THE CONVERSION OF LACTYL CoA TO ACRYLYL CoA IN PEPTOSTREPTOCOCCUS ELDENII: A NEW α-PHOSPHOLACTYL CoA INTERMEDIATE

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THE CONVERSION OF LACTYL CoA TO ACRYLYL CoA IN PEPTOSTREPTOCOCCUS ELSDENII: A NEW alpha-PHOSPHOLACTYL CoA INTERMEDIATE

presented by

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ABSTRACT

THE CONVERSION OF LACTYL CoA TO
ACRYLYL CoA IN Peptostreptococcus elsdenii:
A NEW α-PHOSPHOLACTYL CoA INTERMEDIATE

by Donald L. Schneider

Preliminary studies of the direct reductive pathway of propionate formation from lactate in P. elsdenii were not consistent with a simple dehydration of lactyl CoA. Thus the existence of an acrylyl CoA intermediate (the product expected from dehydration) was reexamined. (1) Lactate was incubated with extracts in tritiated water. The propionate which had been produced was found to contain tritium in carbon positions 2 and 3. (2) 3-^{3}H-Lactate was incubated with extracts in the presence of various amounts of ^{14}C-acrylate. The propionate produced contained lesser amounts of ^{3}H and greater amounts of ^{14}C as the amount of ^{14}C-acrylate was increased. (3) Lactate was incubated with extracts and acrylyl CoA aminase of Clostridium propionicum. β-Alanine formation occurred and was dependent on both extracts of P. elsdenii and acrylyl CoA aminase. These experiments were interpreted to mean that acrylyl CoA is an intermediate.

At this point the crux of the problem was that acrylyl CoA is formed from lactyl CoA but not apparently

by a dehydration. An alternate mechanism was considered; the strategy was that since the hydroxyl is a poor leaving group, if phosphorylation occurred to yield α-phospholactyl CoA, then the problem of a leaving group would be overcome. The following experiments were employed to test for the possibility of phosphorylation.

(1) 2-^{18}O-Lactate was prepared and incubated with extracts. The phosphate from the mixture was isolated and analyzed for ^{18}O content. The results showed that ^{18}O is transferred from 2-^{18}O-lactate to phosphate concomitant with propionate formation.

(2) When lactate was incubated with extracts, the acrylate formed was determined by gas chromatographic analysis. Acetyl phosphate and catalytic amounts of thiolester (added in the form of acetyl CoA) were required for acrylate formation. Presumably lactate is converted to lactyl CoA by thiolester interchange as catalyzed by CoA transferase; lactyl CoA is phosphorylated by acetyl phosphate as catalyzed by a phosphotransferase; phosphorylated lactyl CoA undergoes elimination to form acrylyl CoA as catalyzed by a lyase; and finally acrylate is produced by another thiolester interchange.

(3) ^{14}C-Lactate and ^{32}P-acetyl phosphate were incubated with extracts. Analysis of the mixture by paper chromatography of samples withdrawn at various times showed that (a) a ^{14}C- and ^{32}P-labeled compound appeared rapidly, (b) the double-labeled compound had

an R_F value equal to that of chemically synthesized phospholactate, (c) the level of the double-labeled compound decreased as that of lactate decreased. This pattern was suggestive of an intermediate.

(4) The double-labeled compound was partially purified by chromatography on DEAE cellulose and Sephadex G-10. The partially purified material was reincubated with extracts and with added cold lactate. Acrylate and lactate were isolated from the reincubation mixture. Determination of specific radioactivities showed that that of acrylate was greater than that of lactate. This experiment was interpreted to mean that the double-labeled compound, which had been formed from lactate, was converted to acrylate directly (presumably at the level of thiolester).

(5) The partially purified, double-labeled compound was treated with alkaline phosphatase. Analysis showed that equimolar amounts of lactate and phosphate had been released. The lactate was tested as substrate for D- and L-specific lactate dehydrogenases. Only in the case of D-lactate dehydrogenase was activity observed. Thus the isolated compound is probably α-phospho-D-lactate.

(6) Chemically synthesized phospholactate was incubated with extracts in order to test whether it would be converted to acrylate. The rate of acrylate formation was found to be 1/10 the rate obtained with acetyl phosphate and lactate as substrates. The slow

rate may be due to a restricted conversion of phospholactate and added acetyl CoA to phospholactyl CoA and acetate. In fact without catalytic amounts of acetyl CoA, the formation of acrylate from phospholactate does not occur.

The six experiments described above are interpreted to mean that phospholactyl CoA is intermediate between lactyl CoA and acrylyl CoA in the propionate pathway. Thus the conversion of lactyl CoA to acrylyl CoA, which overall is a dehydration, is accomplished by (a) phosphorylation of the hydroxyl group and (b) β-elimination of the phosphate. The advantage of this mechanism is the leaving prowess of phosphate in comparison to the poor leaving properties of hydroxide.

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By

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VITA

Donald L. Schneider was born on January 15, 1941, in a purlieu adjacent to Lake Michigan, Muskegon, Michigan. Though obfuscated by instructors of industrial arts and athletics he graduated from Muskegon High School in June, 1959. Whereupon he made a peregrination to Kalamazoo College and from which institution he received the degree of Bachelor of Arts in June, 1963.

During the summer of 1962 he had the pleasure of Professor Richard Luecke's tutelage at Michigan State University as a U.S. National Science Foundation undergraduate research participant. As a result he found himself inspired to obtain more than a modicum of education. Thus he accepted a graduate research assistantship in the Department of Biochemistry at Michigan State University. Throughout most of the tenure of his Ph.D. work he was supported by a U.S. National Institutes of Health predoctoral fellowship. The requirements for the Ph.D. degree will be completed in the spring of 1969.

Mr. Schneider is a member of the American Chemical Society and Sigma Xi. He is married and without children. In September, 1969, he will commence postdoctoral study with Professor Efraim Racker at Cornell University.

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CHAPTER I

INTRODUCTION

Originally the intent was to study the mechanism of conversion of lactyl CoA to acrylyl CoA as catalyzed by lactyl CoA dehydrase, an enzyme from Peptostreptococcus elsdenii. The mechanism is of particular interest because lactate is difficult to dehydrate by simple chemical means. Further if one considers the reaction in reverse, the hydroxyl group becomes linked to an already electron-rich carbon atom. This is unique in organic and enzymatic reactions inasmuch as a hydroxyl group would be expected to add to the beta-carbon. However preliminary experiments raised serious doubts about the existence of lactyl CoA dehydrase; consequently, the purpose of this project is to discover the individual steps and mechanism involved in the conversion of lactate to propionate in P. elsdenii.

CHAPTER II

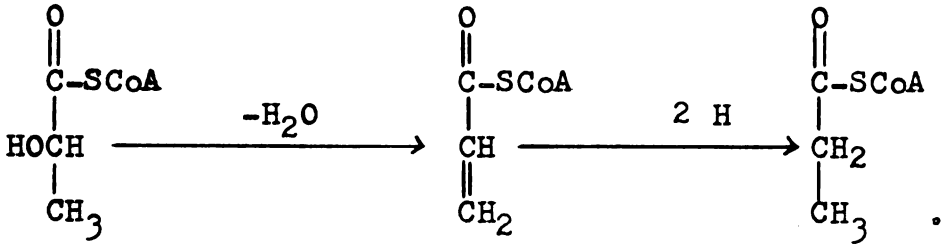
LITERATURE REVIEW

The most ubiquitous pathway of propionic acid formation involves succinate as an intermediate and is called the "dicarboxylic acid pathway" (Leaver et al., 1955; Stadtman and Vagelos, 1957; Wood and Stjernholm, 1961; Swick, 1962). A second pathway involves the direct reduction of lactate to propionate via acrylate without any dicarboxylic acid intermediate (Cardon and Barker, 1947; Johns, 1952; Leaver et al., 1955; Elsden et al., 1956; Ladd, 1957; Ladd and Walker, 1959; Ladd and Walker, 1965; Baldwin et al., 1965). In this pathway lactate-2-^{14}C, for example, is converted to propionate-2-^{14}C; whereas in the dicarboxylic acid pathway the alpha- and beta-carbon atoms randomize due to the symmetry of succinate. Consequently the dicarboxylic pathway is also called the "randomizing pathway," and the direct reductive the "non-randomizing pathway."

THE DIRECT REDUCTIVE PATHWAY

Clostridium propionicum, an anaerobe, metabolizes lactate, pyruvate, and acrylate to propionate, and these fermentations constitute the first evidence of the occurrence of the direct reductive pathway (Cardon and Barker,

1947). Cl. propionicum is not able to decarboxylate succinate and is not able to ferment malate or fumarate (Johns, 1952). Furthermore, lactate-3-^{14}C is fermented to propionate-3-^{14}C by whole cells (Leaver et al., 1955). These observations clearly eliminate succinate as an intermediate and are consistent with the direct reduction of lactate. The propionate = acrylate reaction which is catalyzed by Cl. propionicum extracts occurs at the level of CoA thiolesters (Stadtman and Vagelos, 1957). Such evidence suggests that acyl thiolesters might be the intermediates in the direct reductive pathway:



PEPTOSTREPTOCOCCUS ELSDENII

The most thoroughly studied organism possessing the direct reductive pathway is Peptostreptococcus elsdenii. It is a Gram-negative, strict anaerobe which was isolated from the rumen of sheep (Elsden et al., 1956) and cow (Gutierrez et al., 1956). It ferments acrylate without carbon dioxide fixation, thus ruling out the dicarboxylic acid pathway; furthermore acetone powders are able to utilize acrylate to form propionate and acetate (Lewis and Elsden, 1955).

P. elsdenii also ferments lactate, glucose, fructose, and maltose (Gutierrez et al., 1956); but is not able to ferment succinate, malate, or fumarate (Elsden et al., 1956). Other general properties are (1) lack of spores, (2) non-motility, (3) evolution of carbon dioxide and hydrogen, (4) formation of fatty acids up to n-hexanoate (Elsden et al., 1956), and (5) formation of acetate by the phosphoroclastic reaction (Peel, 1960). The name, Peptostreptococcus elsdenii, was given on basis of the following characteristics (1) chain formation, (2) rapid fermentation of carbohydrates, (3) coccal morphology, and (4) ability to attack organic acids (Gutierrez et al., 1956).

Additional support for the direct reductive pathway in P. elsdenii is that lactate-2-^{14}C is fermented to propionate with the label exclusively in the methylene carbon (Ladd, 1957). These results were confirmed with extracts (Ladd and Walker, 1959). Lactate and acrylate are fermented by cell-free extracts at identical rates and to the same products in identical proportions (Ladd and Walker, 1959). Lactate activation by thiolester formation seems to be necessary because, after five hours of dialysis, the addition of ATP or a source of active phosphate is required in order to restore the ability to form propionate. As in Cl. propionicum, acrylate reduction requires prior thiolester formation (Ladd and Walker, 1965).

Further suggestive evidence for thiolester activation is that, when lactate-^{14}C is incubated with extracts of P. elsdenii, the hydroxamic acids of lactate, propionate, acetate, and perhaps acrylate can be identified (Ladd and Walker, 1959; Baldwin et al., 1962; Baldwin et al., 1965).

All enzymes of the pathway have been demonstrated in P. elsdenii (Baldwin, 1962; Baldwin et al., 1965). CoA transferase activates lactate by catalyzing a general thiolester exchange with, for example, propionyl CoA:

(1) lactate + propionyl CoA = lactyl CoA + propionate.

(2) lactyl CoA == propionyl CoA.

NET REACTION: lactate = propionate.

The acyl CoA dehydrogenase and lactyl CoA dehydrase were also demonstrated and partially purified.

OCCURRENCE AND SIGNIFICANCE OF DIRECT REDUCTIVE PATHWAY

As already discussed, the pathway was first observed in Cl. propionicum. It occurs in P. elsdenii and, in this case, contributes to bloat, a disorder in cattle characterized by distention of the rumen and colon. In a cow afflicted with bloat P. elsdenii becomes very prominent as the microbial population changes. Its association with bloat probably is based on evolution of large quantities of gas, namely carbon dioxide and hydrogen (Gutierrez et al., 1956). P. elsdenii exemplifies the importance of the pathway in another way. As the readily-available carbo-

hydrate in a cow's diet increases, so does the population of P. elsdenii in the rumen. Labeling experiments show that rumen microorganisms form 70-100% of their propionate by the direct reductive pathway (Baldwin, 1962; Baldwin et al., 1963).

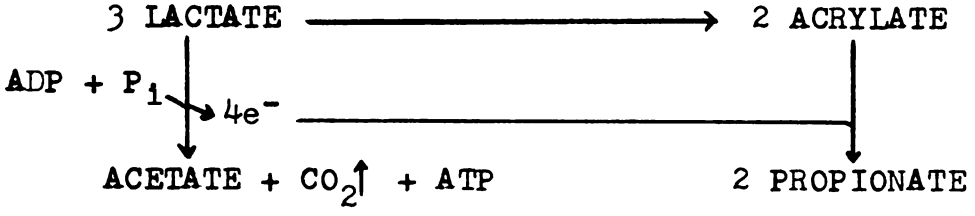
The pathway has been well-documented to occur in legume nodule bacteroids (Jackson and Evans, 1966). Bacteroids produce propionate which is then utilized by the plant, e.g., for heme biosynthesis. Dialyzed extracts of bacteroids require ATP, Mg^{2+}, and NADH as cofactors to convert lactate to propionate.

The fourth known occurrence of the pathway is Bacteroides ruminocola (Wallnöfer and Baldwin, 1967). Apparently this organism, unlike P. elsdenii, becomes predominant when the cow's diet does not readily furnish carbohydrates, e.g., hay.

Most known sources of the pathway consist of strictly anaerobic bacteria which are symbiotic. Whether the pathway occurs in plants or animals has not been thoroughly tested, though pigeon heart muscle has been found to possess acrylyl CoA hydrase activity. In this case however, the reaction is irreversible and produces lactyl CoA (Vagelos et al., 1959). The pathway may function in reverse to form lactate in Moraxella lwoffi (Hodgson and McGarry, 1968), Escherichia coli (Wegener et al., 1967), and in Pseudomonas aeruginosa (Sokatch, 1966).

METABOLISM IN P. ELSDENII

The fermentation products of P. elsdenii grown on lactate are acetate, propionate, CO_2, H_2, and lesser amounts of butyrate, valerate, and hexanoate. Inasmuch as anaerobes, by definition, cannot reduce oxygen to water, electrons which are obtained by oxidation of metabolites in the course of providing high energy compounds are accepted by molecules also formed in the process, e.g., in this case acrylate, crotonate, etc. In otherwords, propionate is formed as a result of acrylyl CoA accepting electrons which are obtained by oxidation of lactate to acetate:



Lactate is oxidized first to pyruvate by NAD-independent dehydrogenase(s) and then to acetate and carbon dioxide by means of the phosphoroclastic reaction (Baldwin, 1962). Since the latter reaction requires phosphate, elimination of phosphate would block oxidation of pyruvate. Under this condition lactate is oxidized to pyruvate with concomitant reduction of acrylyl CoA to propionyl CoA, i.e., all electrons from oxidation of lactate to pyruvate are transferred exclusively to acrylyl CoA and do not contribute to hydrogen formation (Ladd and

Walker, 1959). Though P. elsdenii in the laboratory predominantly encounters L-lactate, its lactate dehydrogenase is specific for the D-isomer (Baldwin, 1962). An explanation may be the prior conversion of L- to D-lactate via a racemase. Such racemases have been found in other organisms: Clostridium acetobutylicum secretes a B_6-Fe^{2+}-dependent racemase into the medium which is thought to catalyze dehydration and rehydration of lactate (Katagiri et al., 1958); Cl. butylicum produces a racemase with an S-lactyl intermediate and an internal hydride shift involved in the mechanism (Dennis and Kaplan, 1959). The D-lactate dehydrogenase of P. elsdenii has been partially purified (Baldwin, 1962).

The phosphoroclastic reaction oxidizes pyruvate to acetyl phosphate and carbon dioxide and is of the clostridial type, i.e., pyruvate decarboxylase forms CO_2 and hydroxyethylthiamine pyrophosphate (TPP); the hydroxyethylTPP is oxidized by ferredoxin to acetyl TPP; acetyl TPP reacts with CoASH to regenerate TPP and to produce acetyl CoA; phosphotransacetylase has been partially purified (Baldwin, 1962) and catalyzes the formation of acetyl phosphate from inorganic phosphate and acetyl CoA, whence the term "phosphoroclastic" reaction (Ladd, 1959; Peel, 1960; Joyner and Baldwin, 1966).

Supposedly acetyl phosphate is the organism's source of energy. The substrate-level phosphorylation

catalyzed by acetokinase would produce one mole of ATP for each mole of lactate oxidized.

The fate of reduced ferredoxin is two-fold. In extracts, a powerful hydrogenase accepts its electron to form $\frac{1}{2}$ H_2 in a reaction which is predominant below pH 7.6 (Ladd and Walker, 1965). Since the rumen is slightly acidic, the electrons from ferredoxin would be expected to be used to evolve hydrogen in nature; instead, in whole cells these electrons are used in formation of higher fatty acids. At slightly alkaline pH the ferredoxin electrons can be transferred to acyl CoA dehydrogenase by way of NAD and an electron carrying protein (Baldwin and Milligan, 1964). Thus in extracts the fermentation normally is:

ADP + 2 lactate = acetate + CO_2 + ATP + H_2 + propionate,

and at pH's above 7.6 is:

ADP + 3 lactate = acetate + CO_2 + ATP + 2 propionate.

Notice that the small amounts of higher fatty acids have been omitted from the above formulation.

The acyl CoA dehydrogenase has been partially purified and may contain a cytochrome cofactor. Coupling of lactate oxidation with acrylyl CoA reduction has been calculated to afford $-\Delta F$ = 18 Kcal/mole which is more than enough for formation of a high energy phosphate bond (Barker, 1956). Furthermore P. elsdenii grows very well for an anaerobe with growth yields as high as 10 g of wet cells/l. Electron transport phosphorylation may occur in

P. elsdenii; however, employing partially purified acyl CoA dehydrogenase and electron carrying protein, ATP formation concomitant with that of propionyl CoA was not demonstrable (Baldwin and Milligan, 1964).

The formation of fatty acids higher than propionate has not been studied, undoubtedly because extracts are not very active in this regard (Ladd and Walker, 1959). ^{14}C-labeling experiments have shown, however, that butyrate is synthesized from two acetates, hexanoate from three, and valerate from one acetate and one propionate with the propionate moiety occupying carbon positions 3, 4, and 5 (Ladd, 1957).

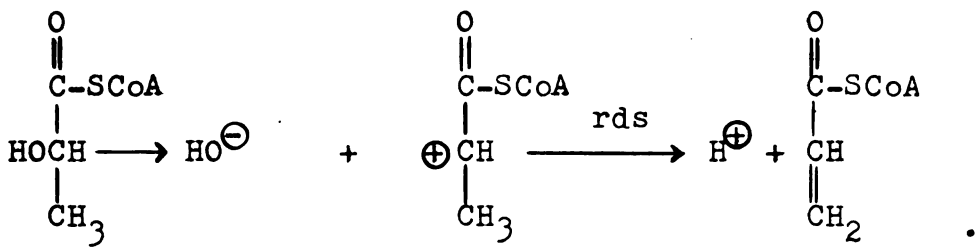
CoA transferase enables P. elsdenii to conserve the energy of active thiolester intermediates and is specific for the CoA moiety, rather non-specific for the acyl group, and has been partially purified (Baldwin, 1962). In this manner lactate may be activated without expenditure of ATP:

propionyl CoA + lactate = lactyl CoA + propionate.

MECHANISM OF LACTATE - ACRYLATE INTERCONVERSION

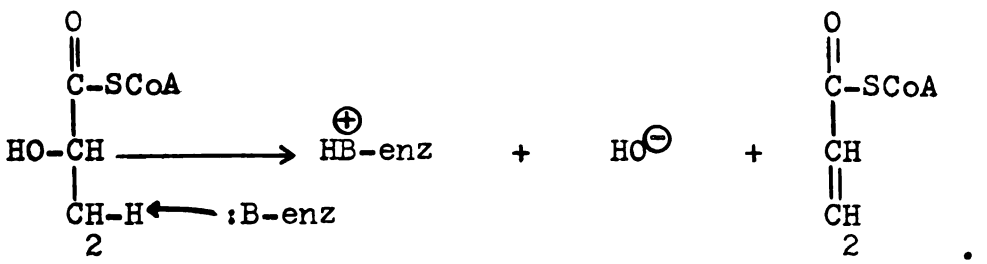
Lactate dehydration requires the elimination of the elements of water. Elimination reactions are known by the organic chemist to occur via two mechanisms (1) unimolecularly and (2) bimolecularly with an assisting molecule of base. The unimolecular elimination is called

E_1 and involves as a first step the removal of a hydroxyl group to form a carbonium ion:



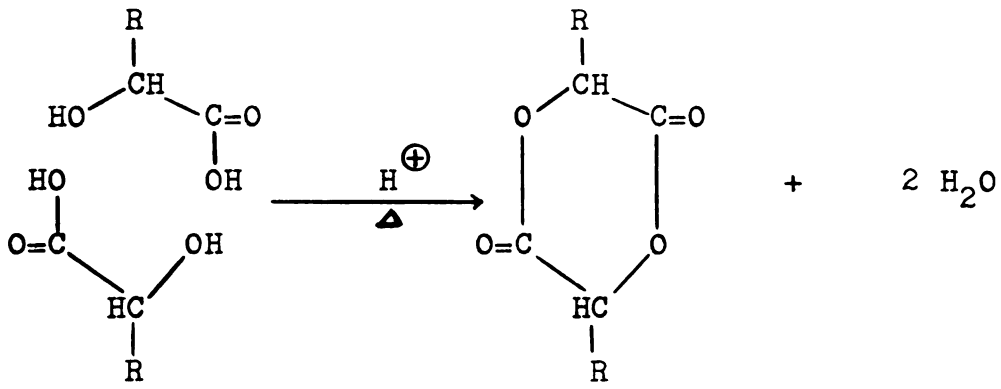
In this instance the reaction is absolutely improbable because the strong electron withdrawal effects of the acid carbonyl and the CoA thiolester would greatly reduce the stability of this carbonium ion. Indeed the CoA is sufficiently electron withdrawing to stabilize an alpha-carbanion to the extent of lowering the alpha-hydrogen's pK_a 4 units below that of the parent acid (Lynen, 1953). This will be discussed more thoroughly below.

The bimolecular eliminations are called E_2 and are dependent on a good leaving group, X, and acidic hydrogens:



Clearly the E_2 mechanism is the more reasonable of the two; nevertheless two difficulties exist. In the first place a beta-hydrogen is not nearly as acidic as an alpha-one at least in the case of lactyl CoA. Second, the

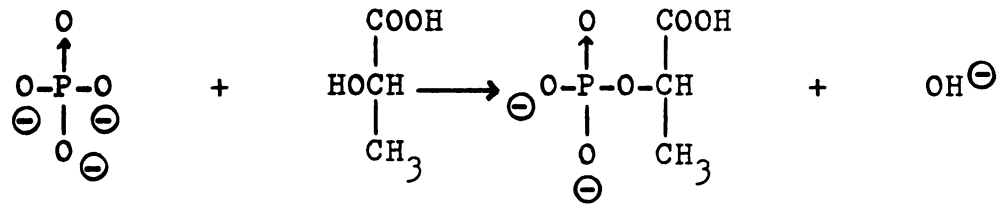
hydroxyl is a poor leaving group (Gould, 1959). In fact lactic acid is difficult to dehydrate chemically: when alpha-hydroxycarboxylic acids are subjected to dehydrating conditions (strong acid, heat) they form cyclic dimers:



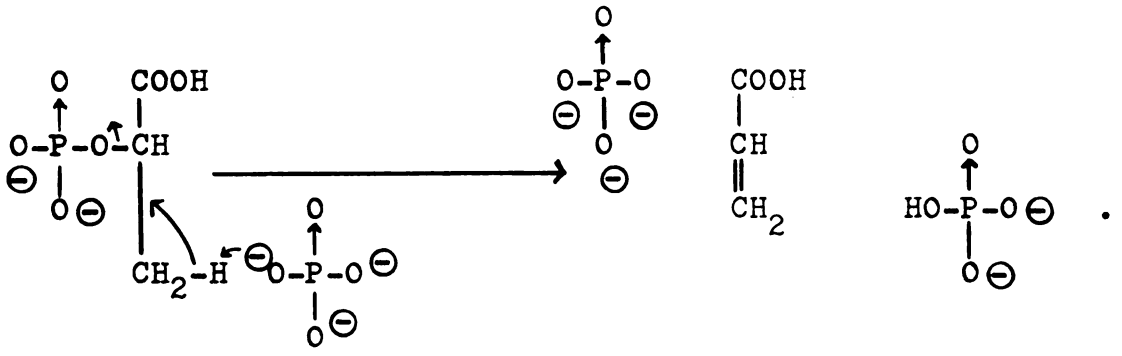
(Morrison and Boyd, 1959).

Concentrated lactic acid catalyzes the reaction itself and thus lactic acid always contains significant amounts of the dimer, called lactide.

alpha-Hydroxyacids are dehydrated to alpha,beta-unsaturated acids industrially by use of heat and catalysts among which sulfate and phosphate are common (Ustavshchikov et al., 1965; Holmen, 1958). Lactate and its oxygen esters have been dehydrated in this fashion. Perhaps these catalysts work by first forming an ester with the hydroxyl group:

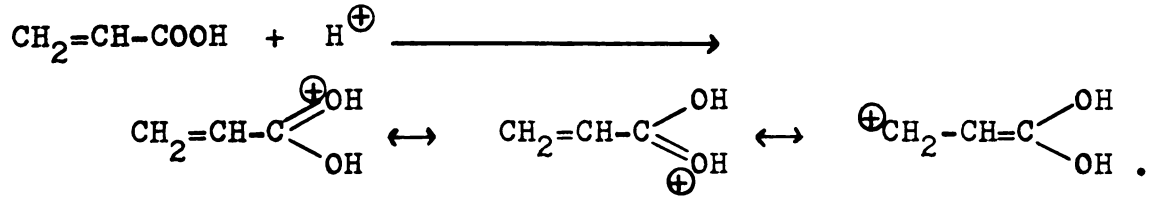


and by eliminating E_2 fashion:



The principal advantage of this scheme is that phosphate is a much better leaving group than the hydroxyl group as will be discussed below.

Consider the dehydration of lactate in reverse. Since acrylate is involved, its chemical properties as well as those of acrylyl CoA are important. Acrylic acid undergoes addition reactions in anti-Markownikoff fashion, e.g., hydrogen bromide yields beta-bromopropionic acid. The first step in the reaction is transfer of a proton to form a resonance hydrid of the following structures (Roberts and Caserio, 1965):



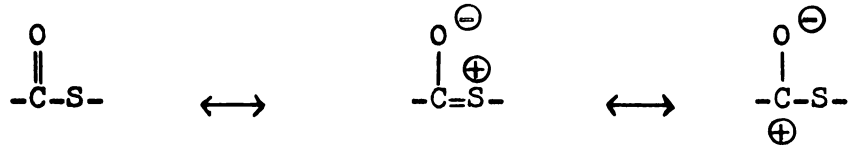
In keeping with this reaction, in which the more electron-withdrawing carboxyl group is protonated rather than the double bond, bromine reacts heterolytically very slowly with acrylic acid on account of the absence of a proton

(Fieser and Fieser, 1956). Acrylic is a stronger acid than propionic:

Acid	pK_a	Ref.
lactic	3.87	Fieser and Fieser, 1956
acrylic	4.26	"
acetic	4.76	"
propionic	4.88	"

Since the properties of free acrylic acid differ from those of saturated acids, acrylyl CoA is expected to be unusual too. A discussion of thiolesters follows.

In a thiolester $p\pi - d\pi$ orbital overlap might be expected (resonance form 1) making the C-S bond stronger; however this form is not significant (Bruice and Benkovic, 1966a).



1 2

The low electronegativity of sulfur does give resonance form 2 greater importance than in oxygen esters (oxygen 3.5; sulfur 2.5 (Pauling, 1960)). Nevertheless both resonance forms would (1) increase the acidity of the alpha-hydrogens of a thiolester, and (2) assist nucleophilic displacement at the carbonyl carbon.

Thiolesters are much more reactive than oxygen ones; Lynen classifies them as acid anhydrides. The acidifying effect of sulfur can be measured in acetoacetic esters by observing the dissociation of a methylene hydrogen (see below). This activation of the alpha-hydrogens accounts for the normal reactions of thiolesters, e.g., acetyl CoA and oxaloacetic acid react to form citric acid as though the alpha-carbon atom of acetyl CoA were a carbanion.

Compound	pK_a	Reference
acetoacetic acid	12.70	Lynen, 1953
ethyl acetoacetate	10.70	"
S-acetoacetyl-N-acetylthioethanolamine	8.50	"

Thiolesters which are alpha,beta-unsaturated absorb at 224 mμ, whereas the saturated ones absorb at 204 mμ. The shift to longer wavelengths is probably due to double bond-CO group resonance, which may explain why unsaturated thiolesters behave like alpha,beta-unsaturated ketones towards reducing agents, e.g., leuco dyes. Furthermore a new band appears at 263 mμ which might be due to π-interaction between the double bond and the -S-CO group. Since the 263-mμ-absorbance peak is unique to unsaturated thiolesters it offers a means of measuring any reaction involving acrylyl CoA (Lynen, 1953).

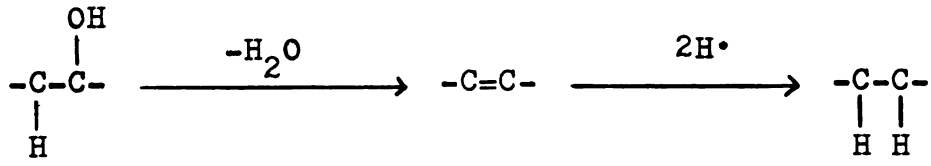
Acrylyl CoA is more reactive in addition reactions

than the free acid, e.g., SH groups will add across the double bond of the ester whereas the acid is unreactive (Dixon and Webb, 1964).

In perspective then the chemistry of acrylyl CoA clearly suggests that in an addition reaction the anionic moiety should go beta because the alpha-carbon is more electron-rich. Thus acrylyl CoA to lactyl CoA is, mechanistically, an unexpected and unusual reaction which merits study and stands in contrast to the normal reactions of acrylyl CoA, e.g., adding an SH group to form 3-thiolpropionyl CoA or an amino group to form beta-alanine (catalyzed by acrylyl CoA aminase).

Consider again the evidence for CoA intermediates: (1) acrylate reduction in Cl. propionicum requires prior thiolester formation, (2) lactate or acrylate fermentation to propionate by P. elsdenii requires catalytic amounts of ATP or thiolester, (3) the radioactive hydroxamates of lactate, acetate, propionate, and perhaps acrylate can be isolated when lactate-^{14}C is fermented by P. elsdenii, and (4) all the enzymes of the pathway have been demonstrated in P. elsdenii, and all involve acyl CoA substrates (Baldwin et al., 1965). In fact the coupled assay, which was developed for the key enzyme, lactyl CoA dehydrase, shows an absolute specificity for acrylyl CoA (Baldwin et al., 1965; Baldwin, 1967). The evidence for thiolester intermediates seems rather clear-cut, but that for an acrylyl one is less so.

Previously, enzymes which replaced a hydroxyl group with a hydrogen atom were believed to (1) eliminate water to form a double bond and (2) to reduce the double bond:



However this generalization is no longer universal. In the case of deoxycytidine diphosphate formation, the concept was thought to apply (Reichard, 1962); and recently the mechanism has been shown to involve hydride ion-like displacement of the 2'-hydroxyl group of cytidine diphosphate (Larsson, 1965; Durham et al., 1965). With this in mind the evidence for acrylyl CoA as an intermediate must be scrutinized anew.

As early as 1942 it was written "Of several mechanisms proposed, that involving removal of water from lactic acid to form acrylic acid which is then reduced to propionic acid has seemed the most probable" (Werkman and Wood, 1942). Despite these expectations the evidence for an acrylyl intermediate remains suggestive, though its existence seems probable. Each datum supporting an acrylyl intermediate is discussed below.

(1) Acetone powders of P. elsdenii reduce acrylyl CoA to propionyl CoA (Lewis and Elsden, 1955). However this activity could be due to the acyl CoA dehydrogenase of fatty acid synthesis (enoyl reductase). Indeed this

was shown to be the case in propionibacteria in which acrylate-^{14}C is converted to succinate-^{14}C (Swick, 1962).

