2. Vitamin B₁ and Bacterial Oxidations

2. The Effects of Magnesium, Potassium and Hexosediphosphate Ions

By J. H. Quastel* and D. M. Webley*, *From the Biochemical Laboratory,
Cardiff City Mental Hospital*

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It was shown in our previous communication [Quastel & Webley, 1941] that vitamin B₁ is essential for the oxidation of acetic acid by propionic acid bacteria. This was demonstrated by growing the bacteria on a vitamin B₁-deficient medium and by investigating the effects of the addition of vitamin B₁ on the amounts of O₂ consumed by suspensions of the vitamin-deficient bacteria in presence of a variety of substrates. In the case of acetic acid it was clear that complete oxidation takes place according to the following equation:

No evidence could be found that pyruvic acid is an intermediate in this reaction, and it was concluded that vitamin B₁ is essential for oxidative attack on the acetic acid molecule. Vitamin B₁ greatly stimulates the oxidation of a variety of substances attacked by propionic acid bacteria, e.g. glucose, glycerol, lactate, succinate, fumarate, but in these cases it was demonstrated that pyruvic acid is formed as an intermediate in their oxidation. The vitamin B₁ effect here, therefore, could be interpreted as due to its catalytic action on pyruvate oxidation.

Suggestions [e.g. Weil-Malherbe, 1940; Lipton & Elvehjem, 1940] have been made as to the mechanism of action of the vitamin, but further experimental evidence is required before the mechanism will be clearly understood. Vitamin B₁-deficient propionic acid bacteria offer very useful material for securing fresh evidence on the factors influencing the action of the vitamin. This paper is concerned with a description of the effects of a variety of substances, especially of the ions of Mg, K and hexosediphosphate on vitamin B₁ activity. The mechanism of action of the vitamin will be considered in the light of these results.

Technique

The technique employed in preparing vitamin B₁-deficient propionic acid bacteria and in investigating the oxidative metabolism of these bacteria in presence of a variety of substrates is fully described in our previous paper [Quastel & Webley, 1941]. When a departure is made from this technique, complete experimental details will be given in the text. The strain of propionic acid bacteria was obtained from the National Collection of Type Cultures, ref. no. 4759.

Action of Mg⁺⁺ and of K⁺, at varying pH, on the O₂ uptake of propionic acid bacteria in presence of acetate and vitamin B₁

A characteristic feature of the velocity of O₂ uptake by propionic acid bacteria respiring in a phosphate buffer solution containing acetate and vitamin B₁, is that the velocity increases with time (see Fig. 1). This is not due to multiplication of the organism during the course of the experiment, as has been shown by bacterial counts [Quastel & Webley, 1941]. The explanation probably is that the pH rises during the course of the oxidation

* Now at the Rothamsted Experimental Station, Harpenden, Herts.

Table 1

O₂ uptakes (μl./mg. dry-weight bacteria per hr.) of propionic acid bacteria, grown on vitamin B₁-deficient medium, in 0.03 *M* Na phosphate buffer containing NaCl, Na acetate (0.01 *M*) and 2 μg. vitamin B₁. Air, 37°.

pH (initial)	Q _{O₂}			
	Mg ⁺⁺ and K ⁺ absent	K ⁺ present (31 μg./ml.)	Mg ⁺⁺ present (8 μg./ml.)	K ⁺ and Mg ⁺⁺ present
6.6	17.4	21.4	29.8	49.4
7.4	37.9	47.1	49.5	73.8
9.0	61.4	63.5	70.2	81.5

owing to the formation of Na₂CO₃, and the velocity of O₂ uptake increases with the pH. Typical results, given in Table 1, show that the O₂ uptake by the bacteria in presence of acetate and a phosphate buffer is much greater at pH 9.0 than at pH 7.4, whilst that at pH 7.4 may be over double that at pH 6.6.

The addition of Mg⁺⁺, as MgCl₂, to the suspension of propionic acid bacteria results in a marked increase in the rate of O₂ uptake in the presence of acetate and vitamin B₁, the percentage increase being greater the lower the pH.

The addition of K⁺, as KCl, also increases the respiration of the bacteria in an acetate-phosphate mixture, the effect being less than that due to the addition of Mg⁺⁺.

When a mixture of Mg⁺⁺ and K⁺ is added to the acetate-phosphate mixture containing vitamin B₁ the respiration of the propionic acid bacteria is very greatly increased, the increase being considerably greater than the sum of the increases due to the Mg⁺⁺ and K⁺ examined separately. This is specially noticeable at pH 7.4 and at higher H⁺ concentrations. Some typical results are shown in Table 1. It will be seen that in presence of K⁺ (31 μg./ml.) and Mg⁺⁺ ions (8 μg./ml.)

the Q_{O₂} of propionic acid bacteria in an acetate-phosphate mixture containing vitamin B₁, at pH 7.4, is almost double that obtained in the absence of added Mg⁺⁺ and K⁺.

The Na⁺ concentration of the phosphate-acetate-saline mixture in which the bacteria respire is approximately 0.16 *M*, and an increase of this concentration has no effect on the bacterial respiration, comparable with that produced by the addition of K⁺.

Fig. 1. Rate of O₂ uptake of vitamin B₁-deficient propionic acid bacteria in 0.01 *M* sodium acetate containing 2 μg. vitamin B₁ and in presence of Mg⁺⁺ (8 μg./ml.) and K⁺ (31 μg./ml.). pH 7.4. Air, 37°.

Effects, on the respiration of vitamin B₁-deficient propionic acid bacteria, of a mixture of Mg⁺⁺ and K⁺, in the presence and absence of vitamin B₁, and in presence of various substrates

(1) *Sodium formate, acetate, propionate and butyrate.* As shown in our earlier paper, vitamin B₁, which exerts little or no effect on the oxidation of formate or butyrate by propionic acid bacteria, has a definite effect with propionate and has a very marked accelerating action with acetate.

In the absence of vitamin B₁ the addition of a mixture of K⁺ and Mg⁺⁺ affects only slightly or not at all the respiration of propionic acid bacteria in presence of the fatty acids. In the presence of vitamin B₁, the addition of the mixture of ions brings about a very large increase in the O₂ uptake when acetate is the substrate, a much smaller one

Table 2

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria in presence of Na salts of fatty acids (0.01 *M*), in the presence and absence of vitamin B₁ and of a mixture of Mg⁺⁺ (8 µg./ml.) and K⁺ (31 µg./ml.).

Each manometer vessel contained 1.0 ml. saline suspension of freshly washed and freshly grown vitamin B₁-deficient *B. acidipropioni*, 0.5 ml. *M*/5 Na phosphate buffer pH 7.4, the substrate and vitamin B₁ solutions and sufficient 0.16 *M* saline to bring the volume to 3 ml. Air, 37°. Experimental run = 1 hr.

Q_{O_2} = µl. O₂ uptake/hr./mg. dry wt. of bacteria.

Substrate (final conc. = 0.01 <i>M</i>)	Q_{O_2}			
	Vitamin B ₁ , K ⁺ and Mg ⁺⁺ absent	2 µg. vitamin B ₁ present	K ⁺ and Mg ⁺⁺ present	2 µg. vitamin B ₁ , K ⁺ and Mg ⁺⁺ present
Nil	14.2	15.8	11.1	12.5
Na formate	23.6	28.0	22.5	24.9
Na acetate	20.5	33.5	24.8	63.1
Na propionate	17.6	26.7	21.4	37.8
Na butyrate	17.2	24.0	19.0	25.1

when propionate is the substrate, and no change within experimental error when formate or butyrate is the substrate. Representative results are given in Table 2.

It is clear from these results as well as from those in Table 1 that:

(a) The addition of Mg⁺⁺ and K⁺ in the absence of vitamin B₁ has no influence on the bacterial oxidation of the lower fatty acids.

(b) The addition of Mg⁺⁺ and K⁺ exerts a large accelerating effect on the bacterial oxidation of acetate, and a much smaller one on that of propionate, when vitamin B₁ is present.

(c) The accelerating action of Mg⁺⁺ on the oxidation of acetate by the bacterial suspension in presence of vitamin B₁ is optimal only in the presence of K⁺.

The effect of Mg⁺⁺ in increasing the O₂ uptake by propionic acid bacteria in presence of acetate, vitamin B₁ and K⁺ is perceptible at as low a concentration as 1 µg. Mg⁺⁺/ml. This is shown in Table 3, where it will also be noted that an increase of concentration of Mg⁺⁺ from 6 to 60 µg./ml. makes no appreciable difference to the Q_{O_2} .

Table 3

Conditions as in Table 2. Na phosphate buffer pH 7.4. Acetate 0.01 *M*. 2 µg. vitamin B₁, and 31 µg. K⁺/ml. present. Air, 37°. Mg⁺⁺ added as MgCl₂, 6H₂O.

Mg ⁺⁺ conc., µg./ml.	Q_{O_2}	Mg ⁺⁺ conc., µg./ml.	Q_{O_2}
Nil	42.6	6.0	58.8
1.2	49.9	60.0	60.8

(2) *Sodium pyruvate, lactate and α-ketobutyrate*. Pyruvate oxidation by propionic acid bacteria is much accelerated by the presence of vitamin B₁, but this acceleration, contrary to what occurs with acetate oxidation, is either slightly or not at all increased by the addition of Mg⁺⁺ and K⁺. A typical result, given in Table 4, shows that a Q_{O_2} of 33.3, obtained in presence of pyruvate and vitamin B₁, is only increased to 37.1 by the addition of Mg⁺⁺ and K⁺. Yet under the same experimental conditions, a Q_{O_2} of 33.5 obtained in

Table 4

O₂ uptakes (Q_{O_2}) by vitamin B₁-deficient propionic acid bacteria in presence of Na salts of pyruvic, lactic and α-ketobutyric acids. Conditions as in Table 2. K⁺ = 31 µg./ml. Mg⁺⁺ = 8 µg./ml.

Substrate (final conc. = 0.01 <i>M</i>)	Q_{O_2}			
	Vitamin B ₁ , K ⁺ and Mg ⁺⁺ absent	2 µg. vitamin B ₁ present	K ⁺ and Mg ⁺⁺ present	2 µg. vitamin B ₁ , K ⁺ and Mg ⁺⁺ present
Na pyruvate	19.9	33.3	23.9	37.1
Na lactate	35.6	52.8	52.2	72.0
Na α-ketobutyrate	19.0	21.7	21.5	32.2

presence of acetate and vitamin B₁ is increased to 63.1 by the addition of Mg⁺⁺ and K⁺. It is clear that if the pyruvate oxidase system of propionic acid bacteria requires the participation of Mg⁺⁺, there must be present in the bacteria, as used in these experiments, sufficient Mg to saturate the system; this cannot be the case with the acetate oxidation system.

Lactate oxidation by vitamin B₁-deficient propionic acid bacteria differs from pyruvate oxidation in the following ways:

(a) The Q_{O_2} with *dl*-lactate as substrate is much higher than that with pyruvate, in the absence of added vitamin B₁ and Mg⁺⁺ and K⁺. It is easy to show that under such conditions pyruvate accumulates during the lactate oxidation [Quastel & Webley, 1941].

(b) The addition of Mg⁺⁺ and K⁺ secures at pH 7.4 a marked increase in the Q_{O_2} without the addition of vitamin B₁. Thus in the experimental result cited in Table 4 a Q_{O_2} of 35.6 is increased to 52.2. An increase of such magnitude does not occur with pyruvate.

