INHIBITIONS BY TETRACYCLINE AND OXYTETRACYCLINE OF THE CONSUMPTION OF PYRUVATE BY *AEROBACTER AEROGENES*

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The effects of tetracycline and oxytetracycline on the production of pyruvate and on its utilisation by a strain of *Aerobacter aerogenes* in glucose-mineral salt media, have been examined. Both antibiotics inhibit the production of formate from pyruvate by non-proliferating suspensions of cells in unaerated, but not in aerated media. The only acidic material accumulated during growth is formic acid and the cessation of growth coincides with the concentration of undissociated formic acid reaching 0·2 mM and 0·3 mM respectively in unaerated and aerated media. At the end of the growth phase of unaerated cultures the reproduction of cells is replaced by the production of lactic acid. The possibility of a common primary site of inhibition being responsible for inhibition of pyruvate and mode II inhibition of growth (Jones and Morrison, 1962) is discussed.

THE molecular forms of both tetracycline and oxytetracycline decrease the rate of growth of unaerated cultures of *A. aerogenes* in glucose-mineral salt media by interfering with the hydrogen-transfer mechanisms (Mode II, Jones and Morrison, 1962). Since the glucose of the media is the source of both the hydrogen and of its ultimate acceptor, the chain of reactions producing this acceptor from glucose may contain the site of origin of mode II inhibition, or its overall functioning be affected by mode II. It is possible also that some reaction in this chain is inhibited independently but not sufficiently severely to make it the rate-controlling reaction of growing cultures.

EXPERIMENTAL METHODS AND MATERIALS

The organism, medium and many of the procedures have been described previously (Jones and Morrison, 1962). Pyruvic acid was released in solution as required from pure lithium pyruvate hydrate (prepared from freshly distilled pyruvic acid) by passing through a column of "Amberlite" IR 120 (H).

Preparation of suspensions of cells. Cells were obtained from fully grown cultures, observed by nephelometry, in which the extent of growth had been limited by the amount of glucose present initially to two-thirds of the maximum obtainable in cultures starting at pH 7·00. They were washed twice in iso-osmotic aqueous buffer, in which they were re-suspended to form a stock suspension which was incubated at 37° for 1 hr. Aliquots of fresh stock suspensions were used in individual experiments.

Concentrations of acids: All acids together. 3·00 ml. of the test liquor was allowed to percolate slowly through 5 cm. of well-washed "Amberlite" C.G. 120 (H) in columns of 1 cm. diameter, the eluate and 12 ml. of

washings with demineralised water being collected. The combined eluate and washings were titrated with 0·100 N NaOH (carbonate-free) from a micro-burette using thymolphthalein as the indicator. The equivalence of the mineral acids released from the buffering salts was determined from samples of the medium and subtracted from the results. The accuracy was ±0·5 m-equiv./litre.

Volatile Acids

The micro-diffusion method was used (Conway, 1961a). The accuracy was ±0·13 m-equiv./litre.

Keto-acids. Differential colorimetric methods based on the reaction with dinitrophenylhydrazine (Friedemann and Haugen, 1943) were used to determine the total concentration of keto-acids, the concentration of pyruvic acid, and hence by difference, the concentration of keto-acids other than pyruvic. The accuracy was ±10 μM, and suspensions of cells produced as described above did not produce measureable concentrations of keto-acids within 3 hr. unless a suitable substrate was added.

Lactic acid. The modification (Hullins and Noble, 1953) of the method devised by Barker and Summerson (1941) was used. The accuracy was ±10 μM.

Malic acid. An E.I.L. direct reading fluorimeter was applied to the method devised by Hummel (1949). Since large concentrations of glucose are troublesome, media containing graded concentrations of glucose were made up and the malate determined in each just as the glucose had been consumed completely. The accuracy was ±2 μM.

RESULTS

Net Rates of Production of Keto-acids and Concentration of Antibiotics

Pyruvate is an essential intermediate in the utilisation of glucose for growth by the test organism (Dagley, Dawes and Morrison, 1951) and small concentrations of it can be measured. It thus provides an opportunity for the examination in two portions of the pathway of reactions in which it takes part between glucose and cellular material. Inhibition of either its production or of its utilisation could decrease the rate of growth, but inhibition of a reaction preceeding the formation of pyruvate must result in a smaller steady-state concentration of pyruvate whereas inhibition of a reaction subsequent to its formation must result in a larger steady-state concentration of it. Preliminary experiments with growing cultures showed that a small constant steady state concentration of other keto-acids was present during the logarithmic phase of growth and consequently they also provide a similar opportunity in the pathway(s) in which they take part.

