The conversion of ethyl alcohol and butyrate to caproate by Clostridium kluvyeri was postulated (1) to involve the following reactions:

\[
\begin{align*}
\text{Ethanol} + \text{inorganic phosphate} & \rightarrow \text{acetyl phosphate} + 4\text{H} \quad (1) \\
\text{Acetyl phosphate} + \text{ADP} & \rightarrow \text{acetate} + \text{ATP} \quad (2) \\
\text{ATP} + \text{butyrate} & \rightarrow \text{butyryl phosphate} + \text{ADP} \quad (3) \\
\text{Butyryl phosphate} + \text{acetate} + 4\text{H} & \rightarrow \text{caproate} + \text{inorganic phosphate} \quad (4)
\end{align*}
\]

Reaction 1 was observed in cell-free enzyme preparations of C. kluvyeri (21), but the participation of the remaining reactions is supported only by indirect evidence obtained from studies on other bacteria (2). Koepsell et al. (12) demonstrated the formation of butyryl phosphate from acetyl phosphate and butyrate by cell-free extracts of Clostridium butylicum; and Lipmann (13, 14) demonstrated the reversible transfer of phosphoryl groups between acetyl phosphate and the adenylic acid system by enzyme preparations of C. butylicum and Lactobacillus delbrueckii, and further postulated that the transfer of the phosphoryl group between acetyl phosphate and butyrate is mediated by adenosine pyrophosphate.

In order to obtain a clearer insight into the mechanisms of fatty acid synthesis we have investigated the occurrence of Reactions 2, 3, and 4 and also several other reactions of acyl phosphates in enzyme preparations of C. kluvyeri.

**Methods**

**Chromatographic Analysis of Acyl Phosphates**

To obtain a quantitative estimation of the various acyl phosphates in a mixture, these substances were converted to their hydroxamic acid derivatives (17), which are easily separated by paper chromatography. After separation, the various hydroxamic acids were located on the paper chromatogram by converting them to their highly colored ferric iron complexes; these were readily extracted from the paper and estimated

---

1 The following abbreviations are used: ADP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, adenosine monophosphate.
quantitatively by a colorimetric procedure. Details of the method are as follows:

Reagents—
Hydroxylamine hydrochloride. A 28 per cent solution of hydroxylamine hydrochloride was mixed with an equal volume of 3.5 N sodium hydroxide just prior to use.

Ferric chloride. Two different solutions were used, a strong solution, made by dissolving 50 gm. of FeCl₃·6H₂O in 1 liter of 95 per cent alcoholic 0.1 N HCl and a weak solution made by mixing 5 gm. of FeCl₃·6H₂O, 25 ml. of concentrated HCl, and 575 ml. of water.

Ethanol, 95 per cent and absolute.

Procedure—From 0.5 to 1.0 ml. of the test solution was added to 0.5 ml. of the hydroxylamine reagent. After 10 minutes at room temperature, 25 ml. of 95 per cent ethanol were added and the precipitated protein was removed by centrifugation. The supernatant was evaporated to a volume of 3 to 4 ml. on a steam bath and finally to dryness at room temperature. The residue was extracted with 2.0 ml. of absolute ethanol and the supernatant was concentrated to 0.1 to 0.2 ml. at room temperature. The sample was centrifuged and the supernatant was used for chromatographic analysis.

From 0.01 to 0.02 ml. of the sample was used on a 50 cm. strip of filter paper. The developing solvent was water-saturated butanol. The technique used in preparing and developing the paper chromatogram was the same as that employed by Hotchkiss (7).

The chromatogram was developed unidimensionally for 5 to 10 hours at 28°. After drying the paper at room temperature, the various hydroxamic acid spots were located by spraying the paper with the concentrated ferric chloride solution.

---

**Table I**

<table>
<thead>
<tr>
<th>Hydroxamic acid derivative</th>
<th>( R_f ) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl phosphate</td>
<td>0.52</td>
</tr>
<tr>
<td>Propionyl phosphate</td>
<td>0.65</td>
</tr>
<tr>
<td>Butyryl phosphate</td>
<td>0.76</td>
</tr>
<tr>
<td>Valeryl &quot;</td>
<td>0.81</td>
</tr>
<tr>
<td>Caproyl &quot;</td>
<td>0.86</td>
</tr>
</tbody>
</table>

* The \( R_f \) value is defined as the distance moved by the compound divided by the distance moved by the solvent. The values are reproducible within ±0.05 unit. The developing solvent was water-saturated butanol; temperature 28°.
The hydroxamic acids were identified by their \( R_f \) values (Table I) and by comparison with synthetic hydroxamic acids.

To obtain a quantitative estimation of the various hydroxamic acids, the individual spots were cut from the paper strip and placed in colorimeter tubes. 6.0 ml of the dilute ferric chloride solution were added and the solution was agitated. After 1 to 2 minutes, essentially all of the hydroxamic acid was extracted. The paper was removed and the optical density of the solution was measured with an Evelyn colorimeter equipped with a 540 nm filter.

**Sensitivity**—Hydroxamic acids in amounts of 0.01 to 0.02 \( \mu \text{M} \) produce easily detectable spots when the above method is used. Satisfactory quantitative estimations can be made when the spots contain 0.1 to 3.0 \( \mu \text{M} \).

**TABLE II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Acyl P added</th>
<th>Acyl P recovered</th>
<th>Average recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{M} )</td>
<td>( uM ) ratio</td>
<td>( uM ) ratio</td>
</tr>
<tr>
<td>Acetyl P</td>
<td>17</td>
<td>1.00</td>
<td>15.3</td>
</tr>
<tr>
<td>Propionyl P</td>
<td>21</td>
<td>1.23</td>
<td>18.2</td>
</tr>
<tr>
<td>Butyryl P</td>
<td>22</td>
<td>1.29</td>
<td>19.5</td>
</tr>
<tr>
<td>Valeryl P</td>
<td>23</td>
<td>1.35</td>
<td>19.0</td>
</tr>
<tr>
<td>Caproyl P</td>
<td>21</td>
<td>1.23</td>
<td>19.2</td>
</tr>
</tbody>
</table>

**Recovery**—Presented in Table II are the results of experiments demonstrating the applicability of the method for estimating the relative amounts of acyl phosphates in mixtures. In these experiments, various combinations of synthetic acyl phosphates having 2 to 6 carbon atoms were analyzed by the above method. As shown in the last column of Table II, the average total recovery of the individual acyl phosphates after chromatographic separation was about 85 per cent.

Fortunately the loss was non-specific; good agreement (100 ± 5 per cent) was obtained between the ratios of added and recovered acyl phosphates. In practice, therefore, the total acyl phosphate concentration of the test solution was determined directly by the hydroxylamine method of Lipmann and Tuttle (17) and a separate aliquot of the test solution was analyzed by the chromatographic method to determine the relative proportions of the different compounds. From these data, it was possible to compute the actual concentrations of the individual acyl phosphates in the test solution.
Other methods used in the experimental work have already been described (15, 20-24).