This effect of a mixture of Mg⁺⁺ and K⁺ in enhancing the oxidation of lactate by vitamin B₁-deficient bacteria is probably to be explained by the action of these ions in accelerating the removal of pyruvate formed as an intermediary, which, it is now well known, inhibits the oxidation of lactate.

Utilization of pyruvate by vitamin B₁-deficient bacteria

Pyruvate is utilized, or broken down, by propionic acid bacteria at a rate much exceeding that which would be expected from the bacterial respiration on the assumption that the O₂ uptake is concerned only with the complete oxidation of the pyruvate present. Taking, for example, the typical results cited in Table 5, a Q_{O_2} of 19.3, obtained in the absence of

Table 5

O₂ uptakes, and utilizations of Na pyruvate by vitamin B₁-deficient propionic acid bacteria.

Conditions as in Table 2. Air, 37°. $-Q_{pyr.}$ = μ l. pyruvate utilized per hr. per mg. dry weight bacteria.

Substrates	Q_{O_2}	$-Q_{pyr.}$	Substrates	Q_{O_2}	$-Q_{pyr.}$
0.01 M pyruvate	19.3	10.2	0.01 M pyruvate + Mg ⁺⁺ (8 μ g./ml.)	23.4	35.2
0.01 M pyruvate	28.2	18.5	+ K ⁺ (31 μ g./ml.)		
+ 2 μ g. vitamin B ₁			0.01 M pyruvate + 2 μ g. vitamin B ₁	31.8	40.5
			+ Mg ⁺⁺ (8 μ g./ml.) + K ⁺ (31 μ g./ml.)		

vitamin B₁ or K⁺ and Mg⁺⁺, should result in a utilization of pyruvate equal to 19.3/2½, since 1 mol. pyruvate requires 2½ mol. O₂ for complete oxidation. This would be equivalent to $-Q_{pyr.}$ = 7.7. The experimental result is $-Q_{pyr.}$ = 10.2, the pyruvate being estimated by the method described in our earlier paper. In the presence of vitamin B₁ the Q_{O_2} is increased to 28.2 and the utilization of pyruvate is increased to 18.5. It is evident, therefore, that complete oxidation of the pyruvate does not occur.

The addition of a mixture of Mg⁺⁺ and K⁺ greatly increases the rate of disappearance of pyruvate by propionic acid bacteria, even in absence of vitamin B₁, without any comparable increase in the Q_{O_2} . Thus, whereas the Q_{O_2} is increased from 19.3 to 23.4 by the addition of these ions, the utilization of pyruvate is increased from 10.2 to 35.2. Such an increased disappearance of pyruvate, effected by the presence of Mg⁺⁺ and K⁺, cannot be brought about only by oxidation of the molecule. Whether a dismutation of the pyruvate takes place, which is greatly catalysed by the presence of Mg⁺⁺ and K⁺ and which is independent of the action of vitamin B₁, has still to be elucidated.

This phenomenon, as has been mentioned already, probably accounts for the accelerating effect of a mixture of Mg⁺⁺ and K⁺ on the oxidation of lactate by vitamin B₁-deficient bacteria.

As stated in our earlier paper the oxidation of α -ketobutyrate is not greatly affected by the presence of vitamin B₁. Nor is it appreciably accelerated by the addition of a mixture

of Mg^{++} and K^+ . When, however, a mixture of vitamin B_1 , Mg^{++} and K^+ is added to the suspension of propionic acid bacteria, the O_2 uptake in presence of α -ketobutyrate is definitely increased. The process resembles that occurring with propionate, and it is possible that the facts may be explained on the assumption that the oxidation of α -ketobutyrate takes place via the intermediate formation of propionate.

(3) *Sodium succinate, fumarate, malate, citrate and α -ketoglutarate.* The O_2 uptake by vitamin B_1 -deficient propionic acid bacteria in presence of Na succinate is very greatly increased by the addition of a mixture of Mg^{++} and K^+ , even in the absence of added

Table 6

O_2 uptakes by vitamin B_1 -deficient propionic acid bacteria in presence of Na salts of succinic, fumaric, malic, citric and α -ketoglutaric acids. Conditions as in Table 2. $K^+ = 31 \mu g./ml.$ $Mg^{++} = 8 \mu g./ml.$

Substrate (final conc. = 0.01 M)	Q_{O_2}			
	Vitamin B_1 , K^+ and Mg^{++} absent	2 $\mu g.$ vitamin B_1 present	K^+ and Mg^{++} present	2 $\mu g.$ vitamin B_1 , K^+ and Mg^{++} present
Na succinate	29.9	49.1	58.4	66.8
Na fumarate	28.3	44.6	53.3	64.8
Na <i>dl</i> -malate	19.3	26.0	21.8	22.8
Na <i>l</i> -malate	18.9	26.1	17.6	20.8
Na citrate	16.5	18.1	12.7	15.5
Na α -ketoglutarate	17.8	16.8	12.8	13.4

vitamin B_1 . A Q_{O_2} of 29.9 obtained in the absence of the vitamin and of Mg^{++} and K^+ is increased to 58.4 by the addition of the ions alone. The same phenomenon occurs with fumarate. The phenomenon is much more pronounced than in the case of lactate oxidation to which reference has already been made.

The addition of vitamin B_1 also increases the O_2 uptake of the propionic acid bacteria in presence of succinate or fumarate, both in the absence and in the presence of a mixture of Mg^{++} and K^+ , but the percentage increase is greater in the absence of the ions. Typical results are shown in Table 6. These results also show that the oxidation of malate (*dl*- and *l*-), citrate or α -ketoglutarate, in the presence of vitamin B_1 and K^+ and Mg^{++} is small compared with that of succinate or fumarate. A possible explanation of the surprisingly small rate of oxidation of malate is given in our earlier paper. There is clearly no accelerating effect of Mg^{++} and K^+ on the oxidation of malate, citrate or α -ketoglutarate.

The accelerating action of a mixture of Mg^{++} and K^+ on succinate oxidation by vitamin B_1 -deficient propionic acid bacteria is considerably greater than the sum of the effects due to the ions taken singly. This is seen in Table 7, where results obtained at

Table 7

O_2 uptakes by vitamin B_1 -deficient propionic acid bacteria in presence of 0.01 M Na succinate at various H^+ concentrations. Effects of Mg^{++} and K^+ . Conditions as in Table 2. No vitamin B_1 present.

pH	Q_{O_2}			
	K^+ and Mg^{++} absent	Mg^{++} present (8 $\mu g./ml.$)	K^+ present (31 $\mu g./ml.$)	Mg^{++} (8 $\mu g./ml.$) and K^+ (31 $\mu g./ml.$) present
6.6	6.6	16.1	13.5	34.8
7.4	17.6	24.8	22.4	43.4
9.0	19.1	22.0	20.3	33.5

different H^+ concentrations are given. Thus it is evident that, as in the case of acetate in the presence of vitamin B_1 , the accelerating effect of Mg^{++} on succinate oxidation by the bacteria is optimal only in the presence of K^+ . The same conclusion applies to the oxidation of fumarate by the bacterial suspensions.

A probable explanation of the high catalytic action of Mg⁺⁺ and K⁺ on succinate oxidation by propionic acid bacteria is to be found in the fact that oxaloacetate breakdown by these bacteria is greatly accelerated by the ions in question. It is known that oxaloacetate inhibits succinic dehydrogenase, and upon this fact the effects of cozymase and of nicotinamide on succinate oxidation by animal tissues have been readily explained [Keilin & Hartree, 1940; Mann & Quastel, 1941]. In the presence of propionic acid bacteria, oxaloacetate undergoes a slow breakdown, which is rapidly catalysed by the presence of a mixture of Mg⁺⁺ and K⁺. A typical result is shown in Table 8. The oxaloacetate was

Table 8

Each manometer vessel contained 1.0 ml. freshly grown and washed vitamin B₁-deficient propionic acid bacteria, 0.5 ml. Na phosphate buffer, pH 7.4 and Na oxaloacetate solution. Solutions containing vitamin B₁, Mg⁺⁺ and K⁺ were added and then saline to 3 ml. Air, 37°.

Substrates	μl. oxaloacetate remaining after 1 hr.	Substrates	μl. oxaloacetate remaining after 1 hr.
194 μl. oxaloacetate	174	194 μl. oxaloacetate and Mg ⁺⁺ (8 μg./ml.) and K ⁺ (31 μg./ml.)	74
194 μl. oxaloacetate and 2 μg. vitamin B ₁	110	194 μl. oxaloacetate and 2 μg. vitamin B ₁ and Mg ⁺⁺ (8 μg./ml.) and K ⁺ (31 μg./ml.)	52

estimated by the procedure given by Greville [1939]. The addition of vitamin B₁, in the absence of added Mg⁺⁺ and K⁺, will also catalyse at a slower rate the breakdown of oxaloacetate in presence of the bacterial suspension. The maximum rate of breakdown is secured by a mixture of vitamin B₁ and Mg⁺⁺ and K⁺.

The catalytic effect of Mg⁺⁺ and K⁺ on oxaloacetate breakdown by suspensions of propionic acid bacteria renders very likely the view that these ions increase bacterial succinate oxidation by removal of the inhibitory intermediate, oxaloacetate. It has been shown earlier [Quastel & Webley, 1941] that there is good evidence for the formation of pyruvate, and therefore of oxaloacetate, during the oxidation of succinate by the propionic acid bacteria. If this view is true, however, it must also be concluded that oxaloacetate inhibits the oxidation of fumarate as well as that of succinate, for Mg⁺⁺ and K⁺ accelerate the oxidation of fumarate by suspensions of the propionic acid bacteria.

These results, with the intact cells of propionic acid bacteria, clearly show that a mixture of Mg⁺⁺ and K⁺ will rapidly catalyse the breakdown both of oxaloacetate and of pyruvate, these phenomena being apparently independent of the presence of vitamin B₁.

Recently, Krampitz & Werkman [1941] have shown that with a lysed preparation of *M. lysodeikticus*, Mg⁺⁺ alone catalyse the breakdown of oxaloacetate to pyruvate. This process is not influenced by vitamin B₁. They were unable to observe this phenomenon with intact living cells, with which these authors (confirming the results of Penrose & Quastel [1930]) have shown that permeability relations play a very important part in determining access of the substrates under investigation to the enzymes. With propionic acid bacteria suspensions there appears to be a small but definite accelerating effect of vitamin B₁ on oxaloacetate removal. Conceivably this may be due, in the intact cell, to a more rapid removal of pyruvate.

(4) *Methyl, ethyl and propyl alcohols.* Methyl alcohol undergoes little or no oxidation by suspensions of propionic acid bacteria at pH 7.4 and neither vitamin B₁ nor Mg⁺⁺ and K⁺ induces any oxidation of this alcohol. With ethyl alcohol, however, there is both a catalysis of O₂ uptake by Mg⁺⁺ and K⁺ in the absence of added vitamin B₁ and a catalysis by vitamin B₁ alone. A representative result is given in Table 9. As shown in our earlier paper, the accelerating effect of vitamin B₁ is probably largely due to the effect of the vitamin in increasing the oxidation of acetic acid produced as an intermediary. This cannot be the explanation of the accelerating effect of Mg⁺⁺ and K⁺ in the absence of

Table 9

O₂ uptakes at pH 7.4 by vitamin B₁-deficient propionic acid bacteria in presence of methyl, ethyl or propyl alcohol. Conditions as in Table 2.