(2) Extracts of P. elsdenii ferment acrylate at the same rate and to the same products as lactate (Ladd and Walker, 1959). This is good evidence, but one could again argue that the fatty acid synthesizing acyl CoA dehydrogenase reduces acrylyl CoA to propionyl CoA which then shuttles back through the direct reductive pathway to lactyl CoA. Such an explanation would require that those enzymes be more active than the enzymes involved in lactate to acetate; the analysis of the end products of the fermentation suggest that this is probably the case (Gutierrez et al., 1956).

(3) Extracts of P. elsdenii catalyze the hydration of ^{14}C-acrylyl CoA to ^{14}C-lactyl CoA (Baldwin et al., 1962). However, since the identification involved paper chromatography of the hydroxamic acids and since acrylyl hydroxamate polymerizes, it can give spots all over the chromatogram from R_F 0.65 (unpolymerized, same as propionate) to R_F 0.20 (Baldwin et al., 1965). Other investigators suggest that acrylyl hydroxamate polymerizes so readily as to give R_F 0.00 (Ladd and Walker, 1959). Since acrylyl hydroxamate polymerizes the identity of any spot is uncertain. Furthermore, even if ^{14}C-lactyl CoA is actually produced, the fatty-acid-synthesis acyl CoA dehydrogenase

possibility, and subsequent reaction of propionate to lactate may apply (cf. above (1)).

(4) In a coupled assay system involving the reactions written below, the formation of ^{14}C-pyruvate is dependent on enzyme, NAD, LDH, and ^{14}C-acrylyl CoA (Baldwin *et al.*, 1965):

1) acrylyl CoA + H_2O $\xrightarrow{\text{15-fold pur. enzyme}}$ lactyl CoA

2) lactyl CoA + acrylate $\xrightarrow{\text{CoA transferase}}$ lactate + acrylyl CoA

3) lactate + NAD $\xrightarrow{\text{LDH}}$ pyruvate + NADH

4) NADH + INT $\xrightarrow{\text{diaphorase}}$ NAD + $\text{INT}^{485\ m\mu}_{\text{reduced}}$.

NET: H_2O + acrylate + INT $\longrightarrow$ pyruvate + $\text{INT}_{\text{reduced}}$.

With this assay, the enzyme could be purified fifteen-fold before activity vanished. However the purified enzyme could not be assayed successfully in a direct manner utilizing the absorption of unsaturated thiol-esters (Lynen, 1953). This could be due to product inhibition, i.e., acrylyl CoA might bind to the enzyme and prevent recycling of the enzyme until the product is consumed in a subsequent reaction. On the other hand, the direct assay has been used for other enzymes which catalyze the acrylyl CoA = lactyl CoA reaction (Vagelos *et al.*, 1959).

(5) The fifteen-fold purified enzyme was shown to be reversible by using acyl CoA dehydrogenase and reduced

safranine as a coupling system and ^{14}C-lactyl CoA as substrate (Baldwin, 1962; Baldwin *et al.*, 1965):

1) ^{14}C-lactyl CoA $\xrightarrow{\text{enzyme}}$ ^{14}C-acrylyl CoA + H_2O

2) ^{14}C-acrylyl CoA + reduced safranine $\xrightarrow[\text{rogenase}]{\text{acyl CoA dehyd-}}$ safranine + ^{14}C-propionyl CoA.

NET: ^{14}C-lactyl CoA + reduced safranine $\longrightarrow$ H_2O + ^{14}C-propionyl CoA + safranine.

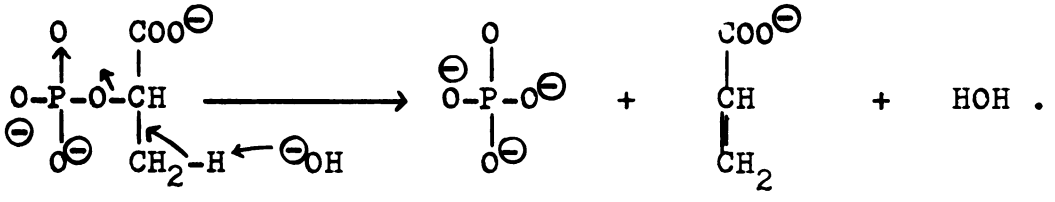
The difficulties are that (1) a fifteen-minute preincubation of the components of reaction 1) is necessary and is unexplainable, and (2) the direct assay of this same reaction does not work.

Two groups have reported that acrylate interferes with the conversion of lactate to propionate (Jackson and Evans, 1966; Whanger and Matrone, 1967). Though the observation is consistent with an acrylyl intermediate, it is, unfortunately, also consistent with acrylate as an inhibitor.

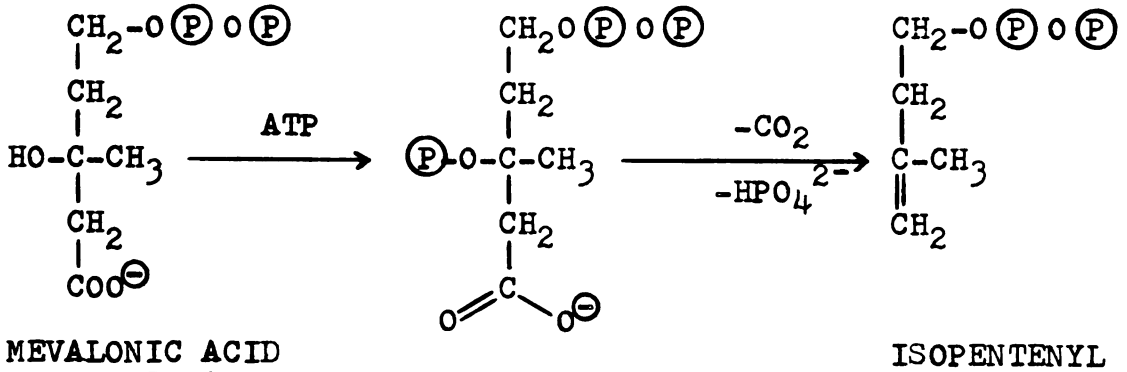
Nonetheless the evidence taken all together, makes the case for acrylyl CoA as an intermediate suggestive. At the same time alternate reactions leading to propionate must be kept in mind. One mentioned previously is hydride displacement as seen in deoxycytidine diphosphate biosynthesis. Another might involve activation of the hydroxyl of lactyl CoA prior to dehydration, e.g., phosphorylation.

Phosphate is a much better leaving group than hydroxyl and in the presence of a *beta*-electron-withdraw-

ing group leads to base catalyzed beta-elimination. Some examples are phosphoserine, 2-cyanoethyl phosphate, 2-sulfoxyethyl phosphate, glyceraldahyde-3-phosphate, adenosine-5'-phosphate, and fructose-1,6-diphosphate (Bruice and Benkovic, 1966b). The elimination of phosphate may be much easier than hydrolysis, e.g., phospholactic acid under optimum conditions for each would be expected to form acrylic acid about 500,000 times faster in alkaline solution (pH 14) than it forms lactic acid in acidic solution (pH 4.5) (Cherbuliez et al., 1962):



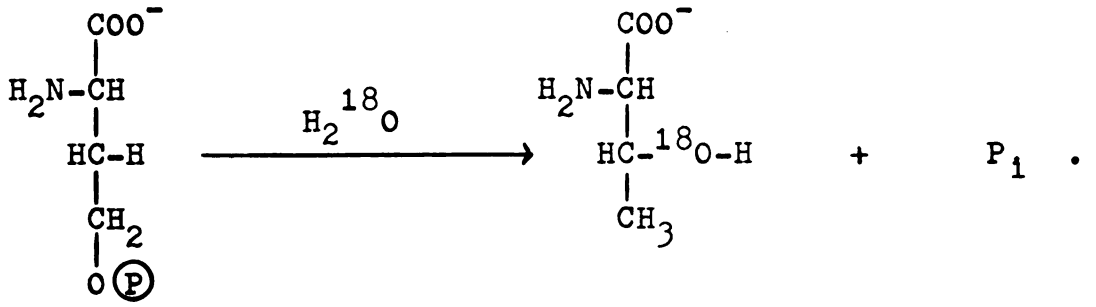
Enzymatic precedent for the aforementioned phosphate-facilitated elimination is mevalonic acid pyrophosphate decarboxylase (ATP: 5-pyrophosphomevalonate carboxylyse 4.1.1.33). ATP and Mg^{2+} are cofactors; the enzyme is involved in steroid biosynthesis (Bloch et al., 1959; Henning et al., 1959; Waley, 1962):



MEVALONIC ACID PYROPHOSPHATE

ISOPENTENYL PYROPHOSPHATE.

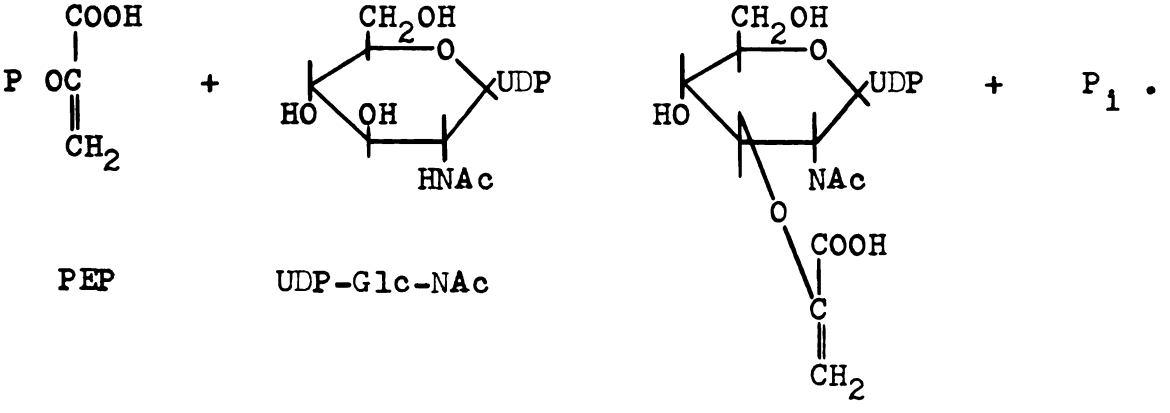
Another example is threonine synthetase in E. coli (Flavin and Kono, 1960):



O-PHOSPHOHOMOSERINE THREONINE

If the reaction is carried out in $H_2{}^{18}O$, one atom of solvant oxygen is incorporated into threonine and none into phosphate. Cleavage occurs at the C-O bond of the phosphate ester, and the phosphate is formed by nonhydrolytic elimination.

The biosynthesis of uridine diphospho-N-acetylmuramic acid demonstrates the leaving prowess of phosphate though a displacement rather than an elimination is involved (Gunetileke and Anwar, 1968):



PEP UDP-Glc-NAc

Suggestion for phospholactyl CoA and/or electron transfer phosphorylation in P. elsdenii is that the interconversion of acrylyl CoA and lactyl CoA is prevented by uncouplers of oxidative phosphorylation, e.g. dinitrophenol (Ladd and Walker, 1959). Yet the system contains no mitochondria and is particulate free. These workers hint at the existence of phospholactyl CoA as they write (Ladd and Walker, 1965), "... the interconversion of lactyl CoA and acrylyl CoA is not the simple reaction shown, but requires a source of energy geared possibly to the transfer of electrons from lactate to acrylyl CoA." Certainly this is in contrast to more commonplace dehydrases, such as 6-phosphogluconic acid dehydrase which requires a divalent metal and free thiols (Meloche and Wood, 1964a). If lactyl CoA is phosphorylated there must be a source of the active phosphate, and since P. elsdenii is an anaerobe this may be a serious problem. The phosphoroclastic reaction is used for energy production and cannot be considered as the source in this case, especially since the fermentation analysis shows that the direct reductive pathway is more active than the clastic reaction (Gutierrez et al., 1956). Another possibility exists, and that is electron transport phosphorylation. Some anaerobes grow beyond the limits of their known substrate phosphorylation, and, at least in the case of Cl. aminobutyricum, electron transport phosphorylation has been demonstrated to occur during the reduction of crotonyl thiolester (Hardman and Stadtman,

1963). This phosphorylation is feasible on a thermodynamic basis because there is a negative free energy change of 18 Kcal under physiological conditions (Barker, 1956). Whether this occurs in P. elsdenii is difficult to test directly because of an extremely active myokinase.

CHAPTER III

MATERIALS AND METHODS

BACTERIOLOGICAL

A culture of P. elsdenii (ATCC no. 17752; strain B 159) was the kind gift of Professor M. Bryant. The organism was maintained in stock culture as described by Elsden and Lewis, except 0.001% resazurin was added as a redox indicator (a red color indicates lack of anaerobiosis) (Elsden and Lewis, 1953). The stock culture has variable viability after prolonged storage and in order to eliminate the danger of losing the culture it was transferred every two weeks. From time to time Gram stains were made to determine cultural purity (Conn, 1957); contaminants were removed by plating either on thioglycollate media with 2% sodium lactate solution (60%) added in ordinary plates in a desiccator under N_2 or on stock culture media with 1% agar added in rolled tubes according to published procedures (Hungate, 1950). When plating, the medium was cooled to 45°C and inoculated; the medium was immediately poured into plates under N_2. P. elsdenii was grown in deep culture on corn steep liquor and lactic acid as described previously (Ladd and Walker, 1959) except that distilled water was employed and trace metals were added

as described for a defined medium (Allison et al., 1966; Bryant and Robinson, 1961); however two attempts to grow P. elsdenii on this defined medium were not successful.

Extracts were prepared by suspending the frozen cells under N_2 in an amount of buffer in milliliters equal to their weight in grams. The buffer was 0.1 M phosphate (K^+) (pH 6.5) and contained 1 mM DTT as reducing agent. The cells were disrupted by either (1) two passages through a French pressure cell or (2) 10 Kc sonication for 20 min. The temperature during disruption was 0-5°C and DNase was added to extracts prepared by the French press. The extracts were stored at -14°C under N_2. Addition of the proteinase inhibitor phenylmethylsulfonyl-fluoride as used by others (Steinman and Jakoby, 1967) was in some instances beneficial for stability.

SUBSTRATES

Acetyl CoA, butyryl CoA, and butyryl glutathione were prepared by the anhydride method as described in METHODS IN ENZYMOLOGY (Stadtman, 1957) as based on an earlier procedure (Simon and Shemin, 1953). Complete reaction of SH groups was ascertained with nitroprusside reagent (Stadtman, 1957) and if necessary for completion of the reaction additional anhydride was added. The reactions were carried out at 0°C and were monitered with a pH meter. The pH was maintained at 6 or above by addition of 1 N KOH and when reaction was complete the pH was

adjusted to 6.

For quantitative determination of thiolester the hydroxamic acid was formed and color developed by addition of ferric chloride. Succinic anhydride afforded the basis of the standard curve even though the color values are slightly different from acid to acid. The original procedure (Stadtman, 1957) was scaled down to 0.30-ml total volume in order to increase sensitivity and the μmoles assayed are equal to 0.9 times net $A_{540\ m\mu}$.

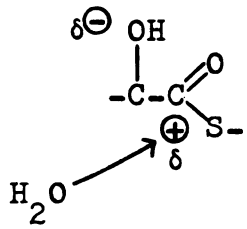
Lactyl CoA was prepared by the mixed-anhydride reaction between ethylchloroformate and free lactic acid and subsequent displacement of ethylformate by CoASH (Flavin, 1963). Inasmuch as the reaction mixture contains large amounts of tetrahydrofuran and pyridine, mixed-anhydride thiolester preparations were always subjected to continuous liquid-liquid extraction with diethylether at 0°C.

Lactyl CoA was found to be very labile to storage and purification. Column chromatography on Bio-Gel P-2 at about 1°C resulted in complete hydrolysis of thiolester. Lactyl thiolesters are surprisingly unstable to storage at -14°C, see Table 1. After 8 days only 14% of the original thiolester remains. When stored at pH 8 they are even more labile as 100% hydrolysis occurs within 3 days. Chromatography of lactyl CoA and lactyl-S-pantheteine on Sephadex G-15 (1 x 50 cm column, flow 4 ml/hr) was the only successful purification step. The

Table 1. Lability of lactyl thiolester to storage

COMPOUND	AMOUNT OF THIOLESTER		AMOUNT OF HYDROLYSIS
	initial	after 8 days at pH 6, -14°C	
Butyryl-S-glutathione	19 μmole/ml	15 μmole/ml	21%
Lactyl-S-glutathione	21	3	81

yields were nearly quantitative if the fractions were concentrated as a liquid on a test tube Rotovap; however, lyophillization resulted in complete hydrolysis. The column was operated as quickly as possible and the temperature was that of the cold room, about 5°C. Lactyl CoA is probably labile due to the neighboring effect of the alpha-hydroxy group. This could occur in at least two ways. First the inductive effect: a hydroxyl is electron-withdrawing and would thus promote attack by water on the carbonyl carbon of the ester:



Second, the hydroxyl might be acting as a nucleophile and displacing the thiolester. alpha-Hydroxycarboxylic acids are very prone to lactone formation (Fieser and Fieser, 1956). However the lability must be more complicated or else lactyl CoA would be stable to lyophillization which was not found to be the case either by Rabin et al. (1965) or in these studies. Many bimolecular reactions have recently been shown to occur much faster in frozen solution than in liquid water at the same temperature, e.g., the mutarotation of glucose (Kiovsky and Pincock, 1966) and the solvolysis of ethylene chlorohydrin (Pincock and Kiovsky, 1966). The explanation seems to be that there is

a concentration effect, i.e., the reactants are localized in small micelles of liquid water within the ice lattice and the effective concentration of reactants increases dramatically.

Acrylyl CoA, or acrylyl-S-pantetheine, must be prepared with precaution to prevent addition of excess CoASH across the double bond of acrylyl CoA. This problem is minimized by employing the mixed-anhydride method with inverse addition, i.e., a small amount of CoASH solution is slowly added to a large excess of acrylyl-mixed-anhydride at 0°C.

Purity of acrylyl thiolester is best measured by taking a difference spectrum in such a way as to give a spectrum of the thiolester alone with no contribution from CoA or other chromophores such as the common contaminate, pyridine, which also absorbs in the 260 mμ region. The procedure is to take two identical aliquots of the acrylyl thiolester, and to hydrolyze #1 in 0.1 N KOH (½ hr, room temp) and then to neutralize by addition of an equivalent amount of HCl. To aliquot #2 one adds equivalent amounts of KOH and HCl simultaneously. The two aliquots are diluted to identical volumes and scanned in a spectrophotometer employing matched cuvettes. Aliquot #1 is placed in the reference beam and #2 in the sample beam. A Cary 15 recording spectrophotometer was used for these determinations. I am grateful to Professor Karl Decker for his suggestion of this procedure.

Acrylyl CoA and other acrylyl thiolesters are stable to storage. However purification proved to be impossible. The thiolester was completely hydrolyzed by passage through Sephadex G-15, DEAE-cellulose, Dowex-1 (HCO_3^-), Dowex-1 (phosphate, pH 6.25), and ECTEOLA-cellulose columns. On other columns, (Dowex-50(H^+), CM-Sephadex, and CM-cellulose), the thiolester linkage remained intact, but extensive polymerization occurred as determined by difference spectra and actual precipitation (turbidity). Such lability is expected as acrylic acid itself polymerizes spontaneously unless stabilizers are added (Feairheller and Katon, 1967). Acrylyl CoA can be made <u>in situ</u> just prior to its use by pre-incubation of buffer, acetyl CoA, acrylate, and CoA transferase. Consistent with the hydrolysis of acrylyl thiolesters during passage through columns with basic functional groups such as DEAE- is that amine buffers are known to catalyze thiolester hydrolysis (Koch and Jaenicke, 1962). The situation is complicated by acid-catalyzed cationic polymerization of acrylyl CoA on acidic columns, e.g., CM-Sephadex.

<u>alpha</u>-Phospholactic acid was prepared as the barium salt by (1) reaction of ethyl lactate with polyphosphoric acid, (2) selective saponification of the ethyl moiety, and (3) alcohol precipitation of the salt as described previously (Cherbuliez and Rabinowitz, 1956; Cherbuliez and Rabinowitz, 1959). Lactic acid cannot be phosphory-

lated directly on account of pronounced dehydration and lack of desired product. Phospholactate is somewhat difficult to hydrolyze. Its half-life in acid-molybdate solution is 46 min at 100°C compared to 24 min for 2,3-phosphoglyceric acid (Rose and Pizer, 1968). Calculations based on the known acid strengths of monoesters of phosphoric acid (Kumler and Eiler, 1943) show that phospholactate should have pK_a's about 1.5, 3.5, and 6.5. These figures correlate well with those for 2-phosphoglyceric acid which are known to be 1.8, 3.63, and 6.64 (Ballou and Fisher, 1954).

Acetyl phosphate was prepared from dipotassium hydrogen phosphate and acetic anhydride and isolated as the dilithium salt as described in METHODS IN ENZYMOLOGY (Stadtman, 1957) according to the procedure of Avison (1955).

3-(S-Glutathionyl)propionyl hydroxamate was prepared by the mixed-anhydride method employing a large excess of glutathione and acrylyl mixed-anhydride (Flavin, 1963) to form 3-(S-glutathionyl)propionyl glutathione; subsequently, the hydroxamate was formed from the thiolester by adding neutralized hydroxylamine. In this instance, advantage is taken of the reactivity of acrylyl thiolester, i.e., the excess glutathione adds to the double bond as soon as acrylyl glutathione is formed. As described in the RESULTS Section, this reactivity provides a means of trapping acrylyl CoA.

Diaphorase, phosphotransacetylasse, and CoA transferase were prepared and assayed as described previously (Baldwin et al., 1965). The optical assay for CoA transferase with butyryl CoA as substrate (Barker et al., 1955) proved to be satisfactory for qualitative determinations such as those employed for purification. 2-Keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) was the gift of my colleague, L. R. Barran. Acrylyl CoA aminase was prepared by rupture of Clostridium propionicum cells grown on beta-alanine. beta-Alanine induces the enzyme to levels 200 times those of cells grown on alpha-alanine and a higher specific activity is difficult to attain (Vagelos et al., 1959); hence the very active extracts were used without purification.

ASSAYS FOR THE INTERCONVERSION OF LACTATE AND ACRYLATE

Throughout the course of this work, a unit is defined to conform with IUPAC recommendations, i.e., 1 unit of enzyme forms 1 μmole of product per minute at a temperature which should be specified (Dixon and Webb, 1964). Assays were performed at room temperature or at 37° as indicated. Protein determinations were performed in triplicate by both the Warburg-Christian and the Waddell methods as described in TECHNIQUES IN PROTEIN CHEMISTRY. The methods usually agree, and the average value was taken.

(1) Coupled Assay

The assay for lactyl CoA dehydrase was assembled from partially purified components as described (Baldwin et al., 1965) and was later modified to consist of the following: (1) 0.08 M phosphate buffer, pH 7.5; (2) 0.80 mM 2-para-iodophenyl-3-para-nitrophenyl-5-phenyltetrazolium chloride (INT); (3) 0.01% gelatin; (4) 0.067 mM phenazine methosulfate (PMS); (5) 13 mM NAD; (6) ca. 0.004% yeast alcohol dehydrogenase (ADH) (1.5 units); (7) 1 mM acrylyl CoA. The modified coupled assay is inherently superior to the original one because none of its components is obtained from P. elsdenii. Yeast ADH recognizes lactyl esters, but not free lactate, as an alcohol; evidently the enzyme has little steric specificity for that portion of the substrate molecule but does require it to be uncharged. The molar extinction coefficient for the formazan produced from INT upon reduction was taken as 14,200 liter per mole-centimeter at 490 mμ (Hirsch et al., 1963). INT is the dye of choice because its formazan is fairly stable to oxygen and is more soluble than others, though gelatin is still desirable to prevent precipitation (Nachlas et al., 1960). During validation of the assay pyruvyl dinitrophenylhydrazone was formed and isolated according to the procedure of Neish (1957).

(2) Direct Assay

All attempts at measuring lactyl CoA dehydrase activity by observing the appearance or disappearance of acrylyl CoA spectrophotometrically at 263 mμ were unfruitful. The variations attempted were (1) 0.06 M triethanolamine-HCl, pH 7.5; 6 mM acrylyl pantetheine; 0.35 mg of enzyme protein; and water to 0.21 ml (Vagelos et al., 1959), (2) the same as above with NAD, LDH, and CoA transferase added individually or in combination and with acrylyl CoA as substrate, (3) 0.05 M phosphate, pH 7.5, or 0.05 M HEPES, pH 7.5, mM EDTA, mM DTT, μM Fe (II) and all combinations thereof with acrylyl CoA and acrylyl pantetheine as substrates, and (4) 0.05 M pyrophosphate buffer, pH 8.5, with purified lactyl CoA as substrate. Furthermore the addition reaction, e.g., of 5 mM DTT and 8.75 mM acrylyl pantetheine in 0.10 M triethanolamine-HCl buffer, pH 7.5, was easily and reproducibly observable; thus the acrylyl thiolesters were present in the assay mixture, and the spectrophotometer, which has the advantage of a linear response to an absorbance of three, is capable of making the necessary measurements.

(3) Manometric Assay

At pH 7.5 or less, extracts form equimolar amounts of H_2 gas and propionate from lactate. Hence measurement of the H_2 evolved in a Warburg apparatus affords an assay

for the lactate to propionate reactions among which is that catalyzed by "lactyl CoA dehydrase." The activity values obtained from this assay are minimal inasmuch as other enzymes in the pathway may be limiting.

The incubations were carried out as described by Ladd and Walker (1959), except that 1 mM DTT was added. Hereafter this will be referred to as incubation system 1. The reactions were stopped after 45 min by addition of an equal volume of 0.1 M H_2SO_4.

Other incubations were carried out as follows: (1) nitrogen atmosphere; (2) room temperature; (3) variable substrate; (4) 0.10 M phosphate buffer (pH 7.0); (5) 5 mM ATP; (6) 5 mM $MgCl_2$; (7) 25 μM acetyl CoA; (8) 0.10 mM NAD; (9) 1.0 mM DTT; and (10) extract. These are essentially the conditions of Evans and Jackson (1966), except DTT was added and dialysis of the extract was normally omitted. These conditions will be referred to as incubation system 2. Distribution of nitrogen to many tubes was facilitated by a needle-valve, aquarium manifold which was purchased from a local pet shop.

(4) Propionate Assay

Gas chromatography of propionate which is formed from lactate is a valid assay if CoA transferase and acyl CoA dehydrogeanse are not rat limiting:

$$\text{lactate} + \text{acetyl CoA} \xrightarrow{\text{CoA-T}} \text{lactyl CoA} + \text{acetate}$$

$$\text{lactyl CoA} \xrightarrow[\text{rds}]{-H_2O} \text{acrylyl CoA}$$

$$\text{acrylyl CoA} + 2e^- + 2H^+ \xrightarrow{\text{ACD}} \text{propionyl CoA}$$

$$\text{propionyl CoA} + \text{acetate} \xrightarrow{\text{CoA-T}} \text{acetyl CoA} + \text{propionate.}$$

$$\text{NET: lactate} + 2e^- + 2H^+ \longrightarrow \text{propionate} + H_2O.$$

Incubations were carried out under N_2 and at 37°C; the assay solution consisted of 0.033 M Tris·HCl (pH 7.6); 0.33 M sodium DL-lactate (pH 7.6); variable ATP as specified; 5 mM $MgCl_2$; 25 μM acetyl CoA; variable NAD(H) as specified; and extract. NADH can be added as a source of electrons for the reduction of acrylate; however, since this assay was only employed with crude extracts the lactate dehydrogenase(s) maintained sufficient levels of reducing equivalents as to make addition of NADH unnecessary. This assay suffers from being dependent on many enzymes for activity and such dependency precludes its use for purification of enzyme activity. The initial idea was that as purification was attempted the enzymes necessary for reduction of acrylyl CoA would separate and then acrylate would appear as the product. Acrylate forms a unique peak on GLC and can be taken as an index of activity. However despite numerous purification attempts this assay, as described above, never gave acrylate as product.

Propionate and other acids were determined quantitatively by gas-liquid chromatography (GLC). The apparatus was a dual column Packard Instrument Co. gas chromatograph equipped with dual hydrogen flame detectors and dual-pen Texas Instrument Co. recorder. The 2 mm-diameter columns were packed with 10% FFAP on Chromosorb W, acid washed DMCS, 80/100, which is produced by Wilkens Instrument Co. The temperatures used were column 120, inlet 145, outlet 190, and detector 155°C. The sensitivity of the system permits determination of 1 nmole of any volatile fatty acid. 50 µl samples of the incubation solution were withdrawn at regular intervals and mixed with 10 µl 1 M H_2SO_4 and 50 µl 0.0105 M sodium isobutyrate which served as an internal standard and eliminated errors due to injection. The peaks were integrated manually by multiplying height by half-height width.

(5) Acrylate Assay

Acrylate was formed as the major product when extracts were added to a solution to give 0.30 ml total volume and the following concentrations of components: (1) 0.033 M HEPES buffer (pH 7.75); (2) 7 mM $MgCl_2$; (3) 0.02% methylene blue; (4) 0.3 mM acetyl CoA; (5) 33 mM DL-lactate (10 µmoles), and (6) 33 mM acetyl phosphate. These assays were performed under N_2 and at 37°C. Acrylate was determined on samples taken at various times and

injected into the gas chromatograph in the same manner as propionate.

LACTYL CoA DEHYDRASE PURIFICATION

When the coupled assay was thought to be valid, lactyl CoA dehydrase was purified according to published procedures (Baldwin et al., 1965; Baldwin, 1967), except that the calcium phosphate gel step was omitted for lack of reproducibility. The partially purified enzyme obtained in this way was applied to various columns in an attempt to achieve additional purification. All column chromatography was done at about 5°C. After addition of sample, a Sephadex G-100 column (1 x 73 cm) was eluted with 0.01 M Tris-HCl buffer, pH 8.5. The eluent was passed through a flow cuvette which was kept at about 5°C and was monitered at 280 and 260 mμ with a DU spectrophotometer equipped with Gilford automatic cuvette and wavelength positioners (Wood and Gilford, 1961). A Bio-Gel P-150 column (2.5 x 50 cm) was poured and operated in similar fashion, except that $5(10)^{-4}$ M DTT and $5(10)^{-4}$ M sodium acrylate were added to the eluting buffer.

DEAE-cellulose was washed and poured into a column (1.2 x 50 cm). Prior to application, the sample was dialyzed against 10^{-6} M Fe^{2+} and 10^{-3} M BAL which is a redox buffer (Wood, 1967). The eluent consisted of a linear gradient of increasing ionic strength with 1 mM DTT throughout: distilled water ⟶ solution of 0.2 M phosphate

and 0.4 M sodium chloride (pH 7.5).

Hydroxylapatite was prepared as described in TECHNIQUES IN PROTEIN CHEMISTRY (Bailey, 1962). The gel was poured into a 3.4 x 45 cm column to a height of 40 cm. Undialyzed enzyme was added and eluted step-wise with increasing concentrations of phosphate buffer pH 6.8.

OTHER ENZYME ASSAYS

Acyl CoA dehydrogenase was assayed as prescribed by Baldwin and Milligan (1964) with the exception that propionyl CoA was substituted for butyryl CoA as substrate.

D-Lactate dehydrogenase was assayed spectrophotometrically by following the disappearance of ferricyanide at 420 mμ upon reduction by lactate. The procedure used was originally that described by Symons and Burgoyne (1967) but was modified to eliminate pyrophosphate and EDTA when a metal requirement was found and consists of mixing the following, in order, 200 μl of 0.1 M Tris (HCl) (pH 8.0) and 0.1 M D-lactate (sodium salt); 50 μl of 0.1 M $K_3Fe(CH)_6$; 15 μl of 20 mM $CoCl_2$; and 35 μl of extract or water. The extinction of ferricyanide was taken as 1,040 liter per mole-centimeter relative to ferrocyanide. The existence of pyruvate produced during the course of an optical assay was correlated with activity by forming the dinitrophenylhydrazone at various times according to the direct method of Friedemann and Haugen (1943), i.e.,

strong alkalai was added to the pyruvyldinitrophenylhydrazone solution (to give 0.5 N NaOH overall) in order to decompose excess dinitrophenylhydrazine and to dissolve precipitated proteins; and the absorbance was measured at 435 mμ without organic-solvent extraction. L-Lactate dehydrogenase activity was determined in a similar manner except that L-lactate was substituted for D-lactate as substrate. Lactate racemase activity was measured by allowing racemase activity to convert D-lactate to L-lactate and the latter was removed by an excess of rabbit muscle L-lactate dehydrogenase, and either NAD at pH 9.9 according to the procedure of Lowry (1957), or 3-acetylpyridine NAD (AcPyAD) at pH 8.5 as described by Dennis and Kaplan (1960). The advantage of the NAD analogue is that it affords a linear assay in the direction of pyruvate formation because the lactate + AcPyAD to pyruvate + AcPyADH equilibrium is shifted to the right relative to NAD. The extinction coefficient of AcPyADH relative to AcPyAD is 7,750 liter per mole-centimeter (Kaplan and Ciotti, 1957).