Results

Transfer of Phosphoryl Group of Acetyl Phosphate to Adenylic Acid—To determine whether the phosphoryl group of acetyl phosphate could be transferred to adenylic acid, various mixtures of acetyl phosphate and adenylic acid were incubated with cell-free extracts. After various periods of incubation, the concentrations of acetyl phosphate and of pyrophosphate (7 minute-hydrolyzable phosphate) were determined. The results, presented in Table III, show that the disappearance of acetyl phosphate is increased by the addition of adenylic acid and that there is a corresponding increase in the amount of pyrophosphate. The data therefore indicate that the phosphoryl group of acetyl phosphate was transferred to adenylic acid (Reaction 5).

\[ \text{Acetyl phosphate} + \text{AMP} \rightarrow \text{acetate} + \text{ADP} \]  

(5)

Comparable changes were not observed in a control sample (not shown in Table III) containing boiled enzyme. In those samples in which the initial acetyl phosphate concentration was 5 to 10 times greater than that of adenylic acid, the molar quantity of 7 minute-hydrolyzable phosphate formed was 1.5 or more times greater than that of adenylic acid added. These results indicate that transphosphorylation does not
stop at the ADP stage but that ATP is formed also. A direct confirmation of this was obtained from an experiment in which acetyl phosphate was incubated with ADP. The results (Table IV) show that when 5.8 \( \mu M \) of ADP were incubated with 30 \( \mu M \) of acetyl phosphate 3.4 \( \mu M \) of 7 minute-hydrolyzable phosphate were formed and there was a corresponding increase in the amount of acetyl phosphate lost. It is evident therefore that the phosphoryl group of acetyl phosphate can be transferred to ADP (Reaction 6).

\[
\text{Acetyl phosphate} + \text{ADP} \rightarrow \text{acetate} + \text{ATP}
\]

\text{(6)}

**Table IV**

*Transfer of Acetyl-Bound Phosphorus to ADP*

20 mg. of cell-free extract (Lot D) and the indicated substrates were incubated in a volume of 2.0 ml. for 130 minutes at 26° in an evacuated Thunberg tube.

<table>
<thead>
<tr>
<th>Acetyl P, initial</th>
<th>ADP (P(_i)) added</th>
<th>P(_f), final</th>
<th>Acetyl P, final</th>
<th>(\Delta ) acetyl P due to ADP added</th>
<th>(\Delta ) P(_f) due to ADP added</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
<td>2.3</td>
<td>23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>5.8</td>
<td>11.5</td>
<td>19</td>
<td>4</td>
<td>3.4</td>
</tr>
</tbody>
</table>

**Table V**

*Transfer of Labile Phosphorus of ADP and ATP to Acetate*

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acetate, initial</th>
<th>ADP, initial</th>
<th>ATP, initial</th>
<th>P(_i), initial</th>
<th>Incubation time</th>
<th>Acetyl P, final</th>
<th>P(_f), final</th>
<th>(\Delta ) P(_f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>min.</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
</tr>
<tr>
<td>1*</td>
<td>155</td>
<td>19.4</td>
<td>0</td>
<td>19.4</td>
<td>95</td>
<td>1.4</td>
<td>15.6</td>
<td>3.8</td>
</tr>
<tr>
<td>2†</td>
<td>155</td>
<td>0</td>
<td>0</td>
<td>0.8</td>
<td>75</td>
<td>0</td>
<td>12.5</td>
<td>3.9</td>
</tr>
</tbody>
</table>

* 50 mg. of Lot D, cell-free extract, and the indicated substrates in 2.0 ml. volume were incubated in an evacuated Thunberg tube, 26°.

† 25 mg. of Lot D and the indicated substrates in 1.5 ml. volume were incubated in an evacuated Thunberg tube, 26°.

**Transfer of Labile Phosphorus of Adenyl Pyrophosphates to Acetate**—To determine whether the transphosphorylations described by Reactions 5 and 6 are reversible, mixtures of ADP and acetate and ATP and acetate were incubated with the enzyme preparation. The results (Table V) show that small but significant amounts of acetyl phosphate were formed. The amounts produced were of the same order of magnitude as those observed by Lipmann (13) in his studies with enzyme preparations of *C. butylicum* and *L. delbrueckii*. From these results it may be concluded that Reactions 5 and 6 are reversible.
It is probable that in these studies a final equilibrium was not attained from either side owing to complicating side reactions. It is obvious, however, that the equilibrium favors the formation of adenyl pyrophosphates.

Data similar to those obtained in the above experiments were used by Lipmann (10, 13) to calculate the equilibrium constant for the reaction. For these calculations, it was assumed that ADP, not adenylic acid, was the true phosphoryl group acceptor (Reaction 3). This assumption was based upon the observation by Meyerhof et al. (18) (see also Bücher (4)) that in a similar reaction between 1,3-diphosphoglyceric acid and the adenylic acid system ADP and not adenylic acid was the true acceptor. Accordingly, Lipmann concluded that when adenylic acid is active, as it was in his enzyme preparations, probably a phosphodismutation, with some ATP present in the preparation, yields ADP in the manner described by Kalckar (9) (Reaction 7), and that the ADP thus formed is the true acceptor of the phosphoryl group of acetyl phosphate.

\[
\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP}
\]  

On the basis of these assumptions, Lipmann obtained an equilibrium constant, \( K = \frac{\text{ATP} \times \text{acetate}}{\text{ADP} \times \text{acetyl P}} = 177 \), for Reaction 6. A similar calculation (see Lipmann (13) for details) from the data of Experiment 2, Table V, gave a value of 360. Considering the many assumptions made in these calculations and the probable failure to achieve a true equilibrium, the agreement between the latter value and that obtained by Lipmann is not unreasonable.

Myokinase Activity—Since the method of calculating the equilibrium constant for the reaction between acetyl phosphate and the adenylic acid system assumes a rapid phosphodismutation of adenosine diphosphate, it seemed desirable to determine experimentally whether the latter reaction actually occurs in the enzyme preparations of \( C. \text{kluuyveri} \). This was done by incubating a mixture of AMP and ATP with the enzyme preparation and looking for a disappearance of AMP (Reaction 7). AMP was estimated by the method of Kalckar (9, 26) and the adenylic compounds in the barium-soluble and insoluble fractions were estimated by a pentose determination after acid hydrolysis (26).

The results were entirely negative. It is therefore probable that in the transphosphorylation between acetyl phosphate and the adenylic acid system both adenylic acid and adenosine diphosphate can function directly as phosphoryl group acceptors. In view of these results, it is obvious that calculations of the type used by Lipmann to determine the equilibrium constant of the transphosphorylation between acetyl phosphate and ADP are inadequate when applied to the \( C. \text{kluuyveri} \) system.
In other experiments it has been shown that the enzyme preparations catalyze the transfer of the phosphoryl group of butyryl phosphate to adenylic acid. However, in view of the fact that a direct transphosphorylation probably occurs between butyryl phosphate and acetate (see below) one cannot conclude that transfer of the phosphoryl group from butyryl phosphate to adenylic acid is direct. It is possible that the small amount of acetate present in the enzyme preparation serves as an intermediate phosphoryl group carrier, as indicated in the following scheme.

\[
\text{Butyryl phosphate} \quad \downarrow \quad \text{Acetate} \quad \uparrow \quad \text{ADP} \quad \uparrow \\
\text{Butyrate} \quad \text{Acetyl phosphate} \quad \text{AMP}
\]

Evidence that such a mechanism may contribute to the transfer of the phosphoryl group from butyryl phosphate to adenylic acid was obtained by showing that the addition of acetate to a mixture of butyryl phosphate and adenylic acid increased the rate of transphosphorylation about 20 per cent. A final decision as to the mechanism of this reaction must await the preparation of a more highly purified enzyme that does not contain catalytic amounts of acetate.

**Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Propionate**

Summarized in Table VI are the results of several experiments showing that enzyme preparations of C. kluyveri catalyze a rapid reversible transphosphorylation between acetyl phosphate and propionate (Reaction 8).

\[
\text{CH}_3\text{COOPO}_4^- + \text{CH}_2\text{CH}_2\text{COO}^- \rightleftharpoons \text{CH}_3\text{COO}^- + \text{CH}_2\text{CH}_2\text{COOPO}_4^- \quad (8)
\]

In Experiments 1 and 2, the enzyme was incubated with acetyl phosphate and propionate. After various periods of incubation, the test solution was analyzed chromatographically for acetyl phosphate and propionyl phosphate. The results show that there was a rapid decrease in the acetyl phosphate concentration and an increase in the concentration of propionyl phosphate. In Experiment 3, propionyl phosphate was incubated with acetate and a rapid transfer of the phosphoryl group to acetate was observed.

In Experiment 4 (Table VI), acyl phosphates were not added but were produced enzymatically by the oxidation of valeric acid (Reaction 9).

\[
\text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + \text{HPO}_4^{2-} + \text{O}_2 \rightarrow \text{CH}_3\text{CH}_2\text{COOPO}_4^- + \text{CH}_3\text{COO}^- + \text{H}_2\text{O} \quad (9)
\]

The steam-volatile acids produced in this oxidation are propionic and acetic. 1 mole of acyl phosphate is also formed. It is not known at present whether the immediate products of the reaction are propionyl...
phosphate and acetate, as indicated in Reaction 9, or acetyl phosphate and propionate. In any event the data of Experiment 4, Table VI, show that a rapid transphosphorylation occurs, forming ultimately both propionyl and acetyl phosphates in almost equal amounts.

These experiments demonstrate very clearly the reversible nature of the transphosphorylation on the acyl phosphate level. The attainment of a true equilibrium is indicated by the fact that essentially the same

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Incubation time</th>
<th>Acetate</th>
<th>Propionate</th>
<th>Propionyl P</th>
<th>Acetyl P</th>
<th>( K = \frac{\text{propionyl P} \times \text{acetate}}{\text{acetyl P} \times \text{propionate}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 min.</td>
<td>1.7</td>
<td>20</td>
<td>0</td>
<td>30</td>
<td>1.06</td>
</tr>
<tr>
<td>1</td>
<td>15 min.</td>
<td>14.1</td>
<td>8.6</td>
<td>11.4</td>
<td>17.6</td>
<td>0.92</td>
</tr>
<tr>
<td>1</td>
<td>30 min.</td>
<td>3.5</td>
<td>40</td>
<td>0</td>
<td>40</td>
<td>1.06</td>
</tr>
<tr>
<td>1</td>
<td>180 min.</td>
<td>22.2</td>
<td>21.3</td>
<td>18.7</td>
<td>21.3</td>
<td>1.10</td>
</tr>
<tr>
<td>2</td>
<td>0 min.</td>
<td>8.9</td>
<td>10.5</td>
<td>10.0</td>
<td>5.8</td>
<td>1.29</td>
</tr>
<tr>
<td>2</td>
<td>5 min.</td>
<td>30</td>
<td>10.7</td>
<td>9.8</td>
<td>6.0</td>
<td>1.17</td>
</tr>
<tr>
<td>2</td>
<td>30 min.</td>
<td>7.5</td>
<td>10.9</td>
<td>9.6</td>
<td>6.2</td>
<td>1.07</td>
</tr>
<tr>
<td>2</td>
<td>60 min.</td>
<td>3.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.08</td>
</tr>
<tr>
<td>2</td>
<td>75 min.</td>
<td>25.3</td>
<td>22.5</td>
<td>19.1</td>
<td>19.8</td>
<td></td>
</tr>
</tbody>
</table>

equilibrium constant for Reaction 8, \( K = \frac{\text{propionyl P} \times \text{acetate}}{\text{acetyl P} \times \text{propionate}} \), was obtained in all of the above experiments (see the last column of Table VI). The value of the equilibrium constant indicates that the phosphate bond energies in propionyl and acetyl phosphates are nearly identical. The rate of the transphosphorylation reaction is evidently very high, since almost complete equilibration of the two acyl phosphates was reached within 5 minutes.

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Butyrate—Koepsell et al. (12) have reported a rapid and extensive trans-
phosphorylation between acetyl phosphate and butyrate by cell-free extracts of *C. butylicum*. It seemed probable, therefore, that a similar transphosphorylation would be catalyzed by extracts of *C. kluyveri*, especially since a rapid reaction occurs between acetyl phosphate and propionate. The data of Table VII show that such a phosphate trans-

**Table VII**

Reversible Transfer of Phosphoryl Group of Acetyl Phosphate to Butyrate

Experiments 1 and 2, 125 mg. of dried cells of *C. kluyveri* (Lot I), pH 7.6, suspended in 5 ml. of water, were incubated anaerobically with the indicated substrates at 26°.

Experiments 3 and 4, 90 mg. of cell-free extract of *C. butylicum* (Koepsell et al. (12)), pH 6.8, were dissolved in 3.6 ml. of water and incubated anaerobically with the indicated substrates at 37°.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Incubation time</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Acetyl P</th>
<th>Butyryl P</th>
<th>( K ) = butyryl P X acetate ( ) / butyryl P X acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>min.</td>
<td>( \mu M ) per ml.</td>
<td>( \mu M ) per ml.</td>
<td>( \mu M ) per ml.</td>
<td>( \mu M ) per ml.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.5</td>
<td>40</td>
<td>40</td>
<td>0</td>
<td>3.5</td>
<td>40</td>
</tr>
<tr>
<td>15</td>
<td>6.3</td>
<td>39.2</td>
<td>37.2</td>
<td>0.8</td>
<td>0</td>
<td>3.5/3.5</td>
</tr>
<tr>
<td>60</td>
<td>9.6</td>
<td>38.9</td>
<td>33.9</td>
<td>1.1</td>
<td>0</td>
<td>3.5/3.5</td>
</tr>
<tr>
<td>120</td>
<td>14.5</td>
<td>39.0</td>
<td>29.0</td>
<td>1.0</td>
<td>0</td>
<td>3.5/3.5</td>
</tr>
<tr>
<td>180</td>
<td>17.9</td>
<td>39.1</td>
<td>25.6</td>
<td>0.9</td>
<td>0</td>
<td>3.5/3.5</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>43.5</td>
<td>2.0</td>
<td>0</td>
<td>38.0</td>
<td>2.0</td>
<td>43.5/43.5</td>
</tr>
<tr>
<td>35</td>
<td>39.4</td>
<td>20.5</td>
<td>4.1</td>
<td>19.5</td>
<td>20.5</td>
<td>39.4/39.4</td>
</tr>
<tr>
<td>90</td>
<td>40.4</td>
<td>30.1</td>
<td>3.1</td>
<td>9.9</td>
<td>30.1</td>
<td>40.4/40.4</td>
</tr>
<tr>
<td>195</td>
<td>40.4</td>
<td>37.9</td>
<td>3.1</td>
<td>2.1</td>
<td>37.9</td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>12.9</td>
<td>58.5</td>
<td>35.6</td>
<td>0</td>
<td>12.9</td>
<td>12.9/12.9</td>
</tr>
<tr>
<td>30</td>
<td>31.1</td>
<td>54.1</td>
<td>27.4</td>
<td>4.0</td>
<td>54.1</td>
<td>31.1/31.1</td>
</tr>
<tr>
<td>90</td>
<td>37.7</td>
<td>53.0</td>
<td>20.8</td>
<td>5.2</td>
<td>53.0</td>
<td>37.7/37.7</td>
</tr>
<tr>
<td>195</td>
<td>44.0</td>
<td>53.0</td>
<td>14.5</td>
<td>5.2</td>
<td>53.0</td>
<td>44.0/44.0</td>
</tr>
<tr>
<td>315</td>
<td>49.6</td>
<td>51.4</td>
<td>8.9</td>
<td>4.1</td>
<td>51.4</td>
<td>49.6/49.6</td>
</tr>
<tr>
<td>4</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>45.0</td>
<td>27.0</td>
<td>0</td>
<td>31.1</td>
<td>27.0</td>
<td>45.0/45.0</td>
</tr>
<tr>
<td>45</td>
<td>44.1</td>
<td>30.5</td>
<td>0.9</td>
<td>27.5</td>
<td>30.5</td>
<td>44.1/44.1</td>
</tr>
<tr>
<td>115</td>
<td>43.4</td>
<td>33.5</td>
<td>1.7</td>
<td>24.5</td>
<td>33.5</td>
<td>43.4/43.4</td>
</tr>
<tr>
<td>215</td>
<td>42.8</td>
<td>36.9</td>
<td>2.2</td>
<td>21.1</td>
<td>36.9</td>
<td>42.8/42.8</td>
</tr>
</tbody>
</table>