Substrate 0.01 M	Q _{O₂}			
	Vitamin B ₁ , K ⁺ and Mg ⁺⁺ absent	2 µg. vitamin B ₁ present	K ⁺ (31 µg./ml.) and Mg ⁺⁺ (8 µg./ml.) present	2 µg. vitamin B ₁ and K ⁺ (31 µg./ml.) + Mg ⁺⁺ (8 µg./ml.) present
Methyl alcohol	15.4	17.0	11.5	13.9
Ethyl alcohol	20.7	28.5	32.2	44.1
Propyl alcohol	23.6	28.2	61.7	66.2

vitamin B₁, since as has been seen earlier (see Table 2) these ions have little or no effect on acetic acid oxidation in absence of the vitamin. The conclusion would be that Mg⁺⁺ and K⁺ catalyse the conversion of ethyl alcohol into acetic acid by resting propionic acid bacteria.

Propyl alcohol oxidation by these bacteria is accelerated very greatly (much more so than in the case of ethyl alcohol) by Mg⁺⁺ and K⁺. This is seen in Table 9, where it will be observed that a Q_{O₂} of 23.6 is raised to 61.7 by the addition of the ions. The addition of vitamin B₁ has only a relatively small effect on the O₂ uptake obtained when propyl alcohol is oxidized with or without Mg⁺⁺ and K⁺.

The catalytic effect of Mg⁺⁺ and K⁺ on the oxidation of ethyl and propyl alcohols may be confined to the activation of the alcohols themselves by their respective dehydrogenases, or to the accelerated formation of a coenzyme, or to the oxidation of intermediates, e.g. the aldehydes. Further experiment is required to settle these points.

(5) *Glycerol, glucose and fructose.* The addition of vitamin B₁ increases the O₂ uptake of vitamin B₁-deficient propionic acid bacteria in presence of glycerol, glucose and fructose. This may be expected from the fact that these substances give rise to pyruvic acid as an intermediate [Quastel & Webley, 1941]. The addition of a mixture of Mg⁺⁺ and K⁺ has no accelerating effect upon the oxidation of glycerol and fructose, but there is a small and definite increase of the O₂ uptake, both in the absence and the presence of vitamin B₁ in the case of glucose (Table 10).

Table 10

O₂ uptakes at pH 7.4 by vitamin B₁-deficient propionic acid bacteria in presence of glycerol, glucose or fructose. Conditions as in Table 2.

Substrate (0.01 M)	Q _{O₂}			
	Vitamin B ₁ , Mg ⁺⁺ and K ⁺ absent	2 µg. vitamin B ₁ present	Mg ⁺⁺ (8 µg./ml.) and K ⁺ (31 µg./ml.) present	2 µg. vitamin B ₁ , Mg ⁺⁺ (8 µg./ml.) and K ⁺ (31 µg./ml.) present
Glycerol	28.8	35.7	28.9	34.1
Glucose	23.5	34.3	32.5	38.9
Fructose	28.4	32.2	20.5	24.2

(6) *Other substances.* Typical results obtained with a variety of substrates are shown in Table 11. Na oxalate is either feebly or not at all oxidized by propionic acid bacteria. Na glycollate is similarly inert. There seems to be a slight vitamin B₁ accelerating effect on the oxidation of Na β-hydroxybutyrate which is increased to a small extent by the addition of Mg⁺⁺ and K⁺. Na α-glycerophosphate oxidation seems not to be affected by vitamin B₁, but this substance shows a peculiarity in that its oxidation is definitely depressed by the addition of Mg⁺⁺ and K⁺. Hexosediphosphate oxidation at pH 7.4 is not affected within the limits of experimental error by vitamin B₁ and is not increased by the admixture of Mg⁺⁺ and K⁺. Glutamate oxidation is slightly increased by the addition of the vitamin, this being further increased by the admixture of Mg⁺⁺ and K⁺. The same phenomena are observed with alanine and glycine, but generally speaking the effects are all small.

Table 11
Conditions as in Table 2.

Substrate (0.01 M)	Q_{O_2}			
	Vitamin B ₁ , Mg ⁺⁺ and K ⁺ absent	2 μ g. vitamin B ₁ present	Mg ⁺⁺ (8 μ g./ml.) and K ⁺ (31 μ g./ml.) present	2 μ g. vitamin B ₁ , Mg ⁺⁺ (8 μ g./ml.) and K ⁺ (31 μ g./ml.) present
Na glycollate	16.7	21.0	15.0	19.0
Na oxalate	16.0	19.4	11.2	13.7
Na β -hydroxybutyrate	18.4	22.7	19.7	25.5
Na α -glycerophosphate	16.9	18.8	9.2	10.4
Na hexosediphosphate	21.0	21.0	17.4	17.5
Na L-glutamate	18.9	25.9	18.9	31.4
Glycine	17.8	25.5	22.4	29.5
Alanine	16.6	20.6	21.6	26.4

Possible interpretation of the effects of Mg⁺⁺ and K⁺

The accelerating effects of vitamin B₁, in the absence of added Mg⁺⁺ and K⁺, on the oxidation of substrates by resting propionic acid bacteria, can be readily explained as due to the catalysed oxidation of pyruvate or acetate formed as intermediaries.

The effects of Mg⁺⁺ and K⁺, however, can be divided into two classes: (a) those which occur in the absence of added vitamin B₁, (b) those which occur only in the presence of vitamin B₁. To the first class belong the oxidations of succinate, fumarate, ethyl alcohol, propyl alcohol, lactate, glucose. It has to be borne in mind, however, that the vitamin B₁-deficient propionic acid bacteria may still contain traces of the vitamin, and the possibility that such traces may be playing an important part in the oxidation of substances which may be catalysed only by the addition of Mg⁺⁺ and K⁺ cannot be disregarded at present. To the second class belong acetate and propionate. A class of substrate whose oxidation seems only to be accelerated by vitamin B₁, and not by the addition of Mg⁺⁺ and K⁺, includes pyruvate and glycerol. Here again, however, it is to be remembered that traces of Mg exist in the bacterial suspensions and that such traces may be ample for the vitamin B₁-catalysed oxidations of pyruvate and glycerol.

It has already been suggested that the effects of Mg⁺⁺ and K⁺ on succinate and fumarate oxidations may be due to the catalysed removal of oxaloacetate formed as an intermediary, and similarly the effect of the ions on lactate oxidation may be due to the catalysed removal of pyruvate. Such an explanation cannot be given, on present evidence, to the effects of the ions on propyl alcohol and ethyl alcohol oxidations. Here, presumably, a dehydrogenase system involved in these oxidations is catalysed by the ions in question.

The effects of the ions on acetate oxidation in presence of vitamin B₁ obviously involves an explanation different from those given above, since the vitamin is definitely implicated. Evidence has already been given by Quastel & Webley [1941] that acetate oxidation by propionic acid bacteria does not proceed mainly via the intermediate formation of succinate, and Krebs & Eggleston [1941] have recently confirmed this conclusion. Hence the acceleration of acetate oxidation by Mg⁺⁺ and K⁺ in presence of vitamin B₁ cannot be due to an effect of the ions on the oxidation of succinate produced as an intermediary. For the interpretation of the effects of the ions on acetate oxidation further experimental evidence is required, and such evidence will be considered later.

The effect of K⁺ in markedly enhancing oxidations by resting propionic acid bacteria in presence of Mg⁺⁺ may be attributed to the action of K⁺ on the permeability of the cell. It is suggested that in presence of K⁺ permeability of the cell to Mg⁺⁺ is increased so that there ensues a more effective oxidation of the substrate, whose combustion necessitates the presence of Mg⁺⁺. This interpretation is supported by evidence to be given shortly.

The results on the oxidations by resting propionic acid bacteria which have been given so far are summarized in Table 12.

Table 12

Substrate	Effect on oxidation of addition of only			Substrate	Effect on oxidation of addition of only		
	Mg ⁺⁺ and K ⁺	Effect on oxidation of addition of only vitamin B ₁	Effect on oxidation of addition of vitamin B ₁ , Mg ⁺⁺ and K ⁺		Mg ⁺⁺ and K ⁺	Effect on oxidation of addition of only vitamin B ₁	Effect on oxidation of addition of vitamin B ₁ , Mg ⁺⁺ and K ⁺
Formate	0	0	0	Malate (<i>dl.</i> and <i>l.</i>)	0	(+)	(+)
Acetate	0	++	++++	α -Ketoglutarate	0	0	0
Propionate	0	+	+++	Citrate	0	0	0
Butyrate	0	0	0	Glucose	+	+	++
Lactate	+	+	++	Glycerol	0	+	+
Pyruvate	0	++	+++	Methyl alcohol	0	0	0
α -Ketobutyrate	0	(+)	+	Ethyl alcohol	+	+	++
Succinate	++++	+	++++	Propyl alcohol	++++	+	++++
Fumarate	++++	+	++++	Hexosediphosphate	0	0	0

Effect of replacement of Mg⁺⁺ by Mn⁺⁺

Mn⁺⁺ cannot replace Mg⁺⁺ as an accelerator of the oxidation of acetate. On the contrary, the addition of Mn⁺⁺ depresses the oxidation of acetate by resting propionic acid bacteria in presence of vitamin B₁ (Tables 13, 14).

Table 13

Conditions as in Table 2. Mn⁺⁺ given as MnSO₄·4H₂O (0.01%).

Substrate	Q _{O₂}			
	2 μ g. vitamin B ₁ present	2 μ g. vitamin B ₁ , Mn ⁺⁺ (25 μ g./ml.) and K ⁺ (31 μ g./ml.) present	2 μ g. vitamin B ₁ and Mn ⁺⁺ (25 μ g./ml.) present	2 μ g. vitamin B ₁ and K ⁺ (31 μ g./ml.) present
Nil	14.6	13.4	—	—
Acetate	41.3	25.7	28.2	41.9

Table 14

Conditions as in Table 2. K⁺ throughout = 0.03 M. Na acetate = 0.01 M. Mg⁺⁺ = 0.067% MgCl₂·6H₂O, Mn⁺⁺ = 0.067% MnSO₄·4H₂O.

Acetate	μ l. O ₂ uptake in 1 hr.		μ l. O ₂ uptake in 1 hr.	
	+ 2 μ g. vitamin B ₁	+ Mg ⁺⁺	Acetate + 2 μ g. vitamin B ₁ and Mg ⁺⁺	+ Mn ⁺⁺
	154.7	654.0	900.0	156.5
	190.0		+ 2 μ g. vitamin B ₁ and Mn ⁺⁺	354.0

Effect of hexosediphosphate on oxidations due to resting propionic acid bacteria

Whilst, as has been stated earlier, hexosediphosphate undergoes little oxidation by propionic acid bacteria, its admixture with certain substrates brings about a large increase in their rates of oxidation. Representative results with acetate and succinate are given in Table 15.

Table 15

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria at pH 7.4 in the presence and absence of Na hexosediphosphate (0.01 M). Conditions as in Table 2. No Mg⁺⁺ present. Air, 37°.