Two kinase assays were employed. The first measures ATP consumption spectrophotometrically in an indirect manner. ATP is provided by PEP, ADP, and pyruvate kinase; as ATP is consumed so is PEP which releases pyruvate; pyruvate appearance is coupled to NADH with rabbit muscle LDH and observed at 340 mμ (Anderson and Wood, 1967). The second depends on disappearance of

acetyl phosphate as measured by hydroxamate formation, e.g., if acetyl phosphate and lactate react to form acetate and phospholactate and since phospholactate gives a negative hydroxamate test, then the reaction can be followed as described. The procedure was to mix in order 100 µl of 0.1 M HEPES buffer (pH 7.75); 35 µl of 60 mM $MgCl_2$; 6 µl of 1% methylene blue; 30 µl of 1 mM dinitrophenol; 42 µl of 1 M acetyl phosphate; 42 µl of 1 M sodium lactate; 30 µl of 5 mM acetyl CoA; and 50 µl of water or extract. The assay was incubated at 37°C under N_2, and at various times aliquots were removed for hydroxamate formation as described above for thiolester determination.

ORGANIC ACID PURIFICATIONS

Propionate and acetate were purified by chromatography on either a Wiseman-Irvin or Swim-Krampitz column as specified (Wiseman and Irwin, 1957) (Swim and Krampitz, 1954). The latter column was modified as described by Kuratomi and Stadtman (1966). The packing of these columns was greatly facilitated by use of a tamper (30 x 1/8 in stainless-steel rod with 7/16 in diameter, perforated, stainless-steel disc on the end). Since these columns do not separate acrylate and propionate, acrylate was, in specified cases, removed by acidifying the solution to 0.4 N HBr and heating in boiling water for 2 min. GLC analysis showed the acrylate to have disappeared.

Both cationic polymerization and hydrobromination probably occur.

Propionic acid was converted to acetic acid by Schmidt degradation and permanganate oxidation according to the procedure of Phares (1951) employing the apparatus described by Krichevsky and Wood (1961).

Lactic and pyruvic acids were purified by chromatography on Celite columns according to the procedure of Swim and Krampitz (1954) as modified by Kuratomi and Stadtman (1966). Lactate was determined in two ways. First, in the Barker and Summerson procedure (1957), lactate was oxidized to acetaldehyde which subsequently reacts with p-hydroxyldiphenyl to form a colored adduct with absorbance at 570 mμ. Lactate was also determined by enzymatic conversion to pyruvate as described above for the lactate racemase assay. The enzymatic determination is the superior method of the two, especially if 3-acetyl-pyridine NAD is employed, because of the ease and reproducibility with which it is performed. In either case a standard curve was run with each set of determinations.

Phospholactic acid was purified for purposes of determining radioactivity by descending paper chromatography on Whatman 3 MM paper with 3:1 95% ethanol-0.1 N acetate buffer (pH 4.0). Several different solvent systems were tested for ability to separate phospholactate,

phosphate, and lactate from one another with the following results:

Solvent	R_F: Phosphate	Phospho-lactate	Lactate
n-Propanol:HCOOH:H_2O (6:3:1)	0.49	0.51	0.78
HCOOH:H_2O:95% EtOH (1:29:70)	.47	.73	.78
2-Butanone:HOAc:H_2O (8:8:1)	.59	streak	1.00
95% EtOH:0.1 N Phthalate pH 3.0 (3:1)	2.6	.63	.68
95% EtOH:Dioxane:H_2O:HOAc (60:20:19:1)	streak	.50	.70
95% EtOH:0.1 N Acetate pH 3.0 (3:1)	.26	.63	.68
95% EtOH:0.1 N Acetate pH 4.0 (3:1)	.25	.40	.60
95% EtOH:0.1 N Acetate pH 5.8 (3:1)	.57	.35	.75

Further purification was accomplished by placing the phospholactate on a DEAE-cellulose column (1 x 40 cm) equilibrated with 0.03 M ammonium carbonate and eluting with a linear gradient consisting of 200 ml of 0.03 M ammonium carbonate to 200 ml of 0.03 M ammonium bicarbonate at a flow rate of 30 ml/hr. Three ml fractions were collected. This column effects very clean separation of phospholactate from lactate. The remaining contaminant was phosphate and was difficult to remove. Specific precipitation with triethylamine-molybdate of orthophosphate is supposed to be possible (Sugino and Miyoshi, 1964);

however all attempts in this regard were unsuccessful because the phospholactate either was destroyed or was precipitated along with the phosphate. The best separation of phospholactate and phosphate was achieved on a Sephadex G-10 column (2.5 x 100 cm) operated at 10 ml/hr, though a second passage was necessary to achieve quantitative separation. Phospholactate was determined either by radioactivity measurements or by alkaline phosphatase treatment followed by determination of the lactate and orthophosphate released. Alkaline phosphatase treatment was performed at pH 8 in the presence of 5 mM $MgCl_2$.

Phosphate was determined by the method of Chen et al. (1956) and, in order to increase sensitivity, the determinations were performed on a 0.80 ml basis instead of 8.0.

beta-Alanine was determined by TLC chromatography on pre-coated Silica Gel plates in 80:20:4 Methanol: Water:Pyridine as described by Brenner et al. (1965). The spots were visualized by spraying with ninhydrin and heating for 10 min at 110°C. In this system beta-alanine has R_F = 0.44 compared to 0.58 for alpha-alanine.

RADIOACTIVITY MEASUREMENTS

Radioactivity was measured in a Packard Tricarb Liquid Scintillometer. For counting tritium and carbon-14 the settings were tritium channel gain 50, window 50-400, and carbon-14 channel gain 10, window 200-1000. For

double-labeling experiments the tritium counts should be about ten times those of carbon-14 because 36.7% of the channel B counts also appear in channel A. Bray's scintillation fluid was used while working with tritium because it works well for samples containing large amounts of water (Bray, 1960). Chemiluminescence is somewhat troublesome; hence the samples should be thoroughly cooled and then counted twice. For counting carbon-14 and phosphorous-32 the settings were carbon-14 channel gain 9.0, window 50-600, and phosphorous-32 channel gain 1.2, window 200-1000. XDC scintillation fluid (xylene, dioxane, cellosolve) was used for all work other than that involving tritium (Bruno and Christian, 1961).

MASS SPECTROMETRY

L-Lactate-2-^{18}O was prepared by exchange between $H_2{}^{18}O$ and pyruvate as catalyzed by KDPG-aldolase and by subsequent reduction with NADH as catalyzed by muscle lactate dehydrogenase (Rose and O'Connell, 1967). The lactate was purified by chromatography on a Celite column as described above. The ^{18}O content of the 2-^{18}O-lactate was determined by converting it to carbon dioxide as catalyzed by mercuric chloride at 500°C according to the procedure of Rittenberg and Ponticorvo (1956); the CO_2 was then analyzed on a mass spectrometer. ^{18}O-Lactate was converted to propionate and ^{18}O-phosphate by reaction with extracts in incubation system 1 (see Manometric

Assay). The phosphate was isolated and purified as described by Boyer and Bryan (1967). The ^{18}O of phosphate was converted to ^{18}O-carbon dioxide by heating with guanidine-HCl according to the procedure of Boyer *et al.* (1961). Carbon dioxide samples were analyzed on a low resolution mass spectrometer by the Department of Chemistry, Michigan State University.

CHEMICALS

Most of the chemicals used in the course of this work are listed below according to their sources. Corn steep liquor was a gift of the A. E. Staley Manufacturing Co., Decatur, Illinois.

CHEMICAL	COMMERCIAL SOURCE
antimycin A	Sigma Chemical Co.
1,10-phenanthroline·H_2O	"
oligomycin	"
glucose-6-phosphate	"
dinitrophenol	"
L(+)-lactic acid	"
pyridoxal·HCl	"
sodium pyruvate	"
yeast alcohol dehydrogenase	"
muscle lactate dehydrogenase	"
rotenone	"
trisodium PEP	"
sodium acrylate	K and K Rare Chem. Co.
alpha-amine red-R	"
dichlorophenolindophenol, sodium salt	"
ethyl chloroformate	"
hydracrylic acid	"
INT dye	"
sodium azide	"
coenzyme A (lithium salt)	P-L Biochemicals, Inc.
ATP, ADP, and AMP	"

CHEMICAL	COMMERCIAL SOURCE
NAD and NADH	P-L Biochemicals, Inc.
NADP	"
glucose-6-phosphate dehydrogenase	"
glutathione, reduced	"
CTP	"
3-acetylpyridine NAD	"
cysteine·HCl	Calbiochem
dithiothreitol	"
D-lactic acid, lithium salt	"
2,2'-dipyridyl	"
Sephadex G-10	Pharmacia Fine Chem., Inc.
Sephadex G-15	"
Sephadex G-100	"
CM-Sephadex C-50	"
60% sodium lactate	Pfanstiehl Lab., Inc.
isobutyric acid	Eastman Organic Chemicals
isovaleric acid	"
acetic anhydride	"
butyric anhydride	"
succinic anhydride	"
succinic acid	"
Bio-Gel P-2	Bio-Rad Labs
Bio-Gel P-150	"
Dowex-1	"
Dowex-50	"
DEAE-cellulose	"
CM-cellulose	"
ECTEOLA-cellulose	"
sodium arsenate	Mallinckrodt
potassium cyanide	"
potassium ferricyanide	"
sodium acetate	"
potassium phosphate, monobasic	"
DL-lactic acid	"
sodium azide	Fisher Sci. Co.
sodium pyrophosphate	"
phosphoglycolic acid, cyclohexylammonium salt	General Biochemicals
disodium EDTA·$2H_2O$	J. T. Baker Chem. Co.
oxalic acid·$2H_2O$	"

CHEMICAL	COMMERCIAL SOURCE
sodium arsenite	Matheson, Coleman, and Bell
methylene blue	Eberbach
intestinal alkaline phosphatase	Worthington.

RADIOCHEMICALS

Lactic acid-2-^{14}C, lactic acid-3-^{14}C, and tritiated water (1 C/gm) were purchased from Volk Radiochemical Co. Acrylic acid-1-^{14}C was obtained from International Chemical and Nuclear Corp. (ICN). Adenosine-5'-triphosphate-gamma-^{32}P was obtained from The Radiochemical Center. Sodium-D-lactate-^{14}C (u) and sodium-DL-lactate-1-^{14}C were from Amersham/Searle.

Lactic acid-3-^{3}H was prepared by taking advantage of the KDPG aldolase-catalyzed exchange of the hydrogens of pyruvate (Meloche and Wood, 1964b). The following reagents were added and stirred (1) 0.50 ml of water; (2) 0.050 ml of tritiated water; (3) 0.500 ml of 0.05 M phosphate buffer (pH 7.5); (4) 110 mg of sodium pyruvate (one millimole); and (5) 0.010 ml of KDPG aldolase (47,000 U/ml). The solution was allowed to incubate at room temperature for three hours to assure complete exchange. Then pyruvate was reduced to lactate by adding rapidly a second solution which was prepared by mixing in order the following (1) 3 ml of 0.05 M phosphate buffer (pH 7.5); (2) 10 mg of NADP; (3) 364 mg of glucose-6-phosphate; (4) 0.100 ml of muscle lactate dehydrogenase crystals;

and (5) 0.050 ml of glucose-6-phosphate dehydrogenase crystals (suspended in ammonium sulfate solution). The combined solution was permitted to incubate at room temperature for sixteen hours. The solution was evaporated to dryness on a test tube Rotovap and the lactic acid was purified on a Swim and Krampitz column. The yield was 72% and the lactic acid-3-^{3}H had a specific activity of about 0.18 μC/μmole. It was stored as a 30 m$\underline{M}$ solution (pH 7) at -14°C.

CHAPTER IV

RESULTS

The experiments presented herein are divided into two sections: (1) those which consider the difficulties of obtaining a lactyl CoA dehydrase assay and the subsequent reinvestigation of acrylyl CoA as an intermediate and (2) those which show a new α-phospholactyl CoA intermediate between lactyl CoA and acrylyl CoA. The first section will involve: (a) validation of the coupled assay; (b) validation of the modified coupled assay; (c) partial purification and properties of lactyl CoA dehydrase; (d) the inconsistencies of the lactyl CoA dehydrase which involve specific activities, inhibitors, and the lack of a direct assay; and (e) further evidence for acrylyl CoA as an intermediate using new types of experiments. The second section will include: (a) the propionate assay and its stimulation by ATP; (b) stabilization of extracts as regards their activity in the assay for propionate formation; (c) purification of the propionate-assay activities; (d) inhibition by dinitrophenol and its reversal by ATP; (e) demonstration of ^{18}O transfer from 2-^{18}O-lactate to orthophosphate concomitant with propionate formation; (f) labeling of phospholactate from gamma-^{32}P-ATP; (g) the assay for acrylate formation

and its requirement for acetyl phosphate; (h) the enzymatic formation of phospholactate from ^{14}C-lactate and ^{32}P-orthophosphate; (i) isolation of the intermediate (phospholactate); (j) the conversion of enzymatically- and chemically-synthesized phospholactate to acrylate; and (k) chemical characterization of phospholactate.

I. DIFFICULTIES WITH THE LACTYL CoA DEHYDRASE ASSAY AND REINVESTIGATION OF ACRYLYL CoA AS AN INTERMEDIATE

The starting point of this investigation was the assembly of the components of the coupled assay for lactyl CoA dehydrase according to the procedure of Baldwin (1962). The coupled assay was subjected to validation tests as described below.

A. Confirmation of Lactyl CoA Dehydrase Activity

The coupled assay assumes ready reversibility of the lactyl CoA-acrylyl CoA interconversion and involves the lactyl CoA dehydrase-catalyzed conversion of acrylyl CoA to lactyl CoA which is observed spectrophotometrically by coupling the formation of lactyl CoA to the reduction of a tetrazolium dye (INT):

1) acrylyl CoA + H_2O $\longrightarrow$ lactyl CoA

2) lactyl CoA + acrylate + INT $\xrightarrow[\text{coupling system}]{\text{CoA transferase}}$

pyruvate + acrylyl CoA + $INT_{reduced}$

The assay was subjected to the following validation tests: (1) the appearance of pyruvate concomitant with the reduction of INT was demonstrated by isolating pyruvyl dinitrophenylhydrazone, and (2) the discrimination of the coupling system against crotonase activity was confirmed by its failure to oxidize beta-hydroxypropionate.

1. Eight different coupled assay mixtures containing lactyl CoA dehydrase, which had been subjected to protamine sulfate treatment and one ammonium sulfate precipitation, were pooled and allowed to react an additional hour. The mixture was incubated with 2,4-dinitrophenylhydrazine reagent and a sample was spotted for chromatography. The paper chromatogram was run in descending fashion with 7:1:2 n-butanol-ethanol-0.5 M ammonia. The spots corresponding to those of authentic pyruvyldinitrophenylhydrazone were visible following incubation but were absent at zero time. This experiment, though admittedly gross, suggests that pyruvate was formed from acrylyl CoA.

2. The following components of the coupled assay system were mixed with beta-hydroxypropionate or lactate, and the absorbance at 485 mμ was recorded: (1) buffer, (2) INT, (3) gelatin, (4) NAD, (5) PMS, and (6) lactate dehydrogenase. The beta-hydroxypropionate solution was prepared by neutralizing 0.10 ml of beta-hydroxypropionic acid and diluting to 1.0 ml. The sodium lactate solution was prepared by diluting 0.10 ml of 60% sodium lactate syrup to 1.0 ml. The data obtained (Table 2) showed that

Table 2. Specificity of coupled assay for conversion of acrylyl CoA to lactyl CoA

SUBSTRATE	AMOUNT ADDED	ΔA/5 MIN
	μmoles	
None	--	0.145
beta-Hydroxypropionate	6.5	0.145
"	13.0	0.060
"	19.5	0.040
Lactate	6.5	3.30
"	13.0	10.0
"	19.5	10.0

Each reaction cuvette contained in 0.250 ml: 0.04 M pyrophosphate buffer (pH 8.5); 0.80 mM INT; 0.01% gelatin; 0.067 mM PMS; 13 mM NAD; substrate; and about 1 unit muscle lactate dehydrogenase.

rabbit muscle lactate dehydrogenase did not utilize beta-hydroxypropionate and hence is specific for the alpha-hydroxy group under these conditions. Therefore the coupled assay is not a measure of crotonase activity.

B. The Modified Coupled Assay for Lactyl CoA Dehydrase

The coupling system of Baldwin's original coupled assay consists of three enzymes, of which CoA transferase and diaphorase were prepared from P. elsdenii:

1) lactyl CoA + acrylate $\xrightarrow{\text{CoA transferase}}$ lactate + acrylyl CoA

2) lactate + NAD^+ $\xrightarrow{\text{L-LDH}}$ pyruvate + NADH + H^+

3) NADH + H^+ + INT $\xrightarrow{\text{diaphorase}}$ NAD^+ + $INT_{reduced}$.

Although not described here the Baldwin assay system was restudied in an effort to eliminate the use of enzymes from P. elsdenii and this resulted in the following modifications (for details see MATERIALS AND METHODS). First the CoA transferase and muscle lactate dehydrogenase were replaced by yeast alcohol dehydrogenase (ADH) because it had been reported by Rabin et al. (1965) that lactyl CoA was oxidized directly by alcohol dehydrogenase whereas lactate was not. Second the diaphorase was replaced by phenazine methosulfate (PMS):

1) lactyl CoA + NAD^+ $\xrightarrow{\text{ADH}}$ pyruvyl CoA + NADH + H^+

$$2)\ NADH + H^+ + INT \xrightarrow{PMS} NAD^+ + INT_{reduced}$$

The modified coupled assay was tested in two ways. First the ability of the coupling system to measure lactyl CoA was compared to that of the original coupling system wherein lactyl CoA dehydrase itself was not of concern but rather whether the product of its action on acrylyl CoA, lactyl CoA, could be rapidly oxidized. Consequently lactyl CoA was added directly. Equivalent amounts of purified lactyl CoA were added to each assay system and the total change in A_{485} was observed (Table 3). The modified system gave slightly lower values, probably because the lactyl CoA was partially hydrolyzed.

In a second test, equivalent amounts of lactyl CoA dehydrase were added to the original and modified assay systems in order to compare activities. The initial rates were about the same in both cases (Table 4).

The modified coupled assay is inherently superior to the original for the following reasons: (1) it eliminates any components which are derived from P. elsdenii other than lactyl CoA dehydrase; (2) whereas CoA transferase is required in the Baldwin assay, the modified assay makes use of alcohol dehydrogenase to oxidize lactyl CoA directly making CoA transferase unnecessary; and (3) since alcohol dehydrogenase is specific for the lactyl portion only, whereas CoA transferase requires CoA esters, it would be possible to use or at least to test the

Table 3. Validation of the modified coupled assay using alcohol dehydrogenase and purified lactyl CoA

ASSAY	TOTAL CHANGE IN A_{485}	
	Experiment	
	1	2
Original	0.75	1.43
Modified	0.60	1.00

Each reaction cuvette contained in 0.250 ml: 0.04 M pyrophosphate buffer (pH 8.5); 0.80 mM INT; 0.01% gelatin; 13 mM NAD; and about 15 μM lactyl CoA. In addition the original assay contained about 1 unit of muscle LDH and 0.1 unit of diaphorase; the modified assay contained 0.067 mM PMS and 0.004% yeast ADH.

Table 4. Comparison of the coupled assays for lactyl CoA dehydrase

EXPERIMENT	ASSAY	
	ORIGINAL	MODIFIED
	units per ml	
Extract		
1	0.0792	0.0477
2	.981	.259
Partially purified fraction		
1	0.054	0.079
2	.150	.082
3	.200	.218

The assays were performed as described in Table 3 except 1 mM acrylyl CoA replaced lactyl CoA as substrate and lactyl CoA dehydrase was added. One unit of enzyme is defined as that which forms 1.0 μmole of product per minute at room temperature (about 28°C inside the spectrophotometer).

pantetheine thiolesters. Both coupled assays have a common shortcoming: dithiolthreitol is required by the dehydrase but it also spontaneously reduces INT thereby giving rise to high blanks.

C. Partial Purification and Properties of Lactyl CoA Dehydrase

Employing Baldwin's procedure (1962; Baldwin et al., 1965), without the final step, the average of ten purifications yielded a specific activity of 0.107 μmole/min/mg protein with a standard deviation of 0.114. This represents an average purification of 20-fold. The highest activity was 0.625 or 109-fold.

Further purification was attempted using Sephadex G-100, Bio-Gel P-150, and DEAE-cellulose. In every case all activity was lost. Recombination of various fractions or addition of boiled enzyme supernatant did not recover the activity. Also no evidence for dissociation of a cofactor was obtained by charcoal treatment of the partially purified enzyme.

The enzyme, as measured by the coupled assay, does require an SH reducing agent, especially during storage. Extracts were prepared in the absence of dithiothreitol (DTT), assayed, and then stored at 5°C. After 43 days, the activity was 15% of the original value and addition of 1 mM DTT resulted in over 100% return of activity (Table 5).

Table 5. Dependence of the coupled assay and of the stability of lactyl CoA dehydrase upon dithiothreitol

TIME	DTT CONCENTRATION	ACTIVITY
Days	mM	units per ml
0	0	0.111
43	0	0.014
43	1.0	0.148

The assay mixture contained 0.08 M Tris-acetate buffer (pH 8.0); 0.80 mM INT; 0.01% gelatin; 13 mM NAD; 1 mM acrylyl CoA; 1 unit of muscle lactate dehydrogenase; about 0.1 unit of diaphorase; and lactyl CoA dehydrase in a final volume of 0.250 ml. One unit is the same as in Table 4.

Up to this point, most of what Baldwin discovered about lactyl CoA dehydrase had been confirmed. However, several difficulties had already presented themselves as discussed below.

D. Evidence Against a Simple Lactyl CoA Dehydrase

The troublesome aspects of the coupled assay were: (a) the failure to demonstrate either the disappearance of acrylyl CoA or the production of acrylyl CoA from lactyl CoA using the direct spectrophotometric assay for the acrylyl thiolester bond; and (b) the extremely low specific activities measured by the coupled assay in P. elsdenii. Also the behavior of the partially purified lactyl CoA dehydrase was unusual. Additional purification attempts, or in some cases storage, resulted in complete loss of activity. Recombination of fractions which might be necessary if a cofactor had been separated was not successful. Most dehydrases fall into one of four groups: (1) no cofactor requiring, (2) divalent metal ion requiring, (3) metal ion and reducing agent requiring, and (4) pyridoxal phosphate requiring (Malström, 1961). Lactyl CoA dehydrase requires a reducing agent, but metal ion cofactors are eliminated inasmuch as EDTA is not an inhibitor and is sometimes beneficial. Pyridoxal phosphate or cobalamin (vitamin B_{12}) does not appear to be required because charcoal-treatment had no effect on

activity, and their function would not be expected on mechanistic grounds. On account of the above difficulties the very existence of lactyl CoA dehydrase was reexamined as discussed below.

The existence of lactyl CoA dehydrase was tested in three ways: (1) Ladd and Walker had shown that 2 x 10^{-5} M cyanide and other inhibitors of electron transport phosphorylation prevent the conversion of lactate to acrylate as well as the reverse reaction. If it is presumed that these inhibit by acting on the "lactyl CoA dehydrase" then the coupled assay should be inhibited to the same extent. (2) The overall rate of propionate formation should be less than or equal to the rate of lactyl CoA dehydration. Hence, if the specific activity of lactyl CoA dehydrase is less than that for the overall reaction, either it cannot be considered as an obligatory enzyme of the acrylate pathway, or the dehydrase assay is defective relative to the fermentation mixture forming propionate. (3) The direct spectrophotometric assay for the appearance or disappearance of acrylyl CoA must be possible with a "lactyl CoA dehydrase" and so a variety of assay conditions were employed in an effort to find one which gave enzyme activity.

(1) The coupled assay was used to measure lactyl CoA dehydrase activity in unpurified extracts in the presence of varying concentrations of sodium cyanide, sodium azide, sodium ethylene diamine tetraacetate (EDTA),

and hydroxylamine hydrochloride. As shown in Table 6, the concentrations necessary to affect a 50% inhibition are much higher than those reported for the interconversion of lactate and acrylate under similar conditions as measured by evolution of hydrogen in the Warburg apparatus (Ladd and Walker, 1965). The manometric assay is valid at pH 7.5 or less because extracts form equimolar amounts of H_2 gas and propionate from lactate. Complete inhibition of the coupled assay was difficult to observe because the response to inhibitor was not linear at high concentrations. The failure to observe inhibition at concentrations as low as those used by Ladd and Walker may be interpreted to mean that the lactyl CoA dehydrase activity observed in the coupled assay is not related to the enzymes of the acrylate pathway.

(2) The specific activity of fresh extracts as measured by the coupled assay was reexamined. Thirteen different fermentations, assayed with the original coupled assay system, gave an average specific activity of 0.00578 μmole/min/mg protein with a standard deviation of 0.00772. Calculation of the minimum specific activity possible assuming that (a) there is log phase growth with no lag, and (b) the enzyme activity increases gradually throughout growth, doubling every generation, gives 0.684 μmole/min/mg protein (for details see APPENDIX, p. 1). The discrepancy between calculated and observed specific activities is greater than 100-fold.

Table 6. Inhibitors of lactyl CoA dehydrase

INHIBITOR	CONCENTRATION NECESSARY FOR 50% INHIBITION OF COUPLED ASSAY	CONCENTRATION NECESSARY FOR 100% INHIBITION OF WARBURG ASSAY*
	mM	mM
Cyanide	2.2	0.02
Azide	8.0	2.0
EDTA	12	-
Hydroxylamine	35	0.1

*Data of Ladd and Walker (1965).

The coupled assays were performed as described in Table 5.

(3) The direct assay for lactyl CoA dehydrase depends on the appearance or disappearance of acrylyl CoA as observed spectrophotometrically at 263 mμ. alpha,beta-Unsaturated thiolesters have a unique absorbance peak at 263 mμ with a molar extinction coefficient of 6,700. The assay was attempted under the following conditions: (1) acrylyl pantetheine as substrate in Tris buffer (pH 7.5) with NAD, LDH, and CoA transferase added individually and in combination, (2) acrylyl CoA as substrate with the above variations, (3) acrylyl CoA or acrylyl pantetheine as substrate in phosphate or HEPES buffer, 1 mM EDTA, 1 mM DTT, 1 μM Fe (II), and all combinations thereof, and (4) lactyl CoA (purified) as substrate in pyrophosphate buffer (pH 8.5). The addition reaction between dithiothreitol and, for example, acrylyl pantetheine was easily and reproducibly observable; thus the acrylyl thiolester was present and observable. Yet in every case there was no enzyme catalyzed appearance or disappearance of acrylyl CoA in the direct assay whether extracts or partially-purified lactyl CoA dehydrase was used. The amount of enzyme assayed from varied from 0.01 to 100 times that required to give average rates in the coupled assay.

With these disturbing facts in mind, it was concluded that the lactyl CoA dehydrase as defined by Baldwin and confirmed in this study is some artifact or side reaction and does not represent the reaction which is the object of the present study. Thus, a new approach was in

order. Before continuing with the purification and study of the key enzyme of the pathway, the following questions ought to be answered with certainty. (1) Is acrylate an intermediate or is the reaction via a totally different mechanism, e.g., hydride displacement as in deoxycytidine diphosphate formation? (2) What are the individual reactions of the pathway, i.e., does the lactyl CoA to propionyl CoA conversion involve one, two, or three separate reactions? (3) How are the enzymes of the individual steps assayed?

As a first step, the existence of acrylyl CoA as an intermediate was reexamined.

E. Confirmation of Acrylyl CoA Intermediate

(1) Larsson showed that when deoxyribonucleotides are formed from ribonucleotides in tritiated water, tritium is not incorporated into the 3' position of ribose. The results were interpreted as evidence of a hydride displacement of the 2' hydroxyl group. This sort of experiment depends on equilibration of the potential hydride hydrogen with the tritiated water. In the case of deoxyribonucleotide formation such was known to be the case. It is likely that in propionyl CoA formation from acrylyl CoA the reducing hydrogens would equilibrate with the solvent since acyl CoA dehydrogenase either utilizes a cytochrome component, in which case the hydrogens would be derived from protons of the solvent, or a flavin component

in which case the reducing hydrogens would exchange with those of the solvent; on the other hand, propionate formation by hydride displacement seemed possible by analogy with deoxyribonucleotide formation. This possibility was tested by: (1) incubating lactate and extracts in tritiated water; (2) isolating the propionate produced; and (3) determining the relative amount of tritium incorporated into positions 2 and 3 of propionate. If hydride displacement of the hydroxyl group occurs, tritium would not be incorporated at carbon 3; and, if elimination of the elements of water occurs, acrylyl CoA would be an intermediate and tritium would be found at carbons 2 and 3. Specifically, lactate-2-^{14}C or lactate-3-^{14}C (120 μmoles; 5.3 x 10^8 cpm/mole) was incubated in system 1 (see MATERIALS AND METHODS) with tritiated water (1.2 x 10^{10} cpm). The propionic acid produced after 45 min was purified by partition chromatography, assayed for radioactivity, and degraded to acetic acid. The acetic acid was purified as above and counted. The percent of the total tritium incorporated into carbon 3 was calculated from the following equation:

$$\%\ ^3H \text{ in C-3} = \left[\frac{\text{counts } ^3H \text{ in AC}}{\text{counts } ^{14}C \text{ in AC}} \div \frac{\text{counts } ^3H \text{ in PRO}}{\text{counts } ^{14}C \text{ in PRO}}\right] \times 100.$$

The ^{14}C affords an internal reference, and hence exact titrations and quantitative transfers are not necessary. The isotope content showed that about 26% of the tritium

incorporated into propionic acid is in the C-3 position (Table 7) and the remainder is in the C-2 position.

Also if one assumes that two tritium atoms should be incorporated per propionate, then the expected $^{3}H/^{14}C$ ratio would be 302; the observed ratio was 60 (see APPENDIX, p. 3, for the details of this calculation). Thus, there is an isotope discrimination of about 5-fold. This is well within the range usually encountered (Melander, 1960).

Since considerable tritium was incorporated at carbon 3 a direct hydride displacement of the hydroxyl group is eliminated as the sole mechanism and the data are consistent with an acrylyl intermediate. Otherwise no tritium would have been found in C-3 as was observed in the deoxycytidine diphosphate case by Larsson (1965) and by Durham et al. (1967). The data are also consistent with the supposition that the reducing protons for the acyl CoA dehydrogenase reaction equilibrate well with the solvent. The fact that tritium is not distributed equally between carbons 2 and 3 may be due to slight differences in isotope effect, i.e., the acyl CoA dehydrogenase may discriminate less at carbon 2 than at carbon 3. The difference may also be due to a loss of some tritium at carbon 3 during the conversion of propionate to acetate inasmuch as the Schmidt degradation and permanganate oxidation impose severe conditions.

Table 7. Tritium incorporation into propionate during its formation from lactate

EXPERIMENT	PROPIONATE CPM			ACETATE CPM			% OF TOTAL TRITIUM IN PROPIONATE	
	^{3}H	^{14}C	RATIO	^{3}H	^{14}C	RATIO ($^{3}H/^{14}C$)	C-2	C-3
1	217,767	56,564	3.85	359,834	238,931	1.505	61	39
2	274,595	70,970	3.87	305,137	202,251	1.51	61	39
3	96,731	16,847	5.74	179,195	83,070	2.16	62	38
4	263,043	33,076	7.95	55,864	248,183	0.225	97	3
5	224,552	19,011	11.8	89,546	39,209	2.28	81	19
6	391,928	28,301	13.8	169,243	58,917	2.87	79	21
Average							73.5 ($\pm$14.7)	26.5 ($\pm$14.7)

The reaction mixture contained the following: 0.08 M phosphate buffer (pH 6.0); extract (about 11 mg protein); 0.048 M DL-lactate (2- or 3-^{14}C, 5.3 x 10^{8} cpm/mole); 0.1 mM acetyl CoA; 0.1 mM DTT; and tritiated water (1.2 x 10^{10} cpm) in a total volume of 2.5 ml. The incubation was at 37°C under N_2 for 45 min. The propionate formed was isolated and degraded as described in MATERIALS AND METHODS.

