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

III. THE OXIDATION OF ALANINE BY *CL. SPOROGENES*.

IV. THE REDUCTION OF GLYCINE BY *CL. SPOROGENES*.

By LEONARD HUBERT STICKLAND[1].

From the Biochemical Laboratory, Cambridge.

(*Received February 16th, 1935.*)

In the first paper of this series [Stickland, 1934] it was shown that washed suspensions of *Cl. sporogenes* can catalyse reactions between various pairs of amino-acids, one amino-acid being oxidised and the other reduced. In the second paper [Stickland, 1935] the reduction of proline was shown to occur by rupture of the ring, giving δ-aminovaleric acid. The present work continues the study of the chemistry of these reactions and deals with the oxidation of alanine and the reduction of glycine.

III. THE OXIDATION OF ALANINE BY *CL. SPOROGENES*.

Experimental.

Preparation of bacterial suspensions.

The culture was the same as that previously used (*Cl. sporogenes* Bellette), and the washed suspensions of bacteria were prepared in the way described in the earlier papers.

The oxidation of alanine.

(*a*) *The degree of oxidation of alanine.* In the first paper it was proved that when alanine is oxidised by cresyl blue two molecules of the dye are reduced for each molecule of alanine oxidised. This result has now been confirmed, using the natural substrate glycine in place of the artificial dye. A series of Thunberg tubes was set up each containing a known amount of *d*-alanine (0·5 ml. of *M*/20 solution), excess of glycine (0·5 ml. of *M*/5 or 0·5 ml. of *M*/2·5 solution), 0·5 ml. of phosphate buffer *M*/2 p_H 7·5 and 1·0 ml. of suspension of bacteria. A control series of tubes contained the same solutions with 0·5 ml. of water replacing the alanine solution. After evacuation and incubation at 40°, pairs of tubes were taken and the ammonia was estimated in 2 ml. of their contents, estimations being carried out from time to time until no further increase in the ammonia (corrected for the blank) was found. Since only the final value was required, samples were usually not taken until after 24 hours, when the reaction was practically complete. The result of one experiment is given in Table I. The ammonia production approaches a limit at 0·74 ml. of *M*/10 per tube. Of this 0·25 ml. is derived from the oxidation of the alanine, so that 0·49 ml. can be attributed to reduction of glycine. Hence 2·0 molecules of glycine are reduced for each molecule of alanine oxidised; two other similar experiments gave values

[1] Beit Memorial Fellow.

Table I.

Time of incubation hours	Ammonia found per tube (corrected for control) ml. $M/10$
22	0·66
34	0·71
47	0·74

of 1·8 and 1·8. Making the assumption, which will be justified experimentally later on, that the reduction of glycine involves the taking up of two equivalents of hydrogen, this result agrees with that obtained by the oxidation of alanine with cresyl blue.

In connection with these experiments it must be mentioned that when thick suspensions of bacteria are used, and the experimental period is rather long, a significant amount of ammonia is produced in tubes containing bacteria and glycine alone, in excess of that found in the blank. This must be attributed to the reduction of a small amount of glycine by the "reducing blank" of the bacteria. This point was already apparent even in the shorter experiments in the first paper of this series (see some results in Table V of that paper [Stickland, 1934, p. 1754]).

(*b*) *The products of oxidation of alanine.* The product of reduction of proline has already been identified as δ-aminovaleric acid [Stickland, 1935], so that from an examination of the products of the reaction between one molecule of alanine and two molecules of proline the oxidation products of alanine can be found. Preliminary experiments showed that ammonia, carbon dioxide and a volatile acid were among the compounds formed.

