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

VII. THE DECOMPOSITION OF PYRUVATE AND *l*-(+)GLUTAMATE BY *CLOSTRIDIUM TETANOMORPHUM*

BY D. D. WOODS[1] AND C. E. CLIFTON

From the Biochemical Laboratory, Cambridge

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IN the preceding paper of this series [Woods & Clifton, 1937], it was shown that washed suspensions of *Cl. tetanomorphum* decompose a number of substances (including seven amino-acids) with evolution of H_2. Carbon dioxide is produced from all substrates attacked by the organism and volatile acid was found whenever tests for this product were made. In the present communication the products of the decomposition of one amino-acid substrate (*l*-(+)glutamate) and one non-amino-acid substrate (pyruvate) will be studied in more detail. *l*-(+)glutamate and pyruvate were chosen since they are attacked more rapidly than any other substrates in their respective groups and are therefore more convenient for large scale work.

GENERAL METHODS

Washed suspensions of *Cl. tetanomorphum* were prepared as before [Woods & Clifton, 1937], precautions being taken to avoid exposing the final suspension to air. H_2, CO_2 and NH_3 were also estimated as described in the previous paper. Total volatile acid was determined by distillation *in vacuo* in the following way. The solution to be tested was made acid with 1 ml. 6*N* phosphoric acid and placed in a Claisen flask connected through a double surface condenser to a Büchner flask acting as receiver. The capillary and a tap funnel in the second neck of the flask were closed with soda-lime guard tubes. The solution and apparatus were freed from CO_2 by drawing through a current of CO_2-free air for 5–10 min. The receiver was rapidly disconnected and replaced after running in an excess of a standard solution of CO_2-free NaOH. The tip of the condenser dipped below the surface of the soda. The test solution was distilled *in vacuo* down to 10 ml. at a temperature not exceeding 40°, 10 ml. water were added to the residue and the distillation continued to the same point. This was repeated with a further 10 ml. water. The contents of the receiver and condenser washings were back-titrated with standard H_2SO_4. With these precautions no trouble is experienced with CO_2 since identical experiments in which baryta replaced the soda in the receiver showed no precipitation of $BaCO_3$. Baryta was not used in the actual determinations since the use of HCl for back-titration had to be avoided as the distillates were often used for Duclaux determinations.

[1] Beit Memorial Research Fellow.

Lactic acid was estimated by the method of Friedemann *et al.* [1927] and formic acid by a method quoted by Woods [1936]. Alcohol was determined by oxidation with acid bichromate and succinic acid by the succinic dehydrogenase method [cf. Elsden, 1937].

All experiments, unless otherwise stated, were carried out in phosphate buffer *p*H 7·2 at 32° in an atmosphere of N_2.

I. Decomposition of pyruvate

Identification and estimation of volatile acids

The Duclaux distillation method [cf. Bertrand & Thomas, 1920] was used to obtain information as to the composition of the volatile acid fraction of the fermentation fluid. Exploratory determinations indicated that only acetic and butyric acids were present so the Duclaux method was carefully calibrated for mixtures of these acids. It is not proposed to give details of the actual procedure, for, in our experience, this method should be recalibrated by each worker with his own apparatus and standard technique. The reader is referred to van Niel [1928] for a general discussion of the method, the precautions to be taken and the method of calculating the tables. We have found the method to give consistent and reliable results. "Homologue-free" acids (Messrs Fraenkel and Landau) were redistilled and the constant boiling fractions used for the calibrations; 10 ml. 0·1 *N* acid was used for each determination. The basal distillation numbers (*d*) obtained for the pure acids are given in Table I. The *d* numbers are obtained

Table I. *Distillation numbers of acetic and butyric acids*

(*d* numbers—% of total acid initially present)

Fraction (ml.)	Acetic acid	Butyric acid
10	6·6	18·3
20	13·2	34·3
30	20·1	48·3
40	27·2	60·5
50	34·9	71·1
60	42·9	79·9
70	51·3	87·0
80	60·5	92·4
90	70·7	96·0
100	83·0	98·4
110	100	100

by expressing the acid distilling over as a percentage of the total acid taken. The figures for acetic acid are in close agreement with those found by van Niel [1928] and Gillespie & Walters [1917]. From these figures the distillation numbers (*d'*) for the pure acids and mixtures given in Table II have been calculated. For convenience in practice these *d'* numbers are expressed as: amount of acid distilling in each fraction × 100/total acid in 100 ml. distillate, and have been calculated in such a way that they refer to the ratios in which the acids are present in the fluid taken for the distillation [v. van Niel, 1928]. In examining an unknown mixture the distillation was carried out in the same apparatus with approximately the same total amount of volatile acid and with the precise experimental procedure used in the calibration.

