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The Fermentation of L-Threonine, L-Serine, L-Cysteine and Acrylic Acid by a Gram-negative Coccus

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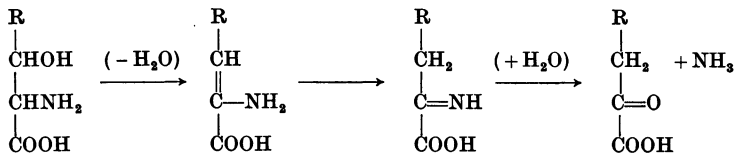
A Gram-negative coccus isolated from the rumen of the sheep (Elsden, Gilchrist, Lewis & Volcani, 1951) ferments a variety of substances with the production of hydrogen, carbon dioxide and volatile fatty acids (Elsden & Lewis, 1953). This organism, referred to as LC, is of considerable interest, since it produces volatile fatty acids (VFA's) higher than butyrate. Its metabolism has been shown to resemble, in many respects, that of *Clostridium kluyveri* (Stadtman & Barker, 1949*a*). During the fermentation of glucose the main products are fatty acids containing an even number of carbon atoms, whereas during growth on lactate considerable amounts of propionate and valerate are formed and little or no hexanoate. The production of fatty acids containing an even number of carbon atoms probably occurs via a condensation of 2-carbon fragments (Elsden & Lewis, 1953).

Lewis, Nisman & Elsden (1952) reported that LC fermented L-serine, L-threonine, L-cysteine and acrylate, and in the present paper we give the results of a more detailed investigation of the anaerobic metabolism of these four compounds by this organism.

During recent years the microbial degradation of serine, threonine and cysteine has been actively studied. Thus, Woods & Clifton (1937) showed that *Cl. tetanomorphum* ferments DL-serine and L-cysteine with the formation of hydrogen, carbon dioxide, ammonia and VFA's. Gale & Stephenson (1938) showed that washed suspensions of *Escherichia coli* under anaerobic conditions rapidly produced ammonia from DL-serine; the progress curve of ammonia formation indicated that one isomer was preferentially attacked. Chargaff & Sprinson (1943), also using *Esch. coli*, showed that the deamination of serine and threonine was accom-

panied by the formation of pyruvate and α-oxobutyrate, respectively. They proposed the mechanism given below, in which R is either H or CH_3, which involves an enzymic dehydration followed by two non-enzymic reactions:



Tarr (1933) showed that hydrogen sulphide is produced from cysteine by washed suspensions of *Proteus vulgaris*, and several investigators have studied the widely distributed enzyme system, cysteine desulphurase, which brings about the formation of hydrogen sulphide, ammonia and pyruvate from cysteine; this work has been reviewed by Smythe (1945) and by Fromageot (1951). There is an obvious parallel between the action of cysteine desulphurase and of serine and threonine deaminase, and Fromageot postulated that the mechanism proposed by Chargaff & Sprinson (1943) for the latter reaction would also account for the former. In the case of the β-hydroxy-α-amino acids the first step is the elimination of water giving an αβ-unsaturated amino acid; in the case of cysteine, hydrogen sulphide is removed giving the same type of compound. There has been considerable discussion on the identity, or otherwise, of these two enzymes. Binkley (1943), using cell-free preparations from yeast, obtained evidence that the two systems were identical, whereas Wood & Gunsalus (1949) made cell-free preparations of *Esch. coli* which attacked both serine and threonine, but not cysteine. Yanofsky & Reissig (1953), using extracts of *Neurospora*, obtained results similar to those of Wood & Gunsalus (1949). They showed that pyruvate and α-oxobutyrate were produced from serine and threonine, respectively, and that ammonia and the α-oxo acid were formed in equivalent amounts. The pH optimum for the deamination was in the range pH 9·0–9·5.

Cardon & Barker (1946, 1947) studied the fermentation of DL-serine, DL-threonine and acrylate by *Cl. propionicum*. Ammonia, carbon dioxide, acetate and propionate were formed from DL-serine; ammonia, carbon dioxide, propionate and butyrate were produced from DL-threonine, and the products from acrylate were carbon dioxide, acetate and propionate. Barker & Wikén (1948) showed that when DL-threonine was fermented by washed suspensions of *Cl. propionicum* in the presence of $^{14}CH_3.^{14}COO-$, the butyrate formed was not radioactive. They concluded, on the basis of these experiments, that acetate was not an intermediary in the synthesis of butyrate from threonine and they suggested instead that, in the conversion of threonine into butyrate, the carbon skeleton remains intact. LC resembles *Cl. propionicum* in that it ferments serine, threonine, lactate and acrylate with propionate as one of the end products. It is perhaps significant for our work that Johns (1952) has shown that the formation of propionate by *Cl. propionicum* does not involve the fixation of carbon dioxide, and that there is a possibility that acrylate, or a derivative thereof, is an intermediary.

METHODS AND MATERIALS

Growth media, washed suspensions and manometric methods. The strain LC3 was used in these experiments; otherwise the procedures were as described by Elsden & Lewis (1953).