For estimation of the volatile acid, 10 ml. of $M/10$ *d*-alanine solution, 10 ml. of $M/5$ proline, 10 ml. of $M/2$ phosphate buffer p_H 7·5 and 20 ml. of suspension of *Cl. sporogenes* were placed in a large (75 ml.) Thunberg tube, which was evacuated and incubated at 40°. A control tube with 20 ml. of water in place of the two amino-acid solutions was treated similarly. For carbon dioxide estimation, a 50 ml. bottle containing 2·5 ml. of $M/10$ alanine, 2·5 ml. $M/5$ proline, 2·5 ml. buffer and 5·0 ml. bacterial suspension was connected in the incubator with a train of three small gas wash-bottles, each containing 5 ml. of $N/10$ baryta and rendered anaerobic by the passage of a stream of oxygen-free nitrogen. A parallel control with neither amino-acid was set up at the same time. A Kjeldahl splash trap was inserted between the experimental bottles and the baryta wash-bottles and the rubber connections were made as short as possible and coated with paraffin wax. The course of the reaction was followed by preparing two sets of small Thunberg tubes containing the solutions in the same proportions as above in a total volume of 2·5 ml. (0·5 ml. $M/10$ alanine, 0·5 ml. $M/5$ proline, 0·5 ml. buffer and 1·0 ml. bacteria, and the corresponding controls) in which the ammonia was estimated from time to time until the whole of the alanine was found to be oxidised.

The reaction was usually complete in from 12 to 24 hours, after which the estimations were carried out.

For the carbon dioxide estimations, the current of nitrogen was increased and allowed to run for 2 hours or more. This was found by trial to be sufficient to remove the whole of the carbon dioxide from the solution even at p_H 7·5. The baryta was then titrated with $N/10$ hydrochloric acid to phenolphthalein.

For volatile acid estimations, 40 ml. of the contents of each large Thunberg tube were freed from chloride by adding them to a mixture of 10 ml. of N sul-

phuric acid and 40 ml. of saturated silver sulphate solution. Volatile acid was estimated in duplicate on 40 ml. portions of the filtrates by distilling *in vacuo* into excess of standard baryta. The manipulations of the acid solutions sufficed to remove the carbon dioxide without further treatment, as was shown by the fact that the baryta in the receiver remained free from barium carbonate.

Ammonia was estimated in duplicate in 2 ml. portions of the residues from both the carbon dioxide and the volatile acid experiments; these two values were always identical, within experimental error.

The complete results of two experiments are given in Table II. Since the product of reduction of proline (δ-aminovaleric acid) is not volatile, all these products must have arisen from the oxidation of alanine, and the figures though

Table II. *Products of the reaction between 10 ml.* M/*10* d-*alanine and 10 ml.* M/*5 proline.*

Expressed as ml. *M*/10.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	9·5	0·5	9·0	9·8	0·4	9·4
Volatile acid	9·4	1·1	8·3	9·7	1·2	8·5
Carbon dioxide	10·4	0·8	9·6	8·1	0·3	7·8

not exact show that by far the greater part of the alanine has been oxidised to one molecule of ammonia, one of a volatile acid and one of carbon dioxide from each molecule of alanine oxidised. (The possibility that the acid titration represents half a molecule of a dibasic acid need not be considered, as all the simpler volatile acids are monobasic.)

(c) *The identity of the volatile acid.* (1) *By the* p-*toluidide.* The identification of a volatile acid is best effected by the formation of the *p*-toluidide. The yield of this derivative by the standard method is however very small, and it has proved impossible to obtain enough of the acid formed by oxidation of alanine by proline to identify it in this way.

(2) *By the lanthanum-iodine reaction.* Krüger and Tschirch [1929; 1930] described a test for acetate depending on the formation of a solution of basic lanthanum acetate, which gives a blue colour with iodine similar to that obtained with starch. The test is specific for acetate and propionate, but is modified or inhibited by many other anions. It is given strongly by the neutralised distillates from the oxidation products of alanine. When the test is carried out at room temperature, it is found that the depth of colour is by no means proportional to the concentration of acetate present but falls off very sharply between acetate concentrations of *M*/100 and *M*/500. (The original authors claim that by gradually heating the mixed solutions a positive reaction may be obtained down to *M*/6000 acetate, but the present author has not been able to detect less than *M*/1000 under any conditions.) The blue colour cannot therefore be used in the ordinary way for colorimetric estimation of acetic acid, but the sharp decrease in intensity between *M*/100 and *M*/500 may be used as the basis of a method of approximate estimation, in the following manner.