Table II. *Standard distillation numbers for mixtures of acetic and butyric acids*

(*d'* numbers—% distilling of total distilling in 100 ml.)

Fraction ml.	Ratio acetic acid : butyric acid					
	Pure acetic	4 : 1	3·5 : 1	3 : 1	2·8 : 1	2·6 : 1
30	24·2	29·9	30·5	31·3	31·6	32·0
40	32·8	39·3	40·0	40·9	41·3	41·8
50	42·1	49·0	49·7	50·6	51·1	51·5
60	51·7	58·4	59·2	60·0	60·6	60·9
70	61·8	67·9	68·5	69·3	69·7	70·2
80	72·9	77·7	78·2	78·8	79·2	79·5
	2·5 : 1	2 : 1	1·5 : 1	1·4 : 1	1·3 : 1	1·2 : 1
30	32·2	33·5	35·2	35·6	36·1	36·6
40	42·0	43·5	45·4	45·9	46·5	47·0
50	51·8	53·4	55·4	55·9	56·5	57·1
60	61·2	62·6	64·7	65·2	65·8	66·4
70	70·4	71·7	73·5	74·0	74·5	75·0
80	79·7	80·7	82·2	82·5	82·9	83·4
	1·1 : 1	1 : 1	1 : 1·5	1 : 2	Pure butyric	
30	37·1	37·7	40·1	41·7	49·1	
40	47·7	48·4	51'2	53·0	61·5	
50	57·7	58·4	61·4	63·3	72·3	
60	67·0	67·7	70·6	72·5	81·2	
70	75·6	76·3	78·8	80·5	88·5	
80	83·8	84·4	86·3	87·7	93·9	

Examination of decomposition products by Duclaux method

Large-scale experiments were carried out in Krebs' pots to obtain a sufficient quantity of volatile acid for Duclaux analysis. Each pot contained 20 ml. *ca.* 0·2 *M* pyruvate, 20 ml. 0·2 *M* phosphate buffer *p*H 7·2 and 8 ml. bacterial suspension in phosphate buffer. For each experiment the total crop of organisms from 2 l. tryptic broth was used (about 180 mg. dry weight). The course of the reaction was followed by measuring H_2 production in manometers containing 1/20 of the above quantities; it will be shown later that no pyruvate remains when H_2 evolution ceases. When the experiment was complete (usually in 2·5–3 hr.) the contents of the Krebs' pot were washed into a centrifuge tube and the suspension brought to about *p*H 2 with phosphoric acid. The supernatant liquid after centrifuging was poured off, the bacterial mass washed on the centrifuge with water and the washings united with the previous supernatant (*A*). The total volatile acid present in *A* was estimated as already described. A suitable quantity of volatile acid (see p. 346) for the Duclaux determinations is obtained if the neutralized distillate from the volatile acid estimation is diluted to 250 ml. (after reliberating the volatile acid) and 110 ml. portions taken for duplicate Duclaux estimations. The results obtained in a number of experiments are given in Table III. The ratio of acetic acid to butyric acid is obtained by comparing the *d'* numbers with the standards (Table II). The distillation numbers are in excellent agreement with those to be expected from a simple mixture of acetic and butyric acids and offer no suggestion of the presence of any other acid. Butyric acid is more volatile than acetic acid (Table I); if therefore we are dealing here with a mixture of these two acids it would be expected that there would be an accumulation of the more volatile acid in the first fractions and of the less volatile in the later fractions. The first 30 ml. and the last 40 ml. fractions of the distillate of Exp. 108 were redistilled separately (after reacidifying and making up to 110 ml.). The resulting *d'* numbers (Table IV) show that a partial separation of the two com-