fer does occur; however, the rate of the reaction was very slow and in no instance was it possible to observe a considerable net formation of butyryl phosphate from acetyl phosphate and butyrate or of acetyl phosphate from butyryl phosphate and acetate. Thus, in Experiment 1, Table VII, starting with 40 \( \mu M \) of acetyl phosphate and 40 \( \mu M \) of butyrate, about 1 \( \mu M \) of butyryl phosphate was produced after 15 minutes incubation, but no further increase was obtained even after 3 hours, although the acetyl phosphate was decreased by phosphatase action. These re-
results could be interpreted to mean that the enzyme catalyzing the transphosphorylation was inactivated after 15 minutes. However, this is probably not true, since the constancy of the butyryl phosphate concentration in a preparation containing an active butyryl phosphatase (Table VII) indicates that transphosphorylation was taking place throughout the experiment. The possibility that acetyl phosphate in relatively high concentration inhibits the hydrolysis of butyryl phosphate by phosphatase action was investigated and no such effect could be observed.

In Experiment 2, Table VII, the reverse reaction, the transfer of the phosphoryl group from butyryl phosphate to acetate, was studied. Again a relatively slow transphosphorylation was observed. After 35 minutes, 4.1 μM of acetyl phosphate had been produced but longer incubation did not cause a further accumulation of acetyl phosphate, although the butyryl phosphate level declined rapidly and only 2.1 μM were left after 195 minutes, at which time there were still 3.1 μM of acetyl phosphate.

The above results show that a true equilibrium was not attained in the acetyl phosphate-butyrate reaction, probably because of the decomposition of the acyl phosphates by phosphatase action. Not only is the rate of hydrolytic cleavage of the acyl phosphates of the same order of magnitude as the rate of transphosphorylation, but in addition the two acyl phosphates are decomposed at markedly different rates. It will be shown below that the hydrolysis of butyryl phosphate is 3.5 times more rapid than the hydrolysis of acetyl phosphate. It should be emphasized that these considerations do not apply to the acetyl phosphate-propionate transphosphorylation, since the rate of transphosphorylation between these substrates is very much greater than the rate of phosphatase action. Moreover, the rates of enzymatic hydrolysis of acetyl phosphate and propionyl phosphate are more nearly the same. The observed equilibrium constant for the acetyl phosphate-propionate reaction is undoubtedly fairly reliable.

Similar studies have shown that a transphosphorylation also occurs between acetyl phosphate and valeric and caproic acids. The rate of transphosphorylation to these acids is somewhat slower than to butyrate.

In studies of the transphosphorylation between acetyl phosphate and butyrate with enzyme preparations of C. butylicum Koepsell et al. (12) found as much as 30 per cent of the total acyl phosphate as butyryl phosphate. This suggested that the true equilibrium for the reaction might be much more in favor of butyryl phosphate than our results with C. kluyveri enzymes indicated. The higher percentage of butyryl phosphate observed with C. butylicum could be due to a more favorable ratio between the rates of transphosphorylation and dephosphorylation.
Fortunately we were able to obtain a small sample of the *C. butylicum* preparation originally used by Koepsell and Johnson\(^2\) (11) and to compare its behavior directly with that of the *C. kluyveri* preparations (Experiments 3 and 4, Table VII). The results demonstrate that the *C. butylicum* preparation, though 7 years old, was still able to catalyze a transphosphorylation between acetyl phosphate and butyrate. The data of Experiment 3 show that after 90 minutes incubation of the enzyme with a mixture of acetyl phosphate and butyrate 20 per cent of the total acyl phosphate was the butyrate derivative. This is a much larger percentage of butyryl phosphate than was obtained with *C. kluyveri* preparations, and also the rate of transphosphorylation was significantly greater. However, the rate was not enough greater than the rate of the interfering hydrolytic reactions to permit a reliable determination of the equilibrium constant. This constant cannot be established with certainty until the transphosphorylating enzyme preparation has been freed of phosphatase activity.

**Rôle of Adenylic Acid in Synthesis and Oxidation of Butyrate**—It has been pointed out already that a postulated mechanism for caproic acid synthesis involves a series of transphosphorylation reactions, as indicated by Reactions 2 and 3. In the preceding experiments it was shown that reactions of this type are catalyzed by enzyme preparations of *C. kluyveri*. It appears possible therefore that these reactions actually are involved in fatty acid synthesis as postulated. In the course of these studies, however, it was found that not all enzyme preparations were equally able to catalyze the transphosphorylations. For example, with the cell-free enzymes of Lots C and D\(^\prime\) (20), the transfer of the phosphoryl group of acetyl phosphate to adenylic acid was very rapid, being almost complete after 10 minutes of incubation. The corresponding transphosphorylation with the dried cell preparation of Lot H was relatively slow and the reaction did not occur at all with the dried cells or cell-free enzymes of Lots G, E, or E\(^\prime\). The possibility that the latter preparations contained an inhibitor was excluded, since the addition of these preparations to the cell-free extracts of Lot D produced no change in the rate of transphosphorylation by the latter. Although the enzyme preparations of Lot G were unable to catalyze a transfer of the phosphoryl group of acetyl phosphate to adenylic acid or adenosine diphosphate, they were able to catalyze the synthesis and oxidation of caproate. It must be concluded that transphosphorylations involving the adenylic acid system do not participate in caproate synthesis or oxidation.

The possibility that adenylic acid served as an intermediate carrier

\(^2\) We are indebted for this preparation to Dr. E. J. Ordal of the University of Washington, who obtained it from Dr. Koepsell.
in the transfer of the phosphoryl group of acetyl phosphate to butyrate
(Reactions 2 and 3) was excluded by the observation that the cell-free
extract of Lot G could catalyze a transphosphorylation between acetyl
phosphate and butyrate but could not catalyze a transfer to adenylic
acid or adenosine diphosphate. These results suggest that the phos-
phoryl group can be transferred directly from acetyl phosphate to other
fatty acids. In any event adenylic acid and adenosine diphosphate are
not obligatory phosphate carriers.