(2) In the second experiment, the competitive effect of acrylate-1-^{14}C on the conversion of lactate-3-^{3}H to propionate by extracts of P. elsdenii was tested in incubation system 2 (see MATERIALS AND METHODS). The reasoning was that added acrylate-1-^{14}C would be converted to acrylyl CoA via CoA transferase and thereby enlarge the pool of acrylyl CoA available for reduction to propionyl CoA. The effect of this would be to diminish the amount of lactate-3-^{3}H converted to propionate. Following incubation, propionate was isolated by partition chromatography and acrylate was removed by treatment with hydrogen bromide as described in MATERIALS AND METHODS. The propionate was then counted for ^{3}H and ^{14}C. Some ^{3}H may have been lost during the conversion of lactate to propionate, but this would not have affected the results because the same relative amount would have been lost in the control without the competition of acrylate. The results show that added acrylate is converted to propionate and that it inhibits the lactate to propionate reaction as would be expected if it were an intermediate (see Table 8). In fact the inhibition is more than expected; however the concentration of acrylate used may be three orders of magnitude greater than would be present as an intermediate.

(3) In the third experiment, production of acrylyl CoA as an intermediate was tested by adding reduced glutathione which acts as a trapping agent by adding across the double bond of acrylyl esters as described by Stadtman

Table 8. Inhibition of the lactate-to-propionate reactions by added acrylate

INCUBATION MIXTURE		PROPIONATE PRODUCED	
LACTATE-3-^{3}H	ACRYLATE-1-^{14}C	FROM LACTATE*	FROM ACRYLATE
µmole	µmole	µmole	µmole
10	0	7.86	-
10	5	2.53	1.37
10	10	2.73	2.78
10	15	2.38	5.36

*Assuming no loss of ^{3}H during the interconversion.

The reaction mixutre contained the following: 0.10 M phosphate buffer (pH 7.0); 2 mM DL-lactate (400,000 cpm of 3-^{3}H-lactate (L); 5 mM ATP; 5 mM $MgCl_2$; 25 µM as indicated (about 0.01 µC/µmole); and extract (27 mg of protein) in a volume of 5 ml. The mixture was incubated at 37°C under N_2 for 30 min. The propionate formed was isolated as described in MATERIALS AND METHODS.

(1957). Production of acrylyl CoA would be established by identification of the adduct chromatographically. The experiment involved incubation of lactate-3-^{3}H (20 μmole) in system 2 (cf. MATERIALS AND METHODS), except that 10 m*M* glutathione was used in place of 1 m*M* DTT. The reactions were stopped by addition of 2 ml of 14% neutralized hydroxylamine and the hydroxamates chromatographed along with authentic 3-(*S*-gluthionyl)propionyl hydroxamic acid. Strips were cut into pieces, each representing an R_F range of 0.05; and assayed for radioactivity. The R_F of authentic 3-(*S*-glutathionyl)propionyl hydroxamate was determined by spraying the remainder of the chromatogram with acidic ferric chloride. The standard was contaminated with polymerized acrylyl hydroxamate as evident from turbidity and this accounts for the second spot observed. Nevertheless, the data show that glutathione did indeed trap an acrylyl intermediate (see Table 9). Furthermore, the lactate to acrylate reaction must occur at the level of thiolester, as reported by Ladd and Walker (1959), or else glutathione would not have been an effective trapping agent as it does not react with free acrylic acid.

(4) Finally, in another experiment designed to establish acrylyl CoA as an intermediate, conditions were set up to trap it enzymatically by conversion to *beta*-alanine. Lactate was mixed with the components of incubation system 2 (cf. MATERIALS AND METHODS) and 0.60 m*M* ammonium chloride was included. Extracts of *P. elsdenii*

Table 9. Chromatographic demonstration of the ability of glutathione to trap an α,β-unsaturated acyl ester intermediate (presumed to be acrylyl CoA)

LANE NUMBER	SAMPLE	METHOD OF DETECTION	R_F
1	3-(S-Glutathionyl)propionyl hydroxamate	Spray	0.42 and 0.38
2	Unknown	Radioactivity	.37
3	3-(S-Glutathionyl)propionyl hydroxamate	Spray	.34 and .30
4	Unknown	Radioactivity	.30
5	3-(S-Glutathionyl)propionyl hydroxamate	Spray	.35 and .29

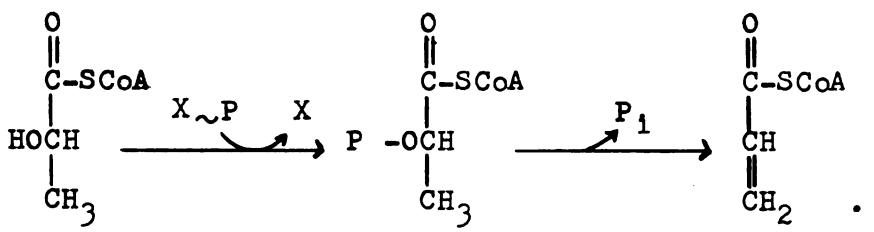
The reaction mixture was the same as in Table 8 except that 1 mM DTT was replaced by 10 mM reduced glutathione. The mixture was incubated at 37°C under N_2 for 30 min. Reaction was stopped by addition of neutralized hydroxylamine, and the hydroxamates so formed were isolated and chromatographed as described in MATERIALS AND METHODS.

and acrylyl CoA aminase of Cl. propionicum were added singlely and in combination. The chromatographic data showed that beta-alanine was formed and that formation was dependent on both P. elsdenii extract and the clostridial acrylyl CoA aminase. Thus P. elsdenii extracts converted lactate to acrylyl CoA and the acrylyl CoA aminase trapped it as beta-alanine. This trapping experiment constitutes definite proof that acrylyl CoA is formed from lactate.

To summarize: (1) the double label experiment demonstrated that tritium from the solvent is incorporated into the C-3 position of propionate; (2) the acrylate competition experiment showed that added acrylyl CoA would be converted to propionate and could slow the rate of lactate conversion; (3) glutathione trapped acrylyl thiolester; and (4) the acrylyl CoA aminase trapped acrylyl CoA. These substantiate more definitively the already existent large body of evidence that an acrylyl intermediate exists. The collective weight of all these experiments clearly says that acrylyl CoA is an intermediate of the direct reductive pathway and that the overall concept of the process is sound. Thus the difficulties must lie in a misunderstanding of the nature of the lactyl CoA-acrylyl CoA conversion.

II. THE HYPOTHESIS OF A NEW ALPHA-PHOSPHOLACTYL CoA INTERMEDIATE IN THE ACRYLATE PATHWAY TO PROPIONATE

At this point, with acrylyl CoA fully reinstated as an intermediate, the difficulties with purification and assay of lactyl CoA dehydrase were almost as perplexing as ever. However Ladd and Walker's observation that dinitrophenol inhibits the interconversion of lactate and acrylate and the fact that the inhibition is reversed by ATP or acetyl phosphate prompted the hypothesis that alpha-phospholactyl CoA was an intermediate between lactyl CoA and acrylyl CoA:



Also preliminary studies with the gas chromatographic propionate assay had on some occasions showed a dependence on ATP.

A. The Propionate Assay

When extracts are incubated with lactate, the propionate formed can be determined simply and sensitively by gas chromatographic analysis. Unlike the troublesome spectrophotometric coupled assay for lactyl CoA dehydrase, the rates obtained in this fashion are

reliable and are not subject to the suspicion of artifacts.

Two reasons were behind the use of the propionate assay: (1) the requirements of the assay might indicate the nature of the reactions involved in the conversion of lactate to propionate. For instance, if catalytic amounts of CoA thiolester are required then some of the reactions would be expected to occur at the acyl CoA level of activation or if ATP is required then the existence of a phospholactyl CoA intermediate would be possible; (2) purification may be possible and might result in the separation of activities, e.g., separation of acyl CoA dehydrogenase activity would be expected to result in the accumulation of acrylate instead of propionate. Furthermore, in the event that conditions are found in which acrylate accumulates then the possibility of separating the individual enzymes responsible for its formation would exist.

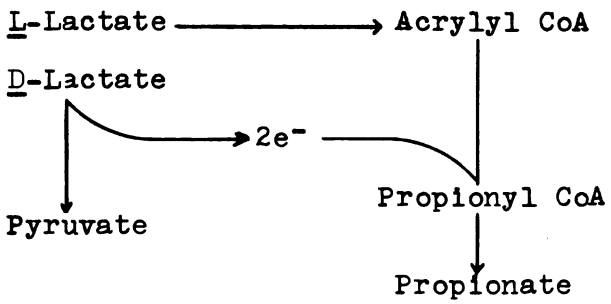
(1) First, the assay requirements were tested by eliminating one component at a time. The components considered initially were catalytic amounts of acetyl CoA, magnesium chloride, NAD, and ATP. In the assay, lactate and catalytic amounts of acetyl CoA were essential for propionate formation; further magnesium chloride was usually stimulatory (Table 10). However, ATP was inhibitory on some occasions and stimulatory on others depending on the age and conditions of the extracts. The ATP effect was most dramatic with aged, partially-inactivated extracts.

Table 10. Requirements for the conversion of lactate to propionate in the standard ("propionate") assay

DELETION	SPECIFIC ACTIVITY	RELATIVE ACTIVITY
	units/mg protein	
None	0.00915	100%
ATP	.060	656
Acetyl CoA	.00106	12
$MgCl_2$	.0102	112
None	0.074	100%
Acetyl CoA	.032	43
$MgCl_2$	.041	55
None	0.00447	100%
ATP	.00117	26

The reaction mixture consisted of the following: 0.033 M Tris buffer (pH 7.6); 0.33 M DL-lactate; 0.033 M ATP (except the last experiment contained 0.023 M ATP); 1 mM $MgCl_2$; 0.25 mM acetyl CoA; 65 µl of extract (about 47 mg protein per ml); and water to give 0.300 ml total volume. Deletions were made as indicated. The mixture was incubated at 37°C under N_2. At various times samples were withdrawn for gas chromatographic analysis as described in MATERIALS AND METHODS.

The stereoisomer of lactate which is converted to propionate may pose an interesting problem when considered in light of the following. P. elsdenii has only D-lactate dehydrogenase; yet D- and L-lactate are converted to propionate and at similar rates (Figure 1). The presence of both isomers gives slightly faster rates than either one alone and since the lactate dehydrogenase is specific for the D-isomer (Baldwin, 1962) this may be interpreted to mean that L-lactate is converted to propionate using electrons derived from the oxidation of D-lactate to pyruvate:

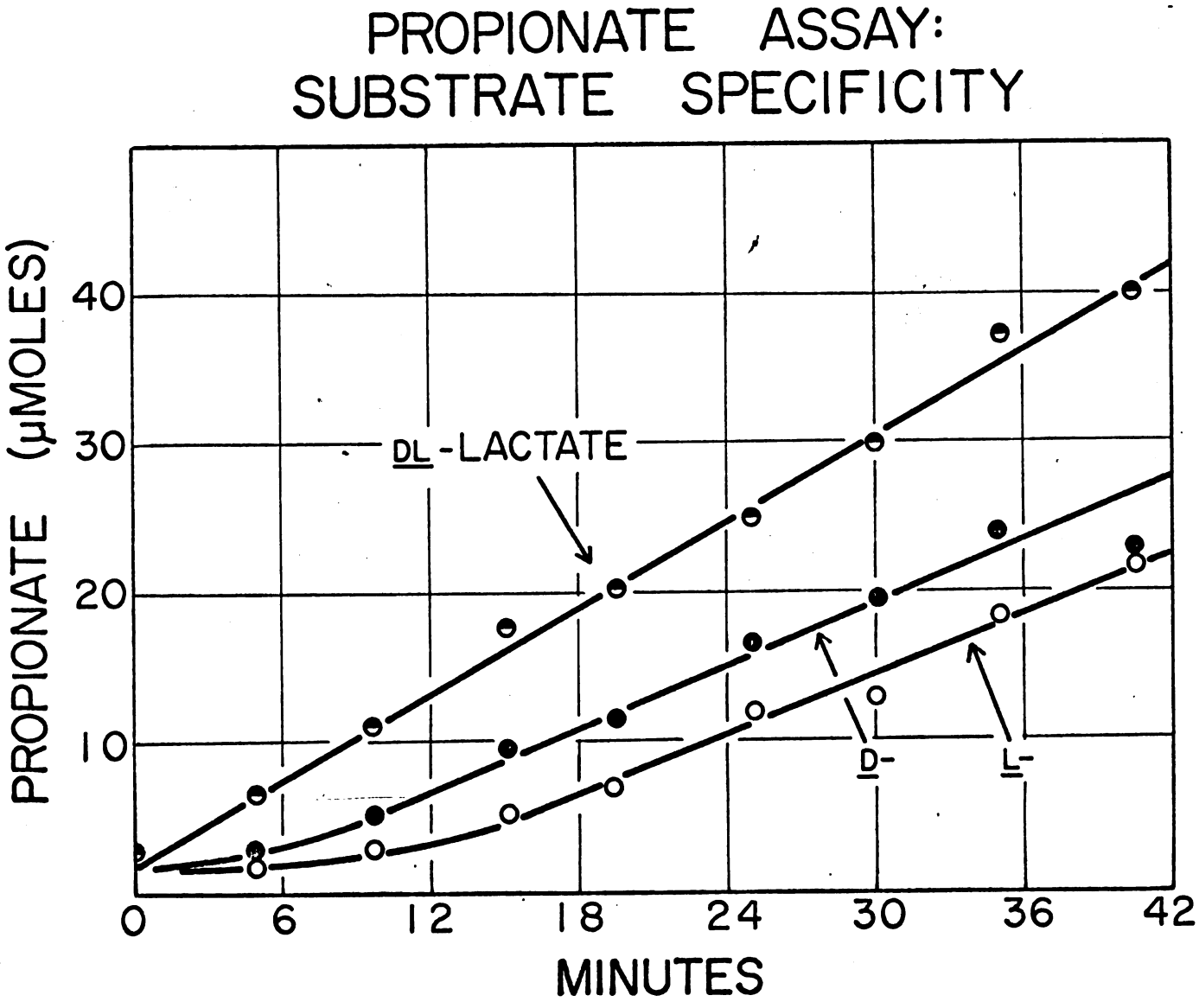


Further the fact that propionate is formed from L-lactate must mean that it is converted (or racemized) to D-lactate inasmuch as (a) propionate formation depends upon lactate dehydrogenase activity as a source of electrons for reduction of acrylyl CoA (Baldwin and Milligan, 1964) and (b) the lactate dehydrogenase is specific for D-lactate.

The specific activity of fresh extracts was usually about 0.1 μmole of propionate formed/min/mg protein and it decreased with increased storage time. This value agrees fairly well with the calculated minimum of 0.68 μmole of

Figure 1. Propionate assay: specificity for stereoisomers of lactate

The reaction mixture consisted of the following: 0.06 M phosphate buffer (pH 7.0); 0.02 M DL-lactate or 0.01 M single isomer as indicated; 1.2 mM $MgCl_2$; 2 mM ATP; 0.4 mM NAD; 0.1 mM acetyl CoA; 250 µl of extract (15 mg protein per ml); and water to give 1.25 ml total volume. The mixture was incubated at 37°C under N_2, and at various times samples were withdrawn for gas chromatographic analysis as described in MATERIALS AND METHODS.


PROPIONATE ASSAY:
SUBSTRATE SPECIFICITY
PROPIONATE (μMOLES)
40
30
20
10
DL-LACTATE
D-
L-
0
6
12
18
24
30
36
42
MINUTES

propionate formed/min/mg protein for a growing culture and is in marked contrast to the value of 0.006 obtained from the coupled assay for lactyl CoA dehydrase.

(2) In an effort to stabilize the enzymes which form propionate from lactate and to eliminate the gradual loss of activity with increased storage, a number of variables were examined. Though none of the results were dramatic (Table 11), high phosphate, 1 mM lactate, and -14°C storage looked promising. Also previous results had suggested that reducing agent was a stabilizing factor. Thus extracts were prepared in high phosphate, 1 mM lactate, and 1 mM DTT and were stored at -14°C. Examination of activity at various times showed little change in specific activity (Table 12).

Storage at various pH's was investigated. Extracts were stored several days at pH 5, 6, 7, and 8 in 0.1 M each phosphate and succinate buffer. Assays showed that at the pH extremes of 5 and 8 activity had been lost (Figure 2).

B. Fractionation of the Enzymes Which Form Propionate From Lactate

After preliminary attempts involving heat and ammonium sulfate steps which were unsuccessful, it was found that calcium phosphate adsorption and elution produced two fractions, both of which were needed for propionate formation. Calcium phosphate gel was formed by

Table 11. Factors affecting stability of "lactyl CoA dehydrase" in extracts of P. elsdenii

TREATMENT	CONCENTRATION	% ORIGINAL ACTIVITY* DAYS OF STORAGE 2	8
	mM		
DL-Lactate	1	32	20
TES (pH 7.5)	100	31	18
TRICINE (pH 8.1)	100	31	16
MES (pH 6.1)	100	31	17
-14°C		30	37
Propionate	1	29	22
HEPES (pH 7.5)	100	28	1
$MgCl_2$	1	26	21
Phosphate (pH 7.0)	100	26	25
Acetate	1	25	20
BICINE (pH 8.3)	100	24	7
EDTA	1	17	17
ADP	1	15	14
25°C		1	0

*Original specific activity was 0.155 in the propionate assay.

The assay reaction mixture consisted of the following: 0.06 M phosphate buffer (pH 7.0); 0.02 M DL-lactate; 1.2 mM $MgCl_2$; 2 mM ATP; 0.4 mM NAD; 0.1 mM acetyl CoA; 250 µl of extract (37 mg protein per ml); and water to give 1.250 ml total volume. The mixture was incubated at 37°C under N_2, and at various times samples were withdrawn for gas chromatographic analysis as described in MATERIALS AND METHODS.

Table 12. Stabilization of enzymes converting lactate to propionate: the extracts were prepared in 0.3 M phosphate at pH 7, 1 mM lactate, and 1 mM DTT and stored at -14°C under N_2

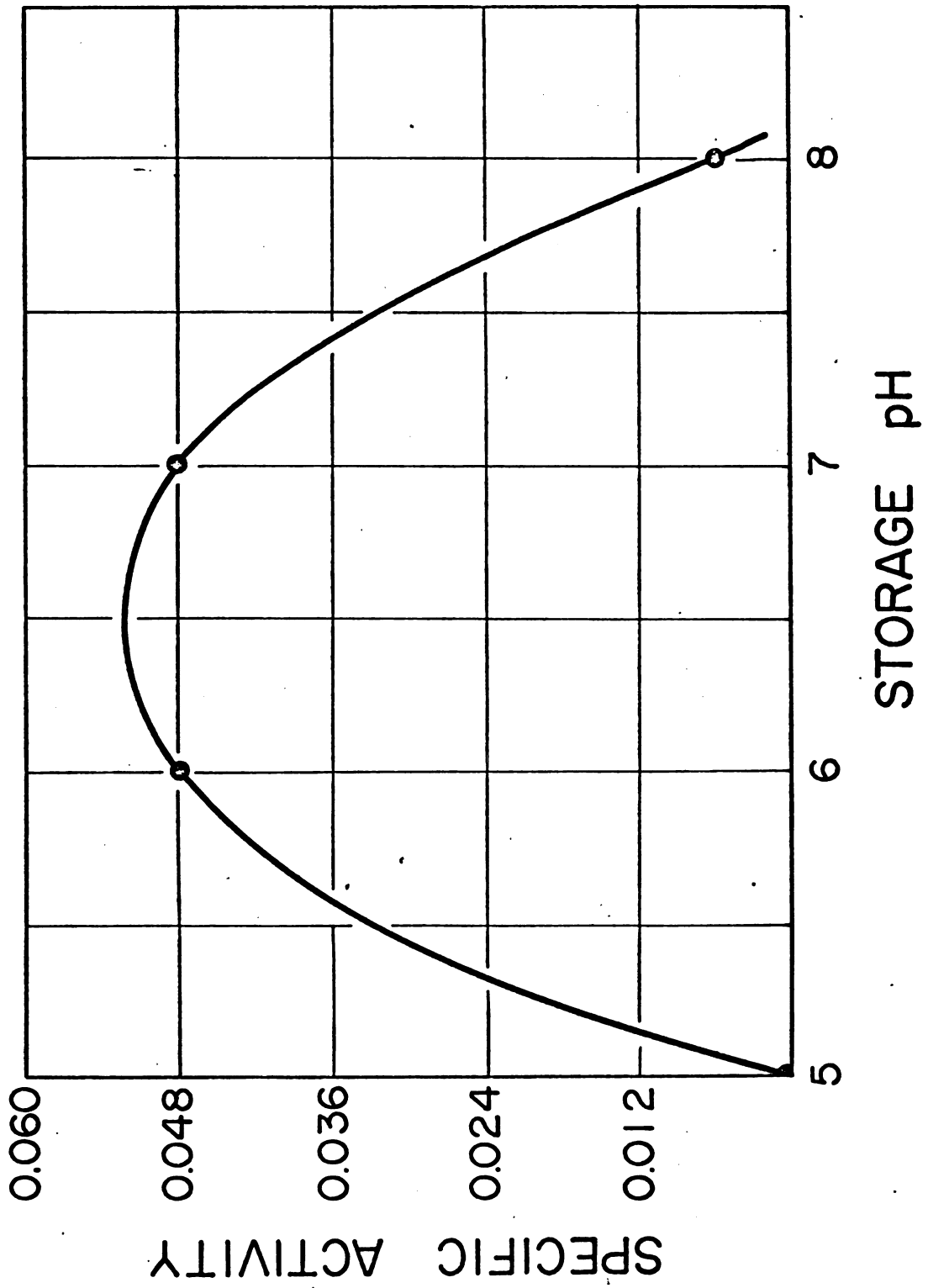
AGE	SPECIFIC ACTIVITY	
	EXPERIMENT 1	EXPERIMENT 2
1 day	0.060	0.035
2		0.055
5	0.074	
7		0.023
9	0.062	
23		0.038*

*Fresh DTT added just prior to assay.

The assay reaction mixture consisted of the following: 0.06 M phosphate buffer, pH 7.0; 0.10 M DL-lactate; 1.2 mM $MgCl_2$; 2.0 mM ATP; 0.4 mM NAD; 0.1 mM acetyl CoA; 2.0 mM reduced glutathione; 500 µl of extract (27 mg protein per ml); and water to 5.0 ml. The mixture was incubated at 37°C under N_2, and at various times samples were withdrawn for gas chromatographic analysis as described in MATERIALS AND METHODS.

Figure 2. Effect of pH of storage on stability of the enzymes converting lactate to propionate

The reaction mixture in a volume of 0.300 ml contained: 0.033 M Tris buffer, pH 7.6; 0.33 M DL-lactate; 0.033 M ATP; 1 mM $MgCl_2$; 0.25 mM acetyl CoA; and 80 µl of extract (45 mg protein per ml). Incubations were performed at 37°C under N_2. Aliquots were removed at intervals and analyzed as described in MATERIALS AND METHODS.


SPECIFIC ACTIVITY
0.060
0.048
0.036
0.024
0.012
5
6
7
8
STORAGE pH

adding $CaCl_2$ and K_3PO_4 to the extract as described by Ochoa (1955). The proportions were modified so that 0.12 volume of 15% $CaCl_2$ solution was added to the extract and 0.24 volume of 10% K_3PO_4 solution was added to the resulting supernatant. The final supernatant after removal of the gel by centrifugation was called calcium phosphate gel "supernatant" fraction, and the eluate of the first precipitate obtained with 0.3 M phosphate buffer (pH 7) was called calcium phosphate gel "eluate" fraction. Both fractions were necessary for production of propionate though each, and especially the second, had residual activity (Table 13). It was concluded that two enzymes had been partially separated. To decide which fraction acted on lactate, presumably to produce an intermediate utilized by the other fraction, the fractions were incubated individually for 1 hr at 37°C under N_2, then the solution was immersed in boiling water for 20 sec. Finally the other fraction was added to the incubation and samples were taken at various times. As shown in Table 14, fraction 1 followed by 2 produced propionate whereas the reverse order did not. It was thus concluded that fraction 1 acts on lactate to produce an intermediate used by fraction 2.

The intermediates possible were lactyl CoA, phospholactyl CoA, acrylyl CoA, or some new and unknown compound. Lactyl CoA was eliminated because both fractions are more active with respect to CoA transferase than they

Table 13. The activity of calcium phosphate gel fractions in converting lactate to propionate

EXPERIMENT	% ORIGINAL ACTIVITY	
	ELUATE AND SUPERNATANT	SUPERNATANT
1	68%	20
2	63	17
3	86	--
4	26	16
5	0*	--
6	0*	--
7	13	0
8	5	0
9	17	0
10	6	0
11	100	0
12	30	47
13	8	--
14	51	12
15	94	208
16	57	25
average	39	22

*PMSF omitted (see APPENDIX, p. 3 and Appendix Figure 1).

Assay reaction mixture consisted of the following: 0.022 M Tris buffer (pH 7.6); 0.022 M DL-lactate; 0.022 M ATP; 0.65 mM $MgCl_2$; 0.16 mM acetyl CoA; 20 µl of each fraction; and water to give 93 µl total volume. Incubation was at 37°C under N_2. Aliquots were withdrawn at intervals for gas chromatographic analysis as described in MATERIALS AND METHODS. The eluate alone usually has less than 1% of the original activity.

Table 14. Determination of order of function of calcium phosphate gel eluate and supernatant fractions

ORDER OF FUNCTION	% OF ORIGINAL ACTIVITY		
	EXPERIMENT		
	1	2	3
Eluate then Supernatant	89	256	20
Supernatant then Eluate	7	0	9

The assay reaction mixture consisted of the following: 0.022 M Tris buffer (pH 7.6); 0.022 M DL-lactate; 0.022 M ATP; 0.65 mM $MgCl_2$; 0.16 mM acetyl CoA; 20 µl of each fraction; and water to give 93 µl total volume. Incubation was at 37°C under N_2. Aliquots were removed at intervals for gas chromatographic analysis as described in MATERIALS AND METHODS.

are for the lactate-to-propionate conversion. Also acrylyl CoA was eliminated because (1) acrylate did not accumulate when fraction 1 and lactate were incubated in the propionate assay system and (2) acyl CoA dehydrogenase activity in both fractions was greater than the rate of the overall system forming propionate.

At this point the earlier observed occasional ATP stimulation and the separation into two fractions by calcium phosphate gel fractionation made the case for a phospholactyl intermediate extremely promising. The next step seemed to be the development of a lactyl CoA kinase assay and its subsequent purification. However the high ATPase activity of extracts precluded demonstrating a lactyl CoA dependent disappearance of ATP (cf. MATERIALS AND METHODS, general kinase assay). The ATPase rate was 6 μmole/min/mg protein or at least 10 times that of the lactate-to-propionate enzymes. Furthermore addition of lactyl CoA slowed rather than increased the loss of ATP. Without an assay for the presumed kinase, purification was not a practical means of demonstrating the reactions involved in the conversion of lactate to propionate and was temporarily abandoned.

C. Indirect Evidence for Phospholactyl Intermediate

Before this work was undertaken Ladd and Walker (1965) had demonstrated that active phosphate compounds

stimulate the interconversion of lactate and acrylate in dialyzed extracts of P. elsdenii. Furthermore the interconversion was inhibited by uncouplers of oxidative phosphorylation. At first their findings were puzzling but as the difficulties with the "lactyl CoA dehydrase" developed they took on new meaning and were reconsidered.

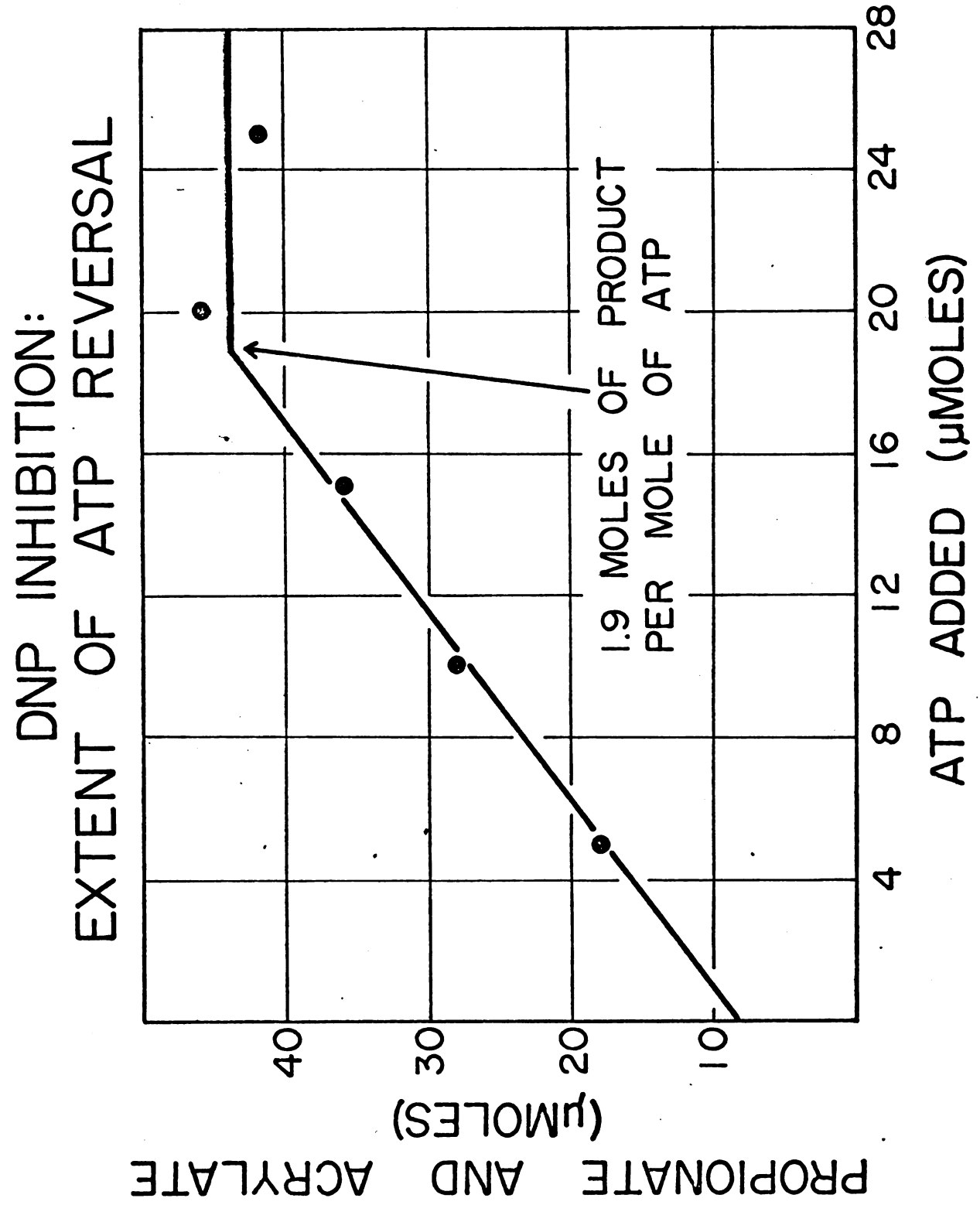
(1) Dinitrophenol (DNP, 10^{-4} M) inhibits 100% the conversion of lactate to propionate by fresh extracts (Ladd and Walker, 1965). There is a slight increase in the levels of propionate during an assay with DNP but it is at the expense of endogenous acrylate. ATP is able to reverse the inhibition of DNP; both the rate and extent of propionate formation increases with increasing amounts of ATP. The effect of ATP on the extent of propionate formation was demonstrated by dialyzing extracts for 4 hr against 0.05 M phosphate buffer (pH 6.5) and by incubating the dialyzed extracts in the propionate system with varying levels of ATP for 24 hr to insure complete reaction; the result is that about 2 moles of product are formed per mole of ATP added (Figure 3). The value is consistent with the existence of phospholactyl intermediate if one assumes that the stoichiometry is

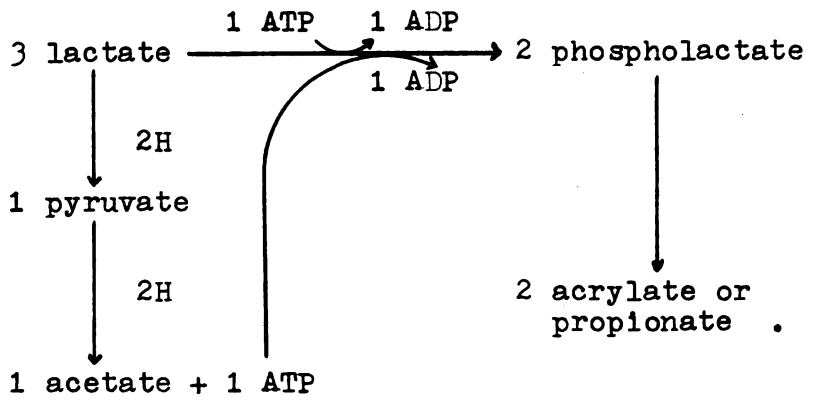
3 lactate $\longrightarrow$ 2 acrylate + 1 acetate,

because the acetate forms 1 ATP or in otherwords the phosphoroclastic system gives 1 ATP per 2 moles of product. This 1 plus the 1 added gives 2 moles of ATP per 2 moles of product, i.e.,

Figure 3. Dinitrophenol inhibition of propionate formation: reversal by ATP

Extracts were dialyzed 4 hr. The reaction mixture contained the following: 0.06 M phosphate buffer (pH 7.0); 0.01 M DL-lactate; 5 mM $MgCl_2$; 25 μM acetyl CoA; 0.1 mM NADH; 1 mM DTT; variable ATP; 2.0 ml of extract (31 mg protein per ml); 10^{-4} M dinitrophenol; and water to 5.00 ml total volume. Incubation was at room temperature under N_2. After 24 hr samples were removed for gas chromatographic analysis as described in MATERIALS AND METHODS.