Table III

Exp. no.	*d'* numbers 64	67	68	108	109	111
Fraction of distillate (ml.)						
30	37·2	36·9	36·7	36·8	36·8	37·0
40	47·7	47·5	47·3	47·4	47·5	47·9
50	57·5	57·5	57·1	57·3	57·4	58·0
60	67·5	66·6	66·3	66·5	66·9	67·1
70	75·5	75·3	74·9	75·2	75·6	75·9
80	83·7	83·3	83·1	83·6	83·9	84·1
Ratio acetic acid : butyric acid	1·1 : 1	1·15 : 1	1·2 : 1	1·15 : 1	1·1 : 1	1·05 : 1
Total volatile acid distilled (ml. *N*/10)	10·09	9·54	9·78	9·88	9·97	9·93

Table IV

Fraction of distillate ml.	*d'* numbers First 30 ml.	Last 40 ml.
20	29·1	20·1
40	53·3	40·9
60	72·7	60·6
80	87·5	79·2
Approx. acetic : butyric ratio	1 : 2	3 : 1

ponents has occurred, for, compared with the original solution (Table III), the first 30 ml. fraction contains a higher proportion of the more volatile acid and the last 40 ml. a higher proportion of the less volatile acid. The exact ratio is not accurately determined in these cases as the total acid distilled was much less than that used for the standards. The approximate ratio is of the expected order assuming the original mixture to be of the composition found in Table III.

Besides yielding information as to the components of a mixture of volatile acids, the Duclaux method, if carefully calibrated, will give a reliable estimation of the ratio in which these acids are present in a simply binary mixture. No other method has so far been described for the estimation of both acetic and butyric acids in a mixture of the two. Check estimations on mixtures of the pure acids gave figures in accord with the standards of Table II. The *d'* numbers obtained in Table III indicate that acetic and butyric acids are present in equal proportions. The acetic : butyric ratio is consistently slightly higher than unity but the deviation is within the limits of experimental error. Since butyric acid is more volatile than acetic it is possible that a small loss of the former may have occurred during the manipulations in acid solution. It should also be pointed out that the volatile acid analysed is derived both from the breakdown of pyruvate and, to a much smaller extent, from unknown substrates in the "blank" reaction of the bacterial suspension alone. This "blank" normally amounted to 5–10 % of the volatile acid formed from pyruvate. For technical reasons it was not possible to obtain sufficient material to subject the blank volatile acids to Duclaux analysis; the proportions of the acids may be different from those obtained from pyruvate. From the data at our disposal it seemed justifiable to conclude (*a*) that acetic and butyric acids are the only volatile acids formed in the decomposition of pyruvate, and (*b*) that these acids are formed in equal proportions.

Separation and identification of acetic and butyric acids

The formation of acetic and butyric acids was confirmed by their separation and identification as salts. The separation was effected by the method of Phelps & Palmer [1917] which depends upon the different solubilities of the quinine

salts of the two acids in carbon tetrachloride. Quinine butyrate is relatively soluble (4 %), whilst quinine acetate is almost insoluble (0·05 %). As there is a difference of over 40° in the melting-points these salts can also be used to characterize the acids.

A number of large-scale fermentations of pyruvate were carried out as in the last section. The supernatant fluids after acidifying and centrifuging were combined and the volatile acid distilled off *in vacuo* and collected in NaOH. The neutralized distillate was reacidified to Congo red with H_2SO_4 and distilled. The distillate was collected in an ice-cooled receiver and titrated with standard baryta. The neutral solution of the barium salts was used for the preparation and separation of the quinine salts essentially by the procedure of Phelps & Palmer [1917]. A total of 33·2 ml. 0·1 *N* volatile acid and 1·26 g. quinine sulphate (theoretical 1·24 g.) were used for the separation. The crude quinine acetate and quinine butyrate fractions amounted to 95 and 90 % respectively of the theoretical yields assuming the original acid to be a mixture of equal parts of acetic and butyric acids. The crude materials were purified in the following way.