Acetone powders. The organism was grown in 10 oz. screw-cap bottles filled to the neck with medium. After autoclaving, the bottles were rapidly cooled, inoculated and incubated at 37° for 20 hr. Under these conditions good growth was regularly obtained without the use of anaerobic jars; thirty bottles were set up at a time. The organisms were harvested on the centrifuge, washed once with and suspended in 100 ml. freshly boiled-out distilled water. The suspension was cooled in ice and squirted into 500 ml. acetone cooled in ice. After standing for 20 min. at 0–2° the precipitate was filtered off (Whatman no. 1 paper) and washed successively with 500 ml. acetone, 500 ml. acetone containing 50% (v/v) ether and finally with 500 ml. ether, all at 0–2°. Care was taken to prevent the residue from becoming dry until the washing was completed. The acetone powder so obtained was dried for 1–2 days in a vacuum desiccator over paraffin wax. Several batches of acetone powder were prepared in this manner and the yields were of the order of 3 g. acetone powder/thirty bottles.

Total nitrogen. This was determined by the Kjeldahl method as described by Chibnall, Rees & Williams (1943) using the Markham steam-distillation apparatus. The ammonia was trapped in the boric acid reagent of Conway & O'Malley (1942) and titrated with standard acid.

Volatile fatty acids were estimated by steam distillation under acid conditions in a Markham still. The distillate was aerated for 3 min. with CO_2-free air and titrated with 0·01 N-NaOH using phenol red as indicator. α-Oxo acids are volatile in steam and thus interfere. The effect of pH on the volatility of VFA's and α-oxo acids was investigated and the results of this study are given in Table 1.

On the basis of these results the following technique was employed for the estimation of the VFA's. A sample (2 ml.) was placed in the Markham still; if a smaller volume was used this was made up to 2 ml. with water. To this was added 2 ml. of M-KH_2PO_4 which had been brought

Table 1. *Effect of* pH *on the steam distillation of fatty acids and* α*-oxo acids*

Distillation carried out in a Markham still using 2 ml. of fatty acid and oxo acid solution (50 μmoles) and 2 ml. buffer (M-KH_2PO_4 adjusted to appropriate pH with 60% (w/v) H_3PO_4). Results expressed as % of that recovered by distilling according to Elsden & Lewis (1953).

pH	Distillate (ml.)	Formic acid	Acetic acid	Pyruvic acid	α-Oxobutyric acid
2·5	50	97	100	20	25
	100	98	100	31	36
	150	100	100	41	50
3·0	50	95	99	8	12
	100	97	100	14	18
	150	98	100	18	25
3·5	50	90	98	3	7
	100	95	99	5	12
	150	96	100	9	16
4·0	50	70	96	1	3
	100	88	98	2	6
	150	92	99	4	8

to pH 3·5 by the addition of 60% (w/v) aqueous H_3PO_4. A volume of 150 ml. distillate was collected, titrated in the usual manner and concentrated *in vacuo* to 1 ml. The concentrate was returned to the Markham still and the volume made up to 2 ml. with washings. The distillation at pH 3·5 was then repeated and 100 ml. of distillate collected and titrated. The subsequent analysis of the fatty acids was carried out by the gas–liquid chromatographic procedure of James & Martin (1952) with some modifications as described by Annison (1954). Using this technique, recoveries of fatty acids added to bacterial suspensions were within the range of 92–98% except in the case of formic acid (86–92%); less than 4% of added pyruvic acid or α-oxobutyric acid appeared in the first distillate.

Ammonia was determined by the method of Conway & O'Malley (1942).

Estimation of amino acids. α-Amino N was determined by measuring the N_2 liberated on treatment with HNO_2 in the Van Slyke-Neil manometric apparatus (Peters & Van Slyke, 1932).

Oxo acids. These were estimated by the method of Friedemann & Haugen (1943).

Reagents. α-Oxobutyrate was obtained from L. Light & Co., Colnbrook, Bucks, England. Crystalline sodium pyruvate was prepared from redistilled pyruvic acid. A standard solution of sodium acrylate was obtained by steam distilling 60% acrylic acid (L. Light & Co., Colnbrook, Bucks) under acid conditions. The distillate was titrated with alkali under CO_2-free conditions and standardized by micro-hydrogenation with colloidal palladium (Harrison, 1939). L-Serine and D-serine were obtained from the California Foundation for Biochemical Research, Los Angeles, California; L-threonine, D-threonine, L-cysteine, DL-alanine, DL-glutamic acid, DL-aspartic acid, L-arginine and DL-isoleucine from L. Light & Co. Ltd., Colnbrook, Bucks; and L-cysteine, DL-methionine, DL-valine, L-histidine, L-leucine and L-proline from Roche Products Ltd., Welwyn Garden City, England. The samples of L-cysteine released ammonia when treated with alkali according to the procedure of Conway & O'Malley (1942) but after recrystallization from aqueous ethanol this did not occur. Consequently, cysteine was recrystallized in this way before use. The amino acids were chromatographically pure, using both collidine saturated with water and butanol saturated with 20% (v/v) aqueous acetic acid as developing solvents. L-Cysteine decomposed during the chromatographic procedure and so its purity could not be assessed in this way. Freshly made solutions of L-cysteine had an optical rotation of $[\alpha]_D^{20} + 6{\cdot}8 \pm 0{\cdot}5°$ in N-HCl (*c*, 6·6).

RESULTS

Experiments with washed suspensions

Deamination by washed suspensions. In preliminary experiments the anaerobic metabolism of the following amino acids by washed suspensions of LC3 was studied; L-serine, L-threonine, L-cysteine, DL-alanine, DL-glutamic acid, DL-aspartic acid, L-cysteine, D-serine, D-threonine, DL-methionine, DL-valine, L-histidine, L-leucine, L-arginine, DL-isoleucine and L-proline. Of these only L-serine, L-threonine and L-cysteine were rapidly deaminated and the remaining amino acids were attacked at less than 10% of the rate of these three; the D isomers of serine, threonine and cysteine were attacked slowly, if at all. We decided, therefore, to confine our attention to L-serine, L-threonine and L-cysteine and the rates of deamination of these three amino acids are given in Table 2.