DNP INHIBITION:
EXTENT OF ATP REVERSAL
PROPIONATE AND ACRYLATE (μMOLES)
ATP ADDED (μMOLES)
1.9 MOLES OF PRODUCT PER MOLE OF ATP
10
20
30
40
4
8
12
16
20
24
28



Oligomycin inhibited propionate formation and again ATP reversed the effect (Table 15): the fact that reversal is not complete may be due to (1) ATP not being the direct phosphoryl donor or (2) an essential cofactor having been removed during dialysis.

(2) ^{18}O Transfer from 2-^{18}O-lactate to orthophosphate. In order to further implicate phospholactate as an intermediate an experiment was designed to determine if ^{18}O of lactate-2-^{18}O would be converted to orthophosphate-^{18}O. As noted previously in phosphoryl transfer P-O cleavage occurs whereas in elimination C-O cleavage would be expected:

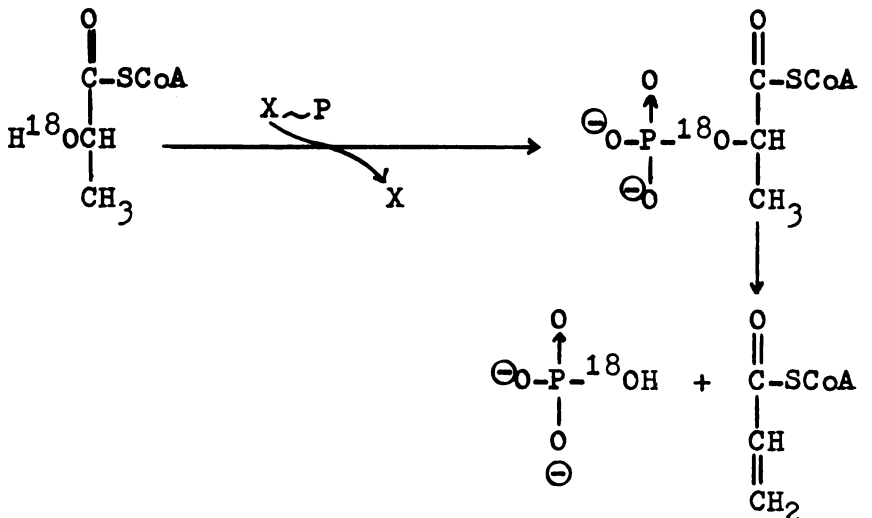


Table 15. Inhibition of propionate formation and its reversal by ATP

INHIBITOR	CONCENTRATION	ATP ADDED	ACTIVITY
	M	μmoles	units
None		0	0.0042
"		5	.0330
"		15	.7000
Oligomycin	10^{-4}	0	0.0
"	"	5	.0041
"	"	15	.0139
Dinitrophenol	10^{-4}	0	0.0
"	"	5	.0020
"	"	15	.0050

The reaction mixture consisted of the following: 0.06 M phosphate buffer (pH 7.0); 0.01 M DL-lactate; 5 mM $MgCl_2$; 25 μM acetyl CoA; 0.10 mM NAD; variable ATP as indicated; variable inhibitor as indicated; 2.0 ml of extract (39 mg protein/ml); and water to 5.0 ml. The reaction was incubated at 37°C under N_2. At intervals samples were removed for gas chromatographic analysis as described in MATERIALS AND METHODS.

The 2-^{18}O-lactate synthesized had an atom % excess of ^{18}O = 1.16%. Since it was formed by equilibrating pyruvate with 5.58% ^{18}O-H_2O the value expected was 1.86; thus during the reduction of pyruvate to lactate some ^{18}O was lost. The 2-^{18}O-lactate was converted to propionate by incubation with extracts according to the conditions of the propionate assay. If it is assumed that the ^{18}O of lactate is transferred to phosphate every time propionate is formed, the expected atom % excess of ^{18}O in phosphate can be calculated:

$$\text{exp.} = \frac{(1.16 \text{ at. \% ex. in lac})(3 \text{ O in lac})(\mu\text{mole PRO formed})}{(4 \text{ O in } P_i)\ (\mu\text{mole } P_i \text{ in system})} .$$

The data show excellent agreement between expected and observed values of ^{18}O in phosphate in some cases. The greater discrepancy in the other values is attributable to the small amount of sample analyzed for ^{18}O and to the oversight of not accurately determining the inorganic phosphate present. However in every case the data suggest at least partial transfer of ^{18}O from lactate to phosphate (Table 16).

(3) gamma-^{32}P-ATP Labeling of the intermediate. Demonstration of the transient accumulation of intermediate was undertaken using ^{32}P-labeled ATP (gamma-labeled) and DL-lactate-^{14}C. Thus the proposed intermediate should be doubly labeled. Specifically 0.25 mC of gamma-^{32}P-ATP and 1 μC of DL-lactate-^{14}C (u) were incubated

Table 16. ^{18}O Transfer from lactate to phosphate concomitant with propionate formation

EXPERIMENT	ATOM % EXCESS ^{18}O IN CO_2 FROM PHOSPHATE	
	EXPECTED	FOUND
1	0.266	0.0397
2	5.22	0.249
3	5.22	5.45
4	5.22	3.48

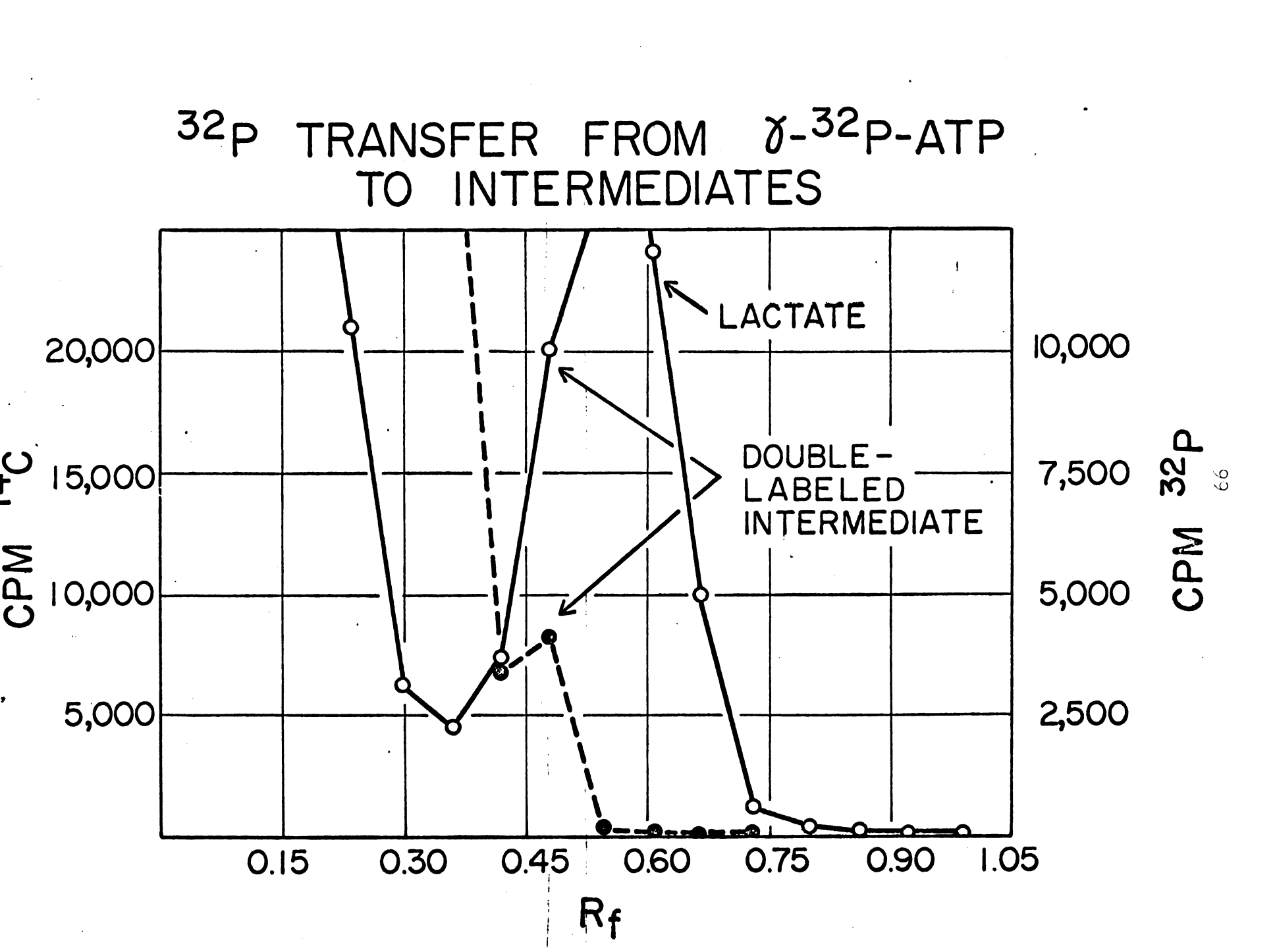
The incubation mixture consisted of: 0.06 M imidazole buffer (pH 7.0); 5 mM ATP; 5 mM $MgCl_2$; 25 μM acetyl CoA; 0.10 mM NAD; 1 mM DTT; 2.6 mM L-lactate-2-^{18}O; 2.0 ml of extract (31 mg/ml); and water to 5 ml. Incubation was at room temperature for 150 min. The orthophosphate was isolated and equilibrated with CO_2 for analysis as described in MATERIALS AND METHODS.

with extracts in the "propionate assay" incubation system. After 30 min the reaction was stopped by addition of ½ volume of ethanol, and the denatured proteins were removed by centrifugation. A small portion of the supernatant was spotted on Whatman 3 MM paper and developed by descending chromatography in 95% ethanol:dioxane:water:acetic acid (60:20:19:1). The levels of ATP were so high that counts were distributed over the entire chromatogram. Therefore the remainder of the incubation mixture (about 4.0 ml) was treated with 50 mg of activated charcoal which had been previously shown to effectively remove gamma-^{32}P-ATP. The resulting supernatant was chromatographed as described above. The radioactivity was located by cutting the strip into pieces each representing delta-R_F spans of 0.0625. Chemically synthesized phospholactate was shown to have an R_F of 0.5 in this system. A ^{32}P labeled peak was found at R_F = 0.5; further there was a slight shoulder at R_F = 0.5 in the ^{14}C-lactate peak at R_F = 0.57 (Figure 4). Though the chromatogram suggested the formation of phospholactate, the evidence was still weak because the ^{14}C-lactate had not been separated and because the radioactive-phosphate peak streaked so broadly that the ^{32}P-phospholactate peak consisted of but one point.

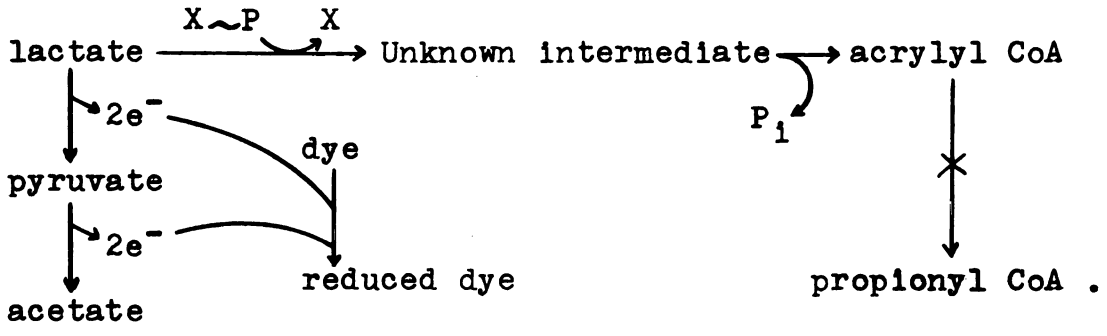
(4) The acrylate assay. A system for conversion of lactate to only acrylate (or acrylyl CoA) was desirable because (a) it would eliminate the requirement for acyl CoA dehydrogenase and lactate dehydrogeanse activities

Figure 4. Transfer of ^{32}P from γ-^{32}P-ATP to an intermediate in the conversion of lactate to propionate

The incubation mixture consisted of the following: 0.06 M phosphate buffer (pH 7.0); 0.01 M DL-lactate and 1 μC DL-lactate-^{14}C (u); 0.01 M ATP and 0.25 mC gamma-^{32}P-ATP; 0.01 M $MgCl_2$; 1 mM reduced glutathione; 1.9 ml of extract (31 mg protein per ml); and water to 5.0 ml. Incubation was at 37°C under N_2 for 30 min. Reaction was stopped by addition of ½ volume of ethanol. After charcoal treatment and centrifugation the sample was applied to Whatman 3 MM paper and developed by descending chromatography in 95% ethanol:dioxane: water:acetic acid (60:20:19:1).


^{32}P TRANSFER FROM γ-^{32}P-ATP
TO INTERMEDIATES
LACTATE
DOUBLE-
LABELED
INTERMEDIATE
20,000
15,000
10,000
5,000
CPM ^{14}C
10,000
7,500
5,000
2,500
CPM ^{32}P
0.15
0.30
0.45
0.60
0.75
0.90
1.05
R_f

which were essential for electron transfer to produce propionate and (b) it should facilitate demonstration of an intermediate. The strategy which permitted development of the assay was that acrylate should accumulate as major product if (1) a source of active phosphate is present and (2) if the electrons produced by oxidation of lactate are removed and then are not available to reduce acrylyl CoA. To prevent the latter the electrons mobilized by oxidation were trapped using an appropriate acceptor:



The oxidation of lactate by NAD-independent dehydrogenase(s) is the primary source of the reducing electrons (see APPENDIX, p. 4) and probably involves flavins. Thus dyes capable of accepting electrons from flavoproteins such as methylene blue and phenazine methosulfate are likely acceptors (Nachlas et al., 1960). The acceptors tested were 2-para-iodophenyl-3-para-nitrophenyl-5-phenyltetrazolium chloride (INT), PMS-ascorbic acid, methylene blue, dichlorophenoindophenol (DCPIP), and ferricyanide. In most cases both acrylate and propionate accumulated when 25 μmoles of the acceptor was added to

the assay (Figure 5). Inasmuch as methylene blue was by far the most effective acceptor (see also Table 17), various amounts were added to the assay in order to optimize its effect. The optimum concentration is 0.02% or 0.160 μmole per assay (Figure 6). However the specific activity with respect to acrylate accumulation was lower than that obtained from the usual propionate assay. Therefore, in an effort to achieve a respectable rate of acrylate formation, a series of phosphoryl donors were screened. Also, in order to dramatize the effect and thereby identify the primary phosphoryl donor, the extracts were dialyzed for 5 hr against 0.05 M phosphate buffer (pH 6.5) and 1 mM DTT prior to testing. In this manner it was found that acetyl phosphate is by far the most effective donor in stimulating acrylate accumulation (Figure 7), and when added the specific activity is higher than that of the usual propionate assay without methylene blue (0.272 μmoles/min/mg vs. about 0.1).

Besides methylene blue and acetyl phosphate, the other requirements of the acrylate assay were expected to be magnesium ion and, by analogy with propionate assay, catalytic amounts of CoA thiolester. At first a magnesium ion requirement was not observable; however, for this and other reasons the assay was modified to contain 10 μmoles of lactate instead of 100 (assay volume 0.300 ml). With this assay as described in MATERIALS AND METHODS under "acrylate assay," the magnesium

Figure 5. Acrylate accumulation from lactate: effectiveness of various electron acceptors

Assay mixtures consisted of the following: 0.033 M Tris buffer (pH 7.6); 0.33 M DL-lactate; 4 mM $MgCl_2$; 6.7 mM ATP; 0.33 mM acetyl CoA; 25 µmole of acceptor; 80 µl of extract (67 mg protein per ml); and water to give 0.300 ml total volume. Incubation was at 37°C under N_2. At intervals samples were removed for gas chromatographic analysis as described in MATERIALS AND METHODS.

(1) Methylene blue, (2) PMS-INT, (3) Ferricyanide, (4) DCPIP, (5) PMS-Ascorbic acid; lines drawn from least squares analysis.

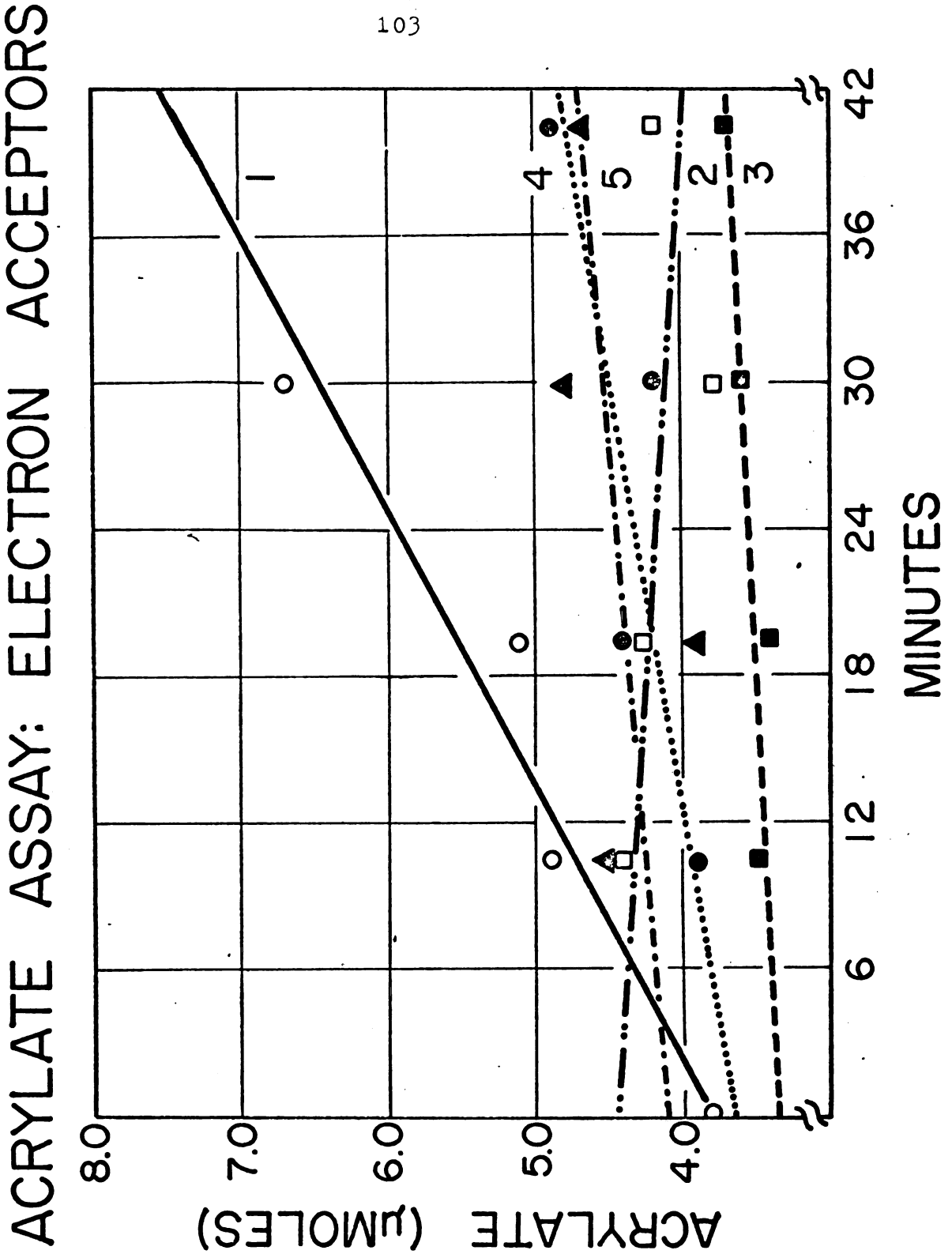

ACRYLATE ASSAY: ELECTRON ACCEPTORS
ACRYLATE (μMOLES)
8.0
7.0
6.0
5.0
4.0
6
12
18
24
30
36
42
MINUTES
1
2
3
4
5

Table 17. Rate of acrylate accumulation from lactate: effectiveness of various acceptors

ELECTRON ACCEPTOR	ACRYLATE ACCUMULATION	E_o'*
	μmole/min	volts
Methylene blue	.089	0.011
PMS-INT	-.011	--
Ferricyanide	.008	-.360
DCPIP	.028	.217
PMS-Ascorbic acid	.015	.080

*E_o' Values from RESPIRATORY ENZYMES, 1949.

Assays were performed as described in Figure 5.

Figure 6. Acrylate accumulation from lactate: effect of methylene blue concentration

Assay mixtures consisted of the following: 0.028 M Tris buffer (pH 7.6); 0.28 M DL-lactate; variable methylene blue; 3.3 mM $MgCl_2$; 0.28 mM acetyl CoA; 5.6 mM ATP; 100 µl of extract (67 mg protein per ml); and water to give 360 µl total volume. Incubations were at 37°C under N_2. At intervals, samples were removed for gas chromatographic analysis as described in MATERIALS AND METHODS.

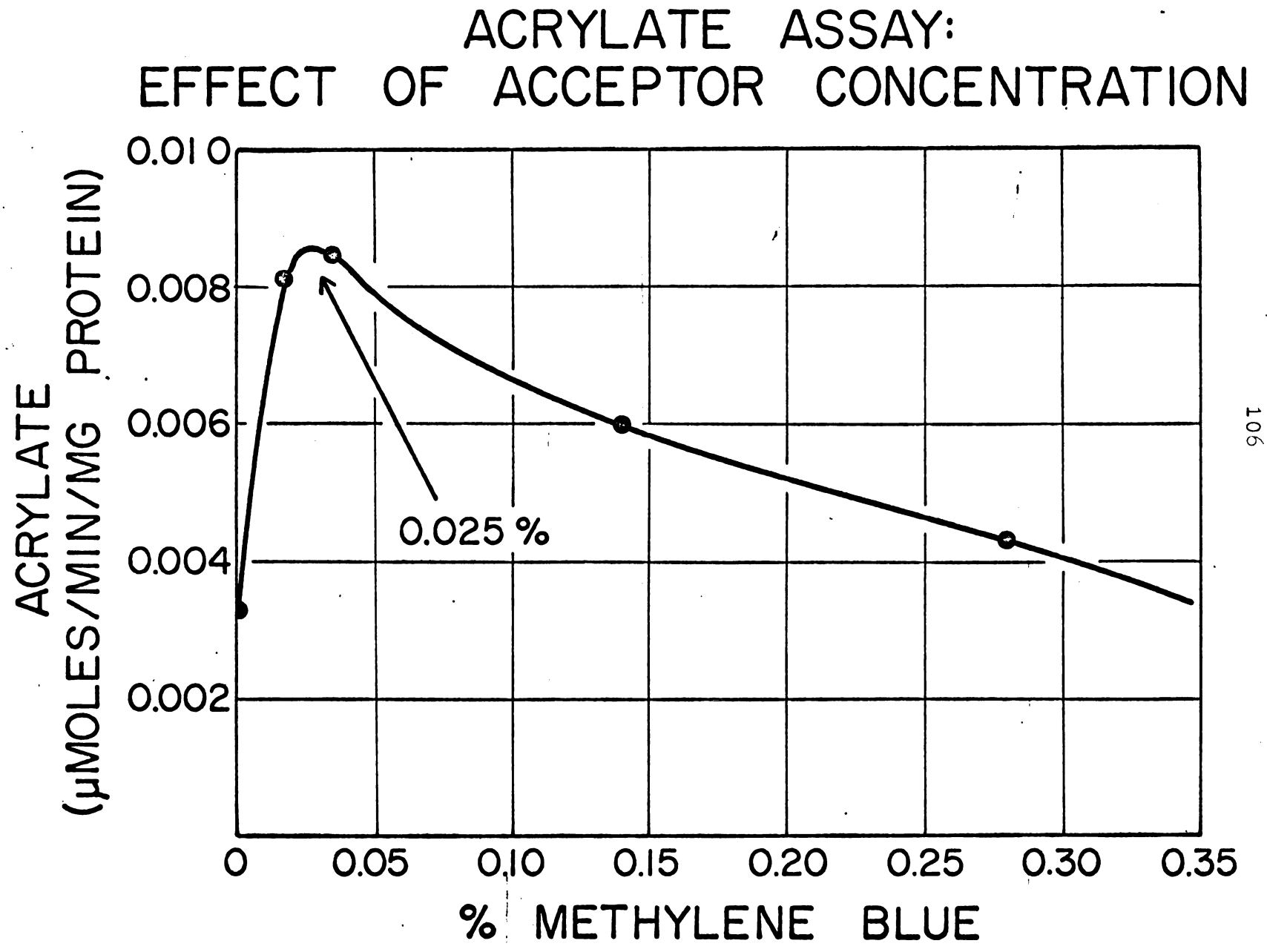
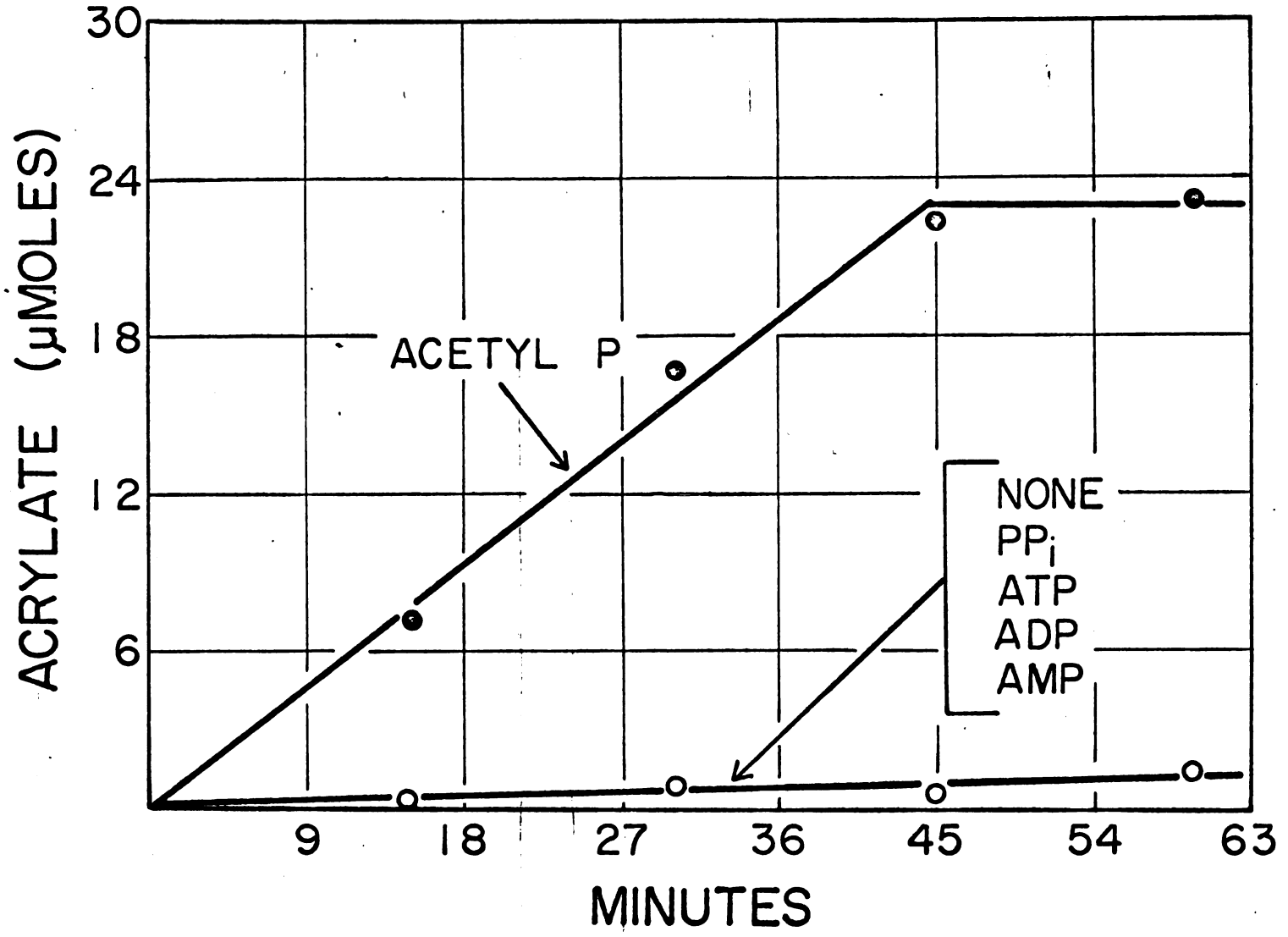

ACRYLATE ASSAY:
EFFECT OF ACCEPTOR CONCENTRATION
ACRYLATE
(μMOLES/MIN/MG PROTEIN)
0.010
0.008
0.006
0.004
0.002
0.025 %
0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
% METHYLENE BLUE

Figure 7. Acrylate accumulation from lactate: phosphoryl donor specificity

Assay mixtures consisted of the following: 0.033 M HEPES buffer (pH 7.75); 0.033 M DL-lactate; 7.0 mM $MgCl_2$; 0.02% methylene blue; 0.033 M phosphoryl donor; 0.42 mM acetyl CoA; 40 µl of extract (dialyzed 5 hr; 62 mg protein per ml); and water to give 300 µl total volume. Incubations were at 37°C under N_2. Samples were removed at intervals and analyzed on the gas chromatograph for acrylate as described in MATERIALS AND METHODS.

EFFECTIVENESS OF PHOSPHORYL DONORS



ion and thiolester requirements were pronounced (Table 18 and Figure 8).

The characteristics of acrylate formation are certainly consistent with involvement of phospholactyl intermediate. Nonetheless the following alternative was considered. Phosphoenolpyruvate (PEP) was tested as an intermediate by adding it alone as substrate to the assay. The specific activity obtained in this manner was but 9% of that on DL-lactate without any phosphoryl donor. Acrylate formation from PEP is probably due to hydrolysis and subsequent reaction of the pyruvate to form acetyl phosphate and lactate which react to form phospholactyl CoA and then acrylate.

Phosphoglycollate was tested as an analogue of phospholactate. There was an absence of any new peak on the gas chromatograph which would have indicated its utilization; further when lactate was added to the assay, its conversion to acrylate was inhibited by phosphoglycollate.