The acetic acid to be estimated is distilled over into excess of alkali, to free it from sulphate and phosphate which inhibit the reaction. The distillate is neutralised, evaporated if necessary and made up to such a volume that it contains roughly *M*/50 acetate (the correct dilution must be determined by trial). Into a series of test-tubes are measured volumes of this solution from 1·0 ml. down to 0·1 ml., and each is made up to 1 ml. Into another series of

test-tubes are measured similar volumes of a standard $M/50$ solution of sodium acetate. To each of these tubes are now added 0·5 ml. of 5 % lanthanum nitrate solution, 0·5 ml. of $N/100$ alcoholic solution of iodine and three or four drops of N ammonia. From a comparison of the blue colours developed in the two series of tubes it is possible to deduce with some accuracy (probably ±10 %) the concentration of acetate in the unknown solution.

This method was applied to the volatile acid distillates obtained from experiments on the oxidation of alanine by proline. The distillates after titration to neutrality were evaporated and made up as accurately as possible in a measuring cylinder to a volume corresponding to that which they had occupied in the original experimental tube (*viz.* 17·7 ml.). If the whole of the volatile acid were acetic acid, the concentration of acetate in this solution should now be $M/50$. The lanthanum-iodine test was carried out on a series of dilutions of this solution and simultaneously on a similar series of dilutions of a $M/50$ solution of sodium acetate. The details and results of two experiments are given in Table III. These figures show that, within the rather wide limits of experi-

Table III.

Volume of standard or unknown ml.	Blue colour			
	Standard acetate	Alanine + 2 proline exp.	Alanine + 2 proline exp.	Control exp.
1·0	+ + + + + +	+ + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + +	+ + + + +	–
0·4	+ + + +	+ + +	+ + + +	–
0·3	+ + +	+ +	+ +	–
0·2	+ +	(+)	+	–
0·1	–	–	–	–
Estimated concentration of acetate		0·015 M	0·018 M	Nil

mental error, the whole of the volatile acid is acetic acid, since propionic acid, which also gives the test, is obviously impossible. The equation representing the oxidation of alanine can therefore be written

$$CH_3.CHNH_2.COOH - 4H + 2H_2O \rightarrow CH_3.COOH + NH_3 + CO_2.$$

(*d*) *Intermediate products in the oxidation of alanine to acetic acid.* Though it is possible that this oxidation proceeds in a single step of four equivalents, it is far more likely to involve two steps of two equivalents each. The most probable course appears to be

(1) $CH_3.CHNH_2.COOH - 2H + H_2O \rightarrow CH_3.CO.COOH + NH_3$,
(2) $CH_3.CO.COOH - 2H + H_2O \rightarrow CH_3.COOH + CO_2$,

pyruvic acid being an intermediate compound. Experiments already quoted [Stickland, 1934] showed that pyruvic acid could not be detected at any stage of the reaction, whether the oxidant were cresyl blue, proline or glycine, within the limits of sensitivity of the nitroprusside test. Therefore if pyruvic acid is indeed the intermediate compound it must be oxidised as quickly as it is formed so that it does not accumulate in the solution. Direct proof of the occurrence of pyruvic acid has not been obtained, but evidence supporting this view has been obtained by proof of the following points:

(1) The rate of oxidation of the intermediate product is greater than that of alanine.

(2) The rate of oxidation of pyruvic acid is greater than that of alanine.

(3) The extent of oxidation of pyruvic acid is in agreement with equation (2) above.

(4) The products of oxidation of pyruvic acid are in agreement with equation (2).