"*Acetate fraction.*" The acetate fraction was re-extracted for 24 hr. with 20 ml. CCl_4 and filtered. The residue was taken up in 10 ml. boiling dry ethyl acetate, filtered from a small amount of insoluble material and allowed to cool slowly. The salt crystallized in glistening rosettes of needles. After twice recrystallizing from hot ethyl acetate and drying *in vacuo* over KOH, the product gave the following M.P. data (uncorrected):

	Experimental product (*a*)	Pure quinine acetate (*b*)	Mixed (*a*) and (*b*)
Shrinks	117°	116°	116°
Softens	122°	121°	121°
Melts	124–126°	124–126°	124–125°

Phelps & Palmer also give 124–6° as the melting-point of quinine acetate. After drying in a high vacuum over P_2O_5 at 40°, the following analytical figures were obtained [Weiler]:

	Experimental product	Pure quinine acetate	Calculated for $C_{20}H_{24}O_2N_2, C_2H_4O_2$
% C	68·35	69·02	68·76
% H	7·51	7·54	7·29
% N	6·72	6·81	7·29

The pure quinine acetate was made by thrice recrystallizing the B.D.H. product from ethyl acetate.

"*Butyrate fraction.*" The butyrate fraction was again taken up in CCl_4 and filtered from some insoluble matter which deposited on standing. The filtrate was precipitated with excess of light petroleum (B.P. 60–80°). The residue after filtering was taken up in a small quantity of ethyl acetate and crystallization induced by the cautious addition of light petroleum to the warmed solution. The material crystallized in glistening rosettes of needles. After twice recrystallizing as above and drying *in vacuo* over KOH the following M.P. data (uncorrected) were obtained:

	Experimental product (*a*)	Pure quinine butyrate (*b*)	Mixed (*a*) and (*b*)
Shrinks	72°	72°	72°
Softens	76°	76°	76°
Melts	77·5–78°	77·5–78°	77·5–78°

Phelps & Palmer [1917] give 77·5°. The following analytical figures were obtained after drying *in vacuo* over P_2O_5 at 40°:

	Experimental product	Pure quinine butyrate	Calculated for $C_{20}H_{24}N_2O_2$, $C_4H_8O_2$
% C	68·79	69·08	69·90
% H	7·58	7·65	7·77
% N	6·69	6·43	6·80

The pure quinine butyrate was prepared by adding the calculated quantity of quinine to butyric acid, evaporating to dryness *in vacuo* and purifying as above.

Complete analysis of reaction products

The constitution of the volatile acid fraction having been established it was now possible to make a complete quantitative analysis of the decomposition products. For this purpose large-scale experiments were carried out in Krebs' pots filled with N_2. The following quantities were used: 10 ml. *ca.* 0·1 *M* pyruvate, 20 ml. 0·2 *M* phosphate buffer *p*H 7·1 and 10 ml. bacterial suspension in phosphate buffer. A control vessel in which water replaced pyruvate was also set up. At the same time the total H_2 and CO_2 production was measured manometrically (as described in the previous paper) with 0·3 ml. 0·1 *M* pyruvate; the other reagents were in the same proportions as in the Krebs' pots. These manometers also served as an index of the course of the reaction. For each complete experiment the crop of organisms from 1800 ml. broth (about 180 mg. dry weight) was used. The exact strength of the pyruvate was determined by the carboxylase method [Westerkamp, 1933; Krebs, 1937, 1]. When the reaction was complete the contents of the Krebs' pots were washed out and centrifuged to remove the bulk of the organisms and the latter washed on the centrifuge with 10 ml. water. The supernatant and washings were made up to 100 ml. (*B*). Pyruvate was estimated on a part of *B* by the carboxylase method. No unchanged pyruvate was found in any experiment, confirming the view that the cessation of H_2 formation is a true indication that the decomposition is complete. The fermentation products did not inhibit the carboxylase enzyme. Volatile acid was estimated in duplicate on aliquot parts of *B*. The combined residues after distilling off volatile acids were used for succinic acid determination[1] and, after neutralization and copper-lime precipitation, for the estimation of lactic acid. Formate estimations were performed with the evaporated neutralized volatile acid distillates, whilst alcohol was estimated in the original solution *B*. In every case similar estimations were done with the products obtained from the control experiment without pyruvate. The mean results of four complete experiments are given in Table V.