Deamination was accompanied by the formation of gas, only part of which was absorbed by KOH. On the basis of analyses of the gases produced during growth it was assumed that the remaining gas was hydrogen. An experiment was therefore designed to determine the course of hydrogen output from the three amino acids and in addition acrylate, which Cardon & Barker (1946) had shown to be fermented by *Cl. propionicum*, an organism whose metabolism resembles, in some respects, that of LC. Fig. 1 shows the progress curves for hydrogen production from L-serine, L-threonine, L-cysteine, acrylate and pyruvate under an

atmosphere of hydrogen. Pyruvate was included as a control, since the metabolism of this compound by LC had been studied previously in some detail (Elsden & Lewis, 1953). It will be seen that hydrogen was produced more rapidly from pyruvate and acrylate than from threonine, serine and cysteine and that, in the case of these two substrates, there was a marked secondary uptake of hydrogen. More hydrogen was produced from threonine than from either serine or cysteine. In some experiments with serine a slight reduction in pressure was observed towards the end of the incubation period. Occasionally, with cysteine as the substrate, no hydrogen was formed but there was instead a slight uptake of gas.

Effect of pH. The effect of pH on the metabolism of these amino acids was next examined. For the purpose of these experiments the cells were both washed with and suspended in 0·9% (w/v) sodium chloride containing 0·02% (w/v) $Na_2S,9H_2O$ and the pH adjusted to 7·0 with N-HCl. To 1 ml. of the suspension in each manometer vessel was added 1 ml. of 0·2 M phosphate buffer of the required pH. The rates of both hydrogen and ammonia formation, expressed as μmoles/mg. bacterial N/hr., were measured and plotted against pH (Fig. 2). The experiments lasted 30 min. and in the case of hydrogen output, the rate was calculated from the figures obtained over the period 10–30 min. following the addition of the substrate. The rates of ammonia formation were calculated from the amounts produced in the 30 min. incubation period. In the case of ammonia formation there appears to be an optimum in the range pH 6·5–7·0.

Table 2. *Ammonia production from amino acids by washed suspension of* LC 3

Experiments carried out in Warburg manometers with single side bulbs which contained 2 ml. washed suspension (0·84 mg. bacterial N) in 0·1 M phosphate buffer (KH_2PO_4–K_2HPO_4 mixture), pH 6·5, and 0·02% (w/v) with respect to $Na_2S,9H_2O$. Side bulb contained 50 μmoles substrate in 0·5 ml. water. Gas phase N_2; temp., 37°; time 40 min. Results expressed as μmoles NH_3 produced/40 min.

Substrate	NH_3 formed (μmoles)
Control	2·7
L-Serine	15·9
L-Threonine	16·6
L-Cysteine	8·9

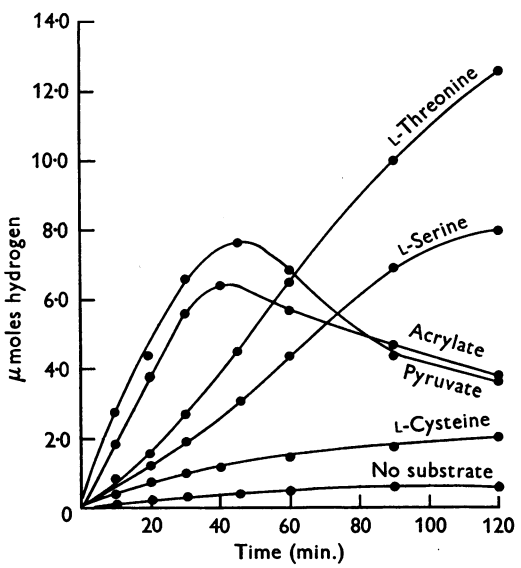


Fig. 1. Rate of production of hydrogen from pyruvate, acrylate, L-cysteine, L-serine and L-threonine by washed suspensions of LC 3. Each flask contained 2 ml. bacterial suspension (0·68 mg. bacterial N) in 0·1 M phosphate buffer (KH_2PO_4–K_2HPO_4 mixture), pH 6·5, and containing 0·02% (w/v) $Na_2S,9H_2O$; 50 μmoles substrate in 0·5 ml. water placed in side bulb; centre well contained 0·2 ml. 20% (w/v) KOH plus filter paper; gas phase H_2; temp. 37°.

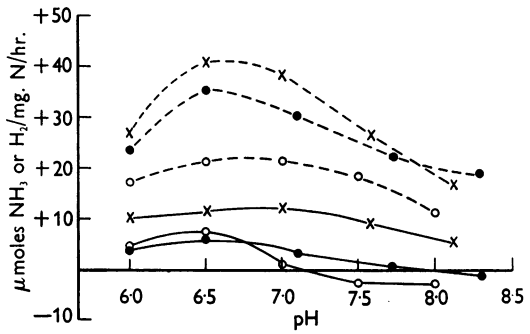


Fig. 2. Effect of pH on the production of hydrogen and ammonia from L-serine, L-threonine and L-cysteine by washed suspensions of LC 3. Experiments carried out in flasks with double side bulbs. Each flask contained 1 ml. of a bacterial suspension (0·4–0·9 mg. bacterial N) in 0·9% (w/v) NaCl containing 0·02% (w/v) $Na_2S,9H_2O$ and pH of suspension adjusted to 7·0; 1 ml. of 0·2 M phosphate buffer (KH_2PO_4–K_2HPO_4 mixtures) of appropriate pH. Centre well contained 0·2 ml. 20% (w/v) KOH; first side bulb 50 μmoles substrate in 0·5 ml. water; second side bulb 0·5 ml. 4 N-H_2SO_4; gas phase H_2; temp. 37°. Reaction stopped 30 min. after addition of substrate by addition of acid from second side bulb. Results expressed as μmoles H_2/mg. bacterial N/hr. (calculated over period 10–30 min.) and as μmoles NH_3/mg. bacterial N/hr. (calculated over period 0–30 min.). Results not corrected for the values obtained in the absence of substrate since these were negligible. ——, H_2; – – – –, NH_3; ○, L-cysteine; ×, L-threonine; ●, L-serine.