(1) *The rate of oxidation of the intermediate compound.* If we consider the reaction between one molecule of alanine and one of proline, it is clear that (*a*) if the intermediate compound is oxidised at a negligible speed, then the whole of the alanine will be oxidised to this compound, and all its nitrogen will be liberated as ammonia; (*b*) if the intermediate product is oxidised very rapidly, then the proline will suffice for the complete oxidation of only one-half of the alanine, and only 50 % of the nitrogen will appear as ammonia. Thus the quantity of ammonia liberated in this reaction serves to indicate the relative velocities of oxidation of alanine and the hypothetical intermediate compound. To test this experimentally, three sets of Thunberg tubes were prepared containing (*a*) 0·5 ml. *M*/10 alanine, 0·5 ml. *M*/10 proline, 0·5 ml. phosphate buffer p_H 7·5 and 1·0 ml. suspension of *Cl. sporogenes*, (*b*) 0·5 ml. *M*/10 alanine, 0·5 ml. *M*/5 proline, 0·5 ml. buffer and 1·0 ml. suspension and (*c*) 1·0 ml. water, 0·5 ml. buffer and 1·0 ml. suspension. After anaerobic incubation at 40° for various periods, the ammonia was estimated in 2 ml. of the contents of one tube from each series, and these estimations were continued until the ammonia produced, corrected for the blank, remained constant. A typical result is shown in Fig. 1.

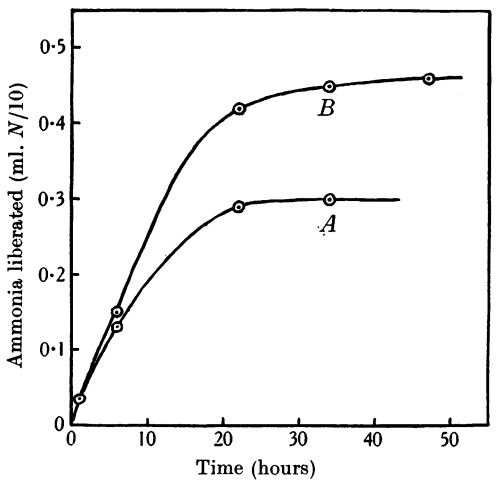

Fig. 1. The course of production of ammonia from: *A*. 0·5 ml. *M*/10 alanine + 0·5 ml. *M*/10 proline; *B*. 0·5 ml. *M*/10 alanine + 0·5 ml. *M*/5 proline.

In this case, while the reaction of alanine with two molecules of proline liberated as usual practically the whole of nitrogen of the alanine as ammonia, the reaction with only one molecule of proline gave only slightly more than half (60 %). Other similar experiments gave values of 58, 57, 68, 57, 66 and 60 % (average 61 %). It can therefore be concluded that if an intermediate compound is formed in the oxidation of alanine it is oxidised more rapidly than alanine itself.

(2) *The rate of oxidation of pyruvic acid.* In the first paper [Stickland, 1934] it was shown that, when cresyl blue is the oxidant, pyruvic acid is oxidised with a velocity about 40 % of that of the oxidation of alanine. Comparison of the rates of oxidation of alanine and pyruvic acid by glycine, however, showed that

in this case the latter is oxidised more rapidly than the former (Table IV). The rates were measured as usual by estimation of the rate of liberation of ammonia from the glycine; allowance is made for the fact that in the alanine oxidation ammonia is formed from both reactants, in the pyruvate from only one.

Table IV.

0·5 ml. $M/10$ glycine +0·5 ml. $M/10$	Ammonia produced in 5 hrs. (ml. $M/10$)	Rate of oxidation (ml. $M/10$ per hr.)	Rate of oxidation of pyruvate / Rate of oxidation of alanine
Alanine	0·32	0·021	2·1
Pyruvate	0·22	0·044	
Alanine	0·32	0·021	2·3
Pyruvate	0·24	0·048	

(3) *The extent of oxidation of pyruvate.* The extent of oxidation of pyruvic acid was measured by incubating a known amount of pyruvate with excess of glycine and determining the amount of ammonia liberated by reduction of the glycine, exactly as in the case of alanine.

Commercial pyruvic acid was distilled *in vacuo*, the fraction boiling at about 66°/15 mm. being collected. This was diluted with ice-cold water to give a roughly 10 % solution and stored in the ice-chest. Titration with alkali and titration of bisulphite-binding capacity agreed exactly, showing the product to be pure. For use in experiments portions of this solution were carefully neutralised immediately before use and diluted to $M/10$.