The test media contained: 12 g. glucose, 5·4 g. KH_2PO_4, 0·082 or 6·56 mmol. magnesium, and 0 to 0·25 μmol. antibiotic, per litre and were adjusted to pH 7·00.

Variation in the concentration of the magnesium or in the concentration of either antibiotic had no effect on the net rate of production of either pyruvate or of keto-acids other than pyruvic by aerated suspensions. Similarly the antibiotics did not affect the net rate of production of keto-acids other than pyruvic by unaerated suspensions, but the net rate of production of pyruvate increased with increasing concentration of antibiotic or with decrease in concentration of magnesium which combines with the antibiotic to form a non-inhibitory complex (Jones and Morrison, 1962). The experimental results for tetracycline are presented in Fig. 1.

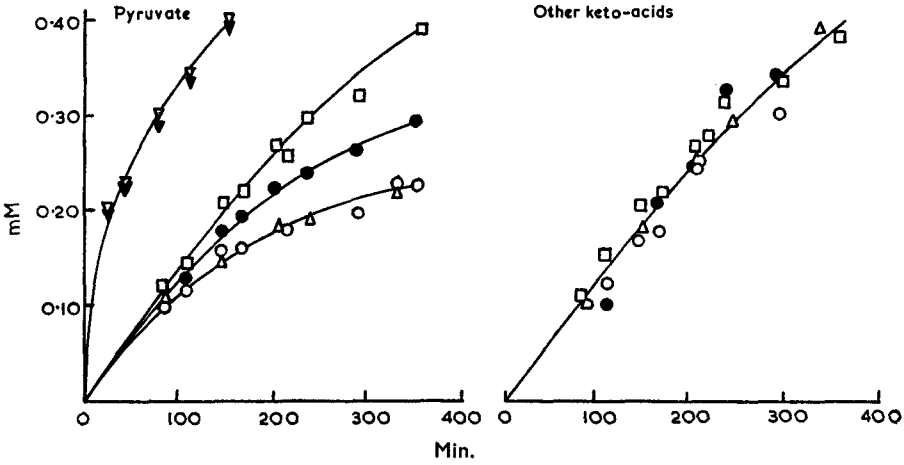

FIG. 1. Effect of tetracycline on the production of keto-acids by suspensions of *A. aerogenes* (0·115 g. (dry) cells per litre).

Concentration of magnesium (mM)	0·082	0·082	0·082	6·56	0·082	0·082
Concentration of tetracycline (μM)	0·000	0·125	0·250	0·125	0·000	0·300
Concentration of formate (N)					0·46	0·46
	○	●	□	△	▽	▼

Thus the utilisation of pyruvate is inhibited more severely than is its production, and the utilisation and production of the other keto-acids are affected equally: since two separate reactions are very unlikely to be equally inhibited at more than one concentration of inhibitor, it is concluded that neither the production nor the utilisation of other keto-acids is inhibited.

Rate of Consumption of Pyruvate and Concentration of Antibiotic

The inhibitions of the consumption of pyruvate by tetracycline and oxytetracycline were examined in test media in which approximately 0·8 mM pyruvate replaced the glucose of the previous experiments. The results are presented in Fig. 2.

Both antibiotics inhibit the consumption of pyruvate in unaerated media but not in aerated media. This inhibition cannot be the origin of mode I inhibition which is effective in aerated conditions (Jones and Morrison, 1962). Also it is not the origin of mode II.

