PART 1 CONVERSION OF LACTATE-C^{14} AND GLUCOSE -C^{14} TO PROPIONATE BY THE RUMEN MICROBIOTA

PART 11 LACTATE METABOLISM BY PEPTOSTREPTOCOCCUS ELSDENII

Thesis for the Degree of Ph. D.

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This is to certify that the

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LACTATE METABOLISM
Part I - Conversion Of Lactate-C^{14} And Glucose-C^{14} To Propionate By The Rumen Microbiota
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ABSTRACT

LACTATE METABOLISM

PART I -- CONVERSION OF LACTATE-C^{14} AND GLUCOSE-C^{14} TO PROPIONATE BY THE RUMEN MICROBIOTA

PART II -- LACTATE METABOLISM BY PEPTOSTREPTOCOCCUS ELSDENII

by Ransom Leland Baldwin Jr.

The problem outlined for investigation was divided into two parts. The first part involved an investigation of the relative contributions of the two alternate pathways (via symmetrical and non-symmetrical intermediates) for the conversion of lactate to propionate in a natural biological system. The second part was concerned with the isolation and characterization of the enzymes responsible for the conversion of lactate to propionate via the acrylate or the so-called non-randomizing pathway.

The biological system chosen for the first phase of the investigation was bovine rumen fluid. Samples removed from cows fed a variety of diets were incubated in vitro with lactate-2-C^{14}, lactate-3-C^{14}, glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14}. The acetic and propionic acids were isolated and degraded and the specific activities of the individual carbon atoms determined. The results obtained in these experiments clearly indicated that (1) the acrylate pathway makes a significant contribution, (2) the contribution of the acrylate pathway increases with the starch content of the diet, and (3) the succinate (randomizing) pathway is the most important since it performs the major role during hexose utilization.

Considerable progress has been made on the problem of identification, isolation and characterization of the enzymes of the acrylate pathway:

(1) acetyl-PO_4 + CoASH $\underset{\leftarrow}{\text{phosphotransacetylase}}$ acetyl-CoA + Pi

(2) acetyl-CoA + lactate $\underset{\leftarrow}{\text{CoA transphorase}}$ lactyl-CoA + acetate

(3) lactyl-CoA $\underset{\leftarrow}{\text{lactylCoA dehydrase}}$ acrylyl CoA + H_2O

(4) acrylyl-CoA + $2e^-$ $\underset{\leftarrow}{\text{acyl-CoA-dehydrogenase}}$ propionyl-CoA

(5) propionyl-CoA + acetate $\underset{\leftarrow}{\text{CoA transphorase}}$ acetyl-CoA + propionate

Lactyl CoA dehydrase (3), the key enzyme of the pathway, has been identified in cell-free extracts of the rumen organism Peptostreptococcus elsdenii and an assay system developed for measuring its activity.

Phosphotransacetylase (1) and CoA transphorase (2, 5) have been purified from extracts of the same organism. Acyl CoA dehydrogenase (4) has been purified 25-fold and partially characterized. The enzyme is freely reversible, has a pH optimum of 7.5 and prefers four carbon to three carbon substrates. Several other enzymes observed in extracts of the organism are: a D lactic dehydrogenase (no pyridine nucleotide requirement), a very stable phosphoroclastic system, and a diaphorase.

The most important result of this work has been the demonstration of lactyl CoA dehydrase. This establishes the fact that the pathway proceeds as postulated.

LACTATE METABOLISM

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PART II -- LACTATE METABOLISM BY PEPTOSTREPTOCOCCUS ELSDENII

By

Ransom Leland Baldwin Jr.

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VITA

Ransom Leland Baldwin Jr. was born in Meriden, Connecticut on September 21, 1935. He graduated from Meriden High School in June, 1953 and received the B. S. degree in agriculture from the University of Connecticut in June, 1957. He accepted a graduate research assistantship in the Dairy department at Michigan State University and received the M. S. degree from that department in 1958 with a minor in physiology. At that time he arranged to do further graduate work jointly between the Departments of Dairy and Biochemistry with a minor in Bacteriology. The requirements for the Ph. D. degree will be completed in the fall of 1962. During the tenure of his Ph. D. work he was supported by a National Science Foundation cooperative fellowship.

Mr. Baldwin is married and has one child. He has accepted an appointment with the Animal Husbandry Department at the University of California at Davis.