Table V

(All values less appropriate controls)

	Mol. (or equiv.) formed per mol. pyruvate			
	Range	Mean	Required by equation (1)	% mean found of required
H_2	0·23 –0·29	0·28	0·33	85
CO_2	0·82 –0·85	0·84	1·0	84
Volatile acid (equiv.)	0·58 –0·62	0·59	0·66	89
Lactic acid	0·059–0·063	0·06	—	—
Formic acid, Succinic acid, Alcohol	Absent	—	—	—

[1] We are grateful to Mr S. R. Elsden for carrying out the succinic acid estimations.

More information as to the quantitative relationship of the original pyruvate and the various products is obtained if the results of individual experiments are expressed as a balance sheet of the various elements involved. This is done for two typical experiments in Table VI. The assumption is made that the

Table VI

	Actual mg.	mg. C	mg. H	mg. O
Exp. 71:				
(*a*) Original pyruvate	78·2	31·99	3·55	42·66
(*b*) Original pyruvate expressed in terms 3 pyruvate + $1H_2O$	83·52	31·99	4·15	47·39
Products:				
H_2	0·42	—	0·42	—
CO_2	31·96	8·72	—	23·24
Acetic acid	15·63	6·26	1·04	8·34
Butyric acid	22·92	12·50	2·09	8·33
Lactic acid	4·70	1·88	0·32	2·50
Total of products	75·63	29·36	3·87	42·41
% total of (*a*)	96·7	91·8	109	99·4
% total of (*b*)	90·6	91·8	93·3	89·5
Exp. 70:				
(*a*) Original pyruvate	62·56	25·60	2·84	34·12
(*b*) Original pyruvate expressed in terms 3 pyruvate + $1H_2O$	66·82	25·60	3·32	37·91
Products:				
H_2	0·36	—	0·36	—
CO_2	26·61	7·26	—	19·35
Acetic acid	13·20	5·28	0·88	7·04
Butyric acid	19·36	10·56	1·76	7·04
Lactic acid	4·00	1·60	0·27	2·13
Total of products	63·53	24·70	3·27	35·56
% total of (*a*)	101·6	96·5	115·1	104·2
% total of (*b*)	95·1	96·5	98·5	93·8

volatile acid consists of equal parts of acetic and butyric acids. The recovery of C (91·8 and 96·5 %) is very satisfactory considering the number of different determinations involved and is of the usual order obtained in this type of work. The recoveries of "total mg." and of H and O are high in comparison with C and is in some cases over the theoretical if pyruvate is considered to be the only initial reactant ("*a*" figures, Table VI). The high recovery of H and O indicates that water is also involved in the reaction. If the original pyruvate is calculated in terms of 3 pyruvate + H_2O ("*b*" figures, Table VI) then the % recoveries of "total mg.", H and O are all in good agreement with the % recovery of C.

The excellent recovery of C shows that it is unlikely that there are any unidentified and unestimated products of the reaction. If the small amount of lactic acid found (which probably arises from a side reaction) is not taken into account the main reaction seems to follow the equation:

$$3CH_3COCOOH + H_2O \rightarrow CH_3COOH + CH_3CH_2CH_2COOH + 3CO_2 + H_2 \ldots (1)$$

The mean production of the various products in a number of experiments fits in well with the requirements of this equation (Table V). The intervention of water in the proportion to pyruvate required is confirmed by the balance sheets, and the occurrence of acetic and butyric acids in equal quantities has been demonstrated in a previous section.