0·204 μM is the critical concentration of tetracycline at which control of the rate of growth of unaerated cultures passes from mode I to mode II,

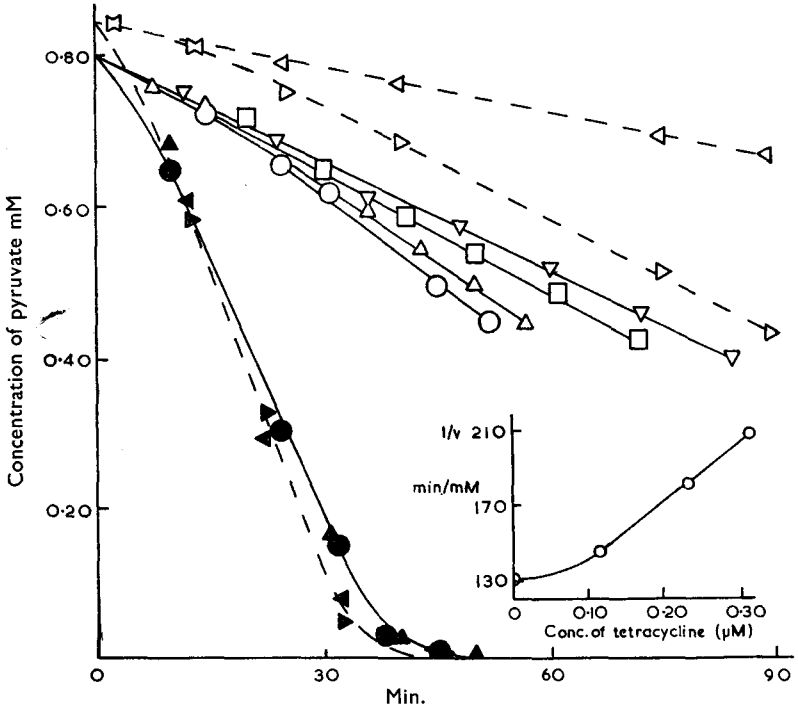

FIG. 2. Consumption of pyruvate by suspension of *A. aerogenes*.
Unaerated media containing 0·100 g. (dry) cells per litre: concentration of tetracycline (μM): 0, ○; 0·014, △; 0·228, □; 0·34, ▽.
Unaerated media containing 0·061 g. (dry) cells per litre: concentration of oxytetracycline (μM): 0, ▷; 0·340, △.
Aerated media containing 0·275 g. (dry) cells per litre: concentration of tetracycline (μM): ●, 0; 0·250, ▲.
Aerated media containing 0·325 g. (dry) cells per litre: concentration of oxytetracycline (μM): 0, ▶; 0·340, ◀.

the mean generation time being 84 ± 2·5 min. (Jones and Morrison, 1962). At this concentration the reciprocal, 1/v, of the rate of consumption of pyruvate is 174 min./mM and this reciprocal increases linearly with increase of concentration of tetracycline (Fig. 2). Thus, if inhibition of the rate of consumption of pyruvate is the cause of mode II, the mean generation time, m, should be given by (84/174) (1/v). In Table I, comparison of the mean generation times at higher concentrations of tetracycline calculated from this expression, with those obtained empirically,

TABLE I

TEST OF INHIBITION OF UTILISATION OF PYRUVATE AS ORIGIN OF INHIBITION OF GROWTH. V IS THE RATE OF CONSUMPTION OF PYRUVATE

Concentration of tetracycline (μM)	1/v for consumption of pyruvate	Mean generation times (min.)	
		Calculated	Measured
0·204	0·174	84	84
0·250	0·188	91	109
0·300	0·203	98	135

shows that inhibition of the consumption of pyruvate by tetracycline does not increase rapidly enough with increase of concentration of tetracycline to account for inhibition of growth by mode II.

A similar test for inhibition of consumption of pyruvate by oxytetracycline gives the same result.

In these experiments the total concentrations of keto-acids were measured and found to be indistinguishable from the concentrations of pyruvate. Thus pyruvate itself is not an intermediate in the production of other keto-acids from glucose. The liquors were examined to establish the nature of the products of consumption of pyruvate: the original concentration of pyruvate (*ca.* 0·8 mM) was replaced by $0{\cdot}8 \pm 0{\cdot}13$ m-equiv./litre of volatile acid. This volatile acid reduced alkaline permanganate and gave a single spot which ran concurrently with formate during partition chromatography on paper using a number of solvent mixtures (Lederer and Lederer, 1957). Standard tests for acetate failed to yield positive results, and neither lactate, nor acetyl phosphate (Lipman and Tuttle, 1945) could be detected. It is concluded therefore that the volatile acid is formic.