TO MARY ELLEN

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INTRODUCTION

The rumen fermentation and the symbiotic relationships between the organisms concerned have been the subject of many investigations in the past decade. Although significant strides have been made, the nutritionist is still unable to solve many of the problems whose solutions were foreseen when the initial studies were undertaken. One of these is the problem of increasing ruminant efficiency. In this particular case, however, sufficient knowledge is available to point the way for further studies. The principle aim of the investigations reported in this thesis was to contribute knowledge about several reactions which occur in the rumen and are related to efficient carbohydrate utilization by the ruminant. In order to carry out these investigations it was necessary to utilize methods and knowledge contributed by biochemists, bacteriologists and nutritionists.

The problem outlined for investigation was divided into two parts. The first part involved an investigation of the relative contributions of the two alternate pathways (via intermediates behaving in a symmetrical and nonsymmetrical fashion with respect to enzyme action) for the conversion of lactate to propionate in bovine rumen fluid. The second part is concerned with the identification, isolation and characterization of the enzymes responsible for the conversion of lactate to propionate via the acrylate, or the so-called non-randomizing pathway.

LITERATURE REVIEW

ORGANIC ACID FORMATION IN THE RUMEN

Carbohydrate utilization by the ruminant has been studied extensively. Several reviews are available which summarize the general nutritional characteristics of the ruminant, the contributions of the rumen fermentation to the host animal, and the characteristics of the rumen microflora (1-10). The purpose of this review is to report some of the developments which are directly related to the data presented in this thesis.

Effect of Diet Upon Volatile Fatty Acid Production

The effect of diet upon the relative proportions of acetate and propionate formed in the rumen has received considerable attention because of the effect these proportions have upon the efficiency of the ruminant (11, 12) and upon the composition of milk (13). Results tabulated by Annison and Lewis (7) show that the molar proportion of acetate to propionate varies from 1 to 4. These data also reveal that there is a high correlation between carbohydrate availability of the ration and the amount of propionate formed. Diets consisting of high proportions of complex (or less available) carbohydrates yield very little propionate while diets containing large amounts of readily available carbohydrates yield higher levels of propionate. For example, Gray and co-workers (14) observed acetate:propionate ratios as high as 4 in the rumen contents of cows fed wheaten hay, while Phillipson (15) reported a ratio of 1 on a diet of flaked maize. Balch and Rowland (16) conducted an intensive study of the diurnal variations in volatile fatty acid (VFA) production, lactate concentration, and pH in the rumens of cows receiving a variety of diets. They found that as the carbohydrate availability of

the diet increased the pH decreased and the concentrations of the organic acids, especially propionate and lactate, increased. They concluded on the basis of these data that the highly acidic conditions existing in the rumen of cows fed high starch diets encourage the proliferation of organisms which produce lower amounts of acetic acid. Recent advances in the development of bacteriological techniques suitable for culturing a large number of the rumen microorganisms should enable investigators to correlate changes in the microbial population with changes in diet (9, 10, 17, 18).

Importance of Lactate

The importance of lactate as a precursor of the volatile fatty acids is not clear. The levels of lactate present in rumen fluid, although usually less than 1.0 mg per cent, are highly correlated with the starch content of the diet and the amounts of propionate present (19, 20). Lactate concentrations as high as 0.270 per cent have been noted in cows fed diets high in flaked maize (16), but only for short intervals immediately after feeding. It has long been known that lactate is an intermediate in carbohydrate dissimilation in the rumen and is quickly degraded to acetate, propionate, butyrate, H_2 and CO_2 (21). Several groups have reported, on the basis of quantitative estimates of the volatile acids formed during lactate fermentation, that lactate is converted mainly to propionate (15, 22, 23). Heuter et al. (24) fed sodium lactate and calcium lactate to cows consuming normal rations and found that lactate stimulated the formation of propionate and butyrate. They also reported that considerable amounts of lactate are absorbed through the rumen wall. Jayasuriya and Hungate (25) studied the fermentation of lactate-2-C^{14} by rumen inocula removed from cows fed several diets. They observed that only small amounts of propionate -C^{14} were formed (less than 20% of

VFA-C^{14}) while large amounts of acetate-C^{14} were formed (70 to 90% of the VFA-C^{14}). No apparent correlation existed between carbohydrate availability in the diet and the fate of lactate. Bruno and Moore (26) reported similar results. It would seem that there is a discrepancy between conclusions based upon quantitative data and those based upon isotopic data. An explanation for these discrepancies will not be forthcoming until more knowledge of the kinetics of carbohydrate fermentation and of the role of lactate and other three-carbon intermediates is available.