D. Direct Demonstration of a New Intermediate (Presumably α-Phospholactyl CoA)

Up to this point the phospholactyl intermediate hypothesis had been established in several indirect experiments. However a direct demonstration of the appearance of the intermediate and of its conversion to acrylate was now desirable. In the experiments described

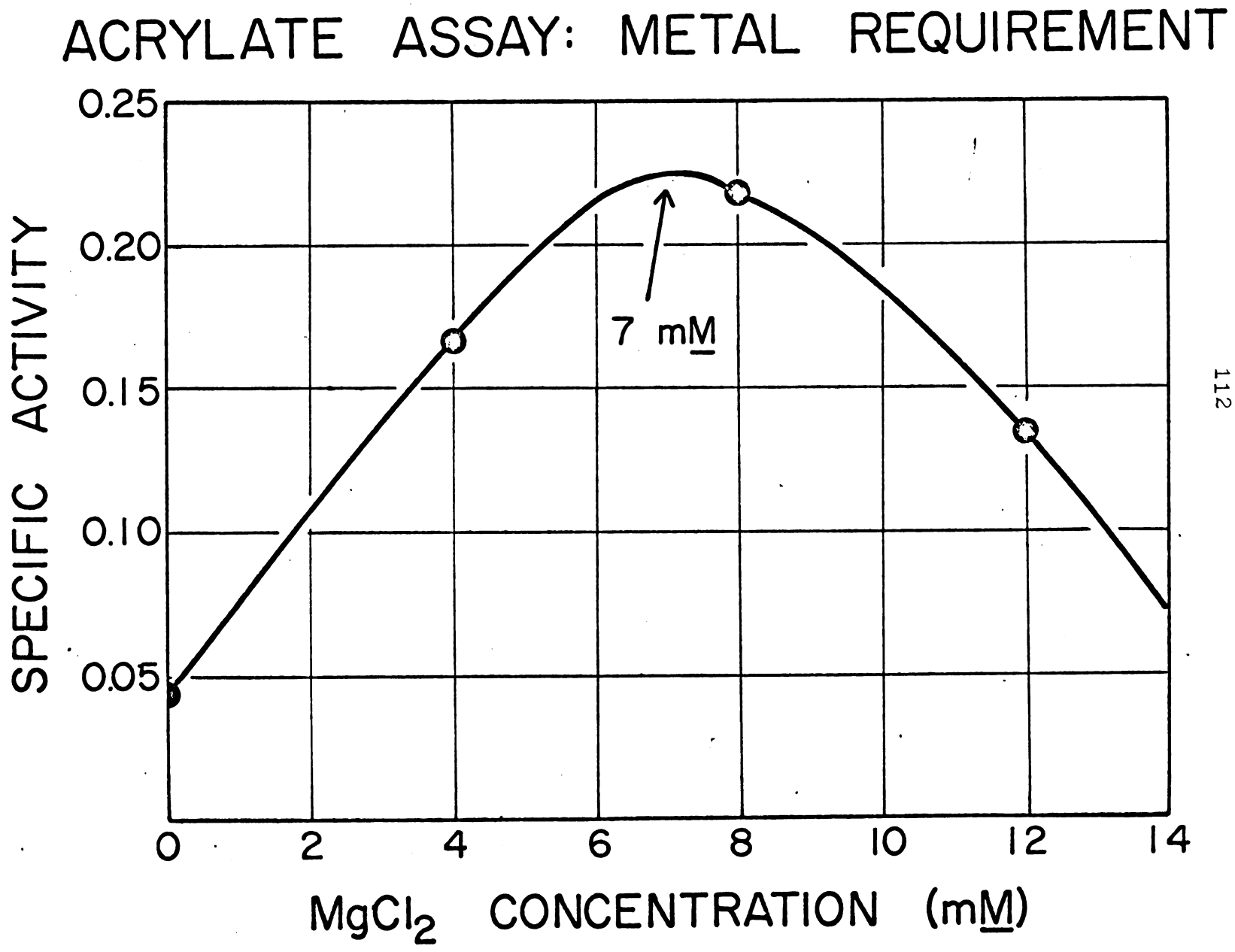
Table 18. Acrylate assay requirements

DELETION	SPECIFIC ACTIVITY
	μmole/min/mg protein
None	0.036
Lactate	0.007
$MgCl_2$	0.042
Acetyl CoA	0.006
Acetyl phosphate	0.015
Methylene blue	0.020

Assays were performed as described in Figure 7 except that acetyl phosphate was the phosphoryl donor (0.033 M).

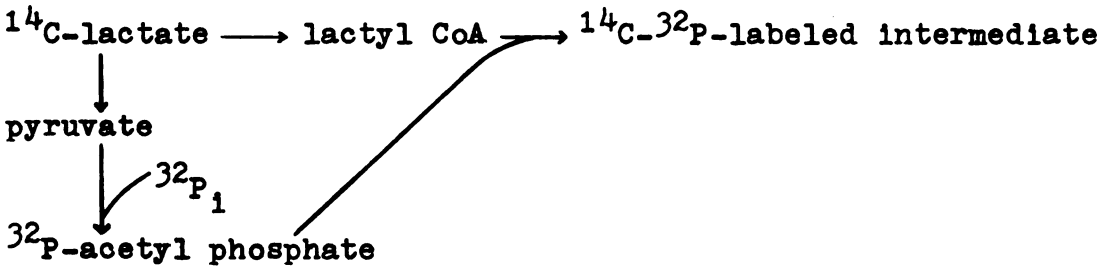
Figure 8. Acrylate formation from lactate: requirement for $MgCl_2$

Assays were performed as described in Figure 7 except that the extract was not dialyzed, and 40 µl of it (53 mg protein/ml) was added to the assay mixture.


ACRYLATE ASSAY: METAL REQUIREMENT
SPECIFIC ACTIVITY
0.25
0.20
0.15
0.10
0.05
7 mM
0
2
4
6
8
10
12
14
MgCl2 CONCENTRATION (mM)

below it is shown that (1) a transient intermediate appears during the course of an incubation, (2) if the intermediate is isolated, purified, and added to another incubation mixture, it is converted presumably via phospholactyl CoA to acrylate, (3) chemically synthesized phospholactate forms acrylate similarly, and (4) the intermediate is characterized to show its identity as phospholactate.

(1) Experiments showing the appearance of a ^{14}C- and ^{32}P-labeled intermediate. (a) The formation of an intermediate was demonstrated by incubating extracts in the "acrylate assay" with L-lactate-^{14}C (u) and ^{32}P-orthophosphate. In all probability the ^{32}P-phosphate is converted to acetyl phosphate:



Following a 60 min incubation, enzymes were inactivated and removed by ethanol precipitation; and, after centrifugation, the entire supernatant was spotted on a TLC plate and developed with (60:20:19:1) 95% ethanol: dioxane:water:acetic acid. A ^{14}C- and ^{32}P-labeled spot was formed with an R_F value of 0.12; chemically synthesized phospholactate displayed an R_F value of 0.13.

However the spot overlapped that of orthophosphate (R_F = 0.03). The incubation and TLC-chromatography was repeated, and then, following drying, a second chromatography was run in formic acid:water:95% ethanol (1:29:70) to the point where the second front was 0.44 as far as the first. The plate was scored into delta-R_F = 0.0526 sections; the silica gel coating was scraped into scintillation vials, one section per vial. Plots of ^{14}C and ^{32}P content versus R_F showed a single double-labeled spot (Figure 9). The ^{14}C in the double-labeled peak represents 12% of the total label added as lactate-^{14}C and the ratio of $^{32}P/^{14}C$ indicates that there are about 0.7 phosphate/lactyl moiety. However the lack of a labeled orthophosphate peak probably means that the second solvent system also failed to resolve the intermediate and phosphate.

In an effort to find an appropriate solvent system, studies were conducted with chemically synthesized phospholactate prepared as described in MATERIALS AND METHODS. The best system proved to be 3:1 95% ethanol:0.1 N acetate buffer (pH 4). Employing this system for chromatography of incubations prepared as above, the separation from orthophosphate was better but still not complete: the R_F-value of orthophosphate is 0.25 compared to about 0.4 for phospholactate. Nevertheless, by plotting changes in radioactivity as a function of time (compared to zero time) vs. R_F in this system the appearance of ^{14}C- and ^{32}P-labeled intermediate was evident (R_F = 0.38) (Figure 10).

Figure 9. Thin-layer chromatography of reaction mixture containing ^{14}C-lactate and ^{32}P-orthophosphate as substrates

The reaction mixture consisted of the following: 0.017 M Tris buffer (pH 7.6); 0.17 M DL-lactate; about 0.13 μC of L-lactate-^{14}C (u); about 0.1 μC ^{32}P-orthophosphate; 2.7 mM acetyl CoA; 30 μl of extract (65 mg protein per ml); and water to give 300 μl total volume. Incubation was at 37°C under N_2 for 60 min. The ratio of counts $^{32}P/^{14}C$ suggests that there are about 0.7 phosphate/lactate in the double-labeled intermediate.

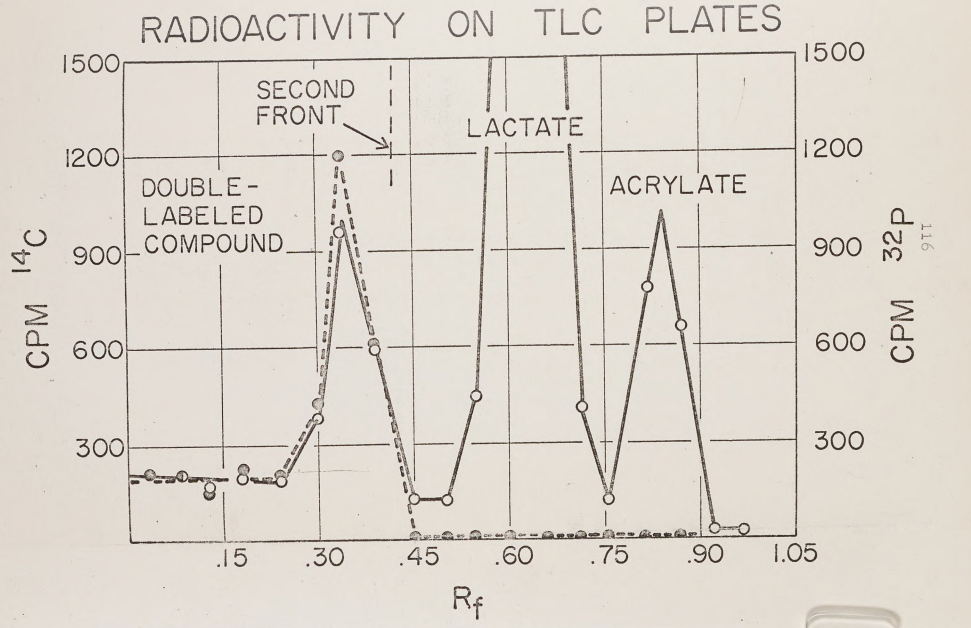

RADIOACTIVITY ON TLC PLATES
1500
1200
900
600
300
CPM 14C
SECOND FRONT
DOUBLE-LABELED COMPOUND
LACTATE
ACRYLATE
CPM 32P
.15
.30
.45
.60
.75
.90
1.05
Rf

Figure 9. Thin-layer chromatography of reaction mixture containing ^{14}C-lactate and ^{32}P-orthophosphate as substrates

The reaction mixture consisted of the following: 0.017 M Tris buffer (pH 7.6); 0.17 M DL-lactate; about 0.13 μC of L-lactate-^{14}C (u); about 0.1 μC ^{32}P-orthophosphate; 2.7 mM acetyl CoA; 30 μl of extract (65 mg protein per ml); and water to give 300 μl total volume. Incubation was at 37°C under N_2 for 60 min. The ratio of counts $^{32}P/^{14}C$ suggests that there are about 0.7 phosphate/lactate in the double-labeled intermediate.

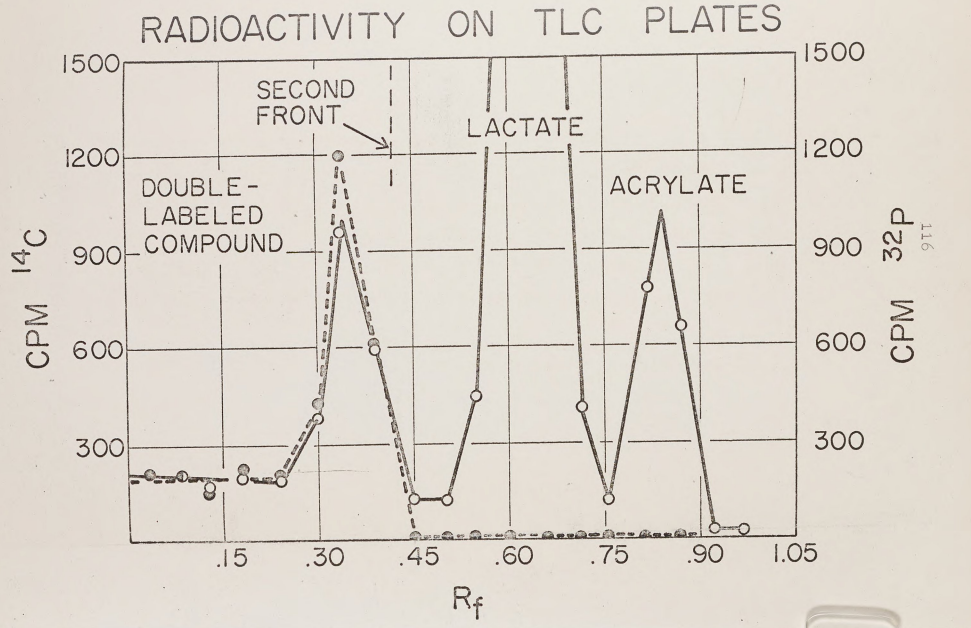

RADIOACTIVITY ON TLC PLATES
1500
1200
900
600
300
CPM 14C
SECOND FRONT
DOUBLE-LABELED COMPOUND
LACTATE
ACRYLATE
CPM 32P
.15
.30
.45
.60
.75
.90
1.05
Rf

Figure 10. Separation of ^{14}C- and ^{32}P-labeled intermediate

The reaction mixture consisted of the following: 0.033 M HEPES buffer (pH 7.75); about 0.01 μC of ^{32}P-orthophosphate; 0.033 M DL-lactate; about 0.07 μC of DL-lactate-1-^{14}C; 7 mM $MgCl_2$; 0.02% methylene blue; 0.5 mM acetyl CoA; 20 μl of extract (53 mg protein per ml); and water to give 150 μl total volume. Incubation was at 37°C under N_2. After deproteinization the samples were analyzed by descending paper chromatography in 3:1 95% ethanol:0.1 N acetate buffer (pH 4); phospholactate standard R_F = 0.39.

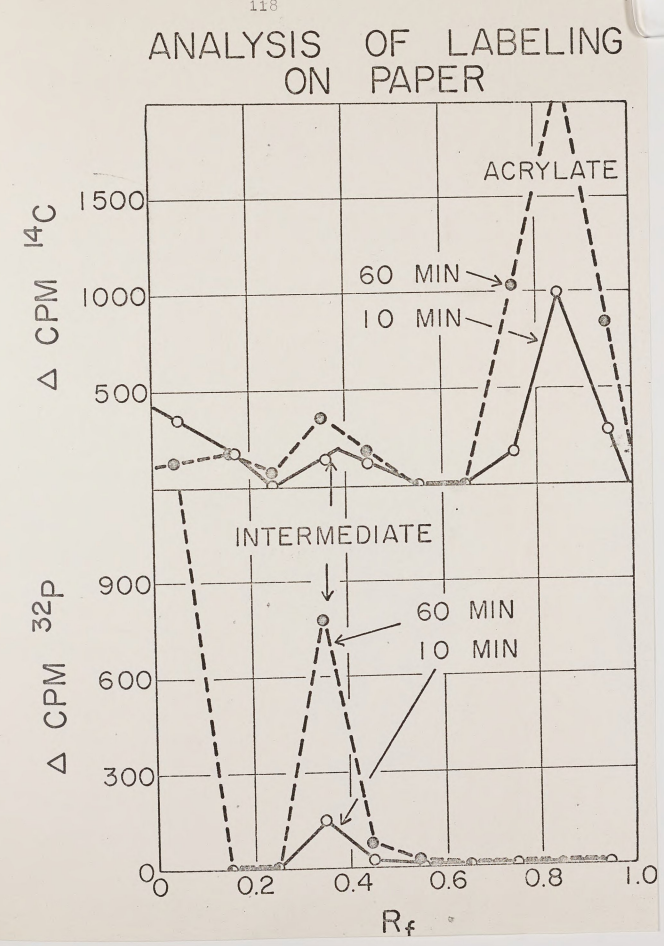
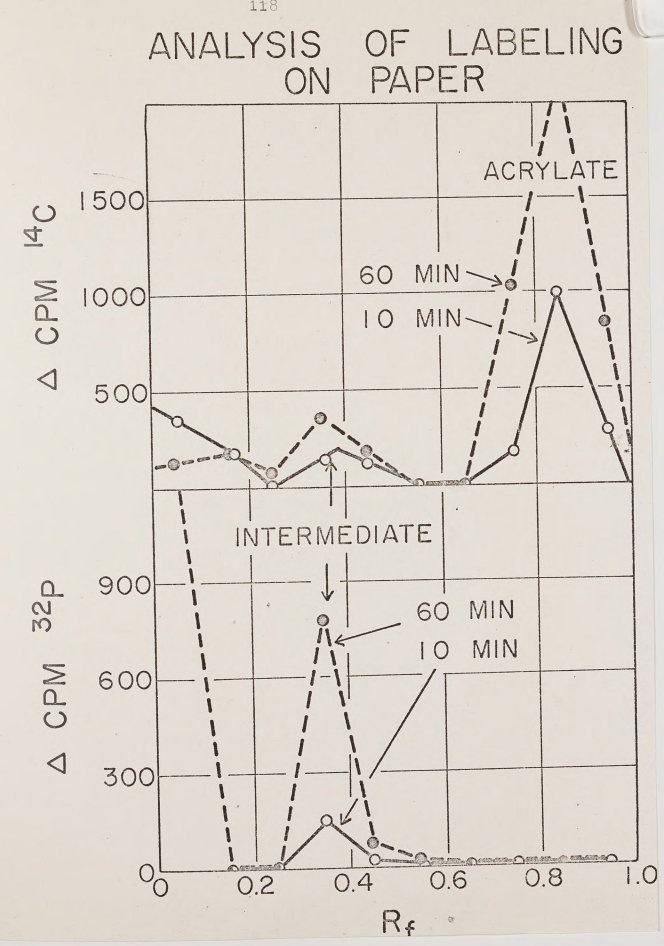

ANALYSIS OF LABELING ON PAPER
ACRYLATE
60 MIN
10 MIN
INTERMEDIATE
60 MIN
10 MIN
Δ CPM 14C
Δ CPM 32P
1500
1000
500
900
600
300
0
0.2
0.4
0.6
0.8
1.0
Rf

Figure 10. Separation of ^{14}C- and ^{32}P-labeled intermediate

The reaction mixture consisted of the following: 0.033 M HEPES buffer (pH 7.75); about 0.01 μC of ^{32}P-orthophosphate; 0.033 M DL-lactate; about 0.07 μC of DL-lactate-1-^{14}C; 7 mM $MgCl_2$; 0.02% methylene blue; 0.5 mM acetyl CoA; 20 μl of extract (53 mg protein per ml); and water to give 150 μl total volume. Incubation was at 37°C under N_2. After deproteinization the samples were analyzed by descending paper chromatography in 3:1 95% ethanol:0.1 N acetate buffer (pH 4); phospholactate standard R_F = 0.39.


ANALYSIS OF LABELING ON PAPER
ACRYLATE
60 MIN
10 MIN
INTERMEDIATE
60 MIN
10 MIN
Δ CPM 14C
Δ CPM 32P
1500
1000
500
900
600
300
0
0.2
0.4
0.6
0.8
1.0
Rf

Fi

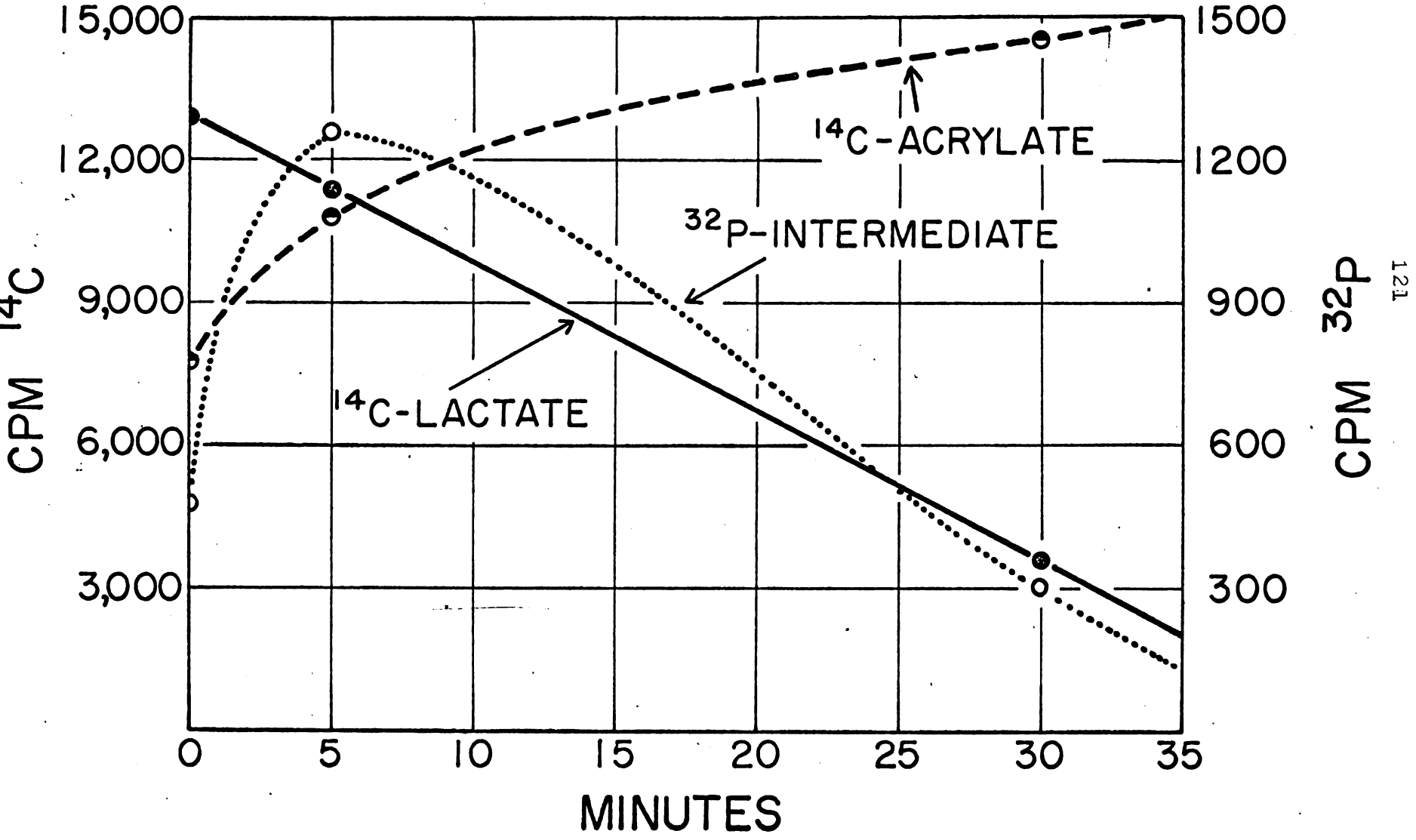
lo
32
DL
mM
an
37
an
et
st

(b) As discussed above, the ^{32}P-labeling of phospholactate from ^{32}P-orthophosphate depends upon ^{32}P-orthophosphate incorporation into acetyl phosphate which is formed by enzymatic oxidation of lactate. Since acetyl phosphate was shown to be the phosphoryl donor in formation of acrylate (presumably the donor forms phospholactyl intermediate) (cf. Figure 7) and since a more rapid labeling of the intermediate is desirable, ^{32}P-acetyl phosphate was synthesized in a manner identical to acetyl phosphate except about 0.1 mC ^{32}P-orthophosphate was added (cf. MATERIALS AND METHODS). Using ^{32}P-acetyl phosphate, instead of gamma-^{32}P-ATP or ^{32}P-orthophosphate, the double-labeled compound could be demonstrated with shorter incubation times even though the specific activity of the prepared ^{32}P-acetyl phosphate was low. Furthermore by following the time course of the labeling it was possible to show that the double-labeled compound appeared and disappeared during the course of an incubation as is typical of an intermediate (Figure 11), i.e., (1) the intermediate accumulates faster than acrylate and (2) when most of the lactate has been converted to acrylate the levels of the intermediate decreases.

(2) Purification of the double-labeled intermediate. A further means of demonstrating that the double-labeled compound is an intermediate would be to reincubate it with extracts and to show its conversion to acrylate. The control in this case must preclude the possibility

Figure 11. Transientness of the compound labeled from lactate-^{14}C and ^{32}P-acetyl phosphate

The reaction mixture consisted of the following: 0.17 M DL-lactate; 0.017 M Tris buffer (pH 7.6); 0.025 μC of DL-lactate-^{14}C (u); 0.02% methylene blue; 0.33 mM acetyl CoA; 0.34 M ^{32}P-acetyl phosphate; 6 μl of extract (51 mg protein per ml); and water to give 60 μl total volumes. Incubation was at 37°C under N_2. The samples were analyzed as described in Figure 10.

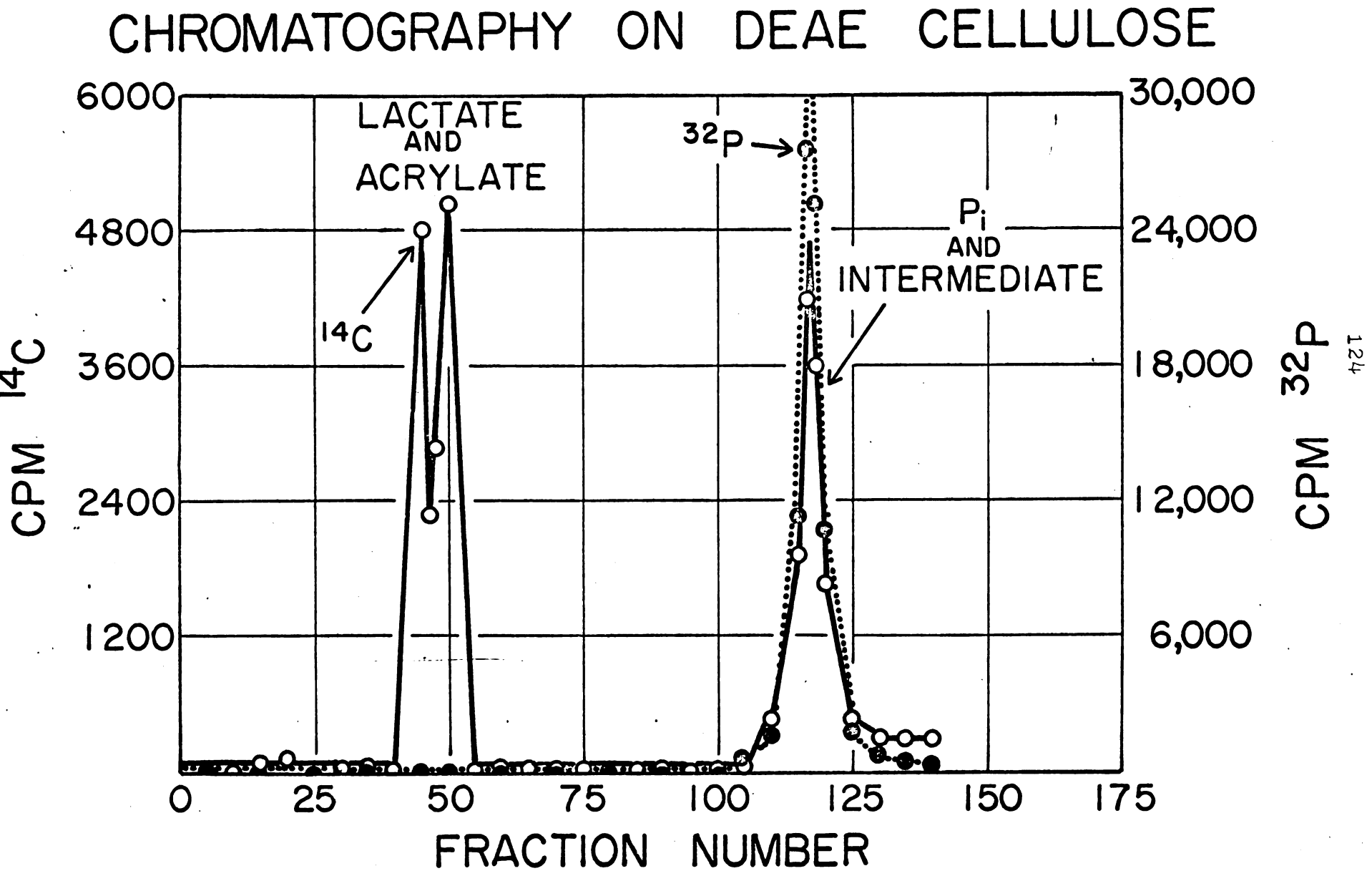

15,000
12,000
9,000
6,000
3,000
CPM 14C
1500
1200
900
600
300
CPM 32P
14C-ACRYLATE
32P-INTERMEDIATE
14C-LACTATE
0
5
10
15
20
25
30
35
MINUTES

that the labeled compound is dephosphorylated to lactate and that the appearance of labeled acrylate is not due to its formation from lactate. Further this sort of experiment necessitates use of labeled-intermediate which is free of any other labeled-organic acid. Accordingly the biosynthesis and isolation of double-labeled intermediate was undertaken so that it could be reincubated with extracts.

Chromatography on DEAE-cellulose was chosen as a first step in purification because preliminary studies with ^{14}C-lactate and ^{32}P-orthophosphate had showed that an ammonium carbonate-ammonium bicarbonate gradient gave complete separation of these contaminants. An incubation with the same components and concentrations as is used in the acrylate assay was run on a 3-ml level and with 10 μC ^{32}P-orthophosphate and 1 μC DL-lactate-^{14}C (u). After a 20 min-incubation period the entire mixture was placed on the DEAE cellulose column. Elution with the gradient separated ^{14}C-lactate from ^{32}P-phosphate; however the phosphate and phospholactate cochromatographed (Figure 12). Fractions 110-125 were pooled and evaporated to near dryness on a Swissco rotovap. The residue was applied to a Sephadex G-10 column (2.5 x 100 cm) and eluted with distilled water. The ^{14}C and ^{32}P emerged from the column together and in a slightly skewed peak; thus either all of the original ^{32}P-phosphate had been converted to phospholactate or the phospholactate was

Figure 12. Purification of enzymatically synthesized intermediate on DEAE-cellulose

The reaction mixture consisted of the following: 0.033 M Tris buffer (pH 7.6); 0.33 M DL-lactate; about 0.2 μC of ^{32}P-orthophosphate; about 1 μC of DL-lactate-^{14}C (u); 0.02% methylene blue; 0.4 mM acetyl CoA; 600 μl of extract (about 50 mg protein per ml); and water to give 3.0 ml total volume. Incubation was at 37°C under N_2 for 20 min. The ratio of counts ^{32}P/^{14}C for the intermediate indicates that there are about 2 phosphates/lactyl moiety.


CHROMATOGRAPHY ON DEAE CELLULOSE
6000
4800
3600
2400
1200
CPM 14C
30,000
24,000
18,000
12,000
6,000
CPM 32P
LACTATE AND ACRYLATE
14C
32P
Pi AND INTERMEDIATE
0
25
50
75
100
125
150
175
FRACTION NUMBER

still impure and contaminated with phosphate. In order to test the purification scheme with respect to its ability to separate phospholactate and orthophosphate, a similar incubation of labeled lactate and unlabeled phosphate was carried out but on 1/10 the original scale and the ^{32}P-phosphate was added after the enzymes had been removed by perchloric acid precipitation; thus any ^{32}P radioactivity in the phospholactate sample will represent contamination by phosphate. The Sephadex column was run first and gave partial separation of phospholactate from phosphate (Figure 13). Fractions 65-77 were concentrated and applied to the DEAE-cellulose column. About 50% of the ^{14}C counts were separated as lactate. Reapplication to the Sephadex column showed that phospholactate was partially separated from more of the phosphate and lactate. Apparently the point of diminishing returns had been reached: the intermediate was being partially hydrolyzed during concentration of fractions due to excessive heat. However this preparation did illustrate that the intermediate and phosphate are separated on Sephadex G-10 (Figure 14).

(3) Reincubation of the double-labeled intermediate and its conversion to acrylate. In order to demonstrate that the labeled compound is an intermediate, the strategy was to reincubate and show that acrylate was labeled before lactate. The partially purified material, extracts, and the components of the acrylate assay were incubated.

Figure 13. Purification of enzymatically synthesized intermediate on Sephadex G-10

The reaction mixture consisted of the following: 0.033 M HEPES buffer (pH 7.75); 7 mM $MgCl_2$; 0.02% methylene blue; 0.88 mM acetyl CoA; 67 mM acetyl phosphate; 67 mM DL-lactate and about 0.5 μC of DL-lactate-^{14}C (u); 400 μl of extract (62 mg protein per ml); and water to give a total volume of 2.21 ml. Incubation was at 37°C under N_2 for 45 min. After deproteinization, about 0.005 μC of ^{32}P-orthophosphate was added to the sample.