II. Decomposition of *l*-(+)glutamate

Identification and estimation of fatty acids

Examination of the fermentation fluid by the Duclaux method. Large-scale experiments were carried out to obtain sufficient volatile acid for Duclaux analysis. The quantities used were: 15 ml. 0·1 *M* *l*-(+)glutamate, 10 ml. phosphate buffer *p*H 7·2 and 10 ml. organisms in phosphate buffer. The experimental procedure was the same as with pyruvate. The *d'* figures obtained in four experiments are given in Table VII. Comparison of these figures with the

Table VII

	d' numbers			
Exp. no.	110	112	113	114
Fraction of distillate (ml.)				
30	31·5	31·4	31·5	31·4
40	41·4	41·4	41·4	41·5
50	51·1	50·9	51·0	51·0
60	60·5	60·2	60·5	60·7
70	69·9	69·6	69·9	70·0
80	79·3	79·0	79·3	79·4
Ratio acetic : butyric acid	2·8 : 1	2·9 : 1	2·8 : 1	2·8 : 1
Total volatile acid distilled (ml. *N*/10)	10·39	10·65	10·13	10·24

standards (Table II) shows that we are dealing with a simple mixture of acetic and butyric acids; there is no indication of the presence of any other volatile acid. The first 30 ml. and the last 40 ml. of the distillate of Exp. 110 were redistilled separately. The accumulation of the more volatile acid in the former and the less volatile acid in the latter was of the order of magnitude required on the assumption that acetic and butyric are the acids in question (Table VIII).

Table VIII

	d' numbers	
Fraction of distillate ml.	First 30 ml.	Last 40 ml.
20	25·6	18·8
40	47·6	37·7
60	66·8	56·6
80	83·7	77·0
Approx. acetic : butyric ratio	1·1 : 1	>4 : 1

As the total acid distilled was much less than that used in the standards the ratio is not accurately determined.

The *d'* numbers given in Table VII indicate that the volatile acid contains three parts of acetic acid to one part of butyric. The ratio is consistently slightly lower than 3 : 1 (2·8–2·9 : 1) but the deviation is within the limit of experimental error. Examination of the standards (Table II) makes it evident that the difference between distillation numbers for successive ratios becomes progressively smaller the more the ratio increases above unity; the experimental error will therefore have a great effect on the computed ratio with the higher concentrations of acetic acid. It must also be remembered that, as with pyruvate, it is not possible to correct for the proportions of volatile acid formed from unknown substrates in the "blank" reaction of the bacterial suspension. It seems justifiable to conclude that acetic and butyric acids are the only volatile

acids produced during the decomposition of *l*-(+)glutamate by *Cl. tetanomorphum* and that these acids are formed in the proportion of 3 acetic to 1 butyric.

Identification of acetic and butyric acids. This was accomplished by the quinine salt method in the same way as described under pyruvate decomposition. A total of 64·5 ml. 0·1 *N* volatile acid and 2·45 g. quinine sulphate (theoretical 2·4 g.) were used for the separation. The yields of crude quinine butyrate and quinine acetate amounted respectively to 95 and 90 % of the theoretical assuming three parts of acetic to one of butyric. The purified products gave the following M.P. data (uncorrected) (Table IX) and analytical figures (Table X).

Table IX

	Shrinks	Softens	Melts
Acetate:			
Experimental product	119–120°	122°	123·5–125·5°
Pure quinine acetate	116°	121°	124 –126°
Mixed	118°	122°	123·5–125·5°
Butyrate:			
Experimental product	72°	74–75°	77 –78°
Pure quinine butyrate	72°	76°	77·5–78°
Mixed	72°	75°	77 –78°

Table X

	% C	% H	% N
Acetate:			
Experimental product	68·34	7·66	7·23
Pure quinine acetate	69·02	7·54	6·81
Calc.: for $C_{20}H_{24}O_2N_2$, $C_2H_4O_2$	68·76	7·29	7·29
Butyrate:			
Experimental product	69·06	7·69	7·03
Pure quinine butyrate	69·08	7·65	6·43
Calc.: for $C_{20}H_{24}N_2O_2$, $C_4H_8O_2$	69·90	7·77	6·80

Complete analysis of reaction products. Balance sheets for the decomposition of *l*-(+)glutamate were computed from the values obtained from large-scale experiments. The procedure was the same as that described for pyruvate except that NH_3 was also estimated on aliquot parts of solution *B*. The quantities used for the Krebs' pots were: 6 ml. 0·1 *M* glutamate or 6 ml. water, 12 ml. phosphate buffer *p*H 7·1 and 6 ml. bacterial suspension. For the manometric determination of H_2 and CO_2 one-twentieth of these quantities was taken. Pure (thrice recrystallized) *l*-(+)glutamic acid was used as starting material. When H_2 and CO_2 production ceased the reaction fluid contained no amino-N (beyond the controls) indicating that the glutamic acid had been completely decomposed. The mean values obtained for the various products in four such complete experiments are given in Table XI.