PROPIONIC ACID METABOLISM

Studies carried out upon animal, plant and bacterial systems indicate that there are at least three distinctly different pathways for the formation and utilization of propionate. Many review articles concerned with various aspects of the problem are available and they have been cited along with the original articles as sources of more detailed information. The three pathways, the dicarboxylic acid pathway, the direct reductive pathway, and the oxidative pathway(s) are treated separately, below.

The Dicarboxylic Acid Pathway

Studies on the mechanism of propionate formation in bacteria and propionate utilization in animals have revealed the existence of a common pathway (27). The various intermediates of the pathway are common to both organisms even though the system described in animals is concerned with the oxidation of propionate whereas the system described in bacteria involves the anaerobic production of propionate. Although the steps involved are the same for both organisms, the characteristics of several of the enzymes are different. The bacterial pathway (Figure 1) involves a condensation of pyruvate and CO_2 to form oxalacetate (reactions I-IV).

DICARBOXYLIC ACID PATHWAY TO PROPIONATE

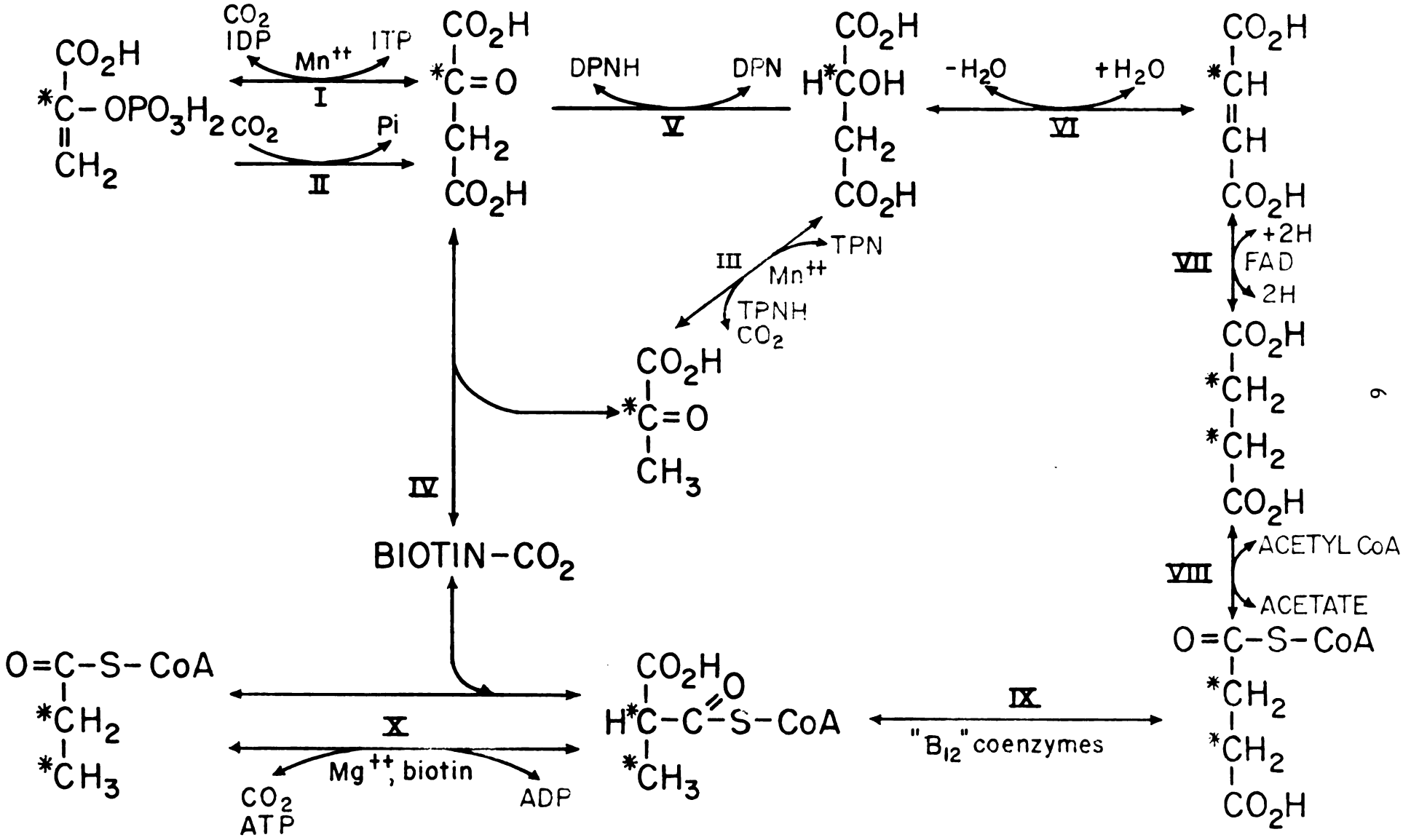


FIGURE 1

The oxalacetate thus formed is then reduced to succinate via malate and fumarate (V-VII). By means of a thiolester exchange reaction (VIII), the coenzyme A ester of succinate is formed. The succinyl CoA is then isomerized (IX) to methylmalonyl CoA, which is then decarboxylated (IV, X) to yield CO_2 (presumably biotin-CO_2) and propionyl CoA. Propionyl CoA can in turn participate in the thiolester exchange reaction (VIII) (27, 47).

Early evidence for the existence of the dicarboxylic acid pathway based upon the carbon balance studies of Van Niel (28) and Wood and Werkman (29) and isotope data (30-32), was reviewed by Werkman and Wood (33). On the basis of the evidence available at that time it was proposed that propionate is formed via succinate.

Isotopic: Isotope experiments employing substrates uniquely labeled with C^{14}, $C^{14}O_2$, and $C^{13}O_2$ to study propionate formation by several propionibacteria and Micrococcus lactyliticus (Veillonella gazogenes or Veillonella alcalescens) have all yielded results consistent with the operation of the above pathway (34-39). The general isotopic distribution patterns of the system are: (a) complete randomization of label originating in C-2 or C-3 of the substrate between C-2 and C-3 of propionate (38); (b) exchange between the carboxyl carbons of succinate and propionate and CO_2 (35, 39); and (c) partial equilibration between CO_2 and C-1 of the substrate. The lack of complete equilibration between the carboxyl group of propionate and $C^{14}O_2$ was formerly considered inconsistent