To measure the degree of oxidation of pyruvic acid, 0·5 ml. $M/10$ or $M/20$ sodium pyruvate solution, 0·5 ml. $M/2{\cdot}5$ or $M/5$ glycine, 0·5 ml. buffer of p_H 7·5 and 1·0 ml. of suspension of bacteria were placed in each of five Thunberg tubes, and in another five tubes the same solutions with 0·5 ml. of water replacing the pyruvate solution. Estimations of ammonia in experimental tubes and controls were carried out from time to time until no further increase was observed. The corrected value for the final ammonia produced showed that for each molecule of pyruvate oxidised one molecule of glycine was reduced (see Table V). This is the degree of oxidation required for the oxidation of pyruvate to acetate as in equation (2) on p. 892.

Table V. *Extent of oxidation of pyruvate.*

Pyruvate	Glycine	NH_3 corrected for blank (ml. $M/10$)	Mols. glycine reduced / Mols. pyruvate oxidised
0·5 ml. $M/10$	0·5 ml. $M/5$	0·39	0·8
0·5 ml. $M/20$	0·5 ml. $M/5$	0·21	0·8
0·5 ml. $M/20$	0·5 ml. $M/2{\cdot}5$	0·23	0·9

(4) *The products of oxidation of pyruvate.* The products of oxidation of pyruvic acid were determined exactly as in the case of alanine, by means of the reaction with proline. For estimation of volatile acid, two 75 ml. Thunberg tubes were prepared containing (*a*) 10 ml. $M/10$ sodium pyruvate, 10 ml. $M/10$ proline, 10 ml. $M/2$ buffer p_H 7·5 and 20 ml. suspension of bacteria and (*b*) 20 ml. water, 10 ml. buffer and 20 ml. suspension. These were evacuated and incubated at 40°. For carbon dioxide estimation two 50 ml. bottles, containing one-quarter of the above quantities of the solutions, were connected in the incubator to a series of three small wash-bottles each containing 5 ml. of $N/10$ baryta. These were rendered anaerobic by the passage of a current of oxygen-free nitrogen, with the precautions mentioned in the alanine experiment. The reaction could

obviously not be followed by estimation of the ammonia produced, so instead a series of small Thunberg tubes was set up with the same solutions in a total volume of 2·5 ml. (no controls were necessary), and the disappearance of the pyruvic acid followed by means of the nitroprusside test (2 % of the initial concentration gave a positive test, so disappearance of the test indicated that more than 98 % of the pyruvate had reacted). When the reaction was finished, the estimations of ammonia, volatile acid and carbon dioxide were carried out as before. The results of two such experiments are given in Table VI and are sufficient to show that from each molecule of pyruvic acid there arise one molecule of volatile acid and one of carbon dioxide.

Table VI. *Products of the reaction between 10 ml.* M/*10 Na pyruvate and 10 ml.* M/*10 proline.*

Results expressed as ml. *M*/10.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	0·5	0·6	0·0	0·6	0·5	0·1
Volatile acid	13·0	2·8	10·2	11·7	2·7	9·0
Carbon dioxide	8·2	0·3	7·9	10·7	0·7	10·0

The volatile acid was identified and roughly estimated by means of the reaction of Krüger and Tschirch [1929], in exactly the same manner as that arising from alanine. The result is given in Table VII, and the figures show that

Table VII.

Volume of standard or unknown ml.	Blue colour		
	Standard acetate	Pyruvate + proline exp.	Control exp.
1·0	+ + + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + +	–
0·4	+ + + +	+ + +	–
0·3	+ + +	+ +	–
0·2	+ +	+	–
0·1	–	–	–
Estimated concentration of acetate		0·018 *M*	Nil

within the experimental error all the volatile acid is acetic acid (propionic acid may again obviously be excluded from consideration). This confirms the equation representing the oxidation of pyruvic acid given on p. 892.

The reduction of methylviologen by alanine.