The curves for hydrogen production are more difficult to interpret, due possibly to the simultaneous occurrence of reactions which involve both the formation and utilization of this gas. Under these circumstances an alteration in the rate of hydrogen output as a result of a change in pH may be due not so much to an inhibition of the 'hydrogenlyase' as to the acceleration of the hydrogen-utilizing system.

Fermentation products. The products formed from the three amino acids, and from acrylate and pyruvate were next determined. The reactions were carried out in vessels made from 100 ml. round-bottomed flasks (Fig. 3).

One of the side bulbs contained 1·0 ml. of a saturated aqueous solution of barium hydroxide to absorb the carbon dioxide produced. This solution was standardized by titration before use. The substrate solution was placed in the second side bulb, and the washed suspension in the main compartment. The gas inlet tube was then attached, the stoppers placed loosely in the sockets of the side bulbs and the flask flushed with hydrogen through the gas inlet tube. When the gassing was completed the cock was turned and the stoppers were pressed firmly into place. Two flasks were set up for each experiment, the second containing no substrate. The flasks were placed in a Warburg bath at 37° and shaken gently for a time, after which the substrate was added from the side bulb. At the end of the experiment, each flask was cooled in an ice bath and 2 ml. of 4N-H_2SO_4 was added through the inlet tube, care being taken to avoid the introduction of air. The flask was then shaken in the bath at 37° for a further 30 min. to ensure that all the carbon dioxide was absorbed by the $Ba(OH)_2$. The carbon dioxide was estimated by titration of the residual $Ba(OH)_2$, using thymol blue as indicator. The amount of hydrogen produced was determined in a parallel series of experiments carried out in Warburg flasks in which all the quantities were reduced to one-fifth of those in the large vessels. The acidified suspension was transferred to a 25 ml. flask and the volume made up to the mark with washings. Samples of this sus-

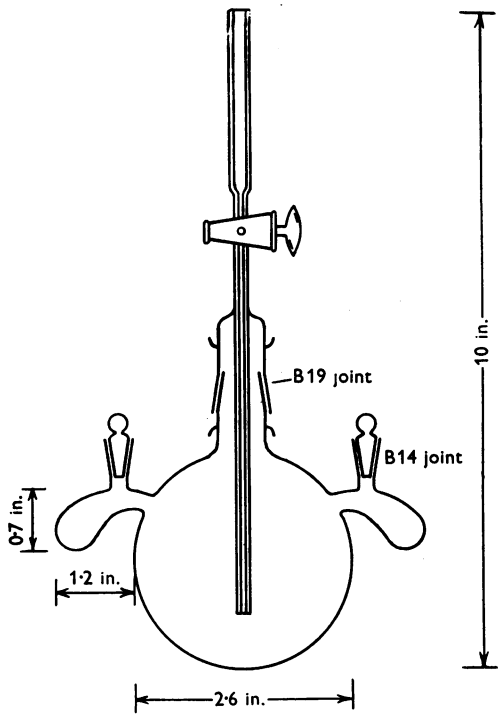


Fig. 3. Large-scale fermentation vessel. Vessel made from a Pyrex 100 ml. round-bottomed flask.

Table 3. *Analysis of products of amino acids and acrylate during their fermentation by* LC

Experiments carried out in flasks shown in Fig. 3. The main compartment contained 10 ml. washed suspension in 0·1M phosphate buffer (KH_2PO_4–K_2HPO_4 mixture) pH 6·5 (8·7 mg. total N). Substrate solution (1 ml.) in first side arm and second side arm contained 1·0 ml. sat. $Ba(OH)_2$. Gas phase H_2, temp. 37°, incubation time 2 hr. Results expressed as μmoles and corrected for control values given. Amounts of amino acids utilized calculated from the amount of ammonia formed corrected for ammonia production in control without substrate.

Substrate	Control	Pyruvate	Acrylate	L-Serine	L-Threonine	L-Cysteine
Added	—	172·8	141·3	106·6	140·0	95·6
Residual substrate	—	0·8	0·0	—	—	—
NH_3 formed	4·6	—	—	47·6	97·1	32·8
Substrate utilized	—	172·0	141·3	47·6	97·1	32·8
Hydrogen	0·6	30·3	28·8	24·0	29·4	2·7
Carbon dioxide	21·3	181·6	72·2	46·6	78·0	33·6
Formic acid	0·6	0·3	0·2	3·4	3·7	4·6
Acetic acid	6·4	32·4	53·9	14·1	6·0	8·4
Propionic acid	0·9	2·0	64·4	3·3	75·6	0·6
Butyric acid	0·5	61·5	1·0	8·6	2·9	10·2
Valeric acid	0	0·9	13·3	1·0	11·0	1·6
Total VFA	8·4	97·1	132·8	30·4	99·2	25·4
Carbon recovery (%)	—	97	105	90	100	107
Redox ratio	—	0·91	0·93	0·98	0·94	0·91