with the pathway (36) but has now been explained (see discussion of succinate decarboxylation below). However, several characteristics are still not understood, for example, the excessive randomization of label into the carboxyl group of propionate from lactate-2-C^{14} and lactate-3-C^{14}, and glucose-1-C^{14} and glucose-6-C^{14}. With respect to the several inconsistencies that have arisen, it has been suggested that the participation

of alternate routes for glucose utilization in addition to the dicarboxylic acid pathway have not been excluded in several organisms (27).

Enzymatic: Enzymatic data obtained through studies employing whole cell, crude, and partially purified preparations of various propionibacteria, Micrococcus lactyliticus and animal tissues using fermentation and direct assay techniques, clearly indicate that the dicarboxylic acid pathway operates in these organisms (40-47). The exact mechanisms by which several of the steps indicated in Figure 1 occur have only recently been elucidated and in some cases are still not entirely known. Therefore, it is of interest to review data pertaining to some of these systems in detail.

Malic dehydrogenase and fumarase (V, VI) are widely distributed and have been demonstrated in many organisms (48). It is interesting to note the demonstration of a lactic-malic dehydrogenase in extracts of M. lactyliticus (49). The reaction catalyzed by this enzyme is freely reversible and no cofactors are required.

The electrons required to carry out the reductive reactions of the pathway are derived from a combination of glyceraldehyde-3-phosphate dehydrogenase, glycerophosphate dehydrogenase, lactic dehydrogenase and the pyruvate oxidase system, depending upon the substrate employed. Glycerophosphate dehydrogenase, glyceraldehyle-3-phosphate dehydrogenase and lactic dehydrogenase (DPN linked) are well-known and have been studied in great detail (44). Participation of the flavin-linked lactic dehydrogenase, which has been reported in yeast (50, 51), and the lactate oxidase and pyruvate dismutation systems of lactobaccillae and mycobacteria has not been demonstrated in the metabolism of propionate-forming bacteria (52). Several pyruvate oxidase systems have been observed in bacteria. These are generally divided into four groups: the lipoic acid-linked; the flavoprotein-linked; the "clastic" cleavage; and the non-coupled type (53). The various systems have been studied

in great detail even though the mechanism of the "clastic" cleavage is still not entirely clear. Several excellent reviews are available on this subject (53-55). A recent report (56) contains a very good discussion of the present status of knowledge of the "clastic" system. In spite of these studies the mode of electron transfer from the donor to the acceptor are not entirely clear.