Rôle of Butyryl Phosphate in Synthesis and Oxidation of Caproate—
While the above experiments appear to rule out adenylic acid and its
derivatives as important components of the system causing the synthesis
and oxidation of fatty acids, it still appeared possible that butyryl phos-
phate was involved in these reactions.

If butyryl phosphate is a normal intermediate in the oxidation of
caproate (the reverse of Reaction 4), one would expect it to accumulate
to some extent when caproate is oxidized aerobically. Therefore, an
experiment was done in which the acyl phosphates produced in the oxida-
tion of caproate were examined for the presence of butyryl phosphate.
20 μM amounts of caproate were incubated with the enzyme preparation
(dried cells, Lot I) in Warburg vessels in air. When one-half (20 μM)
and three-quarters (30 μM) of the theoretical oxygen had been consumed,
14 and 26 μM of acyl phosphate were formed, respectively, and the oxida-
tion was stopped by the addition of hydroxylamine reagent. The enzy-
me mixtures were analyzed chromatographically for acetyl and butyryl
phosphates. In neither sample was it possible to detect even trace
amounts of butyryl phosphate. Practically all of the acyl phosphate
present was acetyl phosphate. A trace amount of caproyl phosphate,
probably formed by secondary transphosphorylation, was also detected.
The absence of butyryl phosphate indicates, but does not definitely
prove, that this compound is not a normal product of caproate oxida-
tion. The possibility remains that butyryl phosphate was preferentially
oxidized further to acetyl phosphate and acetate and therefore failed to
accumulate. To explore this possibility, experiments were done to com-
pare the rates of oxidation of caproate and synthetic butyryl phosphate.
It was found that the two compounds are oxidized at about the same
rate, and therefore it is unlikely that a preferential utilization of butyryl
phosphate can account for the failure to detect this compound as a prod-
uct of caproate oxidation.

Reduction of Acyl Phosphates to Their Corresponding Alcohols—To
determine whether butyryl phosphate could be an intermediate in the
synthesis of caproate (Reaction 4) an experiment was performed in
which butyryl phosphate and acetate were incubated with the enzyme
preparation in an atmosphere of hydrogen. The results showed that hydrogen was taken up. However, almost as much hydrogen was consumed by a sample containing butyryl phosphate alone (36.4 μM) as by a mixture of butyryl phosphate and acetate (49 μM). Since relatively small amounts of acetate (2 to 4 μM) were present in the enzyme preparation, it is obvious that the reduction of butyryl phosphate alone could not be due to the formation of caproate. It appeared probable, therefore, that the butyryl phosphate had been reduced to butanol. Since propanol is found as a normal product of the fermentation of ethanol and propionate by growing cultures of C. kluyveri (3, 25), it also seemed likely that this substance was formed by reduction of propionyl phosphate.

**Table VIII**

Reduction of Acyl Phosphates to Corresponding Alcohols

Each Warburg vessel contained 50 mg. of dried cells (Lot I), 0.1 M trishydroxymethylaminomethane buffer (pH 7.6), and 100 μM of the acyl phosphates. Total liquid volume, 2.0 ml.; gas phase, H₂.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acyl P</th>
<th>Rate of H₂ uptake, initial, per 15 min.</th>
<th>H₂ uptake, total</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>c.mm.</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>1</td>
<td>Propionyl P</td>
<td>132</td>
<td>111</td>
<td>55.5</td>
</tr>
<tr>
<td></td>
<td>Butyryl P</td>
<td>116</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caproyl &quot; &quot;</td>
<td>77</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>2†</td>
<td>Butyryl &quot; &quot;</td>
<td>151</td>
<td>151</td>
<td>75.5</td>
</tr>
</tbody>
</table>

* Estimated by the method of Johnson (8).
† In Experiment 2, the contents of eight similar Warburg vessels were combined in order to provide sufficient material to analyze.

Proof that the acyl phosphates are reduced to the corresponding alcohols was obtained in other experiments. The data of Table VIII show that propionyl, butyryl, and caproyl phosphates are reduced with molecular hydrogen. The rate of hydrogen uptake varies inversely with the length of the carbon chain. Thus the relative rates of reduction of propionyl, butyryl, and caproyl phosphates were 100, 88, and 58, respectively. After hydrogen uptake had ceased, the propionyl and butyryl phosphate samples were adjusted to pH 9.0 and distilled. The volatile alcohols recovered in the distillates were oxidized to their corresponding fatty acids with acid dichromate (8). Duclaux distillation of the fatty acids showed that almost pure propionic and butyric acids were derived from the propionyl and butyryl phosphate samples, respectively. Small amounts of acetate (about 5 per cent) also found were undoubtedly
formed, mainly by overoxidation of the alcohols with the acid dichromate (27). The total amount of acid recovered from the alcohol oxidations was approximately 75 per cent of that to be expected if all of the hydrogen consumed was utilized in the reduction of the acyl phosphates to the corresponding alcohols (Reaction 10).

\[
\text{RCOOPO}_2^- + 2H_2 \rightarrow RCH_2OH + HPO_4^- \quad (10)
\]

The fact that butyryl phosphate is more readily converted to butyl alcohol than to caproic acid is a strong argument against the idea that butyryl phosphate is a normal intermediate in the metabolism of C. kluyveri, because butyl alcohol is never formed as a normal fermentation product.

**Hydrolytic Decomposition of Acyl Phosphates by Phosphatase Action**—Throughout this investigation and in previous studies with enzymes of C. kluyveri (20–24) reference has been made to the fact that the enzyme preparations contain active acyl phosphatases and the failure to observe a perfect stoichiometric relationship in reactions involving acetyl or other acyl phosphates was due to the enzymatic hydrolysis of these compounds. It seemed desirable, therefore, to present the results of experiments designed to demonstrate the magnitude of this effect.

Since the hydrolysis of an acyl phosphate leads to the formation of 1 equivalent of acid (Reaction 11), the reaction can be followed manometrically if it is carried out in a bicarbonate buffer.

\[
\text{RCOOPO}_2^- + H_2O \rightarrow \text{RCOO}^- + HPO_4^- + H^+ \quad (11)
\]

The results of an experiment on the rates of hydrolysis of the acyl phosphates of fatty acids containing from 2 to 6 carbon atoms are given in Table IX. Experiment 1 shows that the rate of hydrolysis increases with the number of carbon atoms, reaching a maximum with valeryl phosphate which is decomposed about 4 times more rapidly than acetyl phosphate. Phosphatase activity is completely destroyed by heating the enzyme preparation at 100° for 10 minutes. In this respect, the enzyme differs from the relatively thermostable animal acylphosphatase obtained from horse muscle (14).