The experiments of the previous section in which glucose was the primary substrate in unaerated media were repeated in the presence of added formate: small concentrations increased the net rate of production of pyruvate and decreased the difference between the rates of production in media containing 0 and 0·3 μM tetracycline. The presence of 0·46 N formate in media at pH 7·00 eliminated the effect of the tetracycline (included in Fig. 1) and lactate was produced. It is concluded that the consumption of pyruvate is by a reversible reaction of the type $MeCO{\cdot}COOH + Y \rightleftharpoons HCOOH + X$, where X is not a volatile acid, and that this reaction is inhibited either directly, or indirectly by inhibition of the utilisation of X, and that tetracycline does not inhibit the production of pyruvate from glucose.

Attempts to follow the consumption of pyruvate by measuring changes in concentration of volatile acid produced using larger initial concentrations of pyruvate, were frustrated by the intrusion of another reaction. When the initial concentration of pyruvate was 8 mM the rate of consumption of pyruvate by 1 g. of cells per litre was 3·6 times as fast as when the initial concentration of pyruvate was 0·8 mM until the concentration of pyruvate had decreased to less than 1 mM. This intruding reaction was not inhibited by tetracycline. The pyruvate was replaced eventually by 14 mM volatile acids including formic, and during the experiments lactate appeared, attained a concentration of 0·1 mM, and then declined to zero. This intruding reaction was not investigated further at the time since it is not affected by tetracycline.

Production of Acids during Growth

Growth of *A. aerogenes* in the glucose-mineral salt medium is associated with the production of acid, and there is a maximum population which is attained before any of the nutrients have been consumed completely, but which depends on the initial pH of the medium (Hinshelwood, 1946).

Measurements of the concentration of total formate produced have lead to the suggestion that this waste product at critical concentrations stops the production of cells, and this suggestion is supported by the fact that additions of formate decreased the extent of growth (Dagley, Dawes and Foster, 1953). Additions of malate, lactate or succinate also decrease the extent of growth, and the dependence of the extent of growth on the initial pH and the ionic strength of the medium can be explained if it is assumed that the undissociated form of an acid produced during growth is the inhibitor (Morrison, 1953).

Unaerated cultures. The concentrations of cells, the pH and the concentrations of (*a*) all acids produced taken together, (*b*) volatile acids, (*c*) malic acid, (*d*) pyruvic acid, and (*e*) lactic acid, were measured at intervals during the growth of cultures starting at different pH values. The results illustrated in Figs. 3 and 4 are:

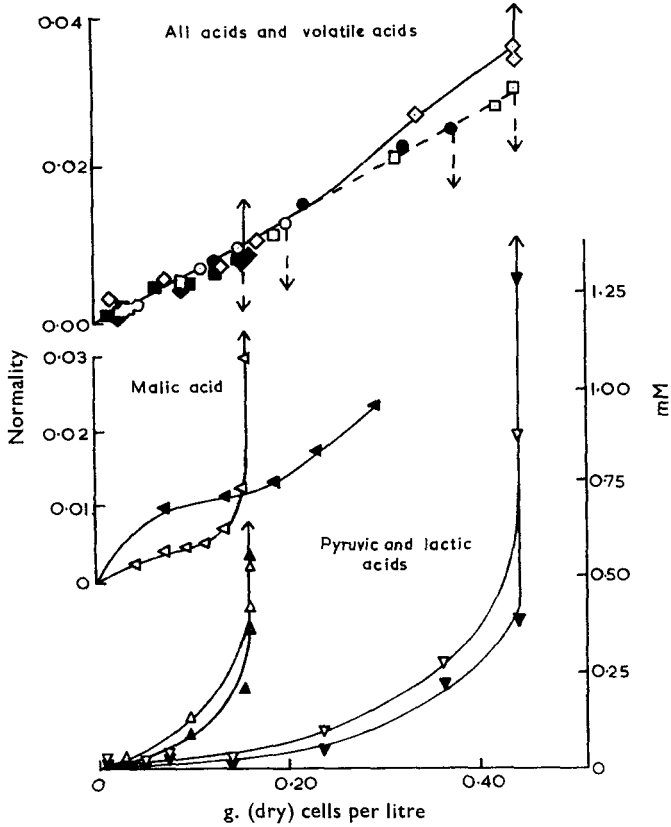

FIG. 3. Production of acids in unaerated cultures of *A. aerogenes*.