Succinic dehydrogenase (VII) is of considerable interest since its characteristics vary widely from organism to organism (57). The dehydrogenases isolated from beef heart mitochondria (58) and from yeast (59) are very similar. The molecular weights of the two aerobic enzymes are approximately 200,000. Four atoms of non-heme iron (probably ferric) are loosely bound and one molecule of FAD is covalently bound. These dehydrogenases are freely reversible under the proper conditions, are very sensitive to malonate and carry out the oxidative reaction at a much faster rate than the reduction reaction.

A second type of succinic dehydrogenase first observed by Peck et al. (60) in extracts of M. lactyliticus and later purified and studied by Warringa et al. (61) and Warringa and Guiditta (62) possesses far different characteristics. Its molecular weight is very high, probably several million; it contains several moles of readily dissociable FAD and 40 atoms of non-heme iron per flavin. This anaerobic dehydrogenase has a very low affinity for succinate and is not inhibited by malonate. It is readily reversible but displays the higher velocity in the direction of reduction.

A succinic dehydrogenase of an intermediate type has been isolated from P. pentosaceum (57). This enzyme carries out the oxidative and reductive reactions with equal facility and shows an intermediate inhibition constant, K_i, for malonate. Singer et al. (63) suggested that the electrode potential characteristics of the M. lactyliticus enzyme indicate that a high energy phosphate bond could be formed if hydrogen or reduced pyridine nucleotide served as the reductant.

The Pyruvate-Carbon Dioxide Condensation Reaction which provides entry into the dicarboxylic acid pathway has not been entirely elucidated. There are at present four enzymes known which could perform this function (Figure 1): phosphoenolpyruvate carboxykinase (I), phosphoenolpyruvate carboxylase (II), the malic enzyme (III), and pyruvate transcarboxylase (IV) (39, 44, 64). The distribution and possibilities for the participation of the first three enzymes in propionate forming systems were discussed by Wood (47). The demonstration of the biotin-containing transcarboxylase (65, 66) has served to clarify the problem of oxalacetate formation from pyruvate in the propionibacteria, but very little is known about the distribution of this enzyme.

Propionate Utilization in Animals involves an initial condensation between CO_2 and propionyl CoA (propionyl carboxylase, X) forming methylmalonyl CoA which is subsequently isomerized to succinyl CoA (IX). This provides for propionate oxidation via the TCA cycle (67-71). Propionyl carboxylase has been purified (72) and crystallized (73). It contains 4 moles of biotin per mole of active enzyme. Evidence for "in vivo" and "in vitro" activation of propionyl carboxylase through the administration of large doses of biotin and biotin, Mg^{++} and ATP respectively, has been reported (74).

It has been known for some time that succinate decarboxylation in propionibacteria and M. lactyliticus proceeded in several steps and that succinyl CoA, propionyl CoA and an active "C_1" complex were intermediates (45, 46, 75, 76), but the demonstration of the key intermediate methylmalonyl CoA, has only recently been reported (64, 77). Methylmalonyl CoA isomerase, an enzyme common to both the animal and bacterial systems, requires the presence of dimethylbenzimidazole cobamide coenzyme (DMBC) (77-79). It has been purified (80) and its mechanism of action studied. It is known that the reaction proceeds via an intramolecular migration of the esterified carboxyl group (81, 82) and

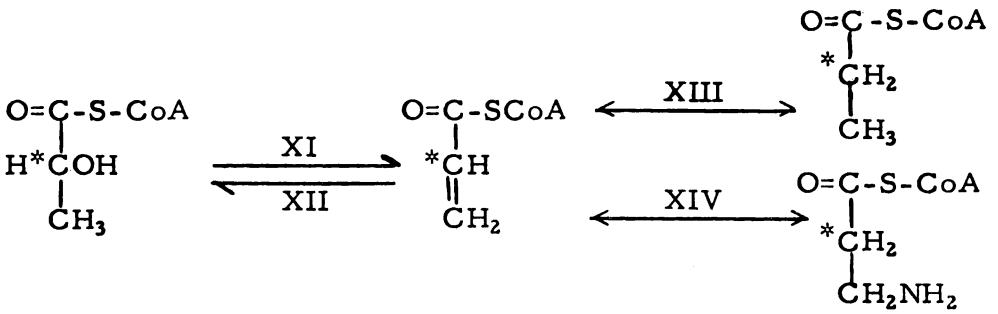
that the B_{12} coenzyme participates as an electron acceptor-donor (83). The possibility of a double bonded intermediate (acrylyl CoA) has been ruled out (82).