Of some significance was the observation that the relative rates of hydrolysis of different acyl phosphates were not the same for different enzyme preparations. For example, in Experiment 1, Table IX, in which dried cells of Lot I were used, the enzymatic hydrolysis of butyryl phosphate was about 3.5 times more rapid than that of acetyl phosphate. However, with the cell-free extract of Lot E (Experiment 2), the rate was roughly 10 times as great. These results suggest that acetyl phosphate and butyryl phosphate are decomposed by different enzymes.
In order to obtain further information on this possibility the rates of hydrolysis of mixtures of acyl phosphates were compared with the rates of hydrolysis of the individual compounds. The results of two such experiments are given in Table X. In Experiment 1, the rates of hydrolysis of acetyl phosphate, valeryl phosphate, and a mixture of the two were compared. The decomposition of the acetyl phosphate was measured manometrically by carbon dioxide evolution from a bicarbonate solution and also by direct colorimetric measurement (17). The data show that the rate of decomposition of the mixture was considerably greater than the rate with either substrate alone, and in fact was almost equal to the sum of the two individual rates. This shows that acetyl phosphate and valeryl phosphate are non-competitive substrates and indicates that they are decomposed by different enzymes. In Experiment 2, the rates of hydrolysis of valeryl phosphate, caproyl phosphate, and a mixture of the two were compared. With these compounds the rate of hydrolysis of the mixture was intermediate between the individual rates, thus indicating a competition of the substrates for a single

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Compound</th>
<th>Enzyme lot</th>
<th>Rate of CO₂ evolution, observed, per 15 min.*</th>
<th>Rate of CO₂ evolution due to phosphatase action, per 15 min.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetyl P</td>
<td>I (Boiled)</td>
<td>2.4</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Propionyl P</td>
<td>&quot; &quot;</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Butyryl P</td>
<td>&quot; &quot;</td>
<td>58</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Valeryl &quot;</td>
<td>&quot; &quot;</td>
<td>64</td>
<td>51</td>
</tr>
<tr>
<td>me ryl P</td>
<td>&quot; &quot;</td>
<td>&quot; (Boiled)</td>
<td>46</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Caproyl &quot; + val-</td>
<td>&quot; &quot;</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>eryl P</td>
<td>&quot; &quot; (Boiled)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>&quot; &quot; &quot;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; &quot;</td>
<td>&quot; &quot;</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Acetyl P</td>
<td>E</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Butyryl P</td>
<td>&quot; &quot;</td>
<td>87</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>&quot; &quot; (Boiled)</td>
<td></td>
<td>4.6</td>
<td></td>
</tr>
</tbody>
</table>

* Based on the second 15 minute period of incubation.
† Data corrected for spontaneous hydrolysis of the acyl phosphate (about 3 c.mm. per 15 minutes) and for the enzyme blank.
enzyme. Considering all the available information, it may be concluded that at least two distinct phosphatases, differing in their ability to hydrolyze acetyl phosphate and the higher acyl phosphates, are active in the enzyme preparations. The simplest interpretation would be that one enzyme is specific for acetyl phosphate, whereas the other can attack all of the acyl phosphates or is specific for only the higher homologues.

Arsenolytic Decomposition of Acetyl Phosphate

Effect of Arsenate on Hydrolysis of Acetyl Phosphate—Mention has already been made of the fact that arsenate is a powerful inhibitor of certain reactions catalyzed by C. kluyveri. For example, arsenate prevents the phosphoroclastic splitting of acetoacetate, the oxidation of butyrate and vinyl acetate, and the reduction of acetyl phosphate to butyrate. However, it has no effect on the reduction of acetoacetate to \( \beta \)-hydroxybutyrate. The latter fact indicates that arsenate does not influence the hydrogen-activating system and its effect is probably more intimately concerned with the reactions of acetyl phosphate.

In addition to the reduction of acetyl phosphate to butyrate, two other reactions involving acetyl phosphate have been demonstrated; namely, the transfer of the phosphoryl group to adenylic acid and adenosine diphosphate and the hydrolysis of acetyl phosphate to acetate and inorganic...
phosphate. Therefore, experiments were done to ascertain whether arsenate has any effect on either of these reactions. Somewhat unexpected was the discovery that arsenate catalyzed the complete and very rapid decomposition of acetyl phosphate. Thus, when 17 μM of acetyl phosphate were incubated with the enzyme preparation in the presence of 0.0025 M arsenate, no acetyl phosphate could be detected after 15 minutes. With 0.02 M arsenate, none was left after 5 minutes. This catalytic effect of arsenate on acetyl phosphate decomposition was completely absent when boiled enzyme preparations were used.

If the disappearance of acetyl phosphate catalyzed by arsenate is due to hydrolysis, 1 mole of acid would be formed (Reaction 11) and the reaction could be followed manometrically in a bicarbonate solution. The data of an experiment carried out to investigate this possibility are plotted in Fig. 1 which shows that the decomposition of acetyl phosphate in the presence of arsenate results in the liberation of 1 equivalent of acid, in agreement with Reaction 11.

Specificity of Arsenolysis of Acyl Phosphates—In view of the large catalytic effect of arsenate on the hydrolysis of acetyl phosphate, it
seemed desirable to determine whether arsenate has a similar effect on the hydrolysis of the higher acyl phosphates. The experimental results presented in Table XI show that a very powerful catalytic effect of arsenate (26- to 65-fold) was attained only with acetyl phosphate. With the higher homologues a much smaller stimulation of the hydrolysis rate (1.1- to 2.5-fold) was observed.

The hydrolysis of propionyl phosphate in the presence of arsenate was increased only slightly by the addition of acetate (Experiment 2, Table XI). Since this enzyme preparation catalyzes a rapid transphosphorylation between propionyl phosphate and acetate (Table VI), one would expect that the acetyl phosphate produced by transphosphorylation would be decomposed rapidly by arsenolysis. The observed slow hydrolysis therefore suggested that propionyl phosphate or propionate, or both, inhibits the arsenolysis of acetyl phosphate. An experiment was performed to test directly the effect of these and other substances on the arsenolysis of acetyl phosphate. The results given in Table XII show clearly that propionyl phosphate is a strong specific inhibitor of the arsenolysis of acetyl phosphate. The other substances tested, namely acetate, propionate, butyrate, and butyryl phosphate, had only slight effects. Propionate is a special case, however. If this substance is added to the enzyme at the same time as arsenate, it has little effect on the arsenolysis of acetyl phosphate (Sample 3, Table XII). If, however, the propionate is allowed to incubate with the enzyme for some time prior to addition of the arsenate (Sample 7), a marked inhibition of

### Table XI

**Arsenolytic Decomposition of Various Acyl Phosphates**

Each Warburg vessel contained 0.11 M NaHCO₃ and 20 μM of acyl phosphate. The total liquid volume was 1.0 ml. Gas phase, 5 per cent CO₂ in nitrogen; temperature, 26°C.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Acyl P</th>
<th>Rate of CO₂ evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No arsenate</td>
</tr>
<tr>
<td></td>
<td>c.mm. per 5 min.</td>
<td>c.mm. per 5 min.</td>
</tr>
<tr>
<td>1. 50 mg. dried cells (Lot I)</td>
<td>Acetyl P</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Butyryl P</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Valeryl ℴ</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Caproyl ℴ</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Propionyl P</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Butyryl P</td>
<td>8</td>
</tr>
<tr>
<td>2. 30 mg. cell-free extract (Lot E)</td>
<td>Propionyl P</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Propionyl P + acetate, 30 μM</td>
<td>9</td>
</tr>
</tbody>
</table>
acetyl phosphate decomposition is observed, owing undoubtedly to the fact that considerable propionyl phosphate is formed by transphosphorylation.

In other experiments that will not be described in detail, it was shown that the inhibition of propionyl phosphate cannot be overcome by the addition of more acetyl phosphate. The inhibition depends upon the relative concentration of propionyl phosphate and arsenate. Inhibition occurs when the molar ratio of propionyl phosphate to arsenate is 2:1 or above; when the ratio is 1:1.5, there is almost no inhibition. The results indicate that propionyl phosphate inhibition of the arseno¯ysis of acetyl phosphate is competitive with respect to arsenate rather than acetyl phosphate.