Initial pH of culture	Concentration of	all acids	Volatile acids	Malic acid	Pyruvic acid	Lactic acid
7·82		◇	□	◀	▽	▼
7·32			●			
6·60			○			
6·32		◆	■	◁	△	▲

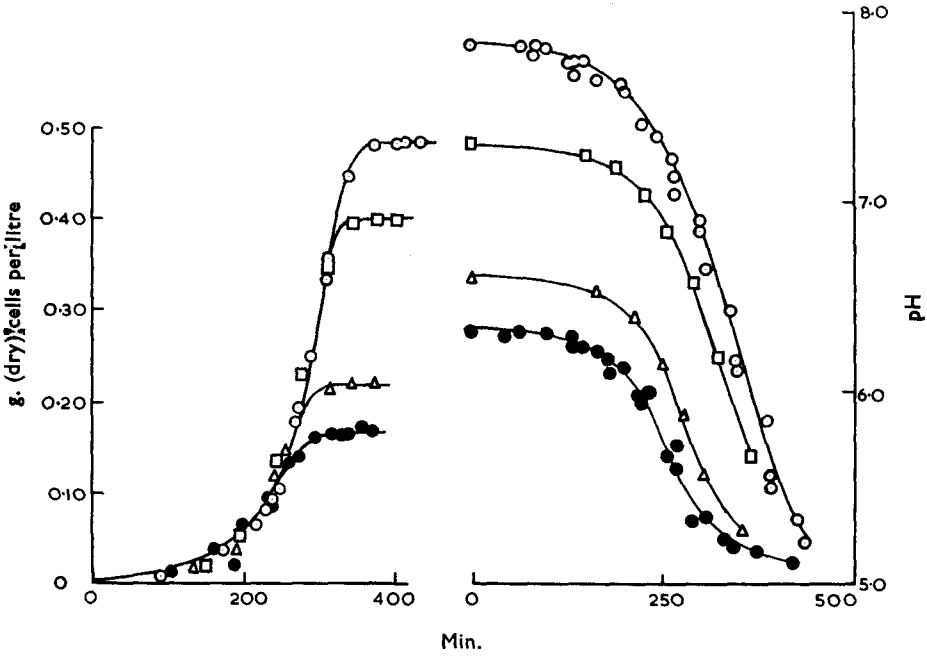

FIG. 4. Cell populations and pH of unaerated cultures of *A. aerogenes*. Initial pH of the culture: 7·82, ○; 7·30, □; 6·60, △; 6·32, ●.

Malate: accounts for only 1/1000th of the total acid produced during growth. Early in the growth period it is almost constant in concentration and hence is probably an intermediate, but at the end of growth it appears to be a waste product to a small extent as its concentration increases.

Pyruvate and lactate: during the early logarithmic phase these acids also account for about 1/1000th of the total acid produced, but later their concentrations increase until they make a decided contribution to the total acidity at the end of growth. After growth stops the production of acids continues and lactate becomes the main product. Clearly growth does not stop because of an inability to dispose of hydrogen since this continues with the formation of lactate.

Volatile acids: throughout the growth phase these acids account for practically all the acidic material produced, and the production of them and the production of cells have an almost constant ratio to each other. Exhaustive examination by standard methods detected only formate and repeated paper chromatography of the ammonium salts using various combinations of alcohols, ammonia and water for development, gave single spots at the R_F expected for formate which ran concurrently with known samples of formate but not with those of acetate. It is concluded that formate is the main acidic waste product during growth.

After growth stops the concentration of volatile acids (formate) decreases slowly with time (Fig. 3) and from this plot the peak concentration of formate, that at the end of growth, can be determined for cultures starting at different pH values. From the first part of Fig. 4 the time at which growth ends can be determined to within 10 min. and the value used to establish from the second part of Fig. 4 the pH within a narrow range of the culture as growth ends. Since the dissociation constant for formic acid is $1{\cdot}74 \times 10^{-4}$ (*Handbook of Chemistry and Physics*, 1959) the concentrations of formate ion and of undissociated formic acid present just as growth stops in cultures starting at different pH values can be calculated. Table II gives the results.