pension were used for the estimation of ammonia, amino-N, VFA's and residual substrate. The results are given in Table 3. The redox balances (Johnson, Peterson & Fred, 1931) were consistently low, and this could be due to low recoveries of either the higher VFA's or of the hydrogen measured manometrically. The main VFA's produced were acetate and butyrate from pyruvate, L-cysteine and L-serine; acetate and propionate from acrylate and propionate from L-threonine. Some valerate was produced from both acrylate and L-threonine. It is interesting to observe that a small though significant quantity of a material which had on the James & Martin (1952) chromatogram the same retention volume as formic acid, was produced from the amino acids. In view of this, the activity of the formic hydrogenlyase of strain LC3 was tested and found to be considerably lower than that reported previously for strain LC1 (Elsden & Lewis, 1953); formate was attacked, but more slowly.

investigating the early stages of the fermentation of amino acids. Preliminary experiments with acetone powders of LC3 confirmed Warner's observations and also showed that both L-serine and L-threonine were attacked with the formation of ammonia and large amounts of oxo acids. A more detailed investigation of the properties of acetone powders was therefore undertaken.

Products of the reaction. The acetone powder was prepared as described above and a series of experiments was designed to determine quantitatively the products of the fermentation of pyruvate, α-oxobutyrate, L-serine, L-threonine and acrylate. The incubations were carried out in manometer vessels using the procedure described by Elsden & Lewis (1953) for experiments with washed suspensions. The results are presented in Table 4. With the exception of α-oxobutyrate and L-threonine the carbon recoveries were adequate, and in view of the variety of end products the redox balances were satisfactory. The oxo acids formed

Table 4. *Fermentation of amino acids, oxo acids and acrylate by acetone powder of* LC3

Experiments carried out in Warburg flasks with double side bulbs (for details of procedure see Elsden & Lewis, 1953). Main compartment contained 30 mg. acetone powder in 2 ml. phosphate buffer (KH_2PO_4–K_2HPO_4 mixture) pH 6·5. One side bulb contained 0·2 ml. CO_2-free 2N-NaOH plus filter paper; the other 0·4 ml. 4N-H_2SO_4. Substrate added from Keilin dangling tube (0·4 ml.). Gas phase H_2; temp. 37°; incubation time 2 hr. Amounts of amino acid utilized calculated from the amount of NH_3 produced corrected for NH_3 produced in the absence of substrate. All results expressed as μmoles and corrected for control values.

Substrate	Control	Pyruvate	α-Oxobutyrate	L-Serine	L-Threonine	Acrylate
Added	0	67·1	49·0	42·7	54·0	70·6
Residual substrate	—	12·3	22·5	—	—	14·3
Ammonia formed	3·4	—	—	34·0	52·9	—
Oxo acid formed	0·8	—	—	11·4	21·2	0·2
Substrate utilized	—	54·8	26·5	34·0	52·9	56·3
Carbon dioxide	4·1	44·8	20·3	20·0	25·0	8·5
Hydrogen	−5·8	23·4	20·0	16·2	22·6	−28·6
Total VFA	12·1	54·6	26·1	22·8	28·3	51·8
Formic acid	0·2	2·2	1·4	0·4	3·4	0
Acetic acid	9·4	44·3	5·3	19·3	1·3	6·5
Propionic acid	1·6	5·2	19·2	3·1	23·6	45·3
Butyric acid	0·9	2·9	0·2	0	0	0
Carbon recovery (%)	—	94	86	101	88	94
Redox balance	—	0·99	0·94	1·03	0·94	0·99

Experiments with acetone powders of LC3

The fermentation of these compounds obviously involves a complex series of reactions, the precise nature of which is difficult to deduce from the results of experiments with whole cells. Mr A. C. Warner, in unpublished experiments, observed that acetone powders of LC attacked pyruvate with the formation of hydrogen and carbon dioxide; acetate was the main VFA produced and it seemed probable that the enzymes concerned with the synthesis of higher acids had been inactivated. Such preparations, because of their restricted activity, seemed to provide a means of

from L-serine and L-threonine were estimated on the assumption that the former gave rise to pyruvate and the latter to α-oxobutyrate. It will be shown later that this assumption was justified. All substrates save acrylate were attacked with the formation of hydrogen and carbon dioxide. In the case of the acrylate there was a large and rapid uptake of hydrogen and only small amounts of carbon dioxide were formed. There was a similarity between products of the fermentation of L-serine and pyruvate on the one hand and between those of L-threonine and α-oxobutyrate on the other. Propionate was the main VFA produced from acrylate, L-threonine and α-oxobutyrate,

whereas acetate was the major acid formed from L-serine and pyruvate. Small amounts of propionate were also produced from this latter pair of substrates and a trace of butyrate was found in the steam distillate from the pyruvate fermentation.