At low enzyme concentrations (0.75 per cent) at which the arseno¯ysis of acetyl phosphate is fairly slow and can be measured accurately, it has been observed repeatedly that the rate of arseno¯ysis changes abruptly after about 50 to 75 per cent of the acetyl phosphate has been decomposed. This is not a gradual change. During the first period of decomposition (usually 20 to 40 minutes) the rate is constant, but then it changes very rapidly, within about 5 minutes, to another rate that is

### Table XII

**Specific Inhibition of Arsenolytic Decomposition of Acetyl Phosphate by Propionyl Phosphate**

Each Warburg vessel contained 15 mg. of cell-free extract (Lot E), 0.11 M NaHCO₃, 0.0025 M arsenate, and 16 µM of acetyl phosphate. The total liquid volume was 2.0 ml. Gas phase, 95 per cent H₂-5 per cent CO₂; temperature, 26°.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Inhibitor</th>
<th>Rate of CO₂ evolution per 15 min.</th>
<th>c.mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td></td>
<td>131</td>
</tr>
<tr>
<td>2</td>
<td>Acetate, 40 µM</td>
<td></td>
<td>106</td>
</tr>
<tr>
<td>3</td>
<td>Propionate, 40 µM</td>
<td></td>
<td>125</td>
</tr>
<tr>
<td>4</td>
<td>Butyrate, 40 µM</td>
<td></td>
<td>103</td>
</tr>
<tr>
<td>5</td>
<td>Butyryl P, 18 µM</td>
<td></td>
<td>113</td>
</tr>
<tr>
<td>6</td>
<td>Propionyl P, 16 µM</td>
<td></td>
<td>38</td>
</tr>
<tr>
<td>7†</td>
<td>Propionate, 40 µM</td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

* In this experiment only 15 mg. of enzyme were used in order to slow down the arseno¯ysis reaction to a point at which accurate rate measurements could be made.

† The propionate and the acetyl phosphate were both incubated with the enzyme during a 25 minute equilibration period prior to dumping the arsenate from the side arm. In all other samples, the acetyl phosphate was incubated with the enzyme during the equilibration period and the inhibitor and arsenate were both placed in the side arm and mixed with the enzyme simultaneously after equilibration.
twice as great. So far no adequate explanation of this effect has been developed. In view of this effect, it should be mentioned that all decomposition rates given in this paper refer to the initial rates.

The catalytic effect of arsenate on the hydrolysis of acetyl phosphate is strongly reminiscent of a similar effect of arsenate on the hydrolysis of glucose-1-phosphate by the glucose-transferring enzyme obtained from the bacterium *Pseudomonas saccharophila* (6). In the latter instance, the course of the arsenolytic decomposition is represented in Scheme I.

According to Scheme I the enzyme is in reality a *trans*-glucosidase whose function is to accept glucose from a suitable donor such as glucose-1-phosphate and transfer it to an appropriate acceptor which may be any one of several monosaccharides, inorganic phosphate, or arsenate. When arsenate is the acceptor, it is believed that glucose-1-arsenate is formed and that this substance undergoes a rapid spontaneous hydrolysis to glucose and arsenate. Thus, the net effort is a hydrolysis of glucose-1-phosphate to glucose and inorganic phosphate.

The arsenolytic decomposition of acetyl phosphate by the cell-free extracts of *C. kluyveri* can be represented in an analogous way (Scheme II) if it is assumed that the reaction is catalyzed by an acetyl transferring enzyme or *trans*-acetylase.

---

**Scheme I**

\[
\begin{align*}
\text{Glucose-1-phosphate + enzyme} & \xrightleftharpoons[\pm \text{arsenate}]{\pm \text{phosphate}} \text{glucose-enzyme} \\
& \downarrow \quad \downarrow \\
& \text{glucose-1-arsenate + enzyme} \\
& \downarrow \\
& \text{glucose + arsenate}
\end{align*}
\]

**Scheme II**

\[
\begin{align*}
\text{Acetyl phosphate + enzyme} & \xrightleftharpoons[\pm \text{arsenate}]{\pm \text{phosphate}} \text{acetyl-enzyme} \\
& \downarrow \\
& \text{acetyl arsenate + enzyme} \\
& \downarrow \\
& \text{acetate + arsenate}
\end{align*}
\]
Exchange of Acetyl-Bound and Inorganic Phosphate—According to Schemes I and II the primary reactions are reversible (Reaction 12 and 13.)

Glucose-1-phosphate + enzyme ⇌ glucose-enzyme + inorganic phosphate (12)
Acetyl phosphate + enzyme ⇌ acetyl-enzyme + inorganic phosphate (13)

Doudoroff, Barker, and Hassid (5) obtained evidence in support of Reaction 12 by incubating glucose-1-phosphate with the enzyme preparation of *P. saccharophila* in the presence of P³²-labeled inorganic phosphate and observed a rapid exchange of phosphate between the ester and the inorganic form.

**TABLE XIII**

Exchange of Inorganic and Acetyl-Bound Phosphorus

Each Thunberg tube contained 25 mg. of cell-free extract (Lot G) and 50 μm each of acetyl phosphate and P³²-labeled inorganic phosphate (specific activity about 30,000 c.p.m. per μm) in a total liquid volume of 3.0 ml. The enzyme used in the control sample had been inactivated by heating at 100° for 10 minutes. pH 8.0. After incubating at 26° for 15 minutes in the evacuated Thunberg tubes, the inorganic phosphate was separated from the acetyl phosphate by calcium precipitation; phosphate determinations (Fiske-Subbarow) and the radioactivity measurements were made on suitable aliquots of the calcium-insoluble fraction (redissolved in dilute HCl) and on the calcium-soluble fraction.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Quantity recovered after incubation</th>
<th>Specific activity after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td>c.p.m. per μM</td>
</tr>
<tr>
<td>Boiled (control) Inorganic P</td>
<td>63</td>
<td>30,500</td>
</tr>
<tr>
<td>&quot; &quot; Acetyl P</td>
<td>45</td>
<td>100</td>
</tr>
<tr>
<td>Active. Inorganic P</td>
<td>61</td>
<td>17,400</td>
</tr>
<tr>
<td>&quot; Acetyl P</td>
<td>42</td>
<td>15,700</td>
</tr>
</tbody>
</table>

A similar experiment was performed with the cell-free extract of *C. kluyveri* to determine whether Reaction 13 is tenable. Acetyl phosphate was incubated with the enzyme in the presence of P³²-labeled inorganic phosphate. After incubation, the acetyl phosphate and inorganic phosphate were separated and analyzed for radioactivity. A similar sample containing an inactive enzyme preparation, obtained by heating at 100° for 10 minutes, was incubated as a control. The data (Table XIII) show that after 15 minutes incubation with the active enzyme the molar specific activity of the acetyl-bound phosphate was 90 per cent of that of the inorganic phosphate. Evidently an almost complete equilibration of the phosphate in the two forms had been achieved. Relatively little radioactivity was observed in the acetyl phosphate isolated from
the control sample, indicating that the phosphate exchange was enzyme-catalyzed. The small amount of $^{32}P$ in the acetyl phosphate fraction of the control sample was probably due to a slight contamination with inorganic phosphate.

A perfect parallelism thus exists between the reactions of acetyl phosphate catalyzed by the extracts of \textit{C. kluyveri} and those of glucose-1-phosphate catalyzed by the glucose-transferring enzyme of \textit{P. saccharophila}. This supports the view that the extracts of \textit{C. kluyveri} contain a \textit{trans-acetylase}.