In the first of these papers it was pointed out that the calculations of Wurmser and Mayer-Reich [1933] indicated that an alanine dehydrogenase system whose end-product was ammonium pyruvate should have a potential in the neighbourhood of r_H 12, while the alanine dehydrogenase of *Cl. sporogenes* actually reduced dyes down to about r_H 2 (methylviologen at p_H 8·0). This discrepancy can now be explained.

A concentration of *M*/50 *d*-alanine at p_H 7·5 gives about 10 % reduction of methylviologen. It was found on further investigation that sodium pyruvate under exactly the same conditions gave a much greater reduction, *viz.* about 70 %. The probable explanation of the reduction in the presence of alanine is that as soon as a trace of oxidation to pyruvate takes place, this product is further oxidised to acetate, and it is this second reaction which produces the

measurable amount of reduction of the indicator. The final equilibrium reached, starting from alanine and methyl viologen, is a very complex one consisting of alanine-NH_4-pyruvate-acetate-bicarbonate-methylviologen-leucomethylviologen, and the quantitative relationships in a case like this have not yet been worked out, but it is evident that a qualitative explanation of the discrepancy mentioned above has been provided.

Discussion.

The end-products of the oxidation of alanine by proline in presence of the enzymes of *Cl. sporogenes* are clearly ammonia, acetic acid and carbon dioxide. Attempts to show directly that pyruvic acid occurs as an intermediate stage in the oxidation have failed; for instance, the addition of sodium arsenite, used successfully by Krebs [1933] for this purpose in the case of mammalian tissue slices, completely inhibited the whole reaction. The indirect evidence obtained, however, is all in favour of pyruvic acid as an intermediate product, and it may be considered as extremely probable that the reaction occurs in the stages given in the equations on p. 892.

IV. THE REDUCTION OF GLYCINE BY *CL. SPOROGENES*.

Experimental.

Estimation of the products of reduction of glycine.

As the products of oxidation of alanine have already been shown to be acetic acid, carbon dioxide and ammonia, the determination of the products of the reaction between alanine and glycine would suffice to decide the fate of the glycine. Experiments were therefore carried out in which one molecule of alanine was oxidised by two molecules of glycine. The experiments were set up as before in three sections: (*a*) for volatile acid estimations, a 75 ml. Thunberg tube containing 10 ml. *M*/10 *d*-alanine, 10 ml. *M*/5 glycine, 10 ml. *M*/2 phosphate buffer p_H 7·5 and 20 ml. bacterial suspension, (*b*) for carbon dioxide estimation, a 50 ml. bottle containing 2·5 ml. *M*/10 alanine, 2·5 ml. *M*/5 glycine, 2·5 ml. buffer and 5 ml. suspension, and (*c*) for following the course of the reaction, a set of five 10 ml. Thunberg tubes each containing 0·5 ml. alanine, 0·5 ml. glycine, 0·5 ml. buffer and 1·0 ml. suspension. In each case control experiments were also prepared, differing by the omission of both amino-acids. The Thunberg tubes in (*a*) and (*c*) were evacuated in the usual way and incubated at 40°. The bottles in (*b*) were connected in the incubator at 40° to a series of three wash-bottles, each containing 5 ml. of *N*/10 barium hydroxide and rendered anaerobic by the passage of a stream of pure nitrogen, with the precautions mentioned in the previous paper. The reaction was followed by estimation of the ammonia in 2 ml. of the contents of the small Thunberg tubes (*c*), and incubation was continued until the quantity of ammonia liberated, corrected for the control, approached the theoretical for the complete reaction. In most experiments the reaction started immediately, was almost complete (about 90 %) in 24 hours and had reached 97–98 % of the theoretical value in 48 hours. Volatile acid was estimated in the contents of the large Thunberg tubes, after removal of the chloride with silver sulphate, by distillation *in vacuo* into standard barium hydroxide, and carbon dioxide by titration of the barium hydroxide in the wash-bottles, as described in the first part of this paper.