Table XI

(All values less appropriate controls)

	Mol. (or equiv.) formed per mol. glutamate			
	Range	Mean	Required by equation (2)	% mean found of required
H_2	0·20–0·30	0·24	0·20	120
CO_2	0·82–0·88	0·85	1·0	85
Volatile acid (equiv.)	1·48–1·53	1·49	1·6	93
NH_3	0·91–0·95	0·92	1·0	92
Lactic acid, Succinic acid, Formic acid, Alcohol	Absent	—	—	—

As with pyruvate the construction of balance sheets for individual experiments yielded further information as to the quantitative relationship between initial and final products. Two typical experiments are analysed in this way in Table XII. The assumption is made (justified in the previous section) that acetic

Table XII

	Actual mg.	mg. C	mg. H	mg. O	mg. N
Exp. 75:					
(*a*) Original glutamate	88·2	36·0	5·4	38·4	8·40
(*b*) Original glutamate expressed in terms of 5 glutamate + $6H_2O$	101·2	36·0	6·84	49·92	8·40
Products:					
H_2	0·36	—	0·36	—	—
CO_2	21·73	5·93	—	15·80	—
NH_3	9·48	—	1·67	—	7·81
Acetic acid	40·06	16·03	2·67	21·37	—
Butyric acid	19·59	10·68	1·78	7·12	—
Total of products	91·22	32·64	6·48	44·29	7·81
% total of (*a*)	103·7	90·7	120·0	115·4	93·0
% total of (*b*)	90·2	90·7	94·7	88·7	93·0
Exp. 72:					
(*a*) Original glutamate	88·2	36·0	5·40	38·4	8·40
(*b*) Original glutamate expressed in terms of 5 glutamate + $6H_2O$	101·2	36·0	6·84	49·92	8·40
Products:					
H_2	0·29	—	0·29	—	—
CO_2	23·15	6·31	—	16·83	—
NH_3	9·69	—	1·71	—	7·98
Acetic acid	41·40	16·56	2·76	22·08	—
Butyric acid	20·24	11·04	1·84	7·36	—
Total of products	94·77	33·91	6·60	46·27	7·98
% total of (*a*)	107·4	94·2	122·2	120·5	95
% total of (*b*)	93·7	94·2	96·5	92·7	95

and butyric acids are present in the ratio of 3 : 1. The recovery of C (90·7 and 94·2 %) is again very satisfactory and of the order expected in this type of experiment. The recoveries of "total mg." and of H and O are high in comparison with C and N and are in all cases over the theoretical if glutamate is taken to be the only initial reactant ("*a*" figures). The greater than theoretical recovery of H and O can only mean that water is also involved in the reaction. A recovery of "total mg.", H and O of the same order as that of C and N is most nearly obtained if the original glutamate is calculated in terms of 5 glutamate + $6H_2O$ ("*b*" figures).

The high % recovery obtained in the balance sheets shows that it is improbable that there remain any unidentified and unestimated reaction products. The proportional mean formation of the various products (Table XI) is in reasonable agreement with the following equation for the main course of the reaction:

$$5C_5H_9O_4N + 6H_2O \rightarrow 6CH_3COOH + 2CH_3CH_2CH_2COOH + 5CO_2 + 5NH_3 + H_2. \quad \ldots\ldots(2)$$

The intervention of water in the proportion required is confirmed by the balance sheets and the acetic and butyric acids are in the ratio indicated by the Duclaux analysis. The mean quantity of free H_2 actually formed is, however, somewhat in excess of the requirements of (2). Some experiments give a value for H_2 closer to that required (see "range", Table XI). The significance of this equation is dealt with in the discussion.