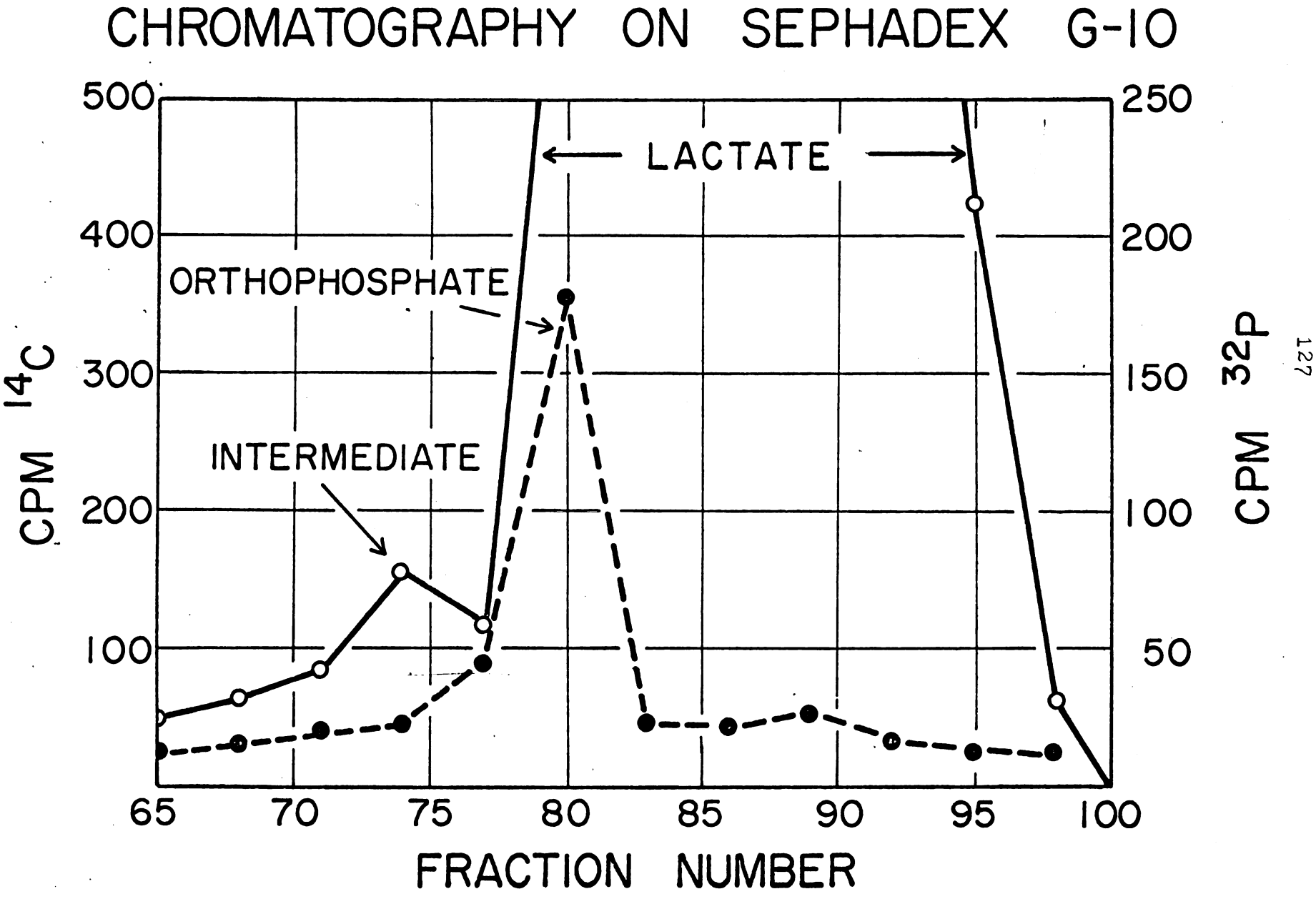
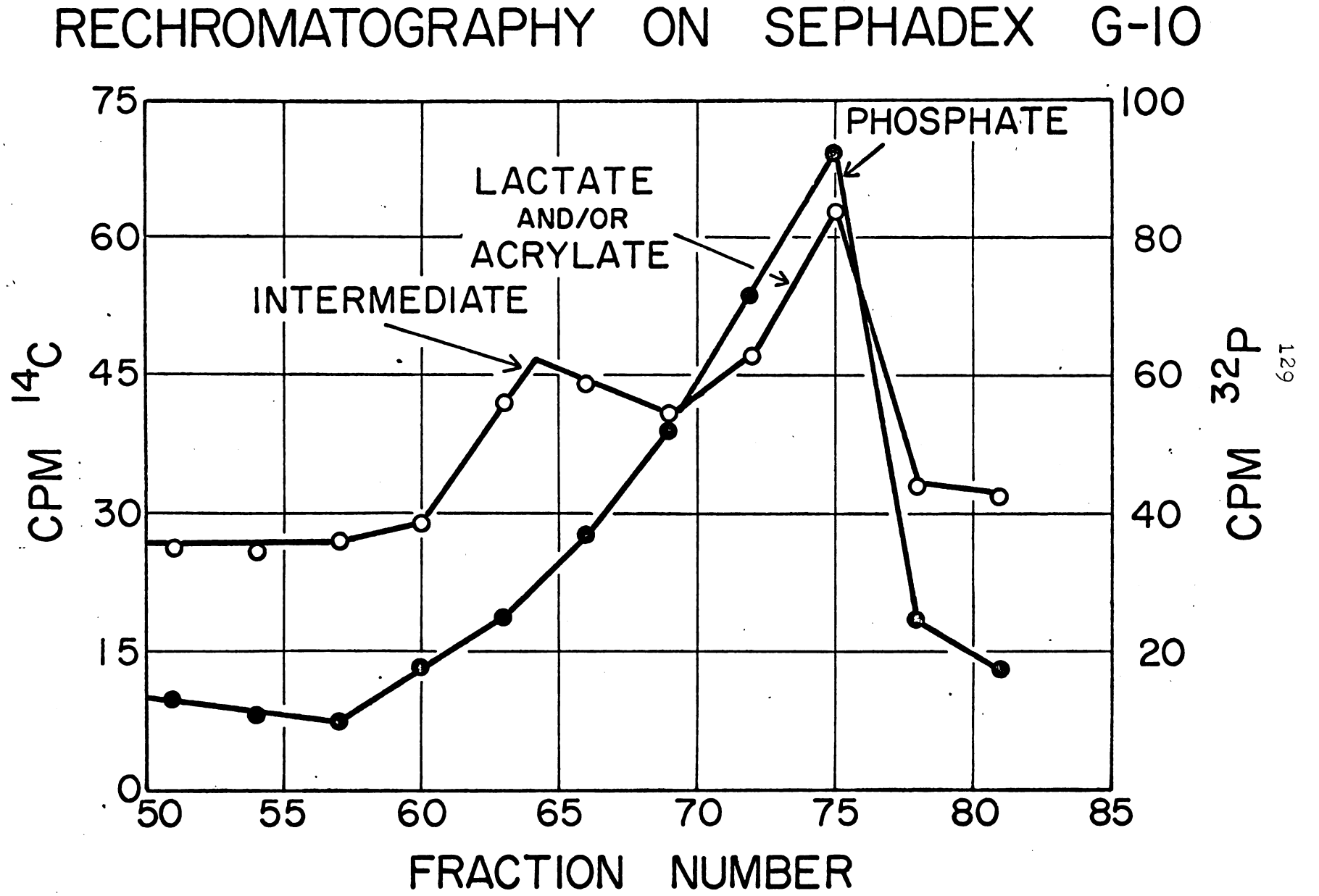

CHROMATOGRAPHY ON SEPHADEX G-10
LACTATE
ORTHOPHOSPHATE
INTERMEDIATE
CPM 14C
CPM 32P
FRACTION NUMBER
500
400
300
200
100
250
200
150
100
50
65
70
75
80
85
90
95
100

Figure 14. Purification of enzymatically synthesized intermediate on Sephadex G-10

The sample consisted of ^{14}C-labeled, biosynthesized phospholactate which was contaminated with ^{32}P-orthophosphate after its formation and had already been partially purified previously by chromatography on Sephadex and DEAE (cf. Figure 13 for details of preparation).


RECHROMATOGRAPHY ON SEPHADEX G-10
75
60
45
30
15
0
CPM 14C
100
80
60
40
20
CPM 32P
PHOSPHATE
LACTATE
AND/OR
ACRYLATE
INTERMEDIATE
50
55
60
65
70
75
80
85
FRACTION NUMBER

After 20 min, the reactions were stopped by addition of perchloric acid. The entire mixture was placed on a Celite column, whereupon acrylate and lactate were eluted separately. The acrylate and propionate were determined quantitatively by gas chromatography, the lactate was determined enzymatically with muscle lactate dehydrogenase, and the radioactivity of both peaks was measured. The results show that acrylate, or propionate inasmuch as they cochromatograph on Celite, was labeled to a greater extent than lactate (Table 19); thus the intermediate, which had been produced from lactate and purified, was converted to acrylate.

Before considering the last point, the reason lactate was labeled to a limited extent in the last experiment should be considered. Conceivably lactate could be labeled when ^{14}C-intermediate was incubated with extracts due to (1) hydrolysis during the course of the incubation and (2) contamination with ^{14}C-lactate. That the latter was the case, i.e., the labeled intermediate was contaminated with ^{14}C-lactate, was shown by paper chromatography of the labeled phospholactate after incubation in the acrylate assay system for 0 and 60 min (Figure 15). The zero time control shows that lactate contamination existed; evidently it formed during the concentration of diluted preparations such as those that elute from columns.

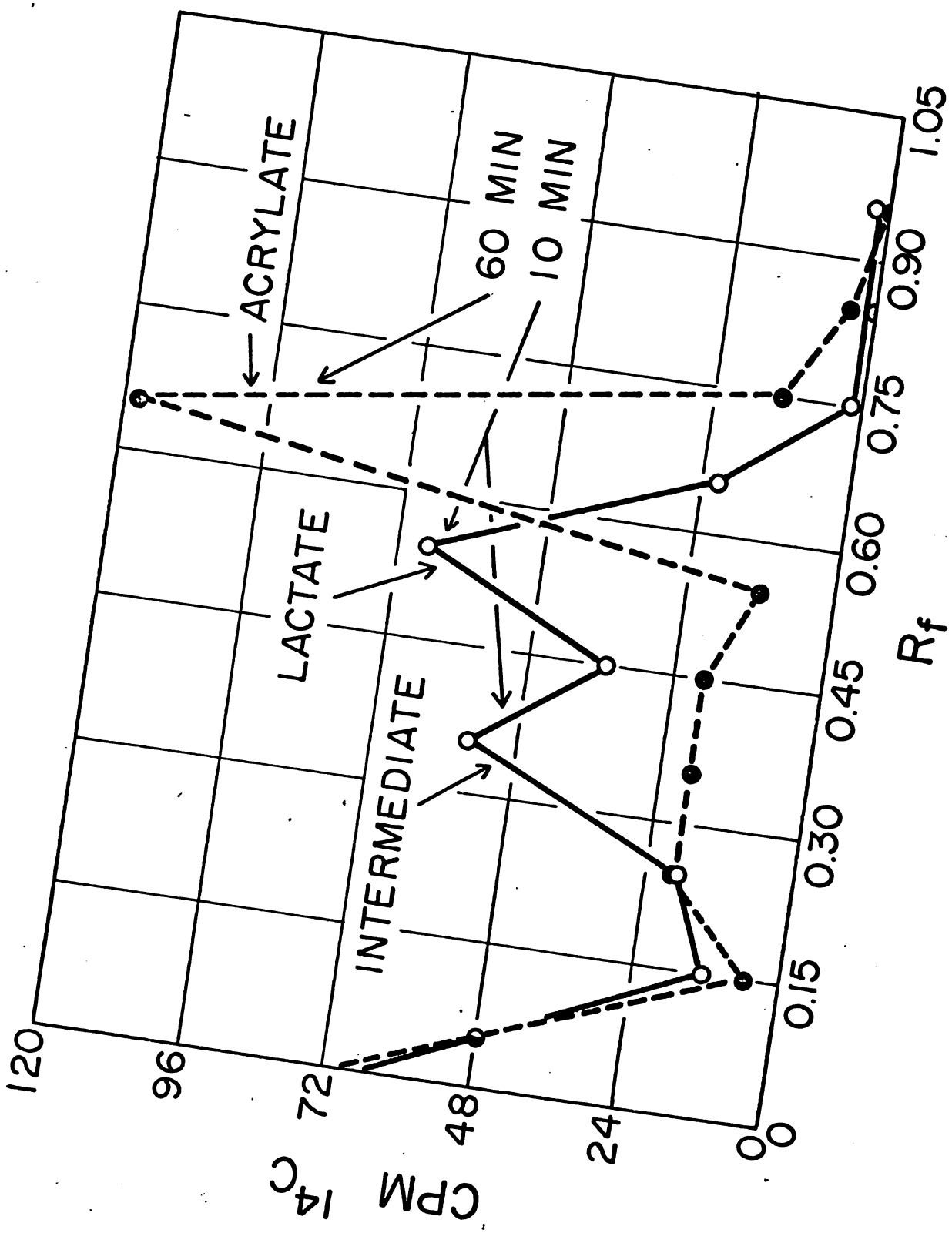
Table 19. Conversion of intermediate to acrylate

EXPERIMENT	COMPOUND	OBSERVED SPECIFIC ACTIVITY		
		CPM	μmole	μμC/μmole
1	Acrylate (or Propionate)	41	0.02	11,200
	Lactate	8	78	0.056
2	Acrylate (or Propionate)	300	17	9.6
	Lactate	25	2	2.6

The reaction mixture consisted of the following: 0.033 M HEPES buffer (pH 7.75); 0.33 M DL-lactate; 7 mM $MgCl_2$; ^{14}C- and ^{32}P-labeled intermediate which had been purified by DEAE and Sephadex chromatography; 0.4 mM acetyl CoA; 80 μl of extract (about 50 mg protein/ml); and water to give 0.60 ml total volume. Incubation was at 37°C under N_2 for 20 min in experiment 1 and 30 min in experiment 2. Acrylate and lactate were purified by partition chromatography on Celite and determined as described in MATERIALS AND METHODS. The specific activity was determined by adding toluene-^{14}C as internal standard (4.098×10^5 dpm/g).

Figure 15. Conversion of the labeled intermediate (which is contaminated with labeled lactate) to acrylate

The reaction mixture contained the following: 0.03 M phosphate buffer (pH 7.0); purified biosynthesized phospholactate; 0.19 mM acetyl CoA; 2.3 mM $MgCl_2$; 0.20 ml of extract (about 50 mg protein per ml); and water to give 1.30 ml total volume. Incubation was at 37°C under N_2. After deproteinization, the samples were analyzed by descending paper chromatography in 3:1 95% ethanol:0.1 N acetate buffer (pH 4).


ACRYLATE
60 MIN
10 MIN
LACTATE
INTERMEDIATE
CPM 14C
120
96
72
48
24
0
0.15
0.30
0.45
0.60
0.75
0.90
1.05
Rf

(4) Characterization of the labeled intermediate as phospholactate. In the remaining experiment to confirm the existence of a new alpha-phospholactyl intermediate in the acrylate pathway, characterization of the double-labeled intermediate was undertaken. First it chromatographed with the same R_F as authentic phospholactate in several solvent systems as discussed above. Second it was labeled with ^{14}C from ^{14}C-lactate and with ^{32}P from either ^{32}P-acetyl phosphate or, more slowly, from ^{32}P-orthophosphate; thus it probably contains the elements of lactate and phosphate. Further the ratio of counts incorporated ($^{32}P/^{14}C$) suggest about 1 phosphate per lactate (0.7 Figure 9 and 2.0 Figure 12). Third the intermediate was treated with alkaline phosphatase and the results show that equimolar amounts of lactate and orthophosphate were released (Table 20). Enzymatic treatment with muscle L-lactate dehydrogenase and NAD-independent D-lactate dehydrogenase from P. elsdenii showed that only D-lactate was formed from the intermediate during incubation with alkaline phosphatase and thus suggests that there is an alpha-phospho-D-lactyl intermediate.

(5) Conversion of chemically synthesized phospholactate to acrylate. Additional evidence that phospholactate is an intermediate of the acrylate pathway was sought by incubating extracts with chemically synthesized phospholactate. A sample of authentic phospholactate was

Table 20. Alkaline phosphatase treatment of labeled intermediate

COMPOUND	AMOUNT		
	EXPERIMENT		
	1	2	3
	µmoles	µmoles	µmoles
Phosphate	1.22	1.39	0.56
Lactate	1.56	.30	.60
D-Lactate	(+)		(+)
L-Lactate	(-)		(-)

The labeled intermediate was incubated with intestinal alkaline phosphatase in 5 mM $MgCl_2$ (pH 8.0) at room temperature for 6 hr. Phosphate and lactate were determined as described in MATERIALS AND METHODS (lactate by the chemical procedure of Neish).

a gift of Professor N. E. Tolbert; phospholactate was also synthesized by this investigator (cf. MATERIALS AND METHODS). Each was incubated in the acrylate assay system except lactate and acetyl phosphate were omitted. Acrylate accumulated (Figure 16), but at rates much less than expected. It is likely that phospholactyl CoA is the phospholactyl intermediate acted upon by the lyase. This idea is supported by the fact that phospholactate is not converted to acrylate unless acetyl CoA is added in catalytic amounts. For instance, the specific activity of acrylate formation with acetyl CoA was 0.0058 μmole acrylate formed/min/mg protein and without acetyl CoA the value was 0.0000. Hence the low specific activities were probably the consequence of the rate of thiol-ester interchange between phospholactate and acetyl CoA as catalyzed by CoA transferase.

E. Lactyl CoA Kinase Assay

If acetyl phosphate is the donor in formation of an intermediate from lactyl CoA then there should be a lactate-dependent disappearance of acetyl phosphate as measured by the hydroxylamine-ferric chloride assay for acetyl phosphate. It should be noted that the phosphoryl group of phospholactyl CoA does not give a hydroxamate:

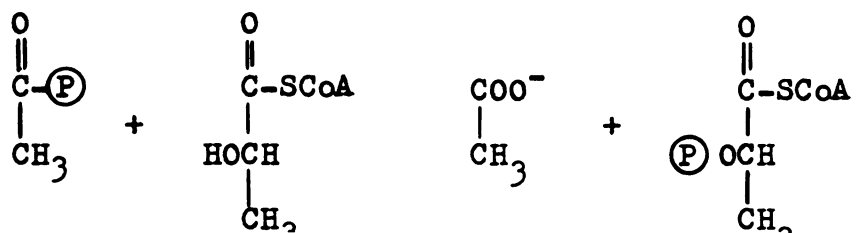
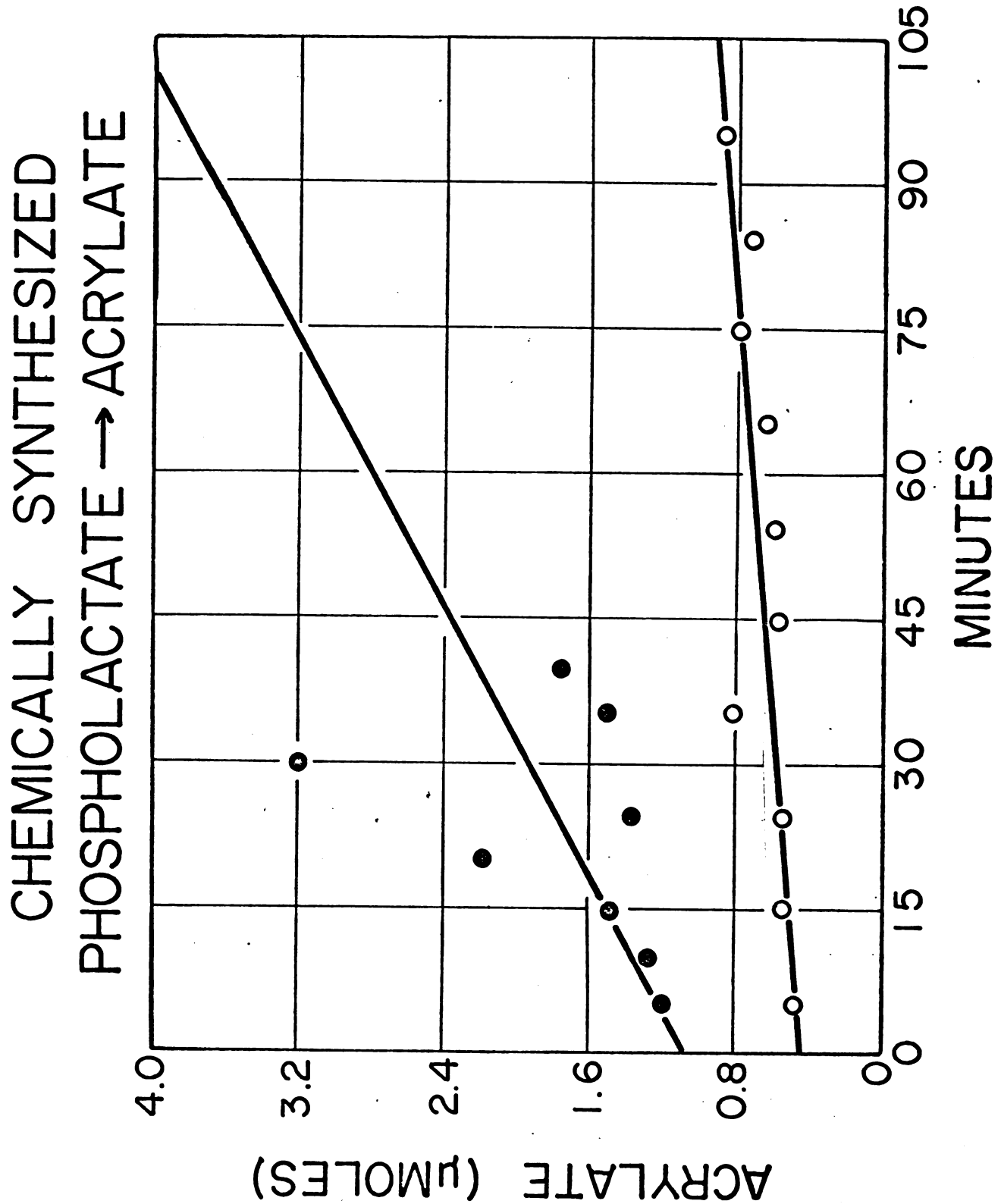


Figure 16. Conversion of chemically synthesized α-phospholactate to acrylate

The assay mixture contained the following: 0.033 M HEPES buffer (pH 7.75); 7 mM $MgCl_2$; 0.02% methylene blue; 0.41 mM acetyl CoA; about 0.033 M α-phospho-DL-lactate; 40 µl of extract (62 mg protein per ml); and water to give 300 µl total volume. The mixture was incubated at 37°C under N_2. At intervals samples were removed for gas chromatographic analysis as described in MATERIALS AND METHODS.


CHEMICALLY SYNTHESIZED
PHOSPHOLACTATE → ACRYLATE
ACRYLATE (μMOLES)
4.0
3.2
2.4
1.6
0.8
0
0
15
30
45
60
75
90
105
MINUTES

Both substrates give a hydroxamate whereas only one product does. Thus there is a net disappearance in the kinase reaction. Many attempts at demonstrating such a lactate-dependent disappearance of acetyl phosphate were unsuccessful. The variations attempted were with lactate and only catalytic amounts of acetyl CoA; with substrate amounts of ethyl lactate, lactyl pantetheine, and lactyl CoA. Extracts and the calcium phosphate eluate were examined for activity in all the variations. However the negative results may be explained by assuming that acetyl phosphate is readily formed from lactate, e.g., assume for argument's sake:

(1) acetyl phosphate + lactyl CoA $\longrightarrow$ acetate + phospholactyl CoA

(2) phospholactyl CoA $\longrightarrow$ P_i + acrylyl CoA

(3) acetate + P_i + acrylyl CoA + $2H\cdot$ $\xrightarrow[\text{phosphorylation}]{\text{electron transport}}$ propionyl CoA + acetyl phosphate

(4) $\frac{1}{2}$ lactate $\longrightarrow$ $\frac{1}{2}$ pyruvate + $H\cdot$

(5) $\frac{1}{2}$ pyruvate + $\frac{1}{2}$ P_i $\longrightarrow$ $\frac{1}{2}$ CO_2 + $H\cdot$ + $\frac{1}{2}$ acetyl phosphate

(6) propionyl CoA + lactate $\longrightarrow$ lactyl CoA + propionate

---

NET: $\frac{1}{2}$ P_i + $1\frac{1}{2}$ lactate $\longrightarrow$ $\frac{1}{2}$ acetyl phosphate + $\frac{1}{2}$ CO_2 + propionate.

Indeed lactate usually slowed the loss of acetyl phosphate as predicted by the above assumptions. Now if it is also

assumed that dinitrophenol uncouples the electron transport phosphorylation of the third reaction, then the net reaction would become:

$$\tfrac{1}{2} \text{ acetyl phosphate} + 1\tfrac{1}{2} \text{ lactate} \longrightarrow \text{acetate} + \tfrac{1}{2} P_i + \tfrac{1}{2} CO_2 + \text{propionate.}$$

Now there should be a lactate-dependent disappearance of acetyl phosphate! As shown in Figure 17 in the presence of 10^{-4} M DNP, the predicted lactate dependence of acetyl phosphate was observed. The difference (between lactate present and absent) in the rate of acetyl phosphate disappearance gives a specific activity of 0.092 μmole/min/mg protein.

F. Reversal of Dinitrophenol Inhibition by Acetyl Phosphate

The initial studies of DNP inhibition of propionate formation showed reversal by ATP. Now that acetyl phosphate is known to be the real phosphoryl donor in formation of the intermediate it must also reverse the effect of DNP. To dramatize the effect of the more direct donor, the experiment was performed with extracts which had been dialyzed 6 hr against 200 volumes of 0.05 M phosphate buffer (pH 6.5) and 1 mM DTT. The assays were done in the "acrylate" system. The results show that acetyl phosphate is effective in reversing the DNP inhibition (Figure 18). This observation thus adds further evidence that acetyl

Figure 17. Lactyl kinase assay. The lactate-dependent disappearance of acetyl phosphate in the presence of 10^{-4} M dinitrophenol

The reaction mixture contained the following: 0.033 M HEPES buffer (pH 7.75); 7 mM $MgCl_2$; 0.02% methylene blue; 0.14 M acetyl phosphate; 0.14 M DL-lactate; 0.5 mM acetyl CoA; 10^{-4} M dinitrophenol; 50 µl of extract (dialyzed 5 hr; 38 mg protein per ml); and water to give 300 µl total volume. Incubation was at 37°C under N_2. Samples were removed at intervals and reacted with hydroxylamine as described in MATERIALS AND METHODS.

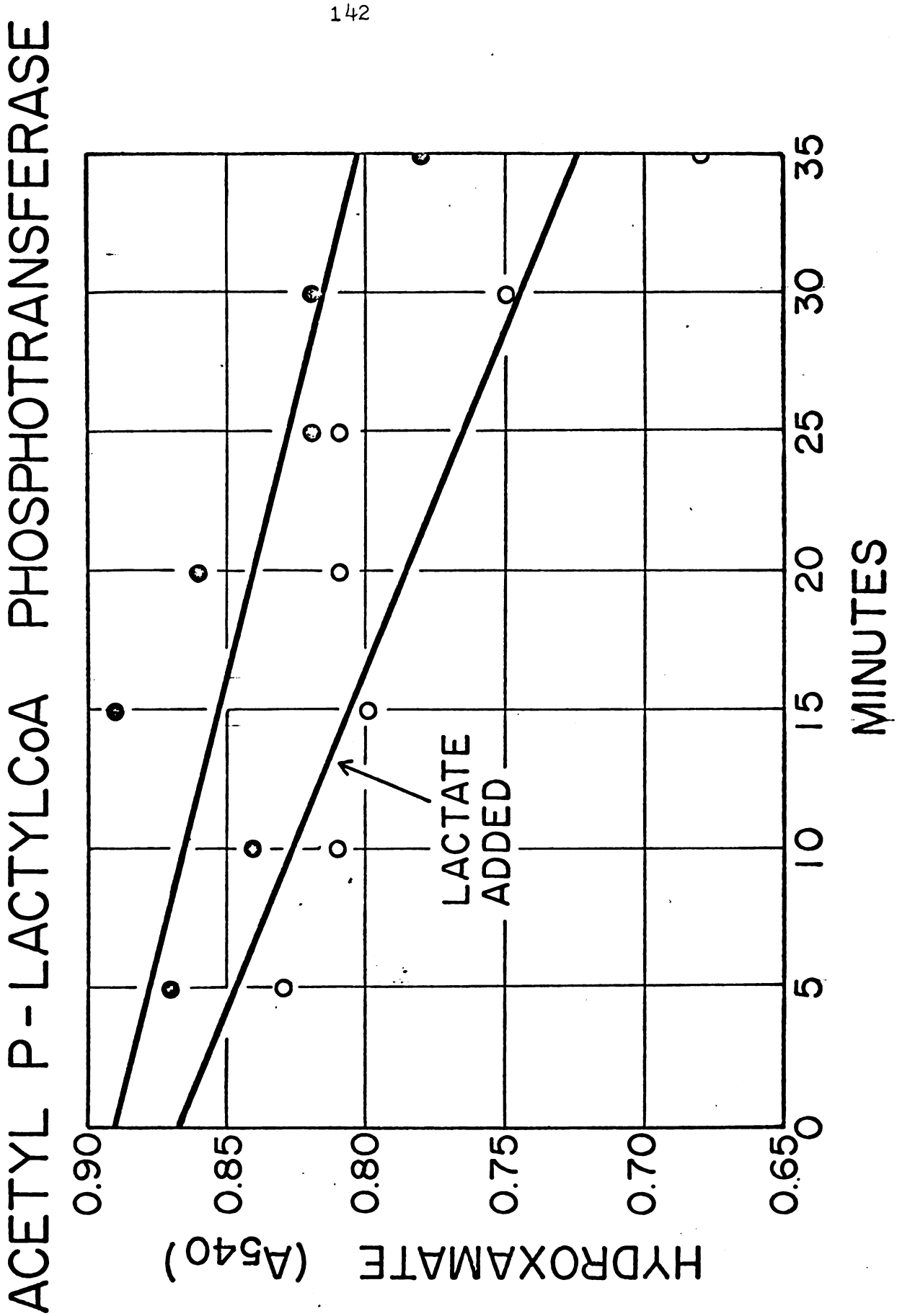
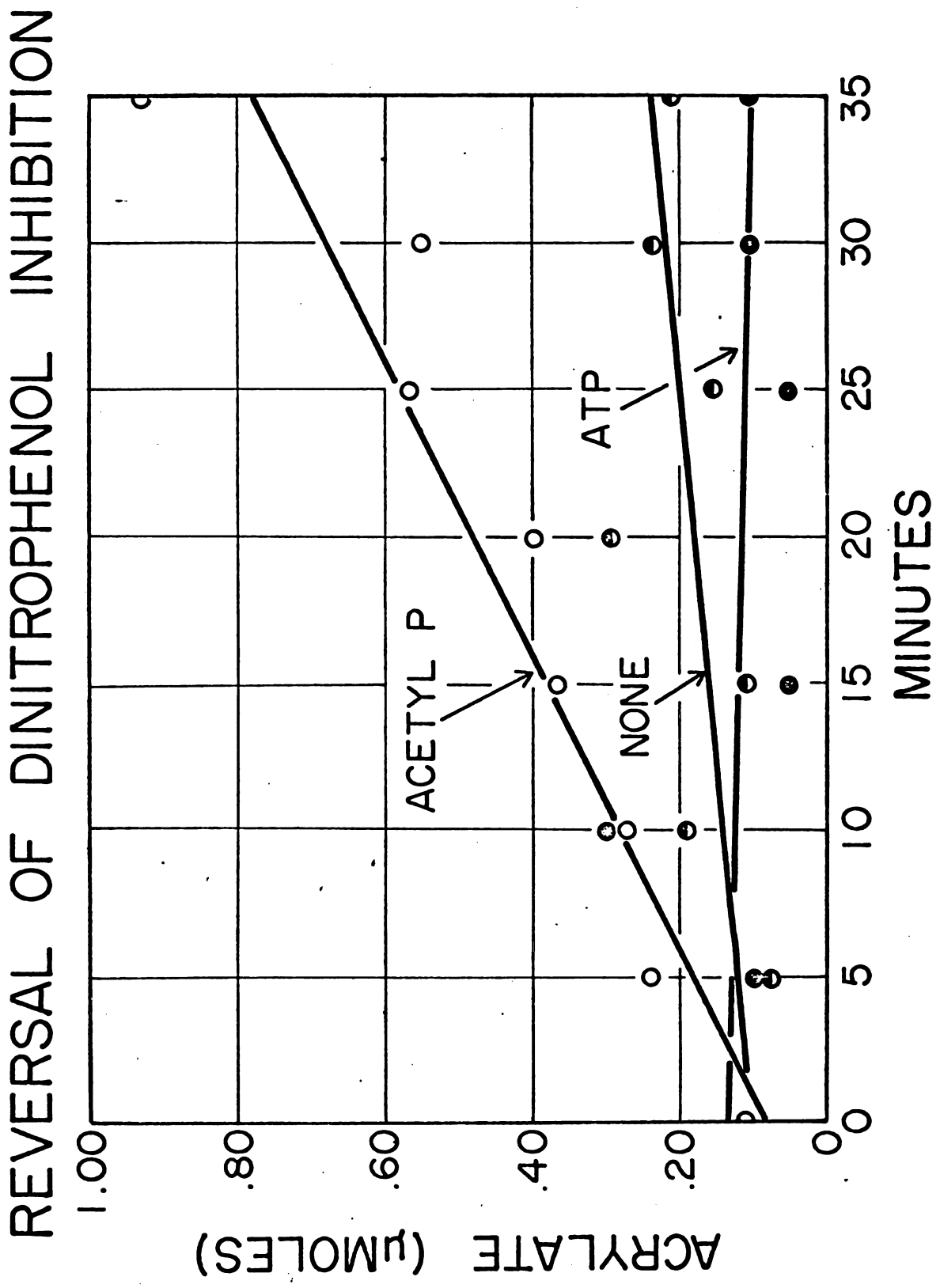

ACETYL P-LACTYLCoA PHOSPHOTRANSFERASE
HYDROXAMATE (A540)
0.90
0.85
0.80
0.75
0.70
0.65
0
5
10
15
20
25
30
35
MINUTES
LACTATE ADDED

Figure 18. Acetyl phosphate reversal of dinitrophenol inhibition

The assay mixture contained the following: 0.033 M HEPES buffer (pH 7.75); 7 mM $MgCl_2$; 0.02% methylene blue; 0.033 M DL-lactate; 0.033 M phosphoryl donor; 10^{-4} M dinitrophenol; 50 µl of extract (dialyzed 5 hr; 38 mg protein per ml); 1 mM acetyl CoA; and water to give 0.300 ml total volume. Incubation was at 37°C under N_2. Samples were removed at intervals for gas chromatographic analysis.