Identification of oxo acids. The oxo acids were isolated as the 2:4-dinitrophenylhydrazones and characterized on the basis of melting points and elementary analyses. The yield of oxo acid from both serine and threonine was found to increase with increasing pH over the range pH 6·0–9·5 and use was made of this in the isolation of the oxo acids produced from these compounds. Also, since the rate of deamination by the acetone powder was rapid, dilute suspensions and an incubation period of only 40 min. were used. The reactions were carried out in the large vessels (Fig. 3), three of which were used, one for L-serine, one for L-threonine and a third which served as a control without substrate. Each flask contained 120 mg. acetone powder in 25 ml. 0·1M borate buffer (boric acid–NaOH–KCl mixture) pH 9·0. One side bulb contained 300 mg. of substrate in 2 ml. of water and the other 2 ml. of 4N-H_2SO_4; the gas phase was hydrogen. After equilibration in the manometer bath, the substrates were tipped in and the vessels incubated at 37° for 40 min. The acid was then added from the second side bulb and the contents of the vessel removed and centrifuged. To the supernatants were added 50 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in N-H_2SO_4. Heavy yellow precipitates formed in the solutions to which serine and threonine had been added but none in the control without substrate. The solutions were allowed to stand overnight at 0°, the precipitates then filtered off and washed with N-H_2SO_4 and dried. On the assumption that pyruvate was produced from serine, the yield of the 2:4-dinitrophenylhydrazone was equivalent to 28% of the added serine and, assuming that α-oxobutyrate was formed from threonine, the yield of the 2:4-dinitrophenylhydrazone amounted to 42% of the threonine used. These preparations were then recrystallized 3 times from aqueous ethanol and dried *in vacuo*. The chemical and physical properties of these crystals were then examined. The 2:4-dinitrophenylhydrazone from L-serine had m.p. 214–215°, mixed m.p. with an authentic sample of the 2:4-dinitrophenylhydrazone of pyruvic acid, prepared by the same procedure from pyruvic acid (m.p. 215°), was 214°. (Found: C, 40·6; H, 3·2; N, 20·7. Calc. for $C_9H_8N_4O_6$: C, 40·3; H, 3·0; N, 20·9%.) The 2:4-dinitrophenylhydrazone from L-threonine had m.p. 194° and the mixed m.p. with an authentic sample of the 2:4-dinitrophenylhydrazone of α-oxobutyric acid, prepared by the same procedure (m.p. 194–195°) was 195°. (Found: C, 42·7; H, 3·7; N, 20·1. Calc. for $C_{10}H_{10}N_4O_6$: C, 42·5; H, 3·5; N, 19·9%.) All m.p.'s are uncorrected and the elementary analyses were carried out by Weiler and Strauss, Oxford. These results confirm that pyruvate is formed from L-serine and α-oxobutyrate from L-threonine.

Effect of pH *on the deamination of* L-*serine and* L-*threonine by acetone powders.* In the previous section it was indicated that maximum accumulation of α-oxo acids occurred at pH 9–9·5. Fig. 4 shows the effect of pH on the rate of ammonia formation by acetone powders of LC3 in the presence of L-serine and L-threonine, respectively. Since the phosphate buffer system would not cover the range required (pH 6–10) the experiments were carried out with both phosphate buffers (KH_2PO_4–K_2HPO_4 mixtures) and borate buffers (H_3BO_3–NaOH–KCl mixtures). The experimental period

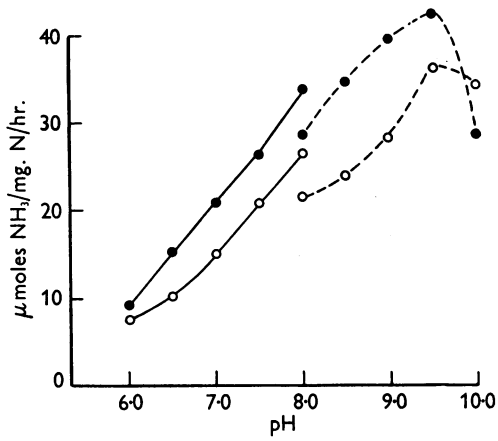


Fig. 4. Effect of pH on the production of ammonia from L-serine and L-threonine by acetone powders of LC3. Experiments carried out in Warburg manometers with double side bulbs. Each flask contained 1 ml. of a suspension of acetone powder (approx. 20 mg. powder/ml., equivalent to approx. 2·3 mg. total N) in 0·9% (w/v) NaCl containing 0·02% $Na_2S,9H_2O$ and pH adjusted to 7·0; 1 ml. of either 0·2M phosphate buffer (KH_2PO_4–K_2HPO_4 mixture) or 0·2M borate buffer (HBO_3–KCl–NaOH mixture). Centre well contained 0·2 ml. 20% (w/v) KOH. First side bulb contained 50 μmoles substrate in 0·5 ml. water and second side bulb 0·5 ml. 4N-H_2SO_4. Gas phase H_2; temp. 37°. Reaction stopped 20 min. after addition of substrate by addition of acid from second side bulb. Results, which are expressed as μmoles NH_3 formed/mg. total N/hr., are not corrected for ammonia formed in absence of substrate which was negligible. ——, Experiments in phosphate buffer; – – –, experiments in borate buffer; ○, L-serine; ●, L-threonine.

was 20 min., and in this time the maximum amount of ammonia produced was equivalent to less than 40% of the substrate added. The results given in Fig. 4, which are expressed as μmoles NH_3/mg. total N/hr., have not been corrected for the ammonia produced in the appropriate controls without substrate since in all cases this amounted to less than 10% of that formed in the presence of the substrate. It will be seen that the optima for both reactions were in the range pH 9–9·5, and that, at pH 8·0, where direct comparison is possible, the reaction is more rapid in phosphate than in borate buffer.