Optical specificity. A few experiments were carried out with *d*-(−)glutamate and the rate of decomposition and quantity of some of the products compared with *l*-(+)glutamate. We are indebted to Dr H. A. Krebs for kindly supplying a sample of *d*-(−)glutamic acid. The mean results of two experiments are summarized in Table XIII. The rate of decomposition (as indicated by Q_{H_2}) is

Table XIII

	Mol. formed per mol. glutamate	
	l-(+)	*d*-(−)
H_2	0·18	0·22
CO_2	0·87	0·79
NH_3	0·87	0·80
	Q_{H_2}	
	17·8	9·6

54 % of that of the natural *l*-(+)isomeride. The slightly smaller absolute amounts of CO_2 and NH_3 from *d*-(−)glutamate are possibly explained by the larger blank deducted (since the reaction is slower). There is some evidence that at least part of the "blank" reaction does not occur in the presence of fermentable substrate. The *d*-(−)isomeride apparently gives a larger amount of H_2 but the excess over that formed from the *l*-(+)isomeride is well within the normal range of variation found with the latter [Woods & Clifton, 1937; Table VI].

Discussion

The decomposition of pyruvate and *l*-(+)glutamate by washed suspensions of *Cl. tetanomorphum* leads to the formation of the same main end products, viz. H_2, CO_2, acetic acid and butyric acid. The proportions of the acids are, however, different with the two substrates and ammonia is also formed from glutamate. A small amount of lactic acid is formed from pyruvate. On the basis of the quantitative data equations have been suggested which express approximately the balance between initial and final products. These equations have no other significance. It seems probable that in these decompositions we are dealing with a series of successive reactions together with side reactions. There is also evidence from the variability in the amount of H_2 formed [Woods & Clifton, 1937] that some intermediates may be further decomposed by two or more methods at different rates. Until it is possible to analyse each of the parts of the reaction separately there is little prospect of obtaining any equation representing truly the final balance of the reactants.

It had been hoped that the identification of all the products might yield some hint as to the chemical mechanisms involved. A large number of experiments with possible intermediates, both alone and in admixture with the initial or final products have so far been unsuccessful.

In the previous paper [Woods & Clifton, 1937] evidence was given that, with this organism, H_2 did not arise via formate. Recently Krebs [1937, 2], on the basis of work by Farkas *et al.* [1934] on the decomposition of formate by *Bact. coli* in the presence of heavy water, has suggested that H_2 arises from formate by the following reaction:

$$H_2\dot{:}O + H.COO' \rightarrow H_2 + HCO_3',$$

that is, the H_2 is derived from water and not from formate. Dr Krebs (personal communication) has suggested that with *Cl. tetanomorphum* we may be dealing

with similar reactions between water and the substrates which give rise to H_2, and that the source of H_2 in each case is water. On this theory there would be two possibilities: (*a*) that there is a direct reaction between water and all the substrates giving H_2, or (*b*) that these substrates give rise to a common, but so far unknown, intermediate which reacts with water. With the two substrates investigated in detail in the present paper it will be noticed that it was necessary to postulate that water entered into the reactions; we have not studied the other H_2-producing substrates from this point of view.

Summary

The products of the decomposition of pyruvate and *l*-(+)glutamate by washed suspensions of *Cl. tetanomorphum* have been subjected to detailed quantitative analysis.

The main products obtained in both cases are: H_2, CO_2, acetic acid and butyric acid; *l*-(+)glutamate also gives NH_3. A small amount of lactic acid is formed from pyruvate but not from glutamate.

On the basis of the quantitative data equations are proposed which express approximately the balance between initial and final products for the main course of the reactions.

d-(−)glutamate is decomposed at about 50 % of the rate obtained with the *l*-(+)isomeride.

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Note added 20 January 1938. Barker (*Enzymologia*, 1937, **2**, 175) has recently described a fermentation of glutamic acid by a new or so far unidentified organism of the genus *Clostridium*. The products obtained in growth experiments are identical with those found by us with suspensions of *Cl. tetanomorphum*. Furthermore, the quantitative relationships are essentially similar to those found in the present paper.