The Direct Reductive Pathway

Several lines of evidence obtained with different genera of bacteria are inconsistent with the dicarboxylic acid pathway to propionate. Evidence for the existence of an alternate pathway has accumulated from experiments with two organisms: Clostridium propionicum (38, 84) and a rumen isolate originally designated micrococcus strain LC (85) (now classified as Peptostreptococcus elsdenii), (86). In view of the observation that the two organisms (a) do not ferment malate, fumarate or succinate (84, 87, 88), (b) ferment lactate, pyruvate, acrylate and several amino acids (84, 88-90), (c) convert position-labeled lactate to propionate without randomizing the label (38, 91), and (d) do not fix $C^{14}O_2$ into the carboxyl carbon of propionate, it was postulated that propionate formation proceeds via a dehydration of lactate and a reduction of the resulting acrylic acid (87, 90). Later when it was known that butyrate formation occurred via the acyl-CoA esters of the respective intermediates (27, 85, 92), it was logical to assume that the intermediates in propionate formation via the direct route involved the CoA esters of lactate, acrylate and propionate (Figure 2). Results of experiments carried out with cell-free extracts of C. propionicum and P. elsdenii thus far provide only partial support for the postulated mechanism (93, 94).

Ladd and Walker (93) studied lactate, pyruvate and acrylate metabolism in the LC coccus very intensively. By taking advantage of the fact that there is a very stable hydrogenase associated with the phosphoroclastic reaction in this organism (95) they were able to measure pyruvate formation manometrically. In fresh preparations, lactate and

Figure 2. The Direct Reductive Pathway



acrylate were rapidly metabolized, but extracts subjected to dialysis, even under anaerobic conditions, soon lost activity. After dialysis for five hours it was possible to reactivate the extracts by adding ATP, ADP, pyruvate or acetyl phosphate thus demonstrating that energy is required. Prolonged dialysis resulted in loss of the ability of the extracts to ferment lactate or acrylate alone under any conditions, however, when equimolar proportions of the acids were added together both were fermented slowly. Employing hydroxylamine as a trapping agent and lactate-C^{14} as the substrate, these workers found radioactive spots on two-dimensional chromatograms corresponding in mobility to the hydroxamates of lactate, acrylate and propionate. The metabolism of threonine by this organism occurs via routes consistent with the mechanism postulated for lactate metabolism (96).

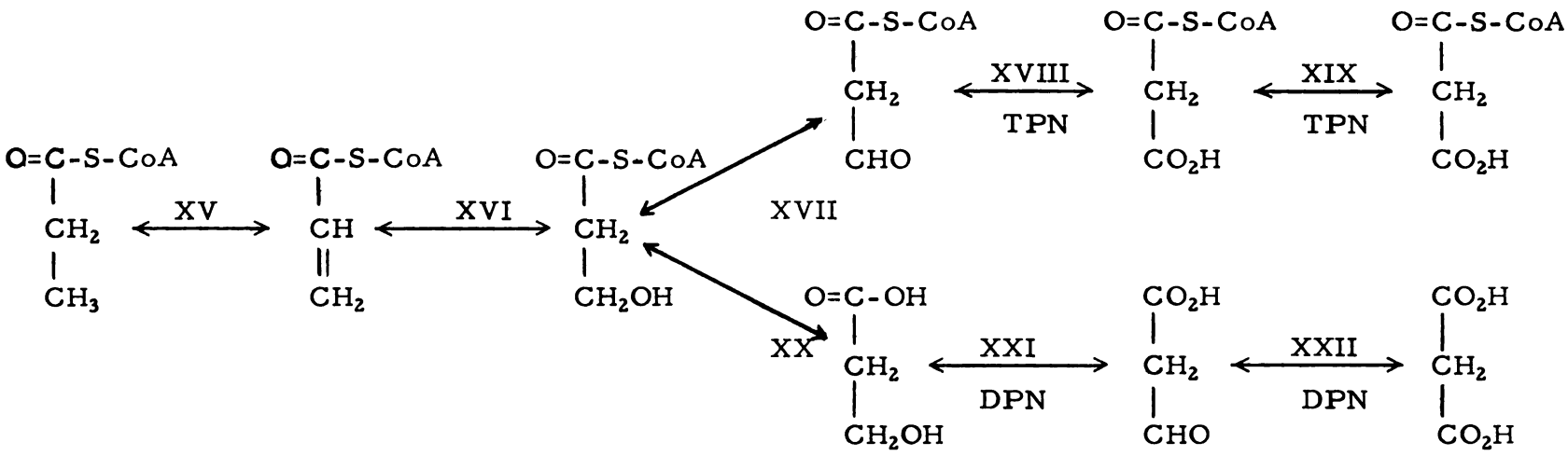
Soon after Stadtman (97) proposed the pathway propionyl CoA dehydrogenase(XIII)and acrylyl CoA aminase (XIV) were identified in extracts of C. propionicum (27, 98). Propionyl CoA dehydrogenase has not been purified nor its properties studied. Acrylyl CoA aminase is present in large amounts in extracts of cells grown on β-alanine and has been purified and fully characterized (99). Goldfine and Stadtman (94) studied β-alanine utilization in extracts of C. propionicum and found a