DISCUSSION

The above experiments show that L-serine, L-threonine, L-cysteine and acrylate are fermented by washed suspensions of LC3 under hydrogen with the formation of hydrogen, carbon dioxide and VFA's. In the case of acrylate, there is an initial evolution of hydrogen which is followed by an uptake of the gas. Acrylate, in this respect, closely resembles pyruvate in its behaviour. Pyruvate, serine and cysteine resembled one another in that acetic and butyric acids were the main acidic end products. In the case of threonine, propionate was the main VFA produced with smaller amounts of acetate and valerate. Acrylate gave approximately equal amounts of acetate and propionate and smaller amounts of valerate. Traces of a VFA which had the same retention volume on the gas–liquid chromatogram as formic acid were produced from the three amino acids.

Experiments with acetone powders of LC3 indicate that pyruvate and α-oxobutyrate are intermediates in the fermentation of L-serine and L-threonine, respectively. The acetone powders did not attack L-cysteine, but in view of work on the metabolism of cysteine by other organisms (e.g. Binkley, 1943; Kallio & Porter, 1950), it seems reasonable to assume that pyruvate is also an intermediate in the fermentation of this amino acid. The fact that acetone powders of LC3 deaminate serine and threonine but not cysteine shows that, in this organism at any rate, cysteine desulphurase is distinct from the enzyme or enzymes which deaminate serine and threonine. It is to be expected that the fermentation of serine and cysteine will proceed by mechanisms similar to that postulated for the fermentation of pyruvate by LC (Elsden & Lewis, 1953). The composition of the end products obtained both with whole cells and acetone powders bears this out. Serine and cysteine differ from pyruvate, however, in that small amounts of a formate-like acid are produced and in that the secondary uptake of hydrogen is small or non-existent.

Since L-threonine gives rise to α-oxobutyrate, the metabolism of this compound can, in part, be explained by the following reactions

$$CH_3.CHOH.CHNH_2.COOH \rightarrow CH_3.CH_2.CO.COOH + NH_3$$

$$CH_3.CH_2.CO.COOH + H_2O \rightarrow CH_3.CH_2.COOH + 2H^+ + 2e + CO_2$$

$$2H^+ + 2e \rightarrow H_2$$

The production of propionate, hydrogen and carbon dioxide, from threonine can be explained in this way and it would seem not unreasonable to postulate propionylcoenzyme A as the intermediary. The formation of acetate and valerate from threonine, in view of what is already known of the metabolism of this organism, suggests that in some way the C_4 chain of threonine is broken to give a 2-carbon fragment which is either converted into acetate or condensed with propionate to give, ultimately, valerate. The fermentation of threonine by this organism seems to differ from that by *Cl. propionicum* which was studied by Cardon & Barker (1946, 1947), who found that growing cultures of this organism converted threonine into a mixture of propionic and butyric acids but no acetic acid was found.

No explanation is offered to account for the appearance of the formate-like compound amongst the fermentation products of the three amino acids.

The fermentation of acrylate by suspensions of LC3 resembles, in its broad outlines, the fermentation of acrylate by *Cl. propionicum* described by Cardon & Barker (1946, 1947), and it is clear from the amounts of acetate and carbon dioxide produced that a considerable part of the substrate disappearing was oxidized. It is not unreasonable to suggest that in this case also the valerate produced was synthesized by the condensation of propionate and acetate, or derivatives thereof.

The experiments with acetone powders present a somewhat different picture. In this case, during the fermentation of acrylate the major VFA was propionate. Small amounts of carbon dioxide and acetate were formed, and there was a large uptake of hydrogen. This suggests that part, at least, of the propionate produced from acrylate was the result of a direct reduction; though whether the acrylate itself is reduced or some derivative of it, e.g. acrylylcoenzyme A, is a question our experiments do not permit us to decide. The reduction of vinylacetate by preparations of *Cl. kluyveri* is very similar to this reduction, and the results of Peel & Barker (1953) suggest that vinylacetylcoenzyme A is the compound actually reduced.

The amount of propionate formed from acrylate was greater than the amount of hydrogen consumed so that other electron donors must be available. This reduction could be coupled with the

oxidation of acrylate to acetate. If the amount of acrylate oxidized in the experiment described in Table 4 is taken as 7·5 μmoles (the mean of the amounts of acetate and carbon dioxide produced), then this will be equivalent to the reduction of 15 μmoles acrylate according to the following equations:

$$CH_2{:}CH.COOH + 2H_2O \rightarrow CH_3.COOH + 4H^+ + 4e + CO_2$$

$$2CH_2{:}CH.COOH + 4H^+ + 4e \rightarrow 2CH_3.CH_2.COOH$$

The amount of hydrogen consumed was 28·6 μmoles, which is equivalent to the formation of 28·6 μmoles propionate. The total propionate formed would thus be 43·6 μmoles (15 μmoles + 28·6 μmoles) and that found directly was 45·3 μmoles. This is reasonable agreement. This dismutation is similar to the dismutation of vinylacetate by extracts of *Cl. kluyveri*, discovered by Stadtman & Barker (1949*b*).

The mechanism of formation of propionate by LC3 has not been established. Whole cells do not decarboxylate succinate, and succinate added to growing cultures is not metabolized. Johns (1952), working with *Cl. propionicum*, an organism which forms propionate from both acrylate and lactate has obtained evidence which makes it most unlikely that succinate is an intermediary in the formation of propionate; this is in direct contrast to the observations with *Veillonella gazogenes* and *Propionibacteria* spp. He proposed the following mechanism: lactate → acrylate → propionate. Such a mechanism could account for the conversion of lactate into propionate by LC3, and our experiments with acrylate support this. This mechanism would also explain the puzzling fact that LC3, when grown upon lactate, produces large amounts of propionate and valerate but, when grown on glucose, produces negligible amounts of propionate and only small amounts of valerate (Elsden *et al.* 1951).

The similarity between Johns's scheme for the formation of propionate and that put forward by Stadtman (1953) to account for the formation of butyrate by *Cl. kluyveri* is obvious. In the latter case the proposed intermediaries are coenzyme A derivatives of β-hydroxybutyrate and vinylacetate (cf. Lynen, 1953; Mahler, 1953). It remains to be seen whether the intermediaries in the formation of propionate by the mechanism proposed by Johns (1952) are derivatives of coenzyme A.

SUMMARY

1. Washed suspensions of LC3, a strain of a Gram-negative, anaerobic organism isolated from sheeps' rumen contents, ferment L-serine, L-threonine, L-cysteine and acrylate with the formation of hydrogen, carbon dioxide and volatile fatty acids. Ammonia was also produced from the amino acids.

2. Small amounts of an acid with the same retention volume as formic acid were produced from all three amino acids but not from acrylate.

3. Propionate and smaller amounts of acetate, butyrate and valerate were formed from threonine. The predominant acids formed from serine and cysteine were acetate and butyrate. Acetate and propionate were the major acidic end products of the fermentation of acrylate along with some valerate.

4. Acetone powders of LC3 decompose L-threonine and L-serine but not L-cysteine. At pH 6·5 the main products from threonine were hydrogen, carbon dioxide, propionate and α-oxobutyrate. Acetate, pyruvate, hydrogen and carbon dioxide were produced from serine at pH 6·5. At pH 9·5 more of the oxo acids accumulated.

5. Acetone powders of LC3 metabolize acrylate. Under a hydrogen-gas phase, hydrogen is taken up and some carbon dioxide is produced, along with propionate and an amount of acetate approximately equivalent to the carbon dioxide formed.

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The Physical Instability of Human Red Blood Cells*

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It is well established that the internal contents of the red cell are not in thermodynamic equilibrium with its environment, but are maintained at a steady level by active processes. While some of the structural components, and the haemoglobin are metabolically inert (Muir, Neuberger & Perrone, 1952), it has been shown that the cell lipids are in a condition of rapid metabolic turnover (Muir, Perrone & Popják, 1951; Altman, 1953). The exchange of cholesterol between the cells and their plasma by diffusion has also been reported (Gould, 1951). The integrity of the cell depends upon the presence of the lipids; if it is assumed that these substances are free to diffuse away, then the cell itself can be considered as a steady-state system maintained intact by metabolic activity. This notion receives support from the observed changes in red cells during cold storage.

Lovelock (1954) reported that cells stored at temperatures between 0 and $-79°$ may lose a considerable proportion of their membrane lipids, and that this loss precedes their haemolysis. Cold storage at $-20°$ or lower is an effective means of arresting metabolic activity, but diffusion processes can still continue at low temperatures although at a reduced rate.

The investigation of the dissolution of red cells at temperatures as low as $-79°$ is necessarily slow, and complicated by the presence of ice and the high viscosity of the suspending medium, which always includes glycerol. This paper reports experiments on the diffusion of lipid components from the red cell and its ensuing dissolution at temperatures above 0°. The loss of components was accelerated by suspending the cells in media maintained unsaturated with these components. This was achieved either by repeated washing or by including a neutral adsorbent substance, namely alumina. The progress of the dissolution of the cells is described and the structure and state of the cell membrane discussed in the light of the experimental observations.

* The experimental data recorded in this paper formed the basis of a dissertation which gained a Ciba Foundation Award (1954–5) for a paper descriptive of Basic Research Relevant to the Problems of Ageing.

METHODS

Red blood cells. Human blood to which anticoagulant had been added was stored at 4° until required for use. The anticoagulant solution was composed of trisodium citrate, 0·073 M; citric acid, 0·035 M; and glucose, 0·01 M. It was added to fresh blood in the proportion of 15 ml. of anticoagulant to 100 ml. of blood. Blood older than 10 days was not used.

Alumina. Commercial chromatographic alumina was used; the particles of this substance were approximately 100 μ. in diameter.

Procedure for repeatedly washing red cells. Stored blood was centrifuged at 3000 *g* for 20 min. The supernatant plasma and the top layer of cells were then pipetted off. The cells were then suspended in an equal volume of lightly buffered solution (NaCl, 0·15 M; Na_2HPO_4, 0·05 M; KH_2PO_4, 0·05 M; and glucose, 0·01 M) centrifuged, and the supernatant and top layer of cells pipetted off. This procedure was used in order to remove as much as possible of the plasma with the minimum of washing. The volume of supernatant trapped with the cells at each of the above stages was less than 10%, so that the final concentration of the original plasma remaining with the packed cells was less than 1·0%. Packed cells (10 ml.) were then transferred to graduated centrifuge tubes and 11 ml. of the buffered glucose NaCl solution added to suspend them. The suspension was left for 10 min. in a water bath at 37°, then centrifuged for 5 min. at 3000 *g*, and 10 ml. of the supernatant were removed for analysis and the volume of the cells observed. A further 10 ml. of sodium chloride solution at 37° were then added and the suspension was returned to the water bath for a further 10 min. at 37°. This procedure was repeated 20 times.

Exposure to alumina. Powdered alumina was weighed into test tubes and 9·0 ml. of sodium chloride solution (NaCl, 0·15 M; Na_2HPO_4, 0·05 M; glucose, 0·01 M) added. After standing for 15 min. during which the suspension was gently agitated sufficient 0·1 M-KH_2PO_4 solution was added to lower the pH to 7·0. The final phosphate ion con-