^{-2·5}$ *M*), so that maximum velocity is not obtained unless the concentration of alanine is about *M*/30.

(*c*) *Stereochemical specificity.* *l*-Alanine hydrochloride was prepared from the commercial *dl*-product by the method of Fischer [1899]. The sample of *l*-alanine prepared was too small to permit of a measurement of its rotation, which is very low, but the rotation of the benzoyl-*l*-alanine was measured before hydrolysis, and found to be of the right order (found $[\alpha]_{5461}^{16·5°} = -36°$; Fischer [1899] gives $[\alpha]_D^{20°} = -37·4°$). As the result was a complete negative, exact proof of the optical purity of the specimen was unnecessary. A neutralised *M*/10 solution was added to a Thunberg tube in the usual way and was found not to reduce methylene blue. To eliminate the unlikely possibility of some toxic material being present in the preparation, a control experiment was carried out as below:

	Reduction time (min.)
1 ml. *d*-alanine *M*/10	7½
1 ml. *l*-alanine hydrochloride *M*/10	<120
1 ml. *d*-alanine + 1 ml. *l*-alanine hydrochloride	9½

Specimens of the enantiomorphs of other natural amino-acids have not yet been obtained, but it seems likely that only the natural isomerides are oxidised.

(*d*) *The course and extent of oxidation of alanine.* The usual method of measuring the extent of oxidation of a substrate, *viz.* manometric measurement of oxygen uptake, was not available in this case. Even in the presence of a dye as hydrogen carrier no oxygen was used. This might be due to peroxide formation, but addition of purified catalase to the solutions in the manometer vessel did not enable the oxidation to proceed. The only alternative method of following the oxidation was to allow the bacteria to reduce a strong solution of an indicator, and for this purpose brilliant cresyl blue was chosen in preference to methylene blue for three reasons: (*a*) it is more quickly reduced, (*b*) it is less toxic, (*c*) its leuco-form is more soluble in water. The potential of cresyl blue (+0·043 v.) is slightly higher than that of methylene blue (+0·011 v.).

The method employed was as follows. In a 100 ml. Büchner flask were placed 5 ml. *M*/5 phosphate buffer p_H 7·5, 10 ml. of a thick suspension of *Cl. sporogenes* and a known amount (usually of the order of 0·005 millimol) of *d*-alanine dissolved in 5 ml. of water. The flask was fitted with a rubber stopper carrying a 10 ml. tapped burette with a fine jet. The flask was now thoroughly evacuated, sealed and immersed in a water-bath at 40°. The burette was filled with a freshly de-aerated *M*/200 solution of cresyl blue, and after an interval of 5 minutes to allow the solutions to come to the temperature of the bath, this solution was run in drop by drop, a further drop being added as soon as the previous one was reduced. The time was noted after the reduction of each 0·2 ml. With every such experiment a control was carried out, differing only in the omission of the alanine.

In Fig. 2 are shown graphically the results of one experiment. It will be noted that the reduction in the control is very large, so that the end-point of the oxidation of the alanine is rather vague. Fig. 3 gives the results of a series

of experiments, all corrected for the blank. These curves show that for each molecule of alanine two molecules of the dye are reduced rapidly. In some

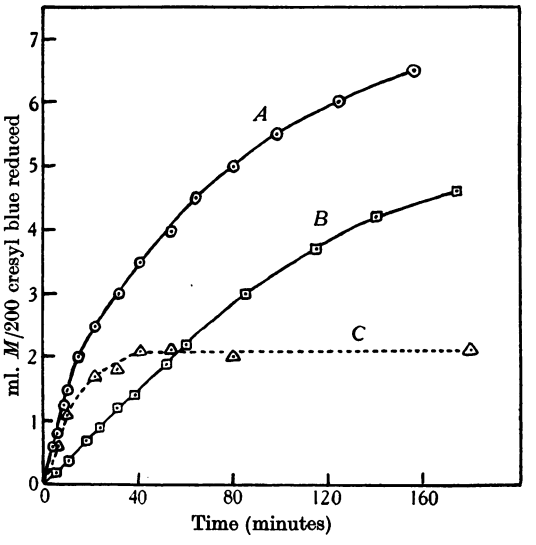

Fig. 2. Course of oxidation of *d*-alanine by cresyl blue. Curve *A*, 1 ml. *M*/200 alanine; curve *B*, no alanine; curve *C*, difference between *A* and *B*.

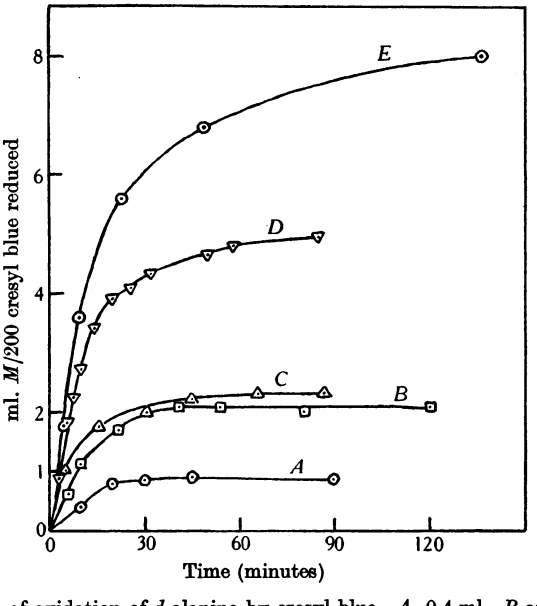

Fig. 3. Course of oxidation of *d*-alanine by cresyl blue. *A*, 0·4 ml., *B* and *C*, 1·0 ml., *D*, 2·0 ml. and *E* 4·0 ml. of *M*/200 *d*-alanine, all corrected for blank.

cases there appears to be a further very slow oxidation, but on account of the large correction that has to be made for the blank it is very doubtful whether this has any significance.

It was found impossible to reduce this "blank" reduction by further washing of the bacteria, or to remove it by chemical treatment of the suspensions. For instance, toluene treatment, which is so useful with *Bact. coli*, completely destroys the enzymes of *Cl. sporogenes*. In these particular experiments the blank is, of course, greatly exaggerated, as the substrate concentration is so small ($M/4000$). At higher substrate concentrations the blank would be proportionately much smaller both in amount and in rate, but higher concentrations of alanine could not be used on account of the toxic properties of large amounts of cresyl blue.

(*e*) *The deamination of alanine during oxidation.* From experiments such as those described in the preceding paragraph it is possible to learn at what stage in the oxidation of alanine its amino-group is eliminated as ammonia. For this purpose an experiment and its control were taken down when the (corrected) amount of dye reduced was rather more than the molecular equivalent of the alanine present. The dye was removed with kieselguhr, and the ammonia estimated in aliquot portions of the filtrates. Other experiments were carried out with excess of alanine in the flask (5 ml. $M/10$). When a certain amount of the dye had been reduced, the solutions were treated as above, and the ammonia was estimated. One result from each type of experiment is given in Table II. It is

Table II.

Alanine present	Cresyl blue reduced (corrected for blank) ml. $M/100$	Ammonia found (corrected for blank) ml. $M/100$
5 ml. $M/100$	7·0	5·3
10 ml. $M/20$	3·75	3·40

clear that in the first case the whole of the alanine had been deaminated while only 40 % excess of dye had been reduced, and in the second case the ammonia corresponded to the amount of dye reduced. The alanine is therefore deaminated during the first step of the oxidation.

Detection of hydrogen acceptors.

A hydrogen acceptor is a substance which, in the presence of cells, will oxidise leuco-dyes. The easiest method of demonstrating such substances is that devised by Quastel and Whetham [1924], in which the bacteria themselves are allowed to reduce the indicator before the substance to be tested is added from a side-tube. In the present work the original U-tube of Quastel and Whetham was replaced by the Keilin modification of the Thunberg tube, which has a hollow stopper capable of holding up to 0·5 ml. of the solution of the substance under investigation.

Each tube contained 1 ml. of buffer, $M/5$ p_H 7·5, 1 ml. of a thick suspension of *Cl. sporogenes*, 1 ml. of indicator (usually $M/2000$, but sometimes less, according to the tinctorial power of the indicator), and 1 ml. of leucine $M/100$ (sometimes, owing to the great reducing blank of the thick bacterial suspensions, the leucine could be omitted). The stopper contained 0·2 ml. of a neutralised $M/2$ solution of the substance to be tested, or else the equivalent amount of the dry solid. The tubes were thoroughly evacuated, special care being taken that no dissolved air remained in the solution in the stopper, and incubated at 40° until the dye was completely reduced. At this point the solution in the stopper was tipped into the tube and the whole left for a further 2 hours to observe any reoxidation of the indicator. In the first series of experiments the traditional methylene blue was used, and no substance was found capable of

oxidising leucomethylene blue in the presence of *Cl. sporogenes*. This meant that, if hydrogen acceptors with respect to *Cl. sporogenes* exist, they must lie at a considerably more negative point on the potential scale than methylene blue, and consequently experiments were carried out with more negative dyes. It was obviously useless to employ dyes so negative that the dehydrogenases of *Cl. sporogenes* were incapable of reducing them, so a rough colorimetric determination was made of the reducing level of the alanine dehydrogenase system. It was found that all dyes down to benzyl viologen were easily reduced (r_H 3 at p_H 7·5), and a partial reduction even of methyl viologen at p_H 8·0 (r_H 1) was observed.

Another series of experiments was therefore carried out with benzyl viologen. It was now found that proline and hydroxyproline caused a rapid and complete reoxidation of this dye, while glycine caused a rapid partial reoxidation. No other substance tried had any effect, and it is noteworthy that, as in the case of hydrogen donators, substances which are active with facultative anaerobes (*i.e.* nitrate and fumarate) are inactive with *Cl. sporogenes* (see Table III).

Table III. *Hydrogen acceptors.*

Substance	Indicator	r_H of indicator	Reoxidation
Sodium nitrate	Benzyl viologen	3 (at p_H 7·5)	−
Sodium fumarate	,,	,, ,,	−
dl-Serine	Benzyl viologen	3 (at p_H 7·5)	−
l-Aspartic acid	,,	,, ,,	−
d-Glutamic acid	,,	,, ,,	−
d-Arginine	,,	,, ,,	−
d-Lysine	Neutral red	,,	−
l-Histidine	Benzyl viologen	,, (at p_H 7·5)	−
l-Tyrosine	,,	,, ,,	−
l-Proline	Benzyl viologen	3 (at p_H 7·5)	+ +
,,	Neutral red	,,	+ +
,,	Rosinduline	4·5	+ +
,,	Phenosafranine	5·5	+ +
,,	Ethyl Capri blue	11·5	−
l-Hydroxyproline	Benzyl viologen	3 (at p_H 7·5)	+ +
,,	Neutral red	,,	+ +
Glycine	Methyl viologen	1 (at p_H 8·0)	+ +
,,	Benzyl viologen	3 (at p_H 7·5)	+
,,	Phenosafranine	5·5	−

+ + indicates complete reoxidation. + indicates partial reoxidation.

A few further experiments were done to determine the oxidising level of glycine and proline (Table III). Glycine oxidised methyl viologen completely, benzyl viologen partially, and phenosafranine not at all, so it lies somewhere near the range of benzyl viologen (r_H 3 at p_H 7·5). Proline reoxidised all the leuco-dyes up to phenosafranine (−0·25 v.), but failed to oxidise leuco-Capri blue (−0·08 v.), so it lies somewhere between these two values. No suitable indicator between these exists, since *Cl. sporogenes* is unable to reduce Nile blue (−0·125 v.) or cresyl violet (−0·15 v.).

The reduction of glycine and proline.

The study of the reduction of hydrogen acceptors by a method analogous to that used in following the oxidation of alanine, *i.e.* by letting the bacteria oxidise a strong standard solution of a leuco-dye, presents great technical difficulties and has not been attempted. There are accounts in the literature however

of the reduction of these substances by mixed cultures of bacteria, which suggest the probable course of the reductions in the present experiments. Brasch [1909] showed that glycine was converted by putrefying bacteria into acetic acid and ammonia; this is indeed the only way in which glycine can be reduced, unless the biologically abnormal reduction of the carboxyl group is considered. Neuberg [1911] allowed mixed putrefying bacteria to act on proline and isolated from the products δ-aminovaleric acid and *n*-valeric acid. The first stage of the reduction in this case apparently breaks the ring, without deamination. Ackermann [1911] also obtained the same products from proline. Keil and Günther [1933], from similar experiments with hydroxyproline, isolated only δ-aminovaleric acid.

Direct reactions between hydrogen donators and hydrogen acceptors.

It has so far been demonstrated that *Cl. sporogenes* is able to activate certain amino-acids, some as hydrogen donators (*e.g.* alanine, valine and leucine) and some as hydrogen acceptors (proline, hydroxyproline and glycine). For the provision of energy for the bacteria it is obviously not sufficient that these substances should react with dyes and leuco-dyes respectively; they must be able to react directly with one another, without the intervention of an artificial hydrogen carrier. For detecting such reactions between hydrogen-donating and hydrogen-accepting amino-acids, a useful test is provided in the fact, already demonstrated, that the amino-group of alanine is eliminated as ammonia during the first stage of oxidation. Hence if, for instance, alanine is oxidised by proline, ammonia will be liberated.

Experiments were carried out in the following way. In a Thunberg tube were placed 0·5 ml. of a *M*/10 solution of the hydrogen donator, 0·5 ml. of a *M*/10 solution of the hydrogen acceptor, 0·5 ml. of *M*/5 phosphate buffer, p_H 7·5, and 1 ml. of a suspension of *Cl. sporogenes*. Control tubes contained (*a*) hydrogen donator alone, (*b*) hydrogen acceptor alone and (*c*) neither amino-acid. The tubes were evacuated and incubated at 40° for a period of usually 2–6 hours. After incubation the free ammonia was estimated in each tube. 2 ml. of the solution were measured into 25 ml. of 50 % alcohol in a Kjeldahl flask, which was connected through a condenser to a receiver containing 5 ml. of *N*/100 sulphuric acid. The solution was made alkaline by the addition of 10 ml. of borate buffer (*M*/5, p_H 10), and raised to the boiling-point, while the ammonia was removed to the receiver by a current of ammonia-free air. The excess of acid was titrated with *N*/100 CO_2-free NaOH. A typical result with alanine and proline is shown in Table IV. This shows that only when hydrogen donator

Table IV.

	Ammonia found ml. *N*/10	Ammonia (corrected for blank) ml. *N*/10
Blank	0·07	—
0·5 ml. *M*/10 *d*-alanine	0·06	0·00
0·5 ml. *M*/10 *l*-proline	0·05	0·00
0·5 ml. alanine + 0·5 ml. proline	0·42	0·35

and hydrogen acceptor were present together was any ammonia liberated, and this is good evidence that the reaction proceeding was indeed an oxidation of alanine and reduction of proline. Further evidence on this point will be given later.

It can further be shown that any one of the hydrogen-accepting amino-acids will react in the same way with any one of the hydrogen-donating amino-acids. Experiments with *d*-alanine, *d*-valine and *l*-leucine on the one hand, and glycine, *l*-proline and *l*-hydroxyproline on the other, carried out in the way described above, gave the results shown in Table V. None of the amino-acids alone is deaminated, but any mixture of hydrogen donator and acceptor produces ammonia.

Table V. *Direct reactions between pairs of amino-acids.*

0·5 ml. of *M*/10 solution of each amino-acid was used.
The figures represent ammonia liberated (ml. *N*/10 per tube) corrected for the blank experiment.
The first figure is for the hydrogen acceptor alone, the second for the hydrogen donator alone, and the third for both together.

	Hydrogen donators		
Hydrogen acceptors	*d*-Alanine	*d*-Valine	*l*-Leucine
l-Proline	0·00	0·00	0·00
	0·00	0·00	0·02
	0·35	0·33	0·20
l-Hydroxyproline	0·00	0·00	0·00
	0·00	0·00	0·02
	0·28	0·41	0·15
Glycine	0·01	0·04	0·04
	0·00	0·00	0·01
	0·26	0·55	0·31

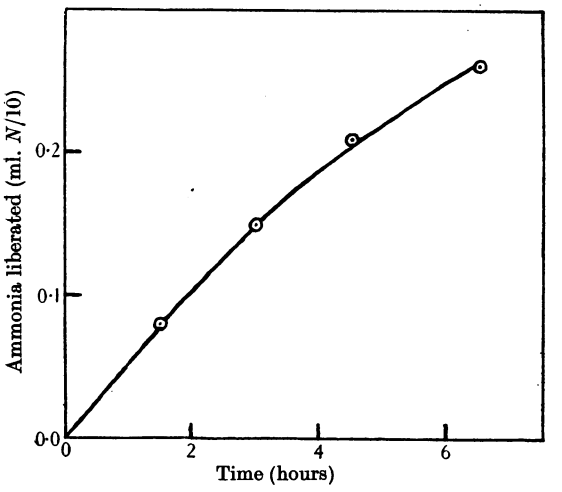

Fig. 4. Course of reaction between glycine and *d*-alanine.

(*a*) *The course of the reaction.* It is necessary to exclude the possibility that the bacteria were growing during these rather long experiments; this is, of course, unlikely as *Cl. sporogenes* has never been shown to grow on such simple mixtures, and has, even when growing on tryptic broth, a lag period of about 24 hours. A set of Thunberg tubes was prepared containing 0·5 ml. of buffer, 0·5 ml. *M*/10 *d*-alanine, 0·5 ml. *M*/10 glycine and 1 ml. of bacterial suspension, and another set with 1 ml. of water replacing the two amino-acids. These were incubated at 40°, and the ammonia liberated was estimated at various times up to 6 hours. The result is shown in Fig. 4 (ammonia liberated, corrected for control,

plotted against time), and it is clear that the reaction started with no lag period and proceeded with slowly diminishing velocity.

Another similar experiment showed that the glycine and alanine were both completely deaminated.

	Ammonia found (6½ hours) (corrected for control) ml. *N*/10
0·5 ml. *M*/10 glycine + 0·5 ml. *M*/10 alanine	0·95

It can further be shown, in the same way, that just as one molecule of alanine can reduce two of cresyl blue, so it can reduce two molecules of glycine.

	Ammonia found (22 hours) (corrected for control) ml. *N*/10
1·0 ml. *M*/10 glycine + 0·5 ml. *M*/10 alanine	1·46

(*b*) *Energy of reaction and rates of reaction.* It is clear now that *Cl. sporogenes* is able to carry out various oxidation-reduction reactions anaerobically between pairs of amino-acids. To decide whether these reactions are of quantitative significance in the metabolism of the bacteria two further points must be considered: (*a*) the amount of energy liberated by the reactions and (*b*) the rates at which they proceed, relative to the amount of cell material present. Unfortunately the data are not available for calculating the change of free energy in any of these reactions, so for the time being this point must be passed over, but experiments on the rates of the reactions show that they proceed at a speed comparable with those of other bacterial oxidations. That some energy is liberated by the reactions is of course demonstrated by the fact that they do proceed spontaneously.

The velocity of the reaction between glycine and *d*-alanine or *l*-proline and *d*-alanine was measured by estimating the rate of ammonia production as described above, and the nitrogen content of the bacterial suspension was determined by a micro-Kjeldahl method. Using the fact that 10 % of the dry weight of *Cl. sporogenes* is nitrogen, the dry weight of the bacteria used in the experiment was calculated. The results are most conveniently expressed in terms of $Q_{O_2}^{\text{alanine}}$, this being taken to mean in the present case the number of μl. of oxygen which would be used per mg. dry weight of bacteria per hour if the oxidation had been aerobic. Allowing for the fact that in the reaction between alanine and glycine only one-half of the ammonia is derived from the alanine, it was found in nine experiments that $Q_{O_2}^{\text{alanine}}$ with glycine as oxidant varied from 5 to 12. Considering the great instability of the enzymes concerned, it appears justifiable to take the highest value recorded as a minimum, and on that assumption the values given in Table VI were obtained. These may be compared with the figures of Cook and Haldane [1931] for aerobic oxidations with *Bact. coli*, *viz.* $Q_{O_2}^{\text{formate}} = 40$, $Q_{O_2}^{\text{lactate}} = 12$ *etc.*

Table VI. *Rates of oxidation in terms of dry weight of bacteria.*

	Hydrogen donators		
	d-Alanine	*d*-Valine	*l*-Leucine
Hydrogen acceptors	Q_{O_2}	Q_{O_2}	Q_{O_2}
Glycine	12	12	8
l-Proline	50	—	30
l-Hydroxyproline	40	—	—

(*c*) *Reactions between other amino-acids.* The fact that glycine when reduced, and alanine when oxidised, liberate ammonia, can be used to confirm the identity of hydrogen donators and hydrogen acceptors. The indicator experiments already described, though useful as a starting-point, are not physiological, and cases are known in which substances which do not react with indicators do react with natural substrates, *e.g. Bact. coli* oxidises acetate rapidly by means of oxygen but not at all by means of methylene blue. A large number of amino-acids were therefore tested as hydrogen donators by estimation of the ammonia produced when they were incubated anaerobically with glycine and a suspension of *Cl. sporogenes*, and as hydrogen acceptors by estimation of the ammonia production when they were incubated with *d*-alanine. The experiments were carried out as before, *i.e.* 0·5 ml. of *M*/10 solution of each amino-acid, 0·5 ml. of buffer at p_H 7·5 and 1 ml. of suspension of bacteria were incubated anaerobically in Thunberg tubes at 40°. After 2–5 hours' incubation, the ammonia was estimated in 2 ml. of the solution. The usual controls with neither amino-acid and with each separately were performed.

The results of incubation of various amino-acids with glycine are shown in Table VII. It will be seen that there is a fairly close agreement between the

Table VII. *Hydrogen donators.*

	By reduction of indicator	By ammonia from glycine		By reduction of indicator	By ammonia from glycine
Glycine	0	0	*d*-Arginine	0	0
d-Alanine	100	100	*d*-Lysine	0	0
d-Valine	60	100	*l*-Histidine	<2	55
l-Leucine	100	70	*l*-Proline	0	0
l-Phenylalanine	10	30	*l*-Hydroxyproline	0	0
l-Aspartic acid	5	25	*l*-Tryptophan	<2	10
d-Glutamic acid	2	15			

indicator method and the glycine method, but that some amino-acids, notably glutamic acid and histidine, react relatively more quickly with glycine than with the indicator.

The tests for hydrogen acceptors by incubation with alanine gave the results shown in Table VIII. The agreement is complete, and only glycine, proline and hydroxyproline can oxidise either alanine or leuco-dyes in the presence of *Cl. sporogenes*.

Table VIII. *Hydrogen acceptors.*

	By oxidation of leuco-indicators	By ammonia from alanine		By oxidation of leuco-indicators	By ammonia from alanine
Glycine	+	+	*d*-Arginine	–	–
d-Alanine	–	–	*d*-Lysine	–	–
d-Valine	–	0	*l*-Histidine	–	–
l-Leucine	–	–	*l*-Proline	+	+
l-Aspartic acid	–	–	*l*-Hydroxyproline	+	+
d-Glutamic acid	–	–	*l*-Tryptophan	0	–

0 = not tested.

Serine and tyrosine.

It was found in the course of the above experiments that serine and tyrosine behaved exceptionally in liberating ammonia when incubated alone with *Cl. sporogenes*. Neither amino-acid reacted with dyes either as a hydrogen

donator or as a hydrogen acceptor, except for a very slight hydrogen-donating activity of tyrosine. The production of ammonia from serine was demonstrated by experiments similar to those already described, but, since only *dl*-serine was available, 0·5 ml. of an *M*/5 solution of this compound was used. The following is a typical experimental result:

	Ammonia production in 7 hours ml. *N*/10 per tube
0·5 ml. *M*/10 glycine	0·04
0·5 ml. *M*/10 *d*-alanine	0·00
0·5 ml. *M*/5 *dl*-serine	0·35
0·5 ml. glycine + 0·5 ml. serine	0·53
0·5 ml. alanine + 0·5 ml. serine	0·35

Thus serine, apart from yielding ammonia by itself, donates hydrogen to glycine to give extra ammonia, but does not accept hydrogen from alanine. The rate of production of ammonia from serine is about the same as that from a mixture of glycine and alanine. Tyrosine gives ammonia at about one-fifth of this rate, and does not yield extra ammonia with either glycine or alanine. The slow rate may be caused by the small solubility of tyrosine in water (this amino-acid was added as a *M*/10 suspension).

The chemistry of the reactions.

Preliminary results show that the complete reaction between *d*-alanine and glycine is

$$CH_3.CHNH_2.COOH + 2CH_2NH_2.COOH + 2H_2O \rightarrow 3CH_3COOH + 3NH_3 + CO_2.$$

The evidence for this will be presented in a later paper. Work is proceeding on the chemistry of the oxidation of *l*-leucine and the reduction of *l*-proline, and also on the application of these results to growth experiments with *Cl. sporogenes*.

Discussion.

The experiments described in this paper show that washed cells of *Cl. sporogenes* are able to catalyse a hitherto unknown type of chemical reaction, *viz.* the oxidation and reduction of certain pairs of amino-acids. Whether these reactions play an important quantitative part in the provision of energy for the growth of the bacteria on protein media has not yet been determined experimentally, but there can be little doubt that they do. The results of Burrows [1933] are in support of this view. He found that *Cl. botulinum* grew well on a mixture of amino-acids containing only glycine, *d*-alanine, *l*-leucine, *l*-proline, *d*-lysine, *l*-cysteine and *l*-tryptophan. It is significant that, excluding the two "essential" amino-acids cysteine and tryptophan, two of the remaining five are hydrogen donators (alanine and leucine) and two hydrogen acceptors (glycine and proline). Burrows [1932; 1933] interpreted his results as showing that all these amino-acids were "essential," *i.e.* the bacteria needed their presence in the medium because they were unable to synthesise them, but this seems improbable, especially in the case of such simple molecules as glycine and alanine. It is far more likely that they are needed to provide energy by their chemical interaction.

It was stated in the introduction to this paper that "it is difficult to picture any reaction by which a single amino-acid molecule could break down anaerobically to yield energy." It now seems possible that the case of serine is an example of this type of reaction, though it is equally possible that this is an example of a reaction between two molecules of the same amino-acid. It is

useless to speculate on the chemistry of the breakdown of serine, and the author hopes to study the matter experimentally.

Bessey and King [1934] have recently described experiments in which washed suspensions of *Cl. sporogenes* were allowed to act aerobically on various amino-acids. They found that the amino-acids were deaminated, some completely. These results are not comparable with those described in the present paper, since, apart from the aerobic conditions, the reactions were very slow (alanine deaminated in 5 days, arginine in 3 days, glutamic acid in 7 days, *etc.*), and there was in every case a considerable lag period before the reaction started. The significance of such deaminations is not yet clear.

Two interesting points should be mentioned in connection with the oxidation of alanine by *Cl. sporogenes*. Krebs [1933] in studying the aerobic oxidation of amino-acids by mammalian tissue slices found that though the natural optical isomerides were oxidised their enantiomorphs were oxidised with much greater velocity by kidney slices. This is the opposite of the case with *Cl. sporogenes*, where the non-natural isomeride is not oxidised at all. Yeast [Ehrlich, 1906] and moulds [Schultze and Bosshard, 1906] also attack only the natural isomerides. Wurmser and Mayer-Reich [1933] calculated the free energy change of the reaction

$$CH_3.CO.COO^- + NH_4^+ + 2H \rightarrow CH_3.CHNH_2.COOH + H_2O$$

and showed that an alanine dehydrogenase system should reduce indicators down to about $-0{\cdot}06$ v. (r_H 12) but no further. The alanine dehydrogenase of *Cl. sporogenes* reduces dyes down to $-0{\cdot}35$ v. (r_H 2), so the only conclusion is that pyruvic acid is not in this case the oxidation product of alanine. Experimental evidence on this point will be given in a later paper.

Summary.

Washed suspensions of *Cl. sporogenes* activate certain amino-acids as hydrogen donators (especially alanine, valine and leucine) and others as hydrogen acceptors (glycine, proline and hydroxyproline).

One molecule of alanine reduces two molecules of cresyl blue; the amino-acid is deaminated during the first step of the oxidation.

Direct reactions occur between the hydrogen-donating and hydrogen-accepting amino-acids, any one of one group reacting with any one of the other.

The rates of these reactions are of the same order as those of aerobic oxidations with other bacteria, *e.g.* $Q_{O_2}^{\text{alanine + proline}} = 50$.

The probable significance of these reactions in the metabolism of the bacteria is discussed.

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