situation similar to that described by Ladd and Walker (93) in extracts subjected to prolonged dialysis, i.e., lactate and/or an α-keto acid was required in order to cause the conversion of acrylate to propionate. It would seem that the link between acrylate and lactate was lacking in their preparations. Vagelos et al. (100) have succeeded in demonstrating the presence of an acrylyl CoA hydrase (VII) in extracts of pigeon breast muscle and in a pseudomonad isolated from propionate enrichment culture, but have been unable to demonstrate either the lactyl CoA dehydrase reaction (XI) or any enzyme capable of utilizing the lactyl CoA formed.

Oxidative Pathways

Studies carried out on animal tissues, peanut cotyledons and Clostridium kluyveri indicate the existence of alternate oxidative pathways for the utilization of propionate (27). Two general mechanisms have been proposed (Figure 3). The first two reactions (XV, XVI) involving a dehydrogenation of propionyl CoA and hydration of the acrylyl CoA formed to yield β-hydroxypropionyl CoA are common to all three organisms. The β-hydroxypropionyl CoA formed in animal (101) and plant tissue (102) is deacylated before further oxidation occurs (XX-XXII), while an alternate route is present in C. kluyveri (103). This route involves a direct oxidation of β-hydroxypropionyl CoA (XVII) to malonyl CoA. The significance of these pathways is unknown. Of some relevance are reports of a malonyl semialdehyde transaminase in kidney tissue (104) and a malonyl semialdehyde dehydrogenase in extracts of Pseudomonas aeruginosa (105).

Figure 3. Oxidative Pathways



Associated Enzyme Systems: The three pathways discussed above all have one feature in common, they all require the participation of thiol esters. Bacteria carry out the required activation in three independent steps:

$$\text{acetate} + \text{ATP} \underset{\text{acetokinase}}{\overset{Mg^{++}}{\rightleftharpoons}} \text{acetyl phosphate} + \text{ADP} \qquad \text{(XXIII)}$$

$$\text{acetyl phosphate} + \text{CoA} \underset{\text{phosphotransacetylase}}{\rightleftharpoons} \text{acetyl CoA} \qquad \text{(XXIV)}$$

$$\text{acetyl CoA} + \text{X} \underset{\text{CoA transphorase}}{\rightleftharpoons} \text{X-CoA} + \text{acetate} \qquad \text{(XXV)}$$

Acetokinase (106), phosphotransacetylase (107) and CoA transphorase (108) have been purified and their properties investigated. Two enzymes, aceto-CoA-kinase and CoA transphorase, carry out the same function in animals:

$$\text{CoA} + \text{acetate} + \text{ATP} \rightleftharpoons \text{acetyl CoA} + \text{AMP} + PP_i \qquad \text{(XXVI)}$$

$$\text{acetyl CoA} + \text{X} \rightleftharpoons \text{acetate} + \text{X-CoA} \qquad \text{(XXVII)}$$

(109, 110, 111).

PART I

CONVERSION OF LACTATE-C^{14} AND GLUCOSE-C^{14} TO PROPIONATE BY THE RUMEN