Substrates (0.01 M)	Q _{O₂}			
	Vitamin B ₁ and K ⁺ absent	K ⁺ (31 μ g./ml.) present	2 μ g. vitamin B ₁ present	2 μ g. vitamin B ₁ and K ⁺ (31 μ g./ml.) present
Nil	14.2	14.7	15.8	15.3
Acetate	15.4	18.7	37.9	47.1
Hexosediphosphate	17.3	17.4	18.4	17.5
Acetate + hexosediphosphate	18.1	19.0	43.0	64.4
Succinate	21.4	26.4	36.3	38.4
Succinate + hexosediphosphate	36.8	59.8	60.8	65.2

The addition of hexosediphosphate to succinate brings about a large increase in the rate of O₂ uptake by propionic acid bacteria, even in the absence of vitamin B₁, Mg⁺⁺ and K⁺. When K⁺ is present the increase in Q_{O₂} brought about by the addition of hexosediphosphate is much greater than in the absence of K⁺. Thus a Q_{O₂} of 26.4, obtained with succinate as substrate, with K⁺ present and vitamin B₁ absent, is increased to 59.8 by the admixture of 0.01 *M* hexosediphosphate.

Using acetate as substrate, hexosediphosphate admixture brings about no stimulation of the O₂ uptake, even in the presence of K⁺, until vitamin B₁ is added. In the presence of vitamin B₁ and K⁺, the addition of hexosediphosphate increases the Q_{O₂} obtained with acetate from 47.1 to 64.4.

The behaviour of hexosediphosphate ions in these oxidations is precisely the same as that of Mg⁺⁺. This makes it likely that both hexosediphosphate and Mg⁺⁺ are ultimately concerned with the same metabolic process in propionic acid bacteria. This view is supported by the fact that the addition of Mg ions to a system already containing hexosediphosphate leads to no further increase in the rate of O₂ uptake (Table 16).

Table 16

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria in presence of 2 μg. vitamin B₁. Conditions as in Table 2. pH 7.4.

Substrate	Q _{O₂}
Acetate (0.01 <i>M</i>)	33.5
Acetate + hexosediphosphate (0.01 <i>M</i>)	40.5
Acetate + hexosediphosphate + Mg ⁺⁺ (8 μg./ml.)	37.4
Acetate + hexosediphosphate + K ⁺ (31 μg./ml.)	64.4
Acetate + hexosediphosphate + K ⁺ (31 μg./ml.) + Mg ⁺⁺ (8 μg./ml.)	63.0

K ions exert the same accelerating action in the presence of hexosediphosphate as they do in the presence of Mg ions (Tables 15–17). It seems most likely that the K behaves similarly in both cases, i.e. it increases the permeability of the cells of propionic acid bacteria to both Mg and hexosediphosphate ions. This conclusion is supported by the fact that the presence of K⁺ has an increasingly greater percentage effect on the O₂ uptake the smaller the concentration of hexosediphosphate ions (Table 17).

Table 17

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria in the presence of succinate and with varying quantities of hexosediphosphate. pH 7.4. Conditions as in Table 2.

Conc. of hexosediphosphate <i>M</i>	Q _{O₂}	
	0.01 <i>M</i> succinate present	0.01 <i>M</i> succinate and K ⁺ (31 μg./ml.) present
Nil	21.4	26.4
0.0003	22.4	41.6
0.01	44.8	59.8

The effect of varying the concentration of hexosediphosphate ions on the O₂ uptake by propionic acid bacteria in presence of acetate, vitamin B₁ and K⁺ is shown in Table 18. It will be observed that the effect of hexosediphosphate under these conditions is not apparent at a concentration of 1.7 × 10⁻⁴ *M* but becomes marked at 10⁻³ *M*.

Table 18

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria in presence of acetate (0.01 *M*), 2 μg. vitamin B₁ and K⁺ (31 μg./ml.). Conditions as in Table 2. pH 7.4.

Conc. of hexosediphosphate (<i>M</i>)	Q _{O₂}	Conc. of hexosediphosphate (<i>M</i>)	Q _{O₂}
Nil	40.3	1.0 × 10 ⁻³	55.3
1.1 × 10 ⁻⁶	40.3	2.0 × 10 ⁻³	59.1
1.7 × 10 ⁻⁴	39.4	5.0 × 10 ⁻³	66.5

Sodium α -glycerophosphate

α -Glycerophosphate exerts little or no catalytic action on the rate of O_2 uptake by propionic acid bacteria in the presence of succinate (Table 19).

Table 19

O_2 uptakes by vitamin B_1 -deficient propionic acid bacteria in presence of Na α -glycerophosphate and Na succinate. 2 μ g. vitamin B_1 present. Conditions as in Table 2.

Substrate (0.01 M)	Q_{O_2}	Substrate (0.01 M)	Q_{O_2}
Nil	15.8	Succinate and α -glycerophosphate	41.3
Succinate	36.3	Hexosediphosphate	18.4
α -Glycerophosphate	18.8	Succinate and hexosediphosphate	60.8

Effects of preliminary exposure of vitamin B_1 -deficient propionic acid bacteria to Mg^{++} and K^+ on subsequent bacterial oxidations

In the experiments now to be described a new technique is adopted. This consists of incubating freshly grown and washed propionic acid bacteria in solutions of known composition in air at 37° , the suspensions being shaken in vessels of the Warburg apparatus. After a period of usually 1 hr. the contents of the vessels are centrifuged at high speed, and the deposit of centrifuged bacteria is suspended in saline and centrifuged again. This deposit of saline-washed bacteria is now suspended in 2 ml. saline which is transferred to a fresh Warburg manometer vessel. To this is added 0.5 ml. 0.2 M sodium phosphate buffer, usually pH 7.4, and solutions of various substrates to make up a total volume of 3 ml. O_2 uptakes at 37° are obtained in the usual way, the experimental run being 1 hr.

Table 20

Substances present in solution in which preliminary incubation was carried out.
pH 7.4. Air, 37° . 1 hr.

O_2 uptake of washed and previously incubated bacteria.
Na acetate (0.01 M). 2 μ g. vitamin B_1 present. No K^+ or Mg^{++} present. pH 7.4. Conditions as in Table 2

	Q_{O_2}
Na phosphate-saline	33.3
Na phosphate-saline + Mg^{++} (8 μ g./ml.)	77.4
Na phosphate-saline + K^+ (31 μ g./ml.)	35.9
Na phosphate-saline + Mg^{++} (8 μ g./ml.) + K^+ (31 μ g./ml.)	92.2

Using this technique, the propionic acid bacteria are exposed to phosphate or saline solutions containing Mg^{++} , or K^+ , or hexosediphosphate ions etc. After centrifuging and washing, the bacteria, exposed in this way, must contain only the smallest traces of the ions in question, and the subsequent O_2 uptakes obtained by these bacteria with substrates such as acetate and succinate indicate whether the ions have affected metabolic processes in the cell independently of the substrates subsequently oxidized.

A typical result illustrating the utility of this technique is shown in Table 20. Here freshly grown and saline-washed vitamin B_1 -deficient propionic acid bacteria were suspended in 3 ml. saline-Na phosphate buffer (0.03 M pH 7.4) containing no added Mg^{++} and K^+ , and in a saline-phosphate medium containing either Mg^{++} , or K^+ , or a mixture of Mg^{++} and K^+ . After incubation, with shaking, in air at 37° for 1 hr. the four suspensions were centrifuged and well washed and suspended in a saline-phosphate medium containing 0.01 M acetate together with 2 μ g. vitamin B_1 , but with no Mg^{++} or K^+ present. The rates of O_2 uptake were measured and the results are given in Table 20. It will be seen that 1 hr. exposure to Mg^{++} (8 μ g./ml.) has resulted in the production of an organism

which oxidizes acetate (in the presence of vitamin B₁) at 2½ times the rate at which acetate is oxidized by an organism not exposed to Mg⁺⁺. Exposure to a mixture of Mg⁺⁺ and K⁺ results in a still more actively oxidizing organism. Exposure only to K⁺ leads to little or no effect on the oxidizing ability of the organism so far as acetate is concerned.

It may be considered that in spite of the washing of the exposed organism sufficient Mg is carried over to the new vessel, with the deposit of centrifuged organism, to effect the accelerations which have already been described. Calculation shows, however, that after incubation of the bacteria in 3 ml. of a solution of Mg⁺⁺ at a concentration of 8 µg./ml. (the concentration which has been usually used to demonstrate the effect of Mg), the largest quantity of Mg⁺⁺ which could be mechanically carried over, after centrifuging and washing, to the 3 ml. of solution in the fresh Warburg vessel would be 0.1 µg. This would yield a Mg⁺⁺ concentration of 0.03 µg./ml. in the solution containing the acetate and vitamin B₁. Such a concentration would be quite unable to effect the large increase in O₂ uptake noted in Table 20 (see also Table 3, where it will be seen that Mg⁺⁺ at a concentration of 1.2 µg./ml. whilst showing a definite increase in Q_{O₂} by no means exerts its maximal effect).

Since it is clear that the large increase in Q_{O₂}, noted in Table 20, which is effected by bacteria exposed to Mg⁺⁺ cannot be due to Mg⁺⁺ mechanically carried over with the centrifuged and washed organism, it follows that the increased activity of the organism must be due either

(a) to Mg⁺⁺, which has been absorbed by the bacterial cell or enzymic structures within the cell, and which is not easily washed away or

(b) to metabolic changes which have taken place in the bacterial cell during the hour's exposure of the cell to the Mg⁺⁺.

It seems likely that both these explanations are possible and they will be considered again when further evidence has been described.

The effects of exposing vitamin B₁-deficient propionic acid bacteria to varying concentrations of Mg⁺⁺ and K⁺ on the subsequent oxidation of acetate in presence of vitamin B₁ are shown in Table 21. It will be seen that, as in the experiments reported earlier,

Table 21

Substances present in medium in which preliminary incubation was carried out. Saline-Na phosphate (0.03 M) pH 7.4. Mg⁺⁺ added as MgCl₂·6H₂O. K⁺ added as KCl. Mn⁺⁺ added as MnSO₄·4H₂O

O₂ uptakes by washed and previously incubated bacteria. Na acetate (0.01 M). 2 µg. vitamin B₁ present. No K⁺ or Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

	Q _{O₂}
K ⁺ (31 µg./ml.)	16.5
K ⁺ (31 µg./ml.) + Mg ⁺⁺ (2 µg./ml.)	32.0
K ⁺ (31 µg./ml.) + Mg ⁺⁺ (8 µg./ml.)	59.3
K ⁺ (31 µg./ml.) + Mg ⁺⁺ (80 µg./ml.)	57.3
Mg ⁺⁺ (8 µg./ml.)	36.2
Mg ⁺⁺ (8 µg./ml.) + K ⁺ (9.2 µg./ml.)	46.0
Mg ⁺⁺ (8 µg./ml.) + K ⁺ (31 µg./ml.)	59.3
Mg ⁺⁺ (8 µg./ml.) + K ⁺ (155 µg./ml.)	61.2
Mg ⁺⁺ (8 µg./ml.) + K ⁺ (310 µg./ml.)	64.3
Mg ⁺⁺ (8 µg./ml.) + K ⁺ (775 µg./ml.)	62.3
Mn ⁺⁺ (4 µg./ml.)	8.9
Mn ⁺⁺ (4 µg./ml.) + K ⁺ (31 µg./ml.)	10.0

K⁺ at a concentration of 31 µg./ml. and Mg⁺⁺ at a concentration of 8 µg./ml. gave optimal effects. Exposure to Mn⁺⁺ (4 µg./ml.) appears only to inhibit the oxidative activity of the bacteria (Table 21).