REVERSAL OF DINITROPHENOL INHIBITION
ACRYLATE (μMOLES)
1.00
.80
.60
.40
.20
0
0
5
10
15
20
25
30
35
MINUTES
ACETYL P
NONE
ATP

phosphate is the phosphoryl donor in formation of an intermediate (presumably alpha-phospho-D-lactyl CoA).

CHAPTER V

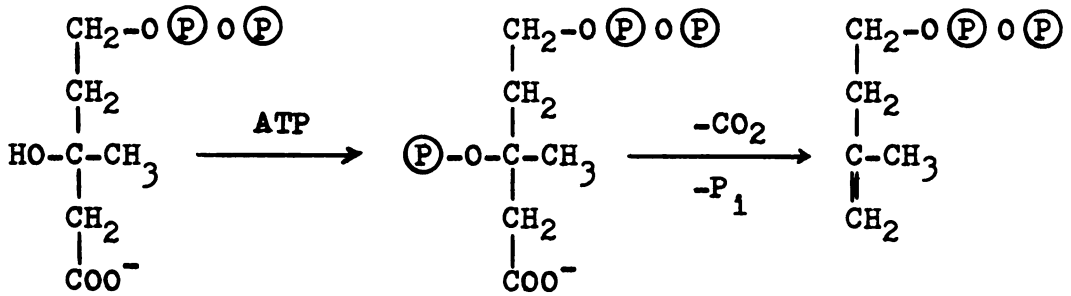
DISCUSSION

The central finding of this research was the fact that phospholactyl CoA is an intermediate between lactyl CoA and acrylyl CoA in the pathway of propionate formation from lactate. This finding is a consequence of the following observations: (1) ^{18}O is transferred from 2-^{18}O-lactate to orthophosphate concomitant with propionate formation; (2) a double-labeled intermediate accumulates during incubation of ^{14}C-lactate and ^{32}P-acetyl phosphate; (3) the labeled intermediate is converted to acrylate; and (4) the labeled intermediate was confirmed to be phospholactate by alkaline phosphatase treatment which released equimolar amounts of lactate and phosphate.

Whereas a phospholactyl CoA intermediate contradicts Baldwin's simple dehydration reaction catalyzed by lactyl CoA dehydrase, it does explain his failure to observe the interconversion of lactyl CoA and acrylyl CoA by a direct spectrophotometric assay. It also explains the sudden loss of activity upon purification (Baldwin, 1962). Furthermore a phospholactyl CoA intermediate dovetails very well with Ladd and Walker's observation of the ability of dinitrophenol to inhibit the lactate-acrylate interconversion and its reversal by ATP or

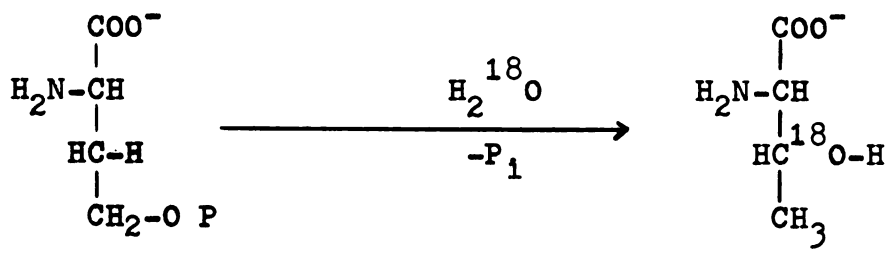
acetyl phosphate (Ladd and Walker, 1965).

The elimination of the phosphate from phospholactyl CoA to form acrylyl CoA presumably by a phospholactyl CoA lyase represents another example of phosphate-facilitated leaving of a hydroxyl group. The other cases of enzymatic reaction of similar reaction mechanism are (1) threonine synthetase and (2) ATP:5-pyrophosphomevalonate carboxy-lyase:



MEVALONIC ACID PYROPHOSPHATE

ISOPENTENYL PYROPHOSPHATE



O-PHOSPHO HOMOSERINE

THREONINE

Model system studies have also shown that the phosphoryl group enhances elimination reactions (Cherbuliez *et al.*, 1962).

Uniquely acetyl phosphate is the phosphoryl donor in the case of *P. elsdenii*. With few exceptions, enzyme-

catalyzed phosphorylations involve nucleoside triphosphates as the phosphorylating agents. Acetyl phosphate has been found to be the donor in formation of D-glucose-6-phosphate from D-glucose as catalyzed by an enzyme from Aerobacter aerogenes (Kamel and Anderson, 1964). In this case the enzyme also utilizes hexose phosphates as donors, e.g., D-mannose-6-phosphate, and other compounds such as carbamyl phosphate and phosphoramidate (Kamel and Anderson, 1967), leading to the conclusion that this is a nonspecific phosphotransferase which can utilize acetyl phosphate as well as other donors. Studies with Clostridium kluyveri which is an anaerobe like P. elsdenii have demonstrated the phosphoryl-donating properties of acetyl phosphate in several reactions (Decker, 1959). Thus the use of acetyl phosphate to form phospholactyl CoA is not an unprecendented example of its use as a phosphoryl donor.

A very important aspect of this new reaction sequence is the implication of the present results with respect to the possibility of electron transport mediated phosphorylation in the anaerobe P. elsdenii. Inasmuch as an acetyl phosphate, a potential source of ATP, is consumed during formation of phospholactyl CoA, an additional phosphorylation besides that of the phosphoroclastic system must occur otherwise the organism would not be able to grow:

(with electron transport mediated phosphorylation)

1. lactate + X CoA $\longrightarrow$ lactyl CoA + X

2. lactyl CoA + acetyl P ⟶ phospholactyl CoA + acetate
3. phospholactyl CoA ⟶ P_i + acrylyl CoA
4. Y + P_i + 2 H + acrylyl CoA ⟶ Y∼P + propionyl CoA
5. Y∼P + acetate ⟶ acetyl P + Y
6. lactate ⟶ pyruvate + 2 H
7. pyruvate + CoA ⟶ $H_2\uparrow$ + $CO_2\uparrow$ + acetyl CoA
8. acetyl CoA + P_i ⟶ acetyl P + CoA
9. ADP + acetyl P ⟶ acetate + ATP
10. propionyl CoA + X ⟶ propionate + X CoA

NET: 2 lactate + ADP ⟶ propionate + acetate + CO_2 + H_2 + **1 ATP** .

(without electron transport mediated phosphorylation)

1. lactate + X CoA ⟶ lactyl CoA + X
2. lactyl CoA + acetyl P ⟶ phospholactyl CoA + acetate
3. phospholactyl CoA ⟶ P_i + acrylyl CoA
4. 2 H + acrylyl CoA ⟶ propionyl CoA
5. lactate ⟶ pyruvate + 2 H
6. pyruvate + CoA ⟶ $H_2\uparrow$ + $CO_2\uparrow$ + acetyl CoA
7. acetyl CoA + P_i ⟶ acetyl P + CoA

NET: 2 lactate ⟶ propionate + acetate + CO_2 + H_2 + **0 ATP** .

The existence of anaerobic electron transport phosphorylation has been speculated for some time. There is precedent for this sort of phosphorylation (E. R. Stadtman, 1966). Direct evidence of anaerobic ATP generation in

clostridia by a mechanism which does not involve substrate phosphorylation was found in the reductive deamination of glycine as in Clostridium sticklandii and Clostridium lentoputrescens (Stadtman and Elliott, 1956). The system was resolved into an electron transport protein, ferredoxin, an acidic and low molecular weight protein, and a quinone (Stadtman et al., 1958; Stadtman, 1962; Stadtman, 1966):

$$NADH + H^+ + \text{glycine} + P_i + ADP \longrightarrow NAD^+ + NH_3 + ATP + \text{acetate}$$

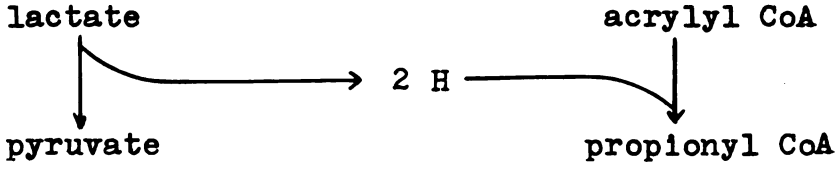
In the similar case of Clostridium aminobutyricum growth studies have shown that 7.6 mg of dry cells are derived from 1 mmole of gamma-aminobutyrate compared to 5.0 mg which would be expected from the substrate-level phosphorylation reactions predicted to occur.

Second, in the case of anaerobic streptococci, electron transport phosphorylation has been implicated. Streptococci are facultative anaerobes which do not possess cytochromes. Thus any electron transport mediated phosphorylation must be different from that which occurs in mitochondria. Studies with Streptococcus faecalis have revealed growth beyond the limits of the substrate-level phosphorylation reactions known to occur, and the additional growth suggests a P/O ratio of 0.6 (Smalley et al., 1968). In S. agalactiae, ATP formation has been demonstrated with cell-free extracts:

$$P_i + NADH + ADP + O_2 \longrightarrow ATP + H_2O + NAD.$$

With respect to the equation above it should be pointed out that the usual acceptor is nitrate not oxygen and that oxygen was used to facilitate assay (oxygen uptake was determined manometrically). In this system a P/O ratio of 0.15-0.42 was observed (Mickelson, 1968).

Preliminary studies with P. elsdenii suggest the existence of a soluble, electron transfer system derived from the coupling of the lactate dehydrogenase with the acyl CoA dehydrogeanse:



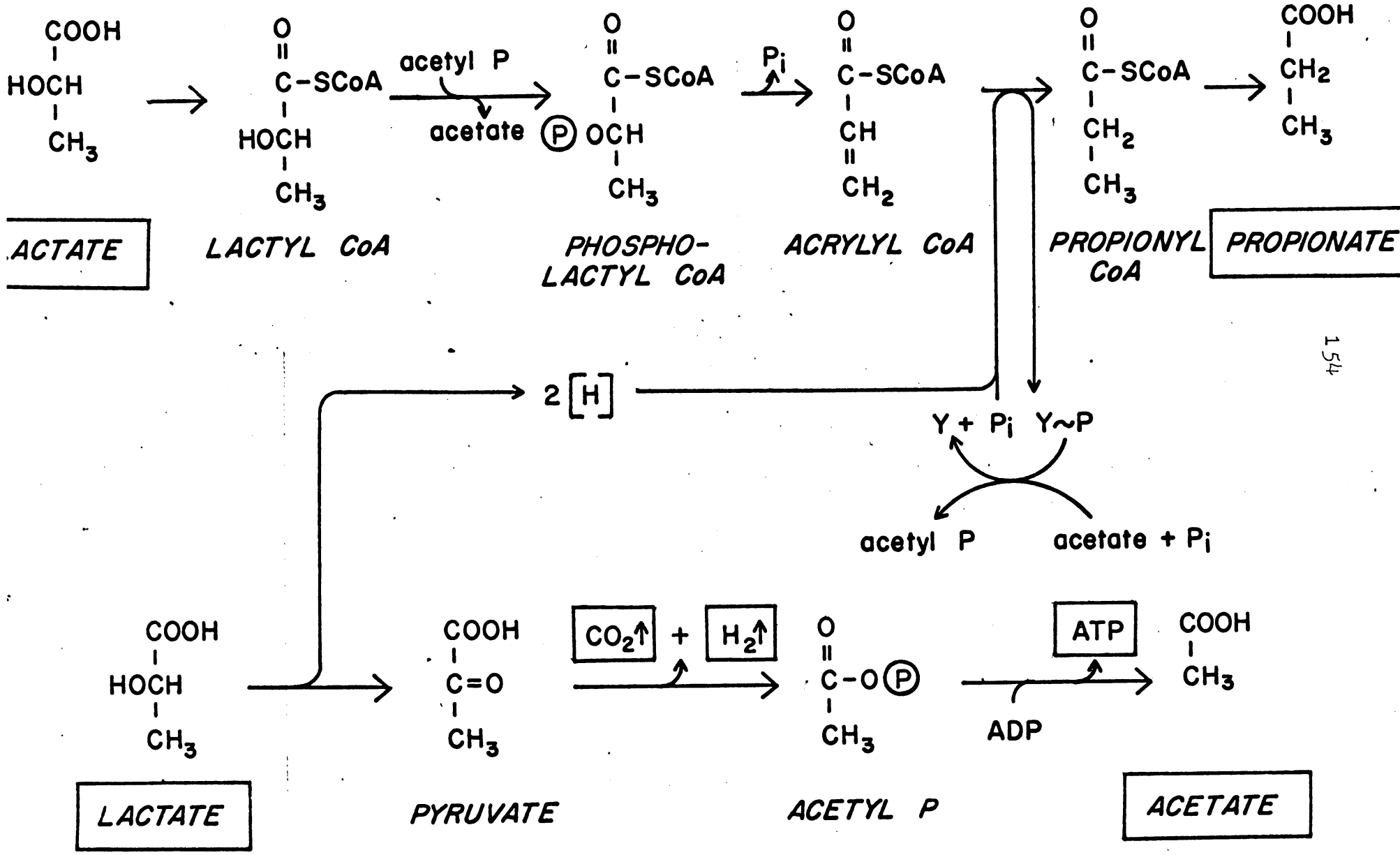
The enzymes are not precipitated by ultracentrifugation for several hours at greater than 100,000 times gravity. Further evidence that the system is not particulate is that solubilizing agents, such as glycerol, and phospholipids are uniformly inhibitory (cf. APPENDIX, evidence for soluble system). Whether phosphorylation accompanies the electron transfer described above is not known. However electron transfer from the lactate dehydrogenase to the acyl CoA dehydrogenase cannot be tightly coupled inasmuch as the fermentation balance reveals that for growth on 100 mmole of lactate 71 mmole of acetate is formed (though most of it is converted to higher fatty acids, especially butyrate and valerate) and 39 mmole of propionate is formed (half of it is converted to

valerate). Inotherwords, the pathway to acetate operates twice for every time that to propionate does (Elsden *et al.*, 1956). Determination of ATP formation by means of the usual hexokinase trap (Pinchot, 1957) as a method of verifying electron transfer phosphorylation is precluded by the presence of a very active adenylate kinase (Baldwin and Milligan, 1964). An alternate approach would be to: (1) isolate the components of the electron transport system; (2) reconstitute the system; and (3) isolate coupling factor which possibly would restore phosphorylation.

Finally, judging from the available data, the basic metabolic system for lactate utilization in *P. elsdenii* is as shown in Figure 19; thus the net reaction for extracts is:

$$2 \text{ lactate} + ADP + P_i \longrightarrow \text{propionate} + \text{acetate} + CO_2\uparrow + H_2\uparrow + ATP.$$

Figure 19. The basic metabolic system for lactate utilization in P. elsdenii


COOH
HOCH
CH3
O
C-SCoA
HOCH
CH3
acetyl P
acetate
P
OCH
CH3
Pi
CH
CH2
CH2
CH3
COOH
CH2
CH3
ACTATE
LACTYL CoA
PHOSPHO-
LACTYL CoA
ACRYLYL CoA
PROPIONYL
CoA
PROPIONATE
2 [H]
Y + Pi
Y~P
acetyl P
acetate + Pi
LACTATE
PYRUVATE
CO2↑
+
H2↑
ACETYL P
ATP
ADP
ACETATE

ABBREVIATIONS USED

ACD	acyl CoA dehydrogenase
AcPyAD	3-acetylpyridine adenine dinucleotide (analog of NAD)
ADH	alcohol dehydrogenase
ATP	adenosine triphosphate
BAL	British anti-Lewisite or 2,3-dimercaptopropanol
CoA	coenzyme A
CoA-T	coenzyme A transferase
DTT	Cleland's reagent or dithiothreitol
Glc	glucose
INT	2-para-iodophenyl-3-para-nitrophenyl-5-phenyl-tetrazolium chloride
LDH	lactic acid dehydrogenase
MB	methylene blue
NAD(H)	(reduced) nicotinamide adenine dinucleotide
NADP(H)	(reduced) nicotinamide adenine dinucleotide phosphate
PEP	phosphoenolpyruvic acid
P_i	orthophosphate
PMS	phenazine methosulfate FW 306.34
PMSF	phenylmethylsulfonylfluoride, a proteinase inhibitor similar to diisopropylfluorophosphate but not poisonous
rds	rate determining step
THF	tetrahydrofuran, a water miscible ether
TPP	thiamine pyrophosphate (vitamin B_1)

APPENDIX

CALCULATION OF MINIMUM SPECIFIC ACTIVITY OF THE ENZYMES OF THE ACRYLATE PATHWAY

During any one time interval:

$$(E)\ (\Delta t) = X$$

where E is enzyme activity in μmole/min/mg protein, t is time in minutes, and X is the amount of material passing through the pathway in μmole/mg protein. Summing the equation over the entire fermentation period gives an integral

$$\int_0^{720} E(t)\ dt = X$$

Before the integral can be evaluated E as a function of time, E(t), and X must be known.

Determination of E(t)

Since the number of cells double each generation and if it is assumed that the amounts of the enzymes of the pathway double in a similar manner, then:

$$E_t = E_o(2)^n$$

where E_t is the enzyme activity at any time t and n is the number of generations. Now, if a 5% inoculum is used, the initial amount of enzyme is:

$$E_o = 0.05\ E_f$$

and number of generations can be calculated:

$$E_{final} = E_f = 0.05\ E_f\ (2)^n$$

$$1.00 = 0.05\ (2)^n$$

$$\frac{1}{.05} = 20 = 2^n$$

$$\log_2 (20) = n$$

$$3.32 \log_{10} (20) = n$$

$$n = 4.31$$

The division time is 720/4.31 or 167 min.

Now E can be expressed as a function of time:

$$E\ (t) = 0.05\ E_f\ (2)^{\frac{4.31}{720}\ t}\ .$$

Remember E_f is final enzyme activity and is not a variable.

Determination of X

Twenty liters of medium containing 270 g of lactic acid when fermented by P. elsdenii yield, among other products, 0.36 moles of propionate and 0.46 moles of valerate (Gutierrez et al., 1956). Both propionate and valerate are products of one pass through the direct reductive pathway; hence, during the whole fermentation at least 0.81 moles (0.36 + 0.46 = 0.82) must represent direct reductive pathway activity. With a five percent inoculum the bacteria reach stationary phase in about twelve hours. Thus the pathway must be as active as 41 μmole/ml per 12 hr $\frac{820{,}000\ \mu\text{moles}}{20{,}000\ \text{ml}} = 41$.

A deep culture of twenty liters of P. elsdenii produce 5,250 mg of protein (average of 25 determinations) or 0.2625 mg protein/ml. Thus the minimum activity is X = 156 μmole/mg protein per 12 hr ($\frac{41 \text{ μmole/ml}}{0.2625 \text{ mg/ml}} = 156$).

Integration

Substituting into the integral gives

$$\int_0^{720} 0.05\, E_f\, (2)^{\frac{4.31}{720}\, t}\, dt = 156$$

or

$$\int_0^{720} (2)^{0.00599\, t}\, dt = \frac{156}{0.05\, E_f}$$

which is in the form

$$\int_b^{au} du = \frac{b^{au}}{a \ln b} + c$$

Hence

$$\frac{156}{0.05\, E_f} = \frac{(2)^{0.00599(720)}}{.00599 \ln 2} - \frac{(2)^{0.00599(0)}}{.00599 \ln 2}$$

$$= \frac{19.9}{.00414} - \frac{1.00}{.00414}$$

$$= 4800 - 242 = 4558$$

or

$$\frac{156}{(.05)\ (4558)} = E_f$$

$$E_f = 0.684 \text{ μmole/min/mg protein.}$$

CALCULATION OF EXPECTED $^{3}H/^{14}C$ RATIO

The tritium used gave $1.16(10)^{10}$ counts per 0.020 ml; in incubation system 1 (cf. MATERIALS AND METHODS) the hydrogens of the water then had a specific activity of:

$$\frac{1.16(10)^{10} \text{ cpm } (18 \text{ g/mole})}{2.6 \text{ g}} = 8.03(10)^{10} \text{ cpm/mole}$$

The lactate had a specific activity of:

$$\frac{63954 \text{ cpm}}{0.12 \times 10^{-3} \text{ moles}} = 5.33(10)^{8} \text{ cpm/mole}$$

Hence the expected ratio in propionate was:

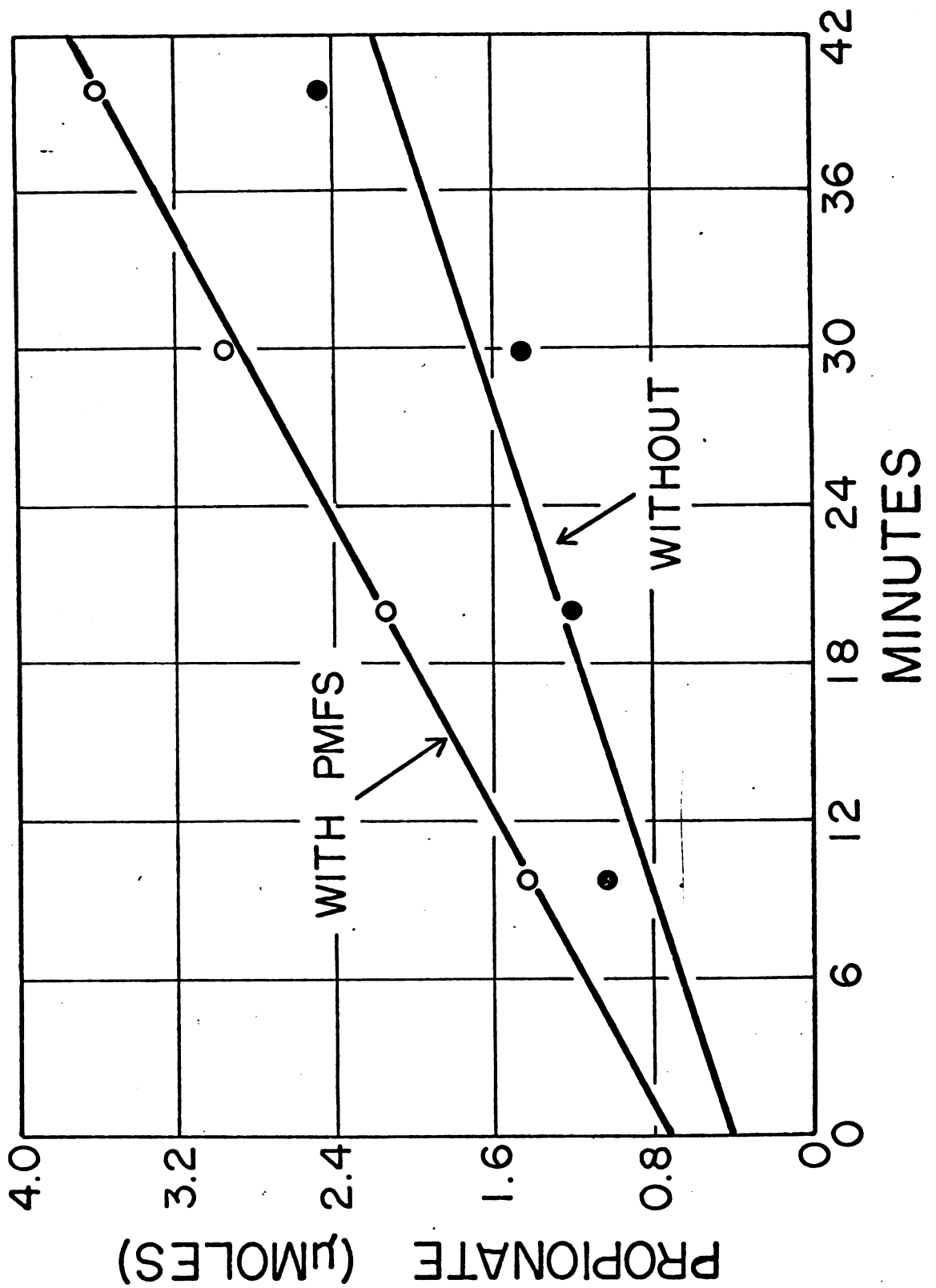
$$\frac{2(8.03)(10)^{10}}{5.33(10)^{8}} = 301 \quad .$$

STABILIZATION OF EXTRACTS BY PROTEINASE INHIBITOR

Activity of extracts in producing propionate from lactate deteriorated upon storage, especially when divalent metals such as Ca^{2+} were present. Such behavior has the appearance of proteolytic digestion. A proteinase inhibitor phenylmethylsulfonylfluoride (PMSF) was effective in preventing this deterioration (APPENDIX Figure 1). It was added to extracts at time of preparation according to the procedure of Steinman and Jakoby (1967). PMSF is better than diisopropylfluorophosphate because it is not detrimental to the nervous system yet has potency in inhibiting proteinases.

Appendix Figure 1. Stabilization of extracts by a proteinase inhibitor, PMSF

The extracts were prepared in the presence and absence of PMSF. After storage at -14°C for 6 days they were assayed in the "propionate" system as described in MATERIALS AND METHODS. The lines drawn represent a least squares treatment of the data.


PROPIONATE (μMOLES)
4.0
3.2
2.4
1.6
0.8
0
6
12
18
24
30
36
42
MINUTES
WITH PMFS
WITHOUT

Lactate Dehydrogenase(s) and Racemase of P. elsdenii

The lactate dehydrogenase (LDH) of P. elsdenii probably utilizes only the D-isomer, the slight activity on L-lactate may be due to a racemase (Appendix Table 1). The D-LDH is activated by divalent metal ions; however there is no correlation of activity with ionic radius (Appendix Table 2). Furthermore these results have not been confirmed with a partially-purified enzyme (Brockman, 1968). Inasmuch as ferricyanide was used as electron acceptor in these studies and Brockman used INT, the discrepancy may be due to (1) this difference in assay procedure or (2) the possibility that purification causes an irreversible change in the enzyme or (3) the possibility that the metal ion effect with unpurified extracts is artifactual. The effect of the metal is to decrease the K_M for lactate about three orders of magnitude (Appendix Table 3). The D-LDH is NAD-independent; since most NAD-independent lactate dehydrogenases are flavoproteins (Snoswell, 1967; Tubbs, 1962), this one may be also.

Lactate racemase activity can be observed in a system in which D-lactate is added as substrate and the L-lactate produced from it by racemization is oxidized to pyruvate by muscle LDH with concomitant reduction of NAD.

The racemase is not stimulated by pyridoxal nor ferrous sulfate unlike that of Clostridium acetobutylicum

Appendix Table 1. Lactate dehydrogenase and racemase activities of *P. elsdenii* extracts

	SPECIFIC ACTIVITY	
ENZYME	EXPERIMENT 1	EXPERIMENT 2
	μmole/min/mg	
D-LDH	0.030	0.075*
L-LDH	.016	.005*
racemase	.048	.026*

*These values all determined at pH 9.9.

The assays were performed as described in MATERIALS AND METHODS.

Appendix Table 2. Divalent metal ion activation of D-lactate dehydrogenase

METAL*	SPECIFIC ACTIVITY	RELATIVE ACTIVITY	IONIC RADIUS
	μmole/min/mg protein	%	Å
$CoCl_2$	0.119	100	0.78
$ZnSO_4$	.072	60	.70
$MnCl_2$	.033	28	.80
$(NH_4)_6Mo_7O_{24}$	.023	19	.68
$FeCl_3$	.022	18	.53
$MgCl_2$	.016	13	.65
$CuSO_4$	.008	7	.69
NONE	.009	8	

*Each metal was tested at a concentration of 1 mM.

The assays were performed as described in MATERIALS AND METHODS.

Appendix Table 3. K_M effect of $CoCl_2$ on D-lactate dehydrogenase with DL-lactate as substrate

ADDITION	CONCENTRATION	K_M	V_{MAX}
	mM	M	
None	--	$52{,}100\ (10)^{-5}$	0.0138
$CoCl_2$	1.0	$7.0\ (10)^{-5}$	0.0129

(Katagiri *et al.*, 1958), and fortunately so because that enzyme is thought to act by a dehydration-rehydration mechanism which would make all discussion of a phospholactyl CoA intermediate unnecessary. The clostridial enzyme is secreted into the culture medium, again unlike that of *P. elsdenii*.

The racemase probably does not involve a pyruvate intermediate because in that case the L-LDH activity would be equal to or greater than the overall racemase activity and this is not observed (cf. Appendix Table 1):

D-lactate = pyruvate = L-lactate.

Dennis and Kaplan report that the racemase of *Cl. butylicum* involves an S-lactyl intermediate and racemization is accomplished by an internal hydride shift (1959); the racemase of *P. elsdenii* may involve a similar reaction mechanism.

Soluble Electron Transfer System of *P. elsdenii*

The possibility of electron transport phosphorylation exists. First there is the dinitrophenol (DNP) inhibition of the lactate-to-acrylate reaction which is reversed by acetyl phosphate. So far every aspect of the DNP inhibition is consistent with the generation of one phosphoryl bond every time acrylyl CoA is reduced to propionyl CoA (cf. RESULTS, lactyl kinase section).

Second if this system exists it may be soluble.

Ultracentrifugation of extracts for 7 hr at greater than 100,000 x gravity did not affect activity (Appendix Table 4). When the cells were broken in 0.5% Triton X-100, 10% glycerol, or 3% sodium deoxycholate the extracts were inactive in forming propionate from lactate. Sonication of whole cells in the presence of 1% lecithin gave extracts which were about 25% as active as usual and the activity was not at all stable to storage. Addition of 20% glycerol to fresh extracts was slightly inhibitory and did not stabilize extracts during storage. Thus the activity is not lost upon ultracentrifugation, and factors which stabilize particulate systems are inhibitory.

Further, a difference spectrum taken on extracts showed no evidence of reduced cytochromes upon addition of dithionite to the sample cuvette, although the cytochrome peak may have been obscured by the high concentration of flavins. The bacteria contain 1.480 μmole flavin per gram dry weight, 73% of which is FAD (Peel, 1958); hence, they appear red colored under the light microscope. Baldwin and Milligan have found evidence for a cytochrome-c-like acyl CoA dehydrogeanse (1964).

Linearity of Acrylate Assays

While the GLC assays are linear with respect to time, they are linear with respect to protein only at very high protein concentrations (Appendix Table 5). This sort of behavior is expected for a multi-enzyme system such as

Appendix Table 4. Effect of ultracentrifugation on lactate-to-propionate activity

EXPERIMENT	ACTIVITY	
	ORIGINAL	AFTER ULTRACENTRIFUGATION*
	μmole/min/mg protein	
1	0.0067	0.0240
2	---	.0275
3	.0122	.0125

*About 1/3 of the protein is removed.

The assays were performed as described in MATERIALS AND METHODS.

Appendix Table 5. Non-linearity of acrylate assays with respect to protein

PROTEIN ASSAYED	SPECIFIC ACTIVITY*
mg	µmole/min/mg protein
0.72	0.076
1.44	.157
2.16	.198
2.88	.392
3.06	.353
3.24	.312
3.42	.374
3.60	.386
3.79	.377

*The last 6 values average 0.365 with a standard deviation of 0.031.

The assays were performed as described in MATERIALS AND METHODS (cf. acrylate assay).

this which depends on CoA transferase, "lactyl CoA kinase," and "phospholactyl CoA lyase" as a minimum.

SUMMARY

Acrylyl CoA was reconfirmed to be an intermediate of the direct reductive pathway of propionate formation from lactate in Peptostreptococcus elsdenii. However the conversion of lactyl CoA to acrylyl CoA was not found to be a simple dehydration: on basis of the results presented herein it is concluded that α-phospho-D-lactyl CoA is intermediate between lactyl CoA and acrylyl CoA.

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