Ammonia was estimated in both the volatile acid experiment (*a*) and the carbon dioxide experiment (*b*), always with concordant results. The results of two experiments are given in Table I. The figures show that when one molecule of

Table I. *Products of the reaction between 10 ml.* M/*10* d-*alanine and 10 ml.* M/*5 glycine.*

Results expressed as ml. *M*/10.

	Exp. 1			Exp. 2		
	Exp.	Control	Diff.	Exp.	Control	Diff.
Ammonia	31·8	2·6	29·2	30·6	1·2	29·4
Carbon dioxide	10·1	1·4	8·7	10·5	0·7	9·8
Volatile acid	30·8	1·8	29·0	30·5	3·7	26·8

alanine is oxidised by two molecules of glycine, there arise three molecules of ammonia, one of carbon dioxide and three of volatile acid. Of these products, one molecule of ammonia, one of carbon dioxide and one of volatile acid (acetic) proceed from the oxidation of the alanine, so that two molecules each of ammonia and of a volatile acid must be the products of the reduction of two molecules of glycine.

The identification of the volatile acid.

(*a*) *By the* p-*toluidide.* The acid distillates from one experiment were collected, evaporated to dryness and dried at 110°. The solid was heated with *p*-toluidine and a little strong hydrochloric acid in the usual way, and from the mixture about 25 mg. of a toluidide were separated. After two recrystallisations from hot water, only a few milligrams of a still slightly impure product remained, and with this a melting-point determination was made and a mixed melting-point with some pure acet-*p*-toluidide. The figures obtained were:

	Melting-point
Acet-*p*-toluidide	147°
Product from action of bacteria	145°
Mixture	146°

These are sufficient to indicate that the volatile acid consisted at any rate chiefly of acetic acid.

(*b*) *By the lanthanum-iodine test.* The acid was identified and roughly estimated by the method already described, based on the reaction of Krüger and Tschirch [1929]. Table II contains the results of one such experiment, with

Table II.

ml. of *M*/50 sodium acetate or of unknown solution	Blue colour produced			
	Standard acetate	Alanine + 2 proline	Alanine + 2 glycine	Control
1·0	+ + + + + +	+ + + + + +	+ + + + + +	–
0·5	+ + + + +	+ + + + +	+ + + + + +	–
0·4	+ + + +	+ + + +	+ + + + + +	–
0·3	+ + +	+ +	+ + + + +	–
0·2	+ +	+	+ + + +	–
0·1	–	–	+ +	–
Estimated acetate concentration	(0·02 *M*)	0·018 *M*	0·055 *M*	Nil

the results of an experiment on the oxidation of alanine by proline for comparison. The results show that, within the rather large limits of experimental error, all the volatile acid produced is acetic acid.

Conclusion.

The formation of one molecule of ammonia and one of a volatile acid from each molecule of glycine reduced, and the identification of the acid as acetic acid, show that the reduction of glycine can be expressed by the following equation:

$$CH_2NH_2COOH + 2H \rightarrow CH_3COOH + NH_3.$$

The formation of acetic acid from glycine by the action of mixed cultures of putrefactive bacteria has already been demonstrated by Brasch [1909].

Summary.

When alanine is oxidised by *Cl. sporogenes*, at the expense of the reduction of proline or glycine, it loses four equivalents of hydrogen and yields one molecule each of acetic acid, carbon dioxide and ammonia.

The reaction probably proceeds in two stages of two equivalents each, ammonium pyruvate being the intermediate product.

Washed suspensions of *Cl. sporogenes* reduce glycine, at the expense of the oxidation of alanine, to acetic acid and ammonia.

REFERENCES.

Brasch (1909). *Biochem. Z.* **22**, 403.
Krebs (1933). *Z. physiol. Chem.* **217**, 191.
Krüger and Tschirch (1929). *Ber. deutsch. chem. Ges.* **62**, 2776.
—— —— (1930). *Ber. deutsch. chem. Ges.* **63**, 826.
Stickland (1934). *Biochem. J.* **28**, 1746.
—— (1935). *Biochem. J.* **29**, 288.
Wurmser and Mayer-Reich (1933). *Compt. Rend. Soc. Biol.* **112**, 1648.