This is not, however, the only possible explanation of the results. The arsenolysis of acetyl phosphate and the phosphate exchange could also occur if acetyl phosphate were reversibly reduced to acetaldehyde and inorganic phosphate. This explanation is not very attractive in view of the fact that no reducing agent was added in the above experiments. It could be argued, however, that such a reduction was catalyzed by trace amounts of a reduced coenzyme already present in the enzyme preparation (Reaction 14).

$$\text{CH}_3\text{C}O\text{OP}_4^- + \text{CoH}_2 \rightleftharpoons \text{CH}_3\text{CHO} + \text{HPO}_4^- + \text{Co} \quad (14)$$

Since arsenate can substitute to a limited extent for inorganic phosphate in the oxidation of acetaldehyde (21), such a reversible reduction of acetaldehyde could explain the arsenolytic action on acetyl phosphate as well as the phosphate exchange.

During the course of studies with various enzyme preparations of \textit{C. kluyveri}, one preparation, the cell-free extract of Lot E, was found to be completely incapable of catalyzing the oxidation of acetaldehyde. However, this preparation could catalyze the rapid arsenolytic decomposition of acetyl phosphate (Fig. 1). Thus the possibility that arsenolysis occurs by means of a reversible reduction of acetyl phosphate by Reaction 14 appears to be excluded.

One other possible mechanism for the arsenolysis reaction should be mentioned; namely, a reversible condensation of acetyl phosphate with acetate (Reaction 15).

$$\text{CH}_3\text{C}O\text{OP}_4^- + \text{CH}_3\text{CO}^- \rightleftharpoons \text{C}_4 \text{ compound} + \text{H}_3\text{PO}_4 \quad (15)$$

That such a condensation of acetyl phosphate and acetate must occur is evident from the fact that acetyl phosphate and acetate can be reduced to butyrate. It has not been established, however, that the primary condensation results in the liberation of inorganic phosphate.

Of some interest in this connection are studies by Lipmann and Tuttle (16) with enzyme preparations of \textit{C. butylicum} and \textit{Escherichia coli}. Their preparations also catalyzed a rapid exchange between acetyl-
bound phosphate and inorganic phosphate. In view of the fact that enzyme preparations of C. butylicum catalyzed the breakdown of pyruvate to acetyl phosphate, CO₂, and H₂ (Reaction 16), while the enzyme preparation of E. coli catalyzed the phosphoroclastic splitting of pyruvate to acetyl phosphate and formate (Reaction 17), Lipmann and Tuttle concluded that the phosphate exchange was due primarily to a reversibility of these reactions.

\[
\begin{align*}
\text{CH}_3\text{COOO}^- + \text{H}_2\text{PO}_4^- & \rightleftharpoons \text{CH}_3\text{COOPO}_3^- + \text{H}_2 + \text{CO}_2 & \text{(16)} \\
\text{CH}_3\text{COOO}^- + \text{H}_2\text{PO}_4^- & \rightleftharpoons \text{CH}_3\text{COOPO}_3^- + \text{HCOOH} & \text{(17)}
\end{align*}
\]

Noteworthy, however, was the fact that the rates of phosphate exchange in both systems were not influenced significantly by the addition of reactants other than acetyl phosphate, e.g., hydrogen, CO₂, or formate. In view of this situation, it would seem that their conclusion that the phosphate exchange was due mainly to a reversibility of Reactions 16 and 17 is inadequately supported. An alternative explanation would be that the exchange was catalyzed by a trans-acetylase according to Scheme II.

**DISCUSSION**

The evidence presented in the experimental section demonstrates clearly that the theory of caproic acid formation described in the introduction is largely incorrect in spite of the fact that three of the four postulated reactions are catalyzed by enzymes derived from C. kluyveri. Reaction 1, the oxidation of ethanol to acetyl phosphate, certainly participates in caproic acid synthesis. Reaction 2, the transphosphorylation between acetyl phosphate and the adenylic acid system, can occur and it may participate in the utilization of phosphate bond energy for the synthesis of cellular constituents, but it is not essential for butyric or caproic acid synthesis. Reaction 3, the transphosphorylation between adenosine triphosphate and butyrate, is also catalyzed by the enzyme system, but it does not appear to have any physiological significance. Indeed, the evidence we have obtained supports the view that butyryl phosphate does not participate in the synthesis or oxidation of caproic acid (Reaction 4) and is not formed in significant quantities during the normal metabolism of C. kluyveri. The latter conclusion is based on the observation that butyl alcohol, the main product of butyryl phosphate reduction by the enzyme preparation, is not formed by living bacteria from ethyl alcohol and acetate, whereas propyl alcohol is formed in quantity from ethyl alcohol and propionate.

The virtual exclusion of butyryl phosphate as a precursor of caproic acid opens the possibility that the reactants in the C₄-C₂ condensation

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are butyrate and acetyl phosphate. If this is correct, the presence of the phosphoryl group in acetyl phosphate must activate the methyl as well as the carboxyl group.

The enzyme that causes the arsenolytic decomposition of acetyl phosphate is of special interest because it appears to catalyze a transfer of acetyl groups from one acceptor to another. The only acceptors so far identified are phosphate and arsenate.\(^3\) However, in view of the obvious importance of C\(_2\) unit condensations in fatty acid synthesis, it seems likely that the transacetylating enzyme also reacts with organic acceptors to form products directly involved in elongation of the carbon chain.

It has long been suspected, on the basis of indirect evidence, that butyl alcohol is formed in bacteria by reduction of butyric acid or a derivative. The conversion of butyric acid to butyl alcohol was clearly demonstrated by Wood et al. (28) using C\(^{13}\) as a tracer. The direct reduction of the free carboxyl group was unlikely, because such a reaction requires a very powerful reducing system. Following the discovery of the carboxyl phosphate compounds (19, 13) Lipmann recognized that the reduction of butyric acid would be greatly facilitated in a thermodynamic sense by phosphorylation of the carboxyl group. Our experiments provide the first direct evidence for the enzymatic reduction of acyl phosphates to the corresponding alcohols. However, it may be noted that this reduction is merely the reverse of the oxidation of alcohols to acyl phosphates, a reaction previously studied with enzyme preparations of C. kluyveri (21).

**SUMMARY**

Enzyme preparations of *Clostridium kluyveri* catalyze a rapid reversible transfer of the phosphoryl group of monoacetyl phosphate to adenylic acid and adenosine diphosphate. The transfer to adenylic acid appears to be direct, since no myokinase activity could be detected.

A quantitative chromatographic procedure has been developed by which acyl phosphate derivatives of the lower fatty acids (2 to 6 carbon atoms) can be estimated in a mixture. By means of this method it was shown that enzyme preparations of *C. kluyveri* catalyze a transfer of the phosphoryl group of acetyl phosphate to fatty acids of 3 to 6 carbon atoms. These transphosphorylations are not mediated by the adenylic acid system.

With molecular hydrogen as the reducing agent, acyl phosphate derivatives of propionate, butyrate, valerate, and caproate are reduced enzymatically to the corresponding alcohols. Evidence is presented that

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\(^3\) Recently amino acids have been found to act as acetyl acceptors under special conditions.
appears to eliminate butyryl phosphate from consideration as an intermediate in the synthesis and oxidation of caproate.

In the presence of arsenate, the enzyme preparations catalyze a rapid and complete hydrolysis of acetyl phosphate to acetate and inorganic phosphate. This arsenolysis reaction is specific for acetyl phosphate; arsenate has little or no influence on the rate of decomposition of other acyl phosphates. Several types of evidence indicate that the arsenolysis reaction is catalyzed by an acetyl-transferring enzyme.

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