*Effect of incubation of propionic acid bacteria with hexosediphosphate
on the subsequent oxidation of acetate*

Exposure of vitamin B₁-deficient propionic acid bacteria for 1 hr. in air (with shaking) at 37° to 0.01 *M* hexosediphosphate results in the production of an organism which oxidizes acetate in the presence of vitamin B₁ at a much higher rate than is accomplished by bacteria not so exposed. Typical results are shown in Table 22. It will be seen that exposure to hexosediphosphate together with K⁺ results in an even more actively

Table 22

Substances present in the phosphate-saline medium in which the preliminary incubation was carried out

O₂ uptakes by washed and previously incubated bacteria. Acetate (0.01 *M*) 2 μg. vitamin B₁ present. No K⁺ or Mg⁺⁺ present. pH 7.4. Conditions as in Table 2

	<i>Q</i> _{O₂}
Na hexosediphosphate (0.01 <i>M</i>)	16.0
K ⁺ (31 μg./ml.)	42.6
Na hexosediphosphate (0.01 <i>M</i>)	18.8
and K ⁺ (31 μg./ml.)	65.1

oxidizing organism. The results are entirely comparable with those obtained after exposure of the bacteria to Mg⁺⁺ (see Table 20) and support the view already put forward that both magnesium and hexosediphosphate influence the same metabolic process leading to increased oxidation of acetate in presence of vitamin B₁.

It may be considered, as in the case of the Mg experiments, that traces of hexosediphosphate are carried over mechanically with the centrifuged organism after washing to the medium containing acetate and vitamin B₁, and that such traces are responsible for the acceleration of oxidation. That this is not the case may be seen from the results in Table 23. Here it will be seen that the bacteria which had been exposed to hexosediphosphate (0.01 *M*), centrifuged and washed three times with 3 ml. saline, gave as high

Table 23

O₂ uptakes in acetate (0.01 *M*) and 2 μg. vitamin B₁. Conditions as in Table 2

	<i>Q</i> _{O₂}	
	After 2 washings	After 3 washings
Bacteria exposed to K ⁺ (31 μg./ml.) only	19.4	18.4
Bacteria exposed to Na hexosediphosphate (0.01 <i>M</i>) and K ⁺ (31 μg./ml.)	61.2	60.7

a *Q*_{O₂} in an acetate medium containing vitamin B₁ (but free from Mg⁺⁺ and K⁺) as that given by the organism similarly exposed but only washed twice with 3 ml. saline. Calculation shows that the concentration of hexosediphosphate, carried over mechanically, in the final medium after the third washing cannot be greater than 3 × 10⁻⁶ *M*, and it will be seen from Table 18 that such a small quantity cannot produce the acceleration of *Q*_{O₂} noted in Table 23.

Curves illustrating the effects of exposure to hexosediphosphate on the oxidizing ability of propionic acid bacteria are shown in Fig. 2.

The effect of exposing the vitamin B₁-deficient propionic acid bacteria to hexosediphosphate on the subsequent oxidation of acetate (with or without the presence of vitamin B₁) is quantitatively the same as that of exposing the organism to Mg⁺⁺. Moreover, the effect is still quantitatively the same if the hexosediphosphate is mixed with Mg⁺⁺ in the initial incubation. This result is seen in Table 24.

If the organism be incubated in a solution containing only vitamin B₁, and then well washed, the organism subsequently oxidizes acetate (in the absence of added vitamin B₁)

as though vitamin B₁ were present. Thus incubation of the organism in a medium containing vitamin B₁ appears to saturate it with the vitamin and this cannot be removed by simple washing. Incubation of the organism with acetate in the presence of vitamin B₁, under aerobic conditions, results in the formation of an organism which subsequently

Fig. 2. A. Rate of O₂ uptake in 0.01 *M* Na acetate after preliminary aerobic incubation for 1 hr. in phosphate-saline. B. Rate of O₂ uptake in 0.01 *M* Na acetate after preliminary aerobic incubation for 1 hr. in phosphate-saline containing 0.01 *M* Na hexosediphosphate. C. Rate of O₂ uptake in 0.01 *M* Na acetate in presence of 2 μg. vitamin B₁ after preliminary aerobic incubation for 1 hr. in phosphate-saline. D. Rate of O₂ uptake in 0.01 *M* Na acetate in presence of 2 μg. vitamin B₁ after preliminary aerobic incubation for 1 hr. in phosphate-saline containing 0.01 *M* Na hexosediphosphate.

oxidizes acetate at a slightly higher rate than if the organism had been previously incubated with vitamin B₁ in the absence of acetate. Incubation of the organism in a medium containing Mg⁺⁺ and K⁺ and vitamin B₁ leads to the production of an organism which,

Table 24

Substances present in the Na phosphate (0.03 *M*)-saline medium (pH 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes of washed and previously incubated vitamin B₁-deficient propionic acid bacteria in acetate, and in acetate solution containing vitamin B₁. Conditions as in Table 2. pH 7.4. No K⁺ or Mg⁺⁺

	Q _{O₂}	
	Acetate (0.01 <i>M</i>)	Acetate (0.01 <i>M</i>) and 2 μg. vitamin B ₁
Mg ⁺⁺ (8 μg./ml.) and K ⁺ (31 μg./ml.)	4.7	13.3
Na hexosediphosphate (0.01 <i>M</i>)	10.4	50.2
2 μg. vitamin B ₁	13.6	48.3
Na acetate (0.01 <i>M</i>) and 2 μg. vitamin B ₁	14.2	13.0
Mg ⁺⁺ (8 μg./ml.), K ⁺ (31 μg./ml.) and Na acetate (0.01 <i>M</i>)	19.3	19.5
Mg ⁺⁺ (8 μg./ml.), K ⁺ (31 μg./ml.) and 2 μg. vitamin B ₁	9.5	54.2
Mg ⁺⁺ (8 μg./ml.), K ⁺ (31 μg./ml.) and 2 μg. vitamin B ₁	59.1	55.2
Mg ⁺⁺ (8 μg./ml.), K ⁺ (31 μg./ml.), Na acetate (0.01 <i>M</i>) and 2 μg. vitamin B ₁	58.2	56.5
Mg ⁺⁺ (8 μg./ml.), K ⁺ (31 μg./ml.) and Na hexosediphosphate (0.01 <i>M</i>)	12.0	50.3

after well washing, subsequently oxidizes acetate (in the absence of the ions and the vitamin) at the same rate as if the ions and vitamin B₁ were present together with the acetate. Representative results illustrating these phenomena are shown in Table 24.

They clearly indicate that the cell after incubation in the presence of Mg^{++} , or hexosediphosphate, and vitamin B_1 becomes saturated with a system bringing about optimal oxidation of acetate, and that this system can be formed in the absence of acetate.

Effect of incubation of vitamin B_1 -deficient propionic acid bacteria with Mg^{++} and K^+ on the subsequent oxidation of pyruvate

It has been shown earlier (see Table 4) that addition of Mg^{++} and K^+ to a pyruvate medium has little influence on the O_2 uptake by the bacterial suspension whether vitamin B_1 be present or not. If, however, the bacteria be incubated with Mg^{++} and K^+ for 1 hr. under the conditions already specified, the organism, after centrifuging and washing, oxidizes pyruvate at a much higher rate than if it had not been exposed to Mg^{++} and K^+ . This result is seen in Table 25. It shows that the effect of incubating the bacteria with Mg^{++} results in the formation of a system (or systems) bringing about the rapid oxidation of both acetate and pyruvate. Incubation with acetate or pyruvate, in the absence of Mg^{++} and K^+ , leads only to slightly increased subsequent rates of oxidation of these substrates (Tables 24, 25).

Table 25

Substances present in the phosphate (0.03 *M*)-saline medium (*pH* 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O_2 uptakes by washed and previously incubated vitamin B_1 -deficient propionic acid bacteria in pyruvate medium containing vitamin B_1 . Conditions as in Table 2. No K^+ or Mg^{++} . *pH* 7.4.

	Q_{O_2}
Na pyruvate (0.01 <i>M</i>) and 2 μ g. vitamin B_1	
—	13.8
Mg^{++} (8 μ g./ml.) and K^+ (31 μ g./ml.)	37.3
0.01 <i>M</i> Na pyruvate and 2 μ g. vitamin B_1	19.4
Mg^{++} (8 μ g./ml.), K^+ (31 μ g./ml.), 0.01 <i>M</i> Na pyruvate and 2 μ g. vitamin B_1	37.0

In the technique which has been described, the organism is incubated in a medium containing phosphate buffer (*pH* 7.4) as well as the substances under investigation. Absence of the phosphate, however, makes no appreciable quantitative difference to the phenomena which have been recorded. Typical results indicating this fact are shown in Table 26.

Table 26

Substances present in the medium in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O_2 uptakes by washed and previously incubated vitamin B_1 -deficient propionic acid bacteria in various media containing vitamin B_1 . No K^+ or Mg^{++} . Conditions as in Table 2. *pH* 7.4

	Q_{O_2}
0.01 <i>M</i> . Na acetate and 2 μ g. vitamin B_1	0.01 <i>M</i> . Na pyruvate and 2 μ g. vitamin B_1
Saline only	4.3
Saline, Mg^{++} (8 μ g./ml.) and K^+ (31 μ g./ml.)	11.9
Na phosphate buffer (0.03 <i>M</i>) <i>pH</i> 7.4-saline	31.7
Na phosphate buffer (0.03 <i>M</i>) <i>pH</i> 7.4-saline, Mg^{++} (8 μ g./ml.) and K^+ (31 μ g./ml.)	13.3
	13.8
	37.3

Effects of incubation of vitamin B_1 -deficient propionic acid bacteria with hexosediphosphate on their subsequent oxidation of lactate and propyl alcohol

A preliminary incubation of vitamin B_1 -deficient propionic acid bacteria with hexosediphosphate results in an organism which, after well washing, not only oxidizes acetate and pyruvate at increased rates in the presence of vitamin B_1 , but which also has increased

oxidative powers on lactate and propyl alcohol. The addition of K⁺ to the hexosediphosphate ions in the initial incubation period brings about increased rates of O₂ uptake with acetate, pyruvate and lactate. These results are seen in Table 27.

Table 27

Substances present in the Na phosphate (0.03 M)-saline medium (pH 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes by washed and previously incubated vitamin B₁-deficient propionic acid bacteria in media containing 2 μg. vitamin B₁, and various substrates. No K⁺ or Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

	Q _{O₂}			
	Acetate (0.01 M) and vitamin B ₁	Pyruvate (0.01 M) and vitamin B ₁	Lactate (0.01 M) and vitamin B ₁	Propyl alcohol and vitamin B ₁
—	16.0	13.2	22.6	15.3
K ⁺ (31 μg./ml.)	18.8	17.2	25.9	16.7
Na hexosediphosphate (0.01 M)	42.6	32.0	40.9	28.1
K ⁺ (31 μg./ml.) and Na hexosediphosphate (0.01 M)	65.1	40.7	69.2	29.8

Effects of incubation of vitamin B₁-deficient propionic acid bacteria with Mg⁺⁺ and K⁺ or with hexosediphosphate on the subsequent oxidation by the bacteria of various substrates in absence of vitamin B₁

Incubation of the organism with Mg⁺⁺ and K⁺, with subsequent thorough washing, leads to greatly increased rates of oxidation by the organism of succinate, fumarate, propyl alcohol and ethyl alcohol (in the absence of vitamin B₁, Mg⁺⁺ and K⁺). Small increases only are noted with acetate, pyruvate and lactate.

When the organism is incubated with hexosediphosphate, it is able subsequently to oxidize succinate (in the absence of vitamin B₁ and Mg⁺⁺ and K⁺) at a greatly increased rate, but the effects with acetate and lactate, in the absence of the vitamin, are small (see Tables 28, 29). Generally speaking the increased rates of oxidation of acetate,

Table 28

Substances present in the Na phosphate (0.03 M)-saline medium (pH 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes by washed and previously incubated vitamin B₁-deficient propionic acid bacteria in the presence of various substrates. No vitamin B₁ or K⁺ or Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

	Q _{O₂}							
	Acetate 0.01 M	Pyruvate 0.01 M	Lactate 0.01 M	Succinate 0.01 M	Fumarate 0.01 M	l-Malate 0.01 M	Ethyl alcohol	Propyl alcohol
—	4.7	9.0	14.4	14.7	19.3	12.4	14.4	17.6
Mg ⁺⁺ (8 μg./ml.) and K ⁺ (31 μg./ml.)	10.4	14.2	21.0	52.7	39.4	13.5	32.5	38.4

succinate etc., by vitamin B₁-deficient propionic acid bacteria, which are secured by adding Mg⁺⁺ and K⁺, or hexosediphosphate ions to these substrates, are also equally well secured by incubating the organism previously with the ions in question and then thoroughly washing the organism. It is suggested that these ions either complete or induce the formation, in the bacterial cell, of a system (or systems) essential for the oxidation of such substrates as acetate, pyruvate, fumarate, ethyl and propyl alcohols. Such an explanation will account for all the facts which have been recorded, and it is unnecessary to postulate that the presence of hexosediphosphate, for instance, is itself required for the accomplishment of the oxidations in question.

Table 29

Substances present in the Na phosphate (0.03 *M*)-saline medium (pH 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes by washed and previously incubated vitamin B₁-deficient propionic acid bacteria in the presence of various substrates. No vitamin B₁ nor K⁺ nor Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

	Q _{O₂}		
	Acetate 0.01 <i>M</i>	Succinate 0.01 <i>M</i>	Lactate 0.01 <i>M</i>
—	4.7	11.2	14.4
Mg ⁺⁺ (8 µg./ml.) and K ⁺ (31 µg./ml.)	10.4	38.6	21.0
Na hexosediphosphate (0.01 <i>M</i>)	13.6	32.4	15.6

Effects of incubation of vitamin B₁-deficient propionic acid bacteria with various substrates on the subsequent rate of oxidation by the bacteria of acetate in presence of vitamin B₁

So far the effects of incubation of vitamin B₁-deficient propionic acid bacteria only in the presence of Mg⁺⁺ and K⁺ and of hexosediphosphate have been considered. These substances have been considered first because of all substances investigated they give rise to the largest and clearest effects. Incubation of the bacteria with certain other substrates, however, give rise to organisms which, after thorough washing, oxidize acetate in the presence of vitamin B₁ at higher rates than if the organism had not received the preliminary incubation.

Fructose. Incubation with fructose for 1 hr. gives rise to an organism which oxidizes acetate in the presence of vitamin B₁ at a markedly high rate. Typical results are noted in Tables 30 and 31. Possibly fructose acts by being converted in the bacterial cell into hexosediphosphate. Glucose (Table 30) incubation has an accelerating effect, but it is smaller than that brought about by fructose incubation. Galactose and mannitol incubations bring about no change in the organisms' oxidizing power on acetate in the presence of vitamin B₁. Similarly, preliminary incubation with Na α-glycerophosphate or with Na acetate in the absence of vitamin B₁ is without effect. On the other hand, glycerol incubation leads to a definite increase of the organisms' oxidizing power (Table 30).

Table 30

Substances (0.01 *M*) present in the Na phosphate (0.03 *M*)-saline medium (pH 7.4) in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes by washed and previously incubated vitamin B₁-deficient propionic acid bacteria in presence of Na acetate and vitamin B₁. No K⁺ or Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

Exp. No. ...	µl. O ₂ uptake in 1 hr., acetate (0.01 <i>M</i>), 2 µg. vitamin B ₁							
	1	2	3	4	5	6	7	8
—	330.7	273.3	235.3	660.4	108.7	442.5	280.4	381.0
Na acetate	—	274.0	—	—	—	—	—	—
Na pyruvate	—	—	—	—	—	552.7	—	—
Na fumarate	—	—	357.1	—	161.0	—	403.0	—
Na hexosediphosphate	585.8	458.7	633.8	930.0	—	748.2	—	—
Na α-glycerophosphate	—	—	192.5	—	—	—	—	—
Glucose	374.7	—	—	795.0	—	—	446.6	—
Fructose	537.8	435.0	—	—	—	—	522.9	—
Galactose	347.5	—	—	—	—	—	—	402.0
Glycerol	—	—	—	—	—	—	493.0	—
Mannitol	—	—	—	—	—	—	—	377.0

Fumarate. It is interesting to note that incubation of the organism with sodium fumarate increases the subsequent rate of oxidation of the organism in presence of acetate and vitamin B₁, and the effect is increased by adding K⁺ to the fumarate (Tables 30, 31). It is evident, therefore, that incubation with fumarate may induce a change in the bacterial cell, similar to that brought about by incubation with hexosediphosphate (though to a much less extent), which secures a higher rate of acetate oxidation than would have

Table 31

Substances present in the phosphate (0.03 *M*)-saline medium pH 7.4 in which the preliminary incubation was carried out. Air, 37°. 1 hr.

O₂ uptakes by washed and previously incubated vitamin B₁-deficient propionic acid bacteria in presence of 0.01 *M* Na acetate and 2 μ g. vitamin B₁. No K⁺ nor Mg⁺⁺ present. Conditions as in Table 2. pH 7.4

	Q _{O₂}
—	14.6
K ⁺ (31 μ g./ml.)	18.8
Fructose (0.01 <i>M</i>)	48.4
Fructose (0.01 <i>M</i>) and K ⁺ (31 μ g./ml.)	59.2
Na fumarate (0.01 <i>M</i>)	22.9
Na fumarate (0.01 <i>M</i>) and K ⁺ (31 μ g./ml.)	30.9
Na adenytriphosphate (1 mg./ml.) and K ⁺ (31 μ g./ml.)	26.8
Adenosine (1 mg./ml.) and K ⁺ (31 μ g./ml.)	26.1
Adenine (1 mg./ml.) and K ⁺ (31 μ g./ml.)	16.5

been secured without such incubation. Fumarate ions may therefore catalyse acetate oxidation indirectly. This type of catalysis will be considered later.

Incubation of the propionic acid bacteria with the following substrates gives rise to no subsequent increase in the rate of oxidation of acetate (in the presence of vitamin B₁): Na phosphoglycerate, sorbitol, ascorbic acid, glutathione, glycine, histidine, arginine, creatine, urea.

'Adaptable' enzymes. It is worth noting at this point that the above observations might be 'explained' by assuming that during the preliminary incubation period there is an increased formation in the cell of the enzymes oxidizing acetate, succinate, propyl alcohol etc., i.e. that these are enzymes which can be built, for example, out of hexosediphosphate or its breakdown products. Such enzymes might then be regarded as adaptable in the sense they have become increased in quantity as a response to exposure to the substrates in the media in which the bacteria were incubated. This hypothesis, however, can scarcely be held in view of the fact that incubation in the presence of Mg⁺⁺ leads to precisely the same increases in oxidative activities as incubation in the presence of hexosediphosphate.

Interpretations

The oxidations of pyruvate and acetate will first be considered. It is now well recognized that cocarboxylase, and not vitamin B₁, is essential for the biological oxidation of pyruvate, and evidence already submitted [Quastel & Webley, 1941] supports the view that cocarboxylase, rather than vitamin B₁, is essential for the oxidation of acetate.

Results secured by Weil-Malherbe [1939] show that with a yeast extract the synthesis of cocarboxylase from vitamin B₁ proceeds only through the mediation of adenytriphosphate, and that in the latter's absence no synthesis of cocarboxylase takes place. Lipton & Elvehjem [1940] have arrived at the conclusion that adenytriphosphate and vitamin B₁ will react in the presence of an alkaline-washed yeast to form adenylic acid and cocarboxylase, a transfer of phosphoric acid groups taking place, Lipschitz *et al.* [1938, 1, 2] had previously demonstrated that a synthesis of cocarboxylase from vitamin B₁ will occur in the presence of washed dried yeast, hexosediphosphate and boiled tissue extract, and suggested that the vitamin was phosphorylated by adenytriphosphate or phosphorylated cozymase.

An explanation of results given in this paper comes from the conclusion that incubation of propionic acid bacteria with hexosediphosphate enriches the cells with adenytriphosphate. Such cells, saturated with adenytriphosphate, phosphorylate, at an optimal rate, vitamin B₁ subsequently added to the cells. With optimal quantities of cocarboxylase, the oxidations of pyruvate and acetate are able to proceed at optimal rates.

The mechanism whereby hexosediphosphate enriches the cell with adenylytriphosphate is not altogether clear. It may be brought about by (a) direct phosphorylation of adenylic acid [Ohlmeyer, 1935] or (b) phosphorylation of adenylic acid coupled with an oxidative reaction secured by hexosediphosphate [Needham & Pillai, 1937].

Thus the following reactions with vitamin B₁-deficient propionic acid bacteria, it is suggested, take place.

(1) *During the preliminary incubation period:* Hexosediphosphate + adenylic acid → adenylytriphosphate (either by direct phosphorylation or by a coupled oxidative reaction).

(2) *During the subsequent period in which the bacteria are incubated with acetate (or pyruvate) in the presence of vitamin B₁:*

(1) Adenylytriphosphate + vitamin B₁ = adenylic acid + cocarboxylase.

(2) Acetic acid | cocarboxylase → Products of
Pyruvic acid | O₂ → oxidation.

Now incubation of the bacteria with Mg⁺⁺ results in an effect on the subsequent oxidation of acetate and pyruvate, which is quantitatively the same as that due to incubation with hexosediphosphate, and the addition of Mg to the hexosediphosphate does not appreciably increase the effect of the latter. Since Mg is to be considered a definite constituent of the pyruvic oxidase system and presumably also of the acetic oxidase system it may be argued that the incubation of the bacteria with Mg⁺⁺ brings about a saturation of the oxidase systems in question so that they are now capable of working at their optimal rates. Whilst no doubt it is true that these systems will take up more Mg if they are not saturated, it must be conceded that the propionic acid bacterial cells must already be saturated with Mg so far as their acetic and pyruvic oxidase systems are concerned since incubation with hexosediphosphate in the absence of added Mg⁺⁺ brings about optimal rates of oxidation. Hence the accelerating effects of added Mg⁺⁺ must be concerned with mechanisms taking place before the action of the pyruvic or acetic oxidase system. Such mechanisms according to the hypothesis just advanced are phosphorylating, i.e. the phosphorylations of adenylic acid and of vitamin B₁. Since it is known that phosphorylase systems are activated by Mg⁺⁺, it is reasonable to assume that the effects of incubation with Mg⁺⁺ are concerned with a catalysis of the formation in the cell of adenylytriphosphate or of cocarboxylase or of both.

Propionic acid bacteria when taken freshly from the vitamin B₁-deficient medium in which they have been grown must contain traces of vitamin B₁, Mg⁺⁺ and of phosphoric esters, for they are still capable of bringing about the oxidation of a variety of substrates (in the absence of added vitamin B₁ or Mg⁺⁺) although at small rates.

Now even if the Mg⁺⁺ concentration in the cell is low, the velocity of phosphorylation in the cell will be increased by increase of the concentration of the phosphate donator (e.g. hexosediphosphate or adenylytriphosphate) until some saturation limit is attained. Similarly, if the concentration of phosphoric esters is low, the velocity of phosphorylation will be increased by increasing the concentration of Mg⁺⁺ to a saturation limit. It may be accepted therefore that, given a sufficiently long preliminary incubation period, admixture of the organism with hexosediphosphate or Mg⁺⁺ will lead to an enrichment of the cell with adenylytriphosphate, whose final concentration will be largely determined by the amount of adenylic acid initially present. Once a limiting concentration of adenylytriphosphate has been attained, admixture of the cell with hexosediphosphate or Mg⁺⁺ will have no further effect on the bacterial oxidations, since as has already been stated, the cell still contains sufficient Mg to complete the acetate and pyruvate oxidase systems.

Summarizing this interpretation of the action of hexosediphosphate and of Mg⁺⁺ on the bacterial oxidation of acetate and pyruvate, it is suggested:

(1) Incubation of the vitamin B₁-deficient propionic acid bacteria with hexosediphosphate or with Mg⁺⁺ leads to an enrichment of the cell with adenylytriphosphate.

(2) Adenyltriphosphate brings about the phosphorylation of vitamin B₁ to cocarboxylase.

(3) The rate of oxidation of acetate and pyruvate is determined by the amount of cocarboxylase present. There is sufficient Mg still present in the bacteria (i.e. without having incubated the organism with Mg⁺⁺) to bring about the accomplishment of processes (2) and (3).

This interpretation accounts for many of the observations concerned with acetate and pyruvate oxidation described in this paper. It does not account for the fact, however, that the addition of Mg⁺⁺ and K⁺ to pyruvate in the presence of vitamin B₁ does not appreciably increase the rate of oxidation by the bacteria (Tables 4, 5), whilst preliminary incubation with Mg⁺⁺ and K⁺ secures a marked increase in the O₂ uptake (Tables 25, 26). The explanation for this anomaly, it is suggested, may lie in an inhibitory effect of pyruvate on the synthesis of adenyltriphosphate, possibly by pyruvic acid competing with adenylic acid as a phosphate acceptor.

Incubation of the bacteria with pyruvate leads to a definite, though small, acceleration of the subsequent O₂ uptake by the vitamin B₁-deficient organism in presence of pyruvate (Table 25) or of acetate (Table 30). The acceleration which is effected would point to an enrichment of the cell with adenyltriphosphate, a process presumably brought about by the phosphorylation being coupled with pyruvate oxidation. Evidence for such a process has been secured by Lipmann [1939] and Ochoa [1940]. It is, however, sure that the acceleration brought about by pyruvate incubation is not nearly so great as that brought about by hexosediphosphate incubation, and this points to the fact that the phosphorylation obtained by the coupling with pyruvate oxidation is much less effective than that effected by incubation with hexosediphosphate.

The marked activating action due to incubation of the bacteria in fructose media (Tables 30, 31) may be due to the transformation of fructose into hexosediphosphate as has already been suggested, but it may be also due to a phosphorylation of adenylic acid coupled with oxidation of fructose. The same explanation may apply to the effects observed on the incubation of the organism under aerobic conditions with glucose, glycerol and fumarate (Tables 30, 31). The results would be in harmony with those described by Kalckar [1939, 1, 2], Lipmann [1939], and Colowick *et al.* [1940].

So far consideration has only been given to the effects of incubation of the propionic acid bacteria with hexosediphosphate, and with Mg⁺⁺ and K⁺, on the subsequent oxidation by the organism of acetate or pyruvate in the presence of vitamin B₁. Large effects, however, due to this incubation, take place on the subsequent oxidation of succinate, fumarate, propyl and ethyl alcohols in the absence of added vitamin B₁ (Tables 28, 29). Moreover, as shown early in this paper, admixture of Mg⁺⁺ and K⁺, or of hexosediphosphate, with succinate etc., results in a large increase of the rate of O₂ uptake by the vitamin B₁-deficient propionic acid bacteria.

It has been shown that the presence of Mg⁺⁺ and K⁺ brings about a marked disappearance (other than by direct oxidation) of oxaloacetate, and it has been suggested that this accounts for the acceleration of O₂ uptake of the bacteria in presence of succinate and fumarate. Whilst this view may account satisfactorily for the action of Mg⁺⁺, it can scarcely be held to provide a satisfactory explanation for the similar activating action of hexosediphosphate.

As has been stated already the effects of hexosediphosphate and Mg⁺⁺ on acetate and pyruvate oxidations can be interpreted as being due to their producing an enrichment of the cell with adenyltriphosphate.

The most obvious manner in which to interpret the results obtained with succinate, fumarate, and ethyl and propyl alcohols is to conclude that adenyltriphosphate is essential for the complete oxidation of these substances, or for their oxidation to that stage (e.g. to pyruvic acid or acetic acid) where cocarboxylase comes into play. On this hypothesis

adenyltriphosphate is a coenzyme for the oxidation of fumarate, ethyl and propyl alcohols, though its method of action in these processes may be indirect (e.g. it may stimulate the formation of cozymase required for alcohol and malate dehydrogenases).

This hypothesis of a central role played by adenyltriphosphate serves to interpret the facts set forth in this paper concerning the effects of Mg^{++} and of hexosediphosphate on the oxidations of vitamin B_1 -deficient propionic acid bacteria.

Effects of adenine compounds on oxidations by vitamin B_1 -deficient propionic acid bacteria

The incubation of vitamin B_1 -deficient propionic acid bacteria in a solution of adenyltriphosphate leads to the production of an organism which definitely oxidizes acetate at a rate higher than is achieved by the organism when it has not so been incubated. A typical result of the incubation with adenyltriphosphate (1 mg./ml.) in presence of K^+ (to increase permeability of the cell) is shown in Table 31. But although the accelerating effect is definite it is not nearly so great as that effected by incubation with hexosediphosphate or Mg^{++} . The explanation may be that the cell is highly impermeable to the triphosphate so that even in presence of K^+ only traces diffuse through, or that the adenyltriphosphate tends to lose phosphate unless there is an excess of phosphate donors present. It is noteworthy that with incubation of the organism adenosine, but not with adenine, leads to an acceleration of acetate oxidation by the bacteria (Table 31).

The incubation of propionic acid bacteria with muscle adenylic acid leads to a small but definite increase in the subsequent oxidation of acetate by the bacteria in presence of vitamin B_1 (Table 32).

Table 32

Substances present in the saline medium (no phosphate) pH 7.4 in which the preliminary incubation was carried out

O_2 uptakes by washed and previously incubated vitamin B_1 -deficient propionic acid bacteria in presence of 0.01 M Na acetate and 2 μ g. vitamin B_1 . No K^+ or Mg^{++} present. pH 7.4

	Q_{O_2}
—	3.2
Muscle adenylic acid (0.4 mg./ml.)	8.0
Mg^{++} (8 μ g./ml.) and K^+ (31 μ g./ml.)	29.8
Mg^{++} (8 μ g./ml.) and K^+ (31 μ g./ml.) and muscle adenylic acid (0.4 mg./ml.)	38.2

Note on the breakdown of adenosine and adenylic acid by propionic acid bacteria

Propionic acid bacteria are capable of breaking down adenosine and (muscle) adenylic acid as can be shown by allowing these bacteria to grow in a glucose-acetate medium containing phosphate, saline, and traces of Fe, Mg and Mn,* the N being supplied by the adenine compounds. After aerobic growth for 44 hr. at 37° on this medium with and without the adenine compounds present, the NH_3 liberated was estimated.

	μ g. NH_3 liberated per 5 ml. medium
After growth on medium in absence of adenosine or adenylic acid	28.0
After growth on medium containing 10 mg. adenosine	191.3
After growth on medium containing 10 mg. adenylic acid	90.0

Propionic acid bacteria are able to deaminate adenosine at a greater rate than (muscle) adenylic acid, a fact already observed by Stephenson & Trim [1938] using *Bact. coli*.

These observations are included to demonstrate that propionic acid bacteria are capable of metabolizing adenine compounds.

* The medium was prepared as follows: Solution A: 1 g. glucose; 0.6 g. Na acetate; 0.25 g. K_2HPO_4 ; 0.25 g. KH_2PO_4 ; to 90 ml. water. Solution B: 1 g. $MgSO_4 \cdot 7H_2O$; 0.05 g. NaCl; 0.05 g. $FeSO_4 \cdot 7H_2O$; 0.05 g. $MnSO_4 \cdot 4H_2O$; to 100 ml. water. To 4.5 ml. solution A were added 0.5 ml. solution B and 4 drops 1% Marmite solution.

Effects on vitamin B₁-deficient propionic acid bacteria, of the addition of fumarate, oxaloacetate and vitamin B₁

The addition of Na fumarate to a suspension of vitamin B₁-deficient propionic acid bacteria which is respiring in the presence of acetate brings about a definite acceleration of the O₂ uptake. This is marked, when Mg⁺⁺ and K⁺ are present (see Table 33, Exp. 2), but is dubious, or absent, when these ions are absent (see Table 33, Exp. 1). The accelera-

Table 33

O₂ uptakes by vitamin B₁-deficient propionic acid bacteria in presence of various substrates. Conditions as in Table 2. (Mg⁺⁺=8 μg./ml., K⁺=31 μg./ml., where present.) pH 7.4 except in Exp. 5, where it was 9.0. Air, 37°.

Substrates		Q _{O₂}
Exp. 1.	Nil	16.4
	Na acetate (0.01 M)	22.3
	Na acetate (0.01 M) + 2 μg. vitamin B ₁	38.9
	Na fumarate (0.003 M)	19.9
	Na acetate (0.01 M) + Na fumarate (0.003 M)	25.4
	Na oxaloacetate (0.003 M)	18.1
	Na acetate (0.01 M) + Na oxaloacetate (0.003 M)	21.5
Exp. 2.	Mg ⁺⁺ + K ⁺	12.6
	Na acetate (0.01 M) + Mg ⁺⁺ + K ⁺	31.3
	Na fumarate (0.0016 M) + Mg ⁺⁺ + K ⁺	23.6
	Na acetate (0.01 M) + Na fumarate (0.0016 M) + Mg ⁺⁺ + K ⁺	52.4
Exp. 3.	Na acetate (0.01 M) + Mg ⁺⁺ + K ⁺	26.9
	Na oxaloacetate (0.003 M) + Mg ⁺⁺ + K ⁺	21.7
	Na acetate (0.01 M) + Na oxaloacetate (0.003 M) + Mg ⁺⁺ + K ⁺	38.1
	Mg ⁺⁺ + K ⁺	12.6
Exp. 4.	Nil	18.8
	Na succinate (0.01 M)	27.7
	Na oxaloacetate (0.002 M)	23.8
	Na succinate (0.01 M) + Na oxaloacetate (0.002 M)	36.0
	2 μg. vitamin B ₁	25.0
	Na succinate (0.01 M) + 2 μg. vitamin B ₁	70.5
	Na oxaloacetate (0.002 M) + 2 μg. vitamin B ₁	37.2
	Na succinate (0.01 M) + Na oxaloacetate (0.002 M) + 2 μg. vitamin B ₁	76.3
Exp. 5.	At pH 9.0	
	Mg ⁺⁺ + K ⁺	13.7
	Na acetate (0.01 M) + Mg ⁺⁺ + K ⁺	29.5
	Na acetate (0.01 M) + Mg ⁺⁺ + K ⁺ + 2 μg. vitamin B ₁	80.6
	Na oxaloacetate (0.002 M) + Mg ⁺⁺ + K ⁺	31.7
	Na acetate (0.01 M) + Na oxaloacetate (0.002 M) + Mg ⁺⁺ + K ⁺	48.3
	Na pyruvate (0.01 M)	30.7
	Na pyruvate (0.01 M) + 2 μg. vitamin B ₁	53.7
	Na pyruvate (0.01 M) + Na oxaloacetate (0.002 M)	38.4

tion is of interest in view of the known acceleration of pyruvate oxidation by fumarate with dialysed pigeon brain suspensions [Banga *et al.* 1939, 1, 2]. Conceivably the 4-C dicarboxylic acid system acts catalytically during the oxidation of acetate but as has been stated earlier there is no evidence that acetate oxidation proceeds through succinate or fumarate. It is significant that incubation of the bacteria with fumarate with subsequent thorough washing gives rise to an organism with an increased ability to oxidise acetate in the presence of vitamin B₁ (see Tables 30, 31). The explanation which is suggested for this phenomenon is that oxidation of fumarate is coupled with a phosphorylation leading to an enrichment of the cell with adenylytriphosphate. It seems likely, therefore, that catalysis of acetate oxidation by propionic acid bacteria by direct admixture with fumarate is due also to the increased formation in the cell of adenylytriphosphate. It may in fact be suggested that some of the catalyses by fumarate, which have been recorded in the literature, are due not so much to the direct interplay of the C₄-dicarboxylic acid system as to an increased rate of formation of adenylytriphosphate. The adoption of a technique similar to that adopted in this work would help to distinguish between the two types of fumarate catalyses.

The addition of sodium oxaloacetate to a suspension of propionic acid bacteria respiring in an acetate medium leads to little or no acceleration of the acetate oxidation (see Exps. 1, 3 and 5; Table 33). Increases of rates of O_2 uptake are observed but they are nearly always the increases to be expected if the oxidations of acetate and oxaloacetate are proceeding additively (or independently). A catalysis due to oxaloacetate is, however, not entirely ruled out, for sometimes more than an additive effect is perceptible. Possibly when a catalysis is observed, it is due to the formation of fumarate from the oxaloacetate by reduction or dismutation [cf. Smyth, 1940].

A suggestion has been made that vitamin B_1 catalyses the formation of oxaloacetate and that the latter acts as a catalytic agent in the oxidation of pyruvate [Krebs & Eggleston, 1940; Smyth, 1940]. Without denying the possibility that vitamin B_1 is involved in oxaloacetate formation, it is easy to show that oxaloacetate cannot replace vitamin B_1 as an accelerator of the oxidation of acetate and propionate by propionic acid bacteria. Results demonstrating this are shown in Table 33 (Exps. 1, 4 and 5). For example a Q_{O_2} of 30.7 obtained when vitamin B_1 -deficient propionic acid bacteria are incubated in the presence of pyruvate, is increased to 53.7 by the addition of vitamin B_1 and only to 38.4 by the addition of oxaloacetate. It is apparent that vitamin B_1 exerts its catalytic effect on acetate and pyruvate oxidations by a process other than by the development of oxaloacetate. Krampitz & Werkman [1941] arrive at a similar conclusion concerning pyruvate oxidation.

Effects of NaF and Na iodoacetate

When propionic acid bacteria are incubated with Na hexosediphosphate in the presence of NaF, no decrease of the subsequent acceleration of acetate oxidation by the bacteria due to the hexosediphosphate takes place (see Exp. 1, Table 34). This is in harmony with the observation of Lutwak-Mann & Mann [1935] that NaF does not influence the formation of adenylyltripphosphate from adenylic acid and hexosediphosphate.

The replacement of NaF by Na iodoacetate leads to a diminution of the ability of the bacteria to oxidize acetate and also greatly inhibits (though it does not eliminate) the accelerating effect of hexosediphosphate. A representative result is shown in Table 34. Since it is known that iodoacetate inhibits oxido-reductions involving hexosediphosphate,

Table 34

Substances present in the phosphate (0.03 M)-saline medium pH 7.4 in which the preliminary incubation was carried out		O_2 uptakes by washed and previously incubated vitamin B_1 -deficient propionic acid bacteria in presence of 0.01 M Na acetate and 2 μ g. vitamin B_1 . No K^+ or Mg^{++} present. pH 7.4. Conditions as in Table 2	
Exp.		Q_{O_2}	
Exp. 1.	—	27.1	
	NaF (0.16 %)	30.9	
	Na hexosediphosphate (0.01 M)	65.8	
	Na hexosediphosphate (0.01 M) + NaF (0.16 %)	66.7	
Exp. 2.	K^+ (31 μ g./ml.)	29.9	
	Na hexosediphosphate (0.01 M)	79.9	
	+ K^+ (31 μ g./ml.)		
	Na iodoacetate (0.01 M) + K^+ (31 μ g./ml.)	10.6	
	Na hexosediphosphate (0.01 M) + Na iodoacetate (0.01 M) + K^+ (31 μ g./ml.)	27.8	

the iodoacetate effect may be held as evidence in favour of the acceleration of acetate oxidation being brought about by adenylyltripphosphate formation coupled with an oxido-

reduction. On the other hand, it is not yet certain how far iodoacetate will inhibit oxidative processes after the formation of adenylyltriophosphate. The full meaning of this observation must, therefore, await further experimental work.

SUMMARY

1. Acetate oxidation by vitamin B₁-deficient propionic acid bacteria is accelerated, in the presence of vitamin B₁, by both Mg⁺⁺ and K⁺, but the effect of the addition of a mixture of both ions is much greater than the sum of the effects of the ions taken singly. Acetate oxidation is increased with increase of pH (till 9.0).

2. The effects of addition of Mg⁺⁺ and K⁺ are to accelerate bacterial oxidation of acetate and propionate in the presence of vitamin B₁, but not that of butyrate or formate. The effect of Mg⁺⁺ (in presence of K⁺) is apparent at a concentration of 1 μg./ml. A similar accelerating effect of Mg⁺⁺ and K⁺ is noted with the Na salts of lactic and α-ketobutyric acids; it is scarcely perceptible with Na pyruvate.

3. The presence of Mg⁺⁺ and K⁺ does not influence the oxidation of acetate and propionate in the absence of vitamin B₁.

4. The presence of Mg⁺⁺ and K⁺ greatly catalyses the oxidation of the following substances in the absence of added vitamin B₁: succinate, fumarate, lactate, ethyl and propyl alcohols, glucose. Here also, the presence of K⁺ augments the effect due to Mg⁺⁺. It is suggested that the effect of K⁺ is to increase the permeability of the cells of propionic acid bacteria to Mg⁺⁺.

5. The presence of Mg⁺⁺ and K⁺ catalyses the breakdown of oxaloacetate and pyruvate by means other than by direct oxidation in vitamin B₁-deficient propionic acid bacteria.

6. The oxidation of glycerol, like that of pyruvate, whilst it is accelerated by the presence of vitamin B₁ is not influenced by the presence of Mg⁺⁺ and K⁺.

7. Na citrate, α-ketoglutarate, oxalate, glycollate, β-hydroxybutyrate, α-glycerophosphate, hexosediphosphate and glutamate are feebly (or not) oxidized by vitamin B₁-deficient propionic acid bacteria, their rates being but little affected by the presence of vitamin B₁ or Mg⁺⁺ and K⁺.

8. The effects of the addition of Mg⁺⁺ cannot be obtained on the addition of Mn⁺⁺. The latter ions at low concentrations exercise an inhibition of the bacterial oxidation of acetate.

9. Accelerating effects on the oxidations by vitamin B₁-deficient propionic acid bacteria, both in the presence and absence of vitamin B₁ can also be obtained by addition to the bacteria of Na hexosediphosphate. The effects of this phosphoric ester are quantitatively the same as those brought about by addition of Mg⁺⁺. The admixture of hexosediphosphate and Mg⁺⁺ does not produce an effect greater than either of the ions. The presence of K⁺ augments the action of hexosediphosphate ions, and a change of cell permeability is held to account for this. Sodium α-glycerophosphate has no accelerating action.

10. When vitamin B₁-deficient propionic acid bacteria are incubated aerobically for 1 hr. at 37° in the presence of Mg⁺⁺ and K⁺, the bacteria being then thoroughly washed, they subsequently exercise greatly increased rates of oxidation of acetate and pyruvate, but only in the presence of vitamin B₁. They also bring about greatly increased rates of O₂ uptake in the presence of succinate, fumarate, ethyl and propyl alcohols and these effects are observable in the absence of vitamin B₁.

11. Preliminary incubation of the vitamin B₁-deficient propionic acid bacteria in the presence of hexosediphosphate, with subsequent thorough washing of the bacteria, brings about the formation of bacteria with identically the same increased oxidative powers, as are obtained after incubation with Mg⁺⁺.

12. It is shown that the increased catalytic powers of the bacteria due to preliminary incubation with Mg^{++} and K^+ or with hexosediphosphate, cannot be due to traces of these substances being carried over mechanically with the washed cells.

13. Preliminary aerobic incubation of the bacteria with pyruvate (in the presence of vitamin B_1) brings about increased catalytic powers on the subsequent oxidation of acetate and pyruvate—but the effect is not as large as with hexosediphosphate or with Mg . Incubation with fructose has a relatively large accelerating action on the subsequent oxidation by the bacteria of acetate in the presence of vitamin B_1 . Incubation with glucose and glycerol has much smaller effects. Preliminary aerobic incubation with Na fumarate also has a marked accelerating action on the subsequent bacterial oxidation of acetate in the presence of the vitamin.

14. Preliminary incubation of the bacteria with adenylytriphosphate produces a small but definite accelerating effect on acetate oxidation in the presence of vitamin B_1 .

15. The above results can be interpreted on the following hypotheses:

(a) Incubation of the organism with Mg or hexosediphosphate ions leads to an enrichment of the cell with adenylytriphosphate. A similar enrichment (but to a smaller extent) occurs by a coupling of phosphorylation with oxidations, when the cells are incubated aerobically with pyruvate, fumarate, and possibly with fructose, glucose and glycerol. In the case of fructose however there may be conversion into hexosediphosphate.

(b) A cell enriched with adenylytriphosphate brings about the optimal rate of phosphorylation of vitamin B_1 to form cocarboxylase.

(c) Cells having optimal amounts of cocarboxylase oxidise acetate and pyruvate at optimal rates, assuming that Mg ions are still present in the cells.

This interpretation is discussed and is held to account satisfactorily for all observed facts.

16. On this view adenylytriphosphate is essential for the oxidation in the intact cell of fumarate, and of ethyl and propyl alcohols.

17. It is shown that propionic acid bacteria are able to deaminate adenosine and adenylic acid, the former molecule being attacked at a greater rate than the latter.

18. It is pointed out that catalysis of oxidations by fumarate may not only be due to the direct interplay of the C_4 -dicarboxylic acid system but to the formation of adenylytriphosphate by a coupled phosphorylation.

19. Addition of oxaloacetate does not bring about accelerating effects comparable with those produced by vitamin B_1 and no evidence has been found with propionic acid bacteria to support the view that the catalytic effects of vitamin B_1 are due to the formation of oxaloacetate.

20. NaF has no inhibitory action on the accelerating effects of hexosediphosphate, but these are greatly retarded by the presence of Na iodoacetate.

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