TABLE II

CONCENTRATIONS OF FORMATE (ACID PLUS FORMATE IONS) AND UNDISSOCIATED FORMIC ACID IN UNAERATED CULTURES AS GROWTH STOPS

Initial pH	Time from inoculation (min.)	pH	As growth stops			
			Cell concentration (g. dry cells/litre)	[HCOOH] + [HCOO⁻] (mM)	[HCOOH] (mM) calculated	Ratio of concentrations of cells and acid produced (g./m.mol.)
7·82	370 ± 5	5·94 ± 0·06	0·460	30·0	0·20 ± 0·03	0·015
7·32	345 ± 5	5·91 ± 0·05	0·387	25·2	0·18 ± 0·02	0·015
6·60	305 ± 5	5·52 ± 0·03	0·207	12·2	0·20 ± 0·02	0·017
6·32	300 ± 10	5·40 ± 0·06	0·155	9·0	0·20 ± 0·03	0·017

The active phase of growth in all cultures ends when the concentration of undissociated formic acid reaches 0·2 mM. Thus attributing inhibition of growth to undissociated formic acid accounts for the dependence of the extent of growth on the initial pH of the medium. Adding formate to media at different pH values in sufficient quantity to give 0·2 mM undissociated formic acid initially prevented all growth for 24 hr. whereas smaller quantities restricted the extent of growth.

Aerated cultures. Changes with time of the concentrations of cells and volatile acids, and of the pH were determined in cultures initially having different pH values. As with unaerated cultures only formate could be detected in the volatile acid produced during growth and for each culture there was a linear relation between the production of cells and the production of volatile acid (formic). The ratio of cells produced to formate produced, expressed in g./litre/mM, however, varied from 0·028 in cultures initially at pH 7·65 and 7·20, to 0·047 in cultures initially at pH 6·20 (Table III below) compared with a value of 0·015 to 0·017 for all unaerated cultures (Table II).

Further, after the active growth phase in aerated cultures a very slow growth could be detected and the rate of consumption of formate was greater than in unaerated cultures. The higher ratio for aerated cultures can be accounted for by an appreciable rate of consumption of formate during aerated growth and the even higher ratio in the case of the culture initially at pH 6·20 may be due to loss of formic acid in the stream of air. The concentrations of undissociated formic acid at the end of the active growth phase calculated as before, are given in Table III.

TABLE III

CONCENTRATIONS OF FORMATE (ACID PLUS FORMATE IONS) AND UNDISSOCIATED FORMIC ACID IN AERATED CULTURES AS GROWTH STOPS

Initial pH	Time from inoculation (min.)	pH	Cell concentration (g. dry cells/litre)	[HCOOH] + [HCOO⁻] (mM)	[HCOOH] (mM) calculated	Ratio of concentrations of cells and acid produced (g./m.mol.)
7·65	355 ± 5	5·78 ± 0·02	0·860	31·0	0·29 ± 0·02	0·028
7·20	345 ± 5	5·67 ± 0·03	0·575	20·9	0·26 ± 0·02	0·028
6·20	245 ± 5	5·08 ± 0·05	0·320	6·9	0·32 ± 0·03	0·047

Thus the active growth phase of aerated cultures ends when the undissociated formic acid produced attains a concentration of approximately 0·3 mM. Adding sufficient formate to media having different pH values to give an initial concentration of 0·3 mM undissociated formic acid prevented all growth for 6–12 hr. whereas normal cultures complete their active growth phase within 4 hr.

DISCUSSION

The production of formate from pyruvate, which was studied with suspensions of *A. aerogenes*, and the production of cells during growth of this bacterium, respond in parallel ways to changes of conditions: both rates increase when the media are aerated, both productions are stopped by the same critical concentration of undissociated formic acid (0·46N formate at pH 7·00—i.e., 0·2 mM undissociated acid), and the inhibition of the pyruvate to formate reaction and mode II inhibition of growth (Jones and Morrison, 1962) by the two tetracyclines are exhibited in unaerated media but not in aerated. This parallelism is emphasised by the linear relation in growing cultures between the production of cells and the production of formate which is the main acidic waste product during growth. Since pyruvate is known to be an essential intermediate for the growth of this strain of *A. aerogenes* in the medium used (Dagley, Dawes and Morrison, 1951), it is probable that the pyruvate to formate reaction is an essential step in the utilisation of pyruvate for growth, i.e., the second product, X, of the reaction $MeCOCOOH + Y \rightleftharpoons HCOOH + X$, is an essential intermediate for growth.

The reversibility of this proposed reaction accounts for the increased rate of production of formate in aerated media: if aeration enhances the rate of consumption of X, the concentration of X when the system is in its steady state will be smaller, and this must stimulate the consumption of pyruvate and the production of formate. Further, since the rate of a reversible reaction depends on the concentrations of all its products, the smaller concentration of X in aerated media will permit a larger concentration of undissociated formic acid before the reaction, and hence, growth, is brought to rest.

Similarly, inhibition of the consumption of X (e.g., by the tetracyclines, mode II) will cause the system to have a steady state in which the concentration of X is larger and its rate of production, and hence the concomitant

rate of production of formate, smaller. Thus the inhibition of the reaction by the two tetracyclines in unaerated media is indirect, which is in accord with the quantitative results that 1/v, where v is the rate of consumption of pyruvate, bears a linear relation to mean generation time of the cultures as the concentration of inhibitor is increased but that 1/v does not increase rapidly enough with increase of concentration of inhibitor to account for mode II inhibition of growth as a consequence of inhibition of the reaction.

A reaction of this type has been proposed for a coliform organism, an *Escherichia coli*, by Chantrenne and Lipman (1950):

$$\text{Me·CO·COOH} + (\text{Co-enzyme A} - \text{apo-enzyme}) \rightleftharpoons \text{HCOOH} + (\text{Me·CO} - \text{enzyme})$$

here the enzyme, "formotransacetylase", combines with co-enzyme A and then accepts the acetyl group from pyruvic acid (acetyl-formate). Thus the co-enzyme A - enzyme complex acts as a carrier for acetyl groups. Some years earlier another reaction was proposed for the same organism

$$\text{Me·CO·COOH} + \text{H}_3\text{PO}_4 \rightleftharpoons \text{HCOOH} + \text{Me·CO·OPO}_3\text{H}_2$$

(Utter, Lipman and Werkman, 1945). This reaction followed by hydrolysis of the acetyl-phosphate could be the reaction which intruded at higher concentrations of pyruvate and which produced twice as much volatile acid as pyruvate consumed.

Since both the inhibition of the pyruvate to formate reaction and mode II inhibition of growth are manifested solely in unaerated media, a common primary site of inhibition may affect a hydrogen-transfer reaction which is by-passed in aerated media. The organism is known to utilise the sequence oxalo-acetate $\rightleftharpoons$ malate $\rightleftharpoons$ fumarate $\rightleftharpoons$ succinate and probably to be able to produce succinate from acetate by an aerobic mechanism (Dagley, Dawes and Morrison, 1951). The sequence of reactions can only operate from left to right under reducing conditions and thus fits the requirements for the involvement of a hydrogen-accepting reaction in a utilisation of X in unaerated media. When two chains of reactions join, in this case the hydrogen-transfer chain and the production of the ultimate acceptor of hydrogen, the kinetic consequences of additions of intermediates to the medium cannot be predicted unequivocally since an excess concentration must inhibit one chain whilst overcoming a shortage for the other: none-the-less, the fact that addition of fumarate to the medium decreases the severity of mode II inhibition (Jones and Morrison, 1962) suggests that the primary site of inhibition is in a chain of reactions which produces fumarate from X or is the fumarate to succinate reaction.

The literature provides some support since inhibition by tetracyclines of carbohydrate oxidation at the tricarboxylic acid level and a sensitiveness of acetate oxidation to tetracyclines, have been noted (Eagle and Saz, 1955). The precise identification of the common site of the inhibition of the consumption of pyruvate by suspensions and of mode II inhibition of growth, or establishing that the phenomena are distinct but coincidentally affected by undissociated formic acid and by the two tetracyclines, requires

detailed investigation of the effects of the inhibitors on the isolated reactions. The kinetic studies however, have directed attention to the parts of metabolism which should be so investigated.

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