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

V. FURTHER EXPERIMENTS ON THE COUPLED REACTIONS BETWEEN PAIRS OF AMINO- ACIDS INDUCED BY *CL. SPOROGENES*.

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IN the first paper of this series Stickland [1934] showed that washed cells of *Cl. sporogenes* were able to bring about the linked oxidation and reduction of pairs of amino-acids. He considered that these reactions were probably the source of energy for the growth of this organism on protein digest media. The amino-acids that were activated as H donators, i.e. oxidized, were: *d*-alanine, *l*-leucine, *d*-valine, and to a less extent *l*-histidine, *l*-phenylalanine and some others. The amino-acids activated as H acceptors, i.e. reduced, were: glycine, *l*-proline and *l*-hydroxyproline. Reactions occurred between any H donator and any H acceptor in the presence of washed cells of *Cl. sporogenes*. Serine, tyrosine and to a small extent, glycine were broken down in the absence of any other amino-acid. In following papers Stickland [1935, 1, 2, 3] worked out in detail the chemistry of some of these linked reactions.

The work of Fildes & Richardson [1935] makes it almost certain that the "Stickland reaction" is in fact the main source of energy for the growth of these organisms. These workers were able to obtain good growth of *Cl. sporogenes* on a medium consisting of pure amino-acids, salts and "*sporogenes* vitamin" only. Furthermore they found that "the conclusions of Stickland upon the sources of energy available for this organism are in agreement with growth experiments".

Bessey & King [1934] have found that arginine, alanine, glutamic acid, tyrosine and cystine are deaminated when incubated alone with washed suspensions of *Cl. sporogenes*. In the cases of alanine and glutamic acid the apparent discrepancy between their results and those of Stickland is possibly explained by the duration of the experiments (3–10 days) and the small amount of substrate used (*M*/300). It seems possible that in Bessey & King's experiments alanine and glutamic acid, which are H donators, were deaminated by reacting with H acceptors formed by the autolysis of the bacterial suspension itself. The case of arginine and cystine will be discussed later.

The work reported in the present paper was undertaken in an attempt to correlate chemical structure with the power of acting as H donator or as H acceptor in the "Stickland reaction". In the course of the experiments it was found that ornithine and arginine acted powerfully as H acceptors and in addition were partially deaminated by *Cl. sporogenes* in the absence of other amino-acids. Similarly it was found that cysteine was deaminated alone in addition to being activated as H donator. The ornithine reaction will be dealt with fully in the present communication; preliminary experiments with cysteine and arginine are also reported.

Preparation of bacterial suspensions.

The organism used was *Cl. sporogenes* (Bellette) and was obtained from the National Collection of Type Cultures (No. 533). This is the same strain as that used by Stickland [1934] and by Fildes & Richardson [1935]. If the suspensions were prepared as described by Stickland [1934] they were found to be occasionally inactive (10 % of cases). The following procedure gave better growth and more consistently active suspensions. 900 ml. tryptic caseinogen digest broth were enriched by the addition of 2 g. wet weight of Lab-lemco (beef extract) and sown immediately after autoclaving and rapid cooling from a culture of *Cl. sporogenes* on Robertson's meat medium. The flask was then put at once into a McIntosh & Fildes's anaerobic jar and incubated at 38°. Under these conditions good growth and active suspensions were obtained after only 20 hours; longer incubation led to spore formation and less active suspensions. The 20-hour cultures were therefore spun down on the centrifuge and the bacteria washed twice on the centrifuge with Ringer's solution. The cells from 900 ml. broth were usually finally suspended in 20 ml. Ringer's solution.

Experimental methods.

In order to determine whether a given substance could be activated by the organism either as H donator or acceptor, use was made of the fact that during these coupled reactions the amino-acids are often deaminated with liberation of ammonia. In testing a substance for activity as H donator it was incubated in the presence of *Cl. sporogenes* with an H acceptor known to give rise to ammonia on reduction (e.g. glycine). Similarly in testing for an H acceptor the substance was mixed with an H donator known to give rise to ammonia on oxidation (e.g. alanine). A control was always done with two amino-acids known to react, such as alanine and proline, in order to be certain that the bacterial suspension was active.

Ammonia was estimated as described by Stickland [1934] by distilling 2 ml. samples from 50% alcohol-borate buffer pH 10 mixture into $N/100$ H_2SO_4 and back-titrating the latter with $N/100$ CO_2 -free NaOH. All experiments were done anaerobically at 37° in evacuated Thunberg tubes.

EXPERIMENTAL RESULTS.

l-Cysteine.

Stickland [1934] found that serine acted as H donator to glycine in addition to being partially deaminated when incubated alone with *Cl. sporogenes*. In view of the close structural relationship of serine and cysteine it was decided to test the action of the organism on the latter amino-acid. Bessey & King [1934] found that cystine was deaminated to the extent of 15 % by washed cells of *Cl. sporogenes* and Tarr [1933] has reported a 43 % formation of H_2S . The cysteine solution used in the following experiments was prepared from *l*-cysteine hydrochloride and was neutralized just before use in order to avoid autoxidation. Thunberg tubes were set up containing 0.5 ml. $M/5$ phosphate buffer pH 7, 1 ml. bacterial suspension, substrates as specified below and water to a total volume of 2.5 ml. Control tubes containing no substrate were also put up. Sets of tubes were taken out of the bath at intervals and the ammonia formed estimated. After 3 hours there was no increase in ammonia in any case and the results obtained are summarized in Table I. In each case the no-substrate control has been deducted (a small amount of NH_3 is always formed from the autolysis of the

Table I.

Substrate	NH ₃ formed as ml. N/100
(1) <i>l</i> -Cysteine	2.57
(2) <i>d</i> -Alanine	0
(3) <i>l</i> -Proline	0
(4) Glycine	0.09
(5) <i>d</i> -Alanine + <i>l</i> -proline	4.78
(6) <i>d</i> -Alanine + <i>l</i> -cysteine	2.59
(7) <i>l</i> -Cysteine + <i>l</i> -proline	3.43
(8) <i>l</i> -Cysteine + glycine	3.72
(9) <i>d</i> -Alanine + <i>l</i> -proline minus (2) and (3)	4.78
(10) <i>d</i> -Alanine + <i>l</i> -cysteine minus (1) and (2)	0.02
(11) <i>l</i> -Cysteine + <i>l</i> -proline minus (1) and (3)	0.86
(12) <i>l</i> -Cysteine + glycine minus (1) and (4)	1.06

Quantities used: cysteine and alanine, 0.5 ml. *M*/10; glycine and proline, 0.5 ml. *M*/5.

suspensions). Qualitative tests for H₂S were positive in all tubes containing cysteine. The results show that cysteine is only 50% deaminated (1), since 0.5 ml. *M*/10 cysteine would yield 5 ml. *N*/100 NH₃ on complete deamination. The proline-alanine control (9) was complete in 3 hours and longer incubation of the cysteine tubes (10 hours) gave no increase in NH₃. This fits in with the approx. 50% formation of H₂S found by Tarr [1933]. In addition to the deamination which occurs when cysteine is incubated alone with the bacterium, some extra ammonia beyond the controls is formed when cystine is mixed with glycine or proline, (11) and (12); cysteine therefore also acts as H donator. No extra ammonia was produced from cysteine-alanine mixtures (10) showing that cysteine does not act as H acceptor. The behaviour of cysteine was entirely analogous to that found for serine by Stickland [1934]. The metabolism of cysteine (and cystine) by *Cl. sporogenes* is being further investigated.

Glycollic acid.

Glycine has been shown by Stickland [1934] to act as a H acceptor. The question arises whether the substitution of a OH group for the NH₂ group of glycine destroys this power. The experimental details were the same as for the cysteine experiments. The results showed that glycollic acid can act neither as donator to glycine nor as acceptor to alanine. The alanine-proline control showed that the bacterial suspension was active. It is evident that the replacement of the NH₂ of glycine by OH completely destroys its capacity as H acceptor in the presence of *Cl. sporogenes*.

β-Alanine.

In order to determine the effect on the activity of alanine as H donator of shifting the NH₂ group from the α- to the β-position, experiments were carried out with β-alanine. The general plan of the experiments was again the same as with cysteine. The results showed (a) that β-alanine was not deaminated when incubated alone with the organism, and (b) that no NH₃ was formed from mixtures of β-alanine and proline or of α-alanine and β-alanine, indicating that β-alanine acts neither as H donator nor as H acceptor in the "Stickland reaction".

Similar experiments with taurine, CH₂NH₂CH₂SO₃H, showed this acid also to be inactive as either donator or acceptor.

d-Ornithine.

l-Proline [Stickland, 1934] acts as H acceptor and gives rise to δ-amino-valeric acid on reduction [1935, 1]. Ornithine has a similar C chain to proline and there is some evidence [Ackermann, 1907-10] that it may also give rise

to δ -aminovaleric acid on putrefaction by mixed cultures of bacteria. The action of washed suspensions of *Cl. sporogenes* on ornithine was therefore tested. The ornithine solutions used throughout this section were prepared from *D*-ornithine hydrochloride (Hoffmann La Roche) and were carefully neutralized before use.

Preliminary experiments. (a) *Activity as H acceptor.* Thunberg tubes were set up containing 1 ml. phosphate buffer pH 7, 1 ml. bacterial suspension, substrates as specified below and water to a total volume of 4 ml. Controls containing (a) no substrate and (b) no bacterial suspension were also put up. After subtracting the no-substrate blank the following results were obtained after 3.5 hours' incubation:

Substrate	NH ₃ formed as ml. N/100
(1) 1 ml. <i>M</i> /10 <i>D</i> -alanine	0.2
(2) 1 ml. <i>M</i> /10 <i>D</i> -ornithine	7.2
(3) 1 ml. <i>M</i> /10 <i>D</i> -alanine + 1 ml. <i>M</i> /10 <i>D</i> -ornithine	15.7
(4) Alanine + ornithine minus (1) and (2)	8.3
(5) Alanine + ornithine (no bacterial suspension)	0

It will be seen (2) that ornithine is deaminated to a considerable extent when incubated alone with the organism, but in addition it forms a large amount of extra NH₃, beyond the controls, when mixed with alanine (4). Ornithine therefore acts as H acceptor to alanine. There is no NH₃ formation in the absence of bacterial suspension (5).

The fact that ornithine is activated by *Cl. sporogenes* as H acceptor was confirmed by the indicator method. It can be shown that ornithine is able to reoxidize (i.e. accept H from) reduced benzylviologen in the presence of the bacterial cells. Hollow-stoppered Thunberg tubes were set up containing in the main tube 1 ml. buffer pH 7, 1 ml. *M*/2000 benzylviologen and 1 ml. bacterial suspension. The stoppers contained 0.5 ml. *M*/5 substrate. The tubes were thoroughly evacuated and incubated until the dye had been completely reduced by the reducing blank of the bacterial suspension. The contents of the stoppers were then tipped in and the time taken for complete reoxidation of the dye noted. The results obtained were:

Substrate	Reoxidation time
Water	Not reoxidized
<i>L</i> -Proline	3 min.
<i>D</i> -Ornithine	3 min.

(b) *Activity as H donator.* Ornithine was also tested for activity as H donator by incubation with the acceptors glycine and proline. The considerable deamination of ornithine when incubated alone with the organism was confirmed, but no extra ammonia was formed in the presence of either glycine or proline indicating that ornithine has no H-donating action. This result was confirmed by methylene blue reduction experiments.

Oxidizing level of ornithine. Alanine, in the presence of *Cl. sporogenes*, is able to reduce oxidation-reduction dyes down to benzylviologen (rH 3 at pH 7.5), while it was shown above that ornithine is able to reoxidize reduced benzylviologen. An oxido-reduction reaction between these two amino-acids is therefore possible and, as has been shown in the preliminary experiments, does in fact take place. The exact oxidizing level of the ornithine system was estimated by indicator reoxidation experiments similar to those described above with benzylviologen; if the reducing blank of the suspension was insufficient to reduce the indicator 0.5 ml. *M*/200 alanine was added. Controls in which water was tipped

in instead of ornithine were done in each case. The following results were obtained:

Indicator	rH of indicator	Reoxidation
Benzylviologen	3 (at pH 7.5)	++
Neutral red	3.0	++
Rosinduline	4.5	++
Phenosafranine	5.5	++
Ethyl Capri blue	11.5	—

++ indicates complete reoxidation.

Ornithine is therefore able to reoxidize all leuco-dyes up to phenosafranine (rH 5.5) but fails to reoxidize leuco-ethyl Capri blue (rH 11.5) so that the rH of the ornithine system lies between these values; no suitable indicator for use with *Cl. sporogenes* between these values exists [Stickland, 1934]. Comparison of these results with those given by proline [Stickland, 1934] shows that the potential levels of these two systems, as far as dye experiments can show, must be fairly close.

Reaction of ornithine with various H donators. Proof has already been given that ornithine will react with alanine. The next series of experiments was designed to test whether ornithine also reacts with the other chief H donators found by Stickland. Thunberg tubes were set up containing 0.5 ml. buffer pH 7, 1 ml. bacterial suspension, 0.5 ml. *M*/10 H donator, 0.5 ml. *M*/10 ornithine and water to 2.5 ml. Controls (*a*) with no substrates, (*b*) with each donator alone, and (*c*) with ornithine alone were also carried out. The results obtained after 5 hours' incubation are summarized in Table II, the appropriate control values having been deducted in each case. In the third column the relative efficiencies of H donators are compared by setting leucine as 100. The results show general correspondence with those obtained by Stickland [1934] in a similar study using glycine as acceptor.

Table II.

H donator	NH ₃ formed, ml. <i>N</i> /100	Leucine = 100	Glycine as ac- ceptor (Stickland)
<i>d</i> -Alanine	2.50	95	100
<i>l</i> -Leucine	2.64	100	70
<i>d</i> -Valine	1.93	73	100
<i>l</i> -Histidine	1.00	38	55
<i>l</i> -Phenylalanine	0.28	11	30

The product of the reduction of ornithine.

Proline, according to Stickland [1935, 1], gives rise to δ -aminovaleric acid on reduction; ornithine has a similar C chain and there is some evidence from old work of Ackermann [1907-10] that it may give rise to δ -aminovaleric acid by the action of mixed putrefactive organisms. It has already been established (p. 1937) that some deamination occurs when ornithine is reduced. As a working hypothesis it may be postulated that the α -amino-group of ornithine is deaminated during reduction and that δ -aminovaleric acid is the product. In such a reaction 2 atoms of H could be accepted:

It is known [Stickland, 1935, 2] that alanine is oxidized in the presence of acceptors according to the following equation:

Since 4H are donated by alanine, 2 mol. of ornithine would be required to oxidize 1 mol. alanine if the above hypothesis is correct, and 3 mol. NH_3 would be liberated.

To test this theory alanine was incubated with excess ornithine and the ammonia produced estimated. In preliminary experiments great difficulty was experienced in obtaining significant results owing to the large formation of NH_3 from ornithine itself, especially when the bacterial suspension was very active. This is best made clear by an example. In a usual Thunberg tube experiment the following results were obtained after various times of incubation, the no-substrate control having been deducted:

Substrate	NH_3 formed as ml. <i>N</i> /100		
	3.5 hours	10.5 hours	24 hours
(1) Ornithine	0.78	1.30	1.80
(2) Alanine	0	0	0
(3) Alanine + ornithine	3.13	6.09	6.21
(4) Alanine + ornithine minus (1) and (2)	2.35	4.79	4.41

If the working hypothesis outlined above is correct, 6 ml. *N*/100 extra NH_3 (4) should have been formed. Actually the extra NH_3 at first increased with time and then decreased. This decrease is due to the increase in the ornithine blank (1), since the total NH_3 from ornithine and alanine (3) increased throughout. It will be seen that the final ornithine blank (1.8 ml. *N*/100 NH_3) was greater than the NH_3 equivalent of the ornithine added in excess of 2 mol. (1 ml. *N*/100 NH_3). A significant result was therefore scarcely to be expected. Before describing similar experiments in which a larger excess of ornithine was added and which gave significant results it will be convenient to discuss the question of the ornithine blank in more detail.

Ornithine, although deaminated to a certain extent when incubated alone with the organism, can also react with alanine. The question arises as to whether the former type of breakdown occurs concurrently with the latter when ornithine-alanine mixtures are treated with *Cl. sporogenes*, or whether the ornithine-alanine oxido-reduction reaction takes place preferentially. In order to test this 1 mol. alanine was treated with exactly 2 mol. ornithine, i.e. the amount required for complete oxidation of the alanine according to the hypothesis. Sets of Thunberg tubes contained (a) water, (b) 0.2 ml. *M*/10 alanine, (c) 0.4 ml. *M*/10 ornithine, (d) 0.2 ml. *M*/10 alanine + 0.4 ml. *M*/10 ornithine, together with 0.5 ml. buffer pH 7, 1 ml. bacterial suspension and water to 2.4 ml. Sets of tubes were taken down at intervals and the experiment continued until the NH_3 in (d) did not increase. The results obtained are summarized in Table III. It will

Table III.

(All values less the appropriate controls.)

Exp.	Ornithine alone	NH_3 formed as ml. <i>N</i> /100		
		Ornithine + alanine	Ornithine + alanine (theoretical)	% found of theoretical
1	2.64	5.62	6.00	94
2	2.38	5.50	6.00	92
3	2.76	5.88	6.00	98

be seen that, assuming the hypothesis to be correct, the ornithine-alanine reaction is almost complete and all the alanine is broken down as well as the ornithine—in spite of the fact that ornithine alone is more than 50 % deaminated (column 2) and that there was only just sufficient ornithine present to oxidize the alanine.

The conclusion seems legitimate that ornithine reacts with alanine preferentially to being decomposed alone and that, provided that ornithine is present in only just sufficient quantity to oxidize the alanine, the only reaction to occur will be that oxidation.

If, however, the working hypothesis were incorrect, and ornithine were deaminated at both amino-groups, then the figures for alanine + ornithine in Table III might be due in part to the ornithine blank and not represent a complete oxidation of alanine. This is unlikely in view of the close approximation of the figures to the theoretical, and also in view of the fact that the complete oxidation of alanine was proved by estimation of volatile acid as well as NH_3 . Large (75 ml.) Thunberg tubes were set up containing (a) 12 ml. water, (b) 8 ml. *M*/10 ornithine, (c) 8 ml. *M*/10 ornithine + 4 ml. *M*/10 alanine, together with 8 ml. buffer pH 7, 8 ml. bacterial suspension and water to 28 ml. The course of the reaction was followed from NH_3 determinations on a series of small tubes containing 1/20 of the above quantities. When these showed the reaction to be complete the large tubes were taken down and NH_3 estimated in duplicate on 1 ml. samples. The remainder of the suspension was used for the estimation (in duplicate) of volatile acid as described by Stickland [1935, 2]. The mean results obtained are given in Table IV.

Table IV.

(All values less the water control.)

	Ornithine alone	Ornithine + alanine	Ornithine + alanine (theoretical)	% found of theoretical
NH_3 produced as ml. <i>N</i> /10	1.60	11.43	12.00	95
Volatile acid formed as ml. <i>N</i> /10	0	3.63	4.00	91

Assuming, according to the hypothesis, that 2 mol. NH_3 come from the reduction of 2 mol. ornithine and 1 mol. NH_3 from the oxidation of alanine, then from Table IV, the NH_3 equivalent to the oxidation of alanine is $11.43/3 = 3.81$ ml. *N*/10. Since Stickland [1935, 2] has shown that 1 mol. alanine gives 1 mol. NH_3 and 1 mol. acetic acid, the volatile acid to be expected should therefore be equal to the NH_3 , i.e. 3.81 ml. *N*/10; 3.63 ml. *N*/10 or 95% of that expected was found. It is unlikely that ornithine gives any volatile acid on reduction; if this were so a much higher figure than that obtained would be expected as ornithine is in excess. So that in spite of the blank formation of NH_3 when ornithine is incubated alone with the organism, 2 mol. ornithine were sufficient to bring about the formation of theoretical quantities of both NH_3 and volatile acid from 1 mol. alanine. The reaction between alanine and ornithine undoubtedly takes place preferentially to the breakdown of ornithine alone. The experiments of Table IV also provide strong evidence in favour of the hypothesis concerning the mode of reduction of ornithine, for the results predicted from it are exactly obtained.

One further point in connexion with the ornithine blank is of interest. The question arises as to whether this breakdown is due (a) to the decomposition of ornithine alone by *Cl. sporogenes*, or (b) to linked reactions with donators derived from the autolysis of the suspensions. In Table IV there was no formation of volatile acid from ornithine alone in the presence of the suspension, and as the chief donators are known to produce volatile acid on oxidation [Stickland, 1935, 2; Author, unpublished] (b) is unlikely. This view was confirmed by experiments in which four different concentrations of ornithine were incubated with the same suspension. Since the donators available from autolysis must be strictly limited

in amount it would be expected that increase in ornithine concentration would not increase the ornithine blank if (b) is correct. The following table shows however that it does increase:

Ornithine	NH ₃ formed as ml. N/100
0.3 ml. <i>M</i> /10	1.88
0.4 ml. <i>M</i> /10	2.35
0.6 ml. <i>M</i> /10	2.95
0.8 ml. <i>M</i> /10	3.45

The evidence is therefore in favour of the partial breakdown of ornithine by a method distinct from linked oxido-reduction reactions.

H equivalence of ornithine. With more detailed knowledge concerning the ornithine blank available it was now possible to design experiments to determine the number of equivalents of H accepted by ornithine during its reduction in the presence of H donors. In order to obtain significant results from NH₃ formation from mixtures of alanine with excess ornithine, it is essential that the ornithine blank shall not exceed the excess ornithine above that required by the hypothesis. In the following experiments twice the theoretical amount of ornithine was used and the ornithine blank in no case exceeded the excess. Sets of Thunberg tubes were set up containing (a) water, (b) 0.2 ml. *M*/10 alanine, (c) 0.8 ml. *M*/10 ornithine and (d) 0.2 ml. *M*/10 alanine + 0.8 ml. *M*/10 ornithine; in addition each tube contained 0.5 ml. buffer pH 7, 1 ml. bacterial suspension and water to 2.5 ml. Sets of tubes were removed at intervals until the NH₃ in (d) no longer increased. The final results obtained in three typical experiments are given in Table V. The value (5) was calculated in the following way. Using the

Table V.

Substrate	NH ₃ formed as ml. N/100		
	Exp. 1	Exp. 2	Exp. 3
(1) Alanine	0.26	0	0.21
(2) Ornithine	3.46	1.96	1.94
(3) Ornithine + alanine	8.80	7.48	7.44
(4) Ornithine + alanine minus (1) and (2)	5.08	5.52	5.29
(5) Theoretical NH ₃ corresponding to (4)	5.22	6.00	5.37
(6) % found of theoretical	97	92	98.5

figures of Exp. 3, there were originally present 2 ml. *M*/100 alanine; of this 0.21 ml. has disappeared by blank deamination of alanine (1), leaving 1.79 ml. for interaction with ornithine. Assuming that 1 mol. alanine reacts with 2 mol. ornithine yielding 3 mol. NH₃, then the extra NH₃ formed from alanine + ornithine (4) should be $1.79 \times 3 = 5.37$ ml. N/100. The agreement between observed and calculated values (6) shows that 1 mol. alanine is oxidized by 2 mol. ornithine. The fact that alanine is oxidized completely in these experiments has already been established by the volatile acid determinations (Table IV). Since 1 mol. alanine donates 4H on oxidation each mol. ornithine must therefore accept 2H on reduction.

Further evidence that 2 mol. ornithine are required is provided by experiments in which 1 and 2 mol. ornithine were made to react with 1 mol. alanine. The results given in Table VI were obtained. The ornithine blank has not been deducted since it has been shown above that the ornithine-alanine reaction is the only one to occur under these conditions (no excess of ornithine). The theoretical values are those required if 1 mol. alanine reacts with 2 mol. ornithine. 1 mol. ornithine (1) is insufficient to oxidize 1 mol. alanine; it does in fact oxidize almost exactly $\frac{1}{2}$ mol.

Table VI.

Substrate	NH ₃ formed as ml. N/100		
	Found	Theoretical	% found of theoretical
(1) 0.3 ml. <i>M</i> /10 alanine + 0.3 ml. <i>M</i> /10 ornithine	4.35	4.50	96.6
(2) 0.3 ml. <i>M</i> /10 alanine + 0.6 ml. <i>M</i> /10 ornithine	8.61	9.00	95.7

Throughout the experiments so far described concerning the ornithine-alanine reaction the NH₃ formed has never exceeded that corresponding to deamination of the alanine and one amino-group only of the ornithine. That one amino-group of ornithine is left unchanged was proved by experiments in which disappearance of amino-N was estimated concurrently with the appearance of NH₃. Thunberg tubes were set up as described for the experiments of Table V except that only 0.4 ml. *M*/10 ornithine was used. The residue in the Kjeldahl flask after the distillation of NH₃ was quantitatively removed and 2 ml. 30 % trichloroacetic acid added to precipitate the bacteria. After filtration and washing, the filtrate and washings were amalgamated, neutralized with 30 % KOH, made slightly acid with acetic acid and made up to 25 ml. with water. This solution was used for the estimation (in duplicate) of amino-N by the Van Slyke manometric method, 30 min. shaking being employed. The ornithine blank was ignored since only just sufficient ornithine to oxidize the alanine had been present (see above). Estimations were also done before incubation to obtain the amino-N originally present. The results given in Table VII were obtained after

Table VII.

	Exp. 1	Exp. 2
(1) Ornithine amino-N originally present (mg.)	1.054	1.002
(2) Total amino-N originally present (mg.)	1.317	1.282
(3) Total amino-N finally present (mg.)	0.540	0.480
(4) Loss of amino-N (mg.)	0.777	0.802
(5) Gain in ammonia-N (mg.)	0.740	0.792
(6) % final amino-N of (1)	51	48
(7) % completion of reaction (from NH ₃ estimations)	88	94

incubation until the NH₃ determinations showed that the reaction was approximately complete; the water blank has been deducted in each case. These results show (6) that 50 % of the amino-N of ornithine remains unchanged at the end of the reaction, although alanine is completely deaminated. The fact that the amino-N disappearing closely approximates to the ammonia-N appearing, (4) and (5), provides confirmation of the accuracy of the experiments.

Isolation of δ-aminovaleric acid. Evidence has been presented that ornithine takes up 2H and loses one amino-group on reduction. The question as to whether it is the α- or the δ-amino-group which is deaminated could only be finally settled by isolation of the product of the reduction. A large Thunberg tube was put up containing 4 ml. *M*/10 alanine, 8 ml. *M*/10 ornithine, 8 ml. *M*/5 buffer pH 7 and 8 ml. bacterial suspension (twice normal strength). The course of the reaction was followed by NH₃ determinations on a series of small tubes. After 29 hours these indicated that the reaction was 93 % complete. The large tube was then taken down and made strongly acid with HCl. It was then possible to isolate δ-aminovaleric acid as the α-naphthylisocyanate derivative by the procedure given by Stickland [1935, 1]. Since less product was to be expected

only 0.2 g. α -naphthylisocyanate was used. The dinaphthylurea formed as by-product was not completely precipitated in 1 hour so the mixture was allowed to stand overnight at this stage. The crude yield of α -naphthylcarbamido-*n*-valeric acid was 93 mg., equivalent to 47 % of the ornithine used.

The crude product was recrystallized three times from 50 % alcohol. The substance then melted with decomposition at 192° (uncorr.) and the solidified decomposition product melted at 231°. The corresponding figures obtained by Stickland [1935, 1] were 191 and 231° respectively, whilst Keil & Gunther [1933] record 197 and 231°. The substance gave the following analytical data (Weiler):

	Calculated for α -naphthylcarbamido- <i>n</i> -valeric acid	Found
C%	67.13	67.10
H%	6.29	6.47
N%	9.79	10.08

It may be concluded that when ornithine is reduced in these reactions it is the α -amino-group which is deaminated. All the experimental evidence obtained agrees with the original hypothesis that when ornithine reacts with H-donating amino-acids in the presence of *Cl. sporogenes* it undergoes reductive deamination at the α -amino-group with formation of δ -aminovaleric acid:

Stereochemical specificity of ornithine. All the experiments so far described have been done with *d*-ornithine. In order to test the optical specificity of ornithine in these linked reactions, the activity of synthetic *dl*-ornithine¹ as acceptor was compared with that of the *d*-form. The NH_3 formation, in the presence of the organism, from 1 mol. alanine and 2 mol. of either *d*- or *dl*-ornithine was estimated after various times together with the usual controls. Table VIII shows that *dl*-ornithine gives precisely similar quantitative results to

Table VIII.

Time	...	...	NH ₃ formed as ml. N/100			
			5 hours		18 hours	
Form of ornithine	...	...	<i>d</i> -	<i>dl</i> -	<i>d</i> -	<i>dl</i> -
Substrate						
Ornithine alone			1.64	1.55	2.56	2.59
Ornithine + alanine			4.42	4.32	5.78	5.80

Quantities used: ornithine, 0.4 ml. *M*/10, alanine 0.2 ml. *M*/10.

d-ornithine, and that 2 mol. *dl*-ornithine are able to oxidize completely 1 mol. alanine (theoretical NH_3 , 6 ml. *N*/100; found, 5.80 ml.). It is obvious therefore that *l*-ornithine as well as *d*-ornithine must act as H acceptor in the "Stickland reaction". Table IX shows that the same lack of optical specificity is true also of the acceptor proline, for *dl*-proline gives similar quantitative results to the natural *l*-form, and 2 mol. *dl*-proline (as with *l*-proline) are sufficient to oxidize completely 1 mol. alanine (theoretical NH_3 , 5 ml. *N*/100; found, 4.76).

Since glycine is optically inactive, it is interesting to note that *Cl. sporogenes* shows no stereochemical specificity toward the three amino-acids, glycine, proline and ornithine, which it activates as H acceptors. With the H donors on the other hand Stickland [1934] has found the unnatural *l*-alanine to be inactive.

¹ I am indebted to Dr H. A. Krebs for the gift of a sample of synthetic *dl*-ornithine.

Table IX.

		NH ₃ formed as ml. N/100			
Time		3 hours		6 hours	
Form of proline	...	<i>l</i> -	<i>dl</i> -	<i>l</i> -	<i>dl</i> -
Substrate					
Proline alone		0	0	0	0
Proline + alanine		3.62	3.62	4.81	4.76

Quantities used: alanine, 0.5 ml. *M*/10; proline, 0.5 ml. *M*/5.

Experiments with *d*-arginine.

In view of the positive results obtained with ornithine it was decided to reinvestigate the action of *Cl. sporogenes* on arginine, which like ornithine is a δ -substituted derivative of α -aminovaleic acid. Stickland [1934] has found that arginine was not attacked, but Bessey & King [1934] reported that this amino-acid was completely deaminated by washed cells of *Cl. sporogenes*. The action of washed suspensions of the organism on arginine was tested in the usual way by estimating NH₃ formed from the amino-acid alone and in admixture with alanine or proline. The results of two typical experiments are shown in Table X. The

Table X.

Substrate	NH ₃ formed as ml. N/100	
	Exp. 1	Exp. 2
(1) Arginine	14.19	13.01
(2) Alanine	0.27	0.16
(3) Proline	0	—
(4) Arginine + proline	13.94	—
(5) Arginine + proline minus (1) and (3)	0	—
(6) Arginine + alanine	17.42	18.09
(7) Arginine + alanine minus (1) and (2)	2.96	4.92
(8) Arginine + alanine (no bacterial suspension)	0	0

Quantities used: 0.5 ml. *M*/10 solution of each substrate.

source of arginine in Exp. 1 was *d*-arginine hydrochloride, and in Exp. 2 the free base; both solutions were neutralized before use. It will be seen that arginine is considerably deaminated when incubated alone with the bacterial suspension (1). The NH₃ formed approaches 3 mol. per mol. arginine; this would require the formation of 15 ml. *N*/100 NH₃. It seems probable that three of the four amino- and imino-groups of arginine are deaminated. But in addition to this breakdown alone, extra NH₃ beyond the controls was found in the presence of alanine (7) proving that arginine also acts as H acceptor. There was no extra NH₃ in the presence of proline (5) showing that arginine does not act as H donor.

The activation of arginine as H acceptor by *Cl. sporogenes* was confirmed by experiments on the reoxidation of benzylviologen (as described for ornithine):

Substrate	Reoxidation time
Water	Not reoxidized
<i>d</i> -Arginine	3 min.
<i>l</i> -Proline	3 min.

Arginine is therefore able to accept H from reduced benzylviologen. Methylene blue reduction experiments confirmed that arginine has no activity as H donor.

At first sight it seemed possible that arginine might be broken down to ornithine and urea by an arginase enzyme and that the ornithine liberated then

acted in the usual way as H acceptor. But the amount of NH_3 actually produced from arginine could only agree with such a theory if the urea formed were also deaminated by the bacterium. Preliminary experiments have shown that this is not the case, for urea was not attacked either alone or in admixture with donors or acceptors. Further work on the arginine problem is in progress.

DISCUSSION.

Fildes & Richardson [1935] obtained slight growth of *Cl. sporogenes* on amino-acid media from which all Stickland's acceptors (glycine, proline and hydroxyproline) had been excluded. These workers considered that the energy for growth in this case might have been derived from serine or tyrosine. In the present paper it has been shown that two other H acceptors, ornithine and arginine, exist in addition to those named by Stickland [1934]. As the medium of Fildes & Richardson contained arginine it seems possible that linked reactions between donors and this acceptor might provide the energy for the slight growth mentioned above.

If the action of *Cl. sporogenes* on amino-acids, as far as is at present known, is considered from the point of view of the relation between the structure of the amino-acids and the method by which they are attacked by the organism, several interesting points emerge. With regard to the method of attack the amino-acids may be divided into two main groups:

(1) Amino-acids which are activated as H donors. This group may be further divided into two sections:

(a) Those amino-acids such as alanine, leucine, valine etc. which are attacked only in the presence of an H acceptor. These acids are characteristic simple α -monoamino-acids.

(b) Amino-acids which, in addition to acting as H donors, can be partly deaminated in the absence of H acceptors. The two examples are cysteine and serine; these are characterized by the presence of a second substituent group which is not an amino-group in the molecule.

(2) Amino-acids which are activated as H acceptors. This group may also be divided into two sections:

(a) Amino-acids which act only as H acceptors and are not attacked in the absence of an H donor. The two examples are proline and hydroxyproline; these are not true amino-acids but imino-acids having ring closure through the amino-group.

(b) Amino-acids acting as H acceptors but also partially deaminated in the absence of H donors. Such acids are glycine, ornithine and arginine. Glycine is an abnormal amino-acid as being the first member of the homologous series; it is for example optically inactive. Arginine and ornithine are characterized by the presence of further amino-groups in the molecule in addition to the α -group.

From this list a few general conclusions may be drawn. Simple α -monoamino-acids, with the exception of glycine which behaves abnormally, appear to act only as H donors. When there is another substituent group in the chain in addition to the α -amino-group then the amino-acids behave abnormally and are broken down by *Cl. sporogenes* in the absence of any other amino-acid. If this extra group is not an amino-group, as in cysteine and serine, the substance also acts as H donor; on the other hand if the extra group is an amino-group or contains an amino-group, as with ornithine and arginine, then the substance also acts as H acceptor. It should be emphasized that these considerations apply only to amino-acids which are known to be attacked in one way or other by *Cl. sporogenes*. The structure of lysine, for example, would place it in group 2 b; however,

lysine is not attacked by the organism in any way [Stickland, 1934]. All that can be said at the present time is that there does seem to be some relationship between the structure of the amino-acids and their mode of activation by *Cl. sporogenes*. It is hoped that such considerations may eventually lead to a clearer understanding of the mechanism of these linked oxidation-reduction reactions.

SUMMARY.

The work of Stickland on coupled reactions between pairs of amino-acids induced by *Cl. sporogenes* has been continued. The following new facts emerged:

1. *l*-Cysteine acts as H donator in these reactions; in addition it is partially deaminated in the absence of other amino-acids.

2. *d*-Arginine and *d*-ornithine are both activated as H acceptors, but in addition are partially deaminated in the absence of H donators. When ornithine reacts with the donator alanine it accepts 2H and undergoes reductive deamination to δ -aminovaleric acid.

3. Glycollic acid, β -alanine and taurine are not attacked by the organism.

The relation between the structure of the amino-acids and their mode of activation by *Cl. sporogenes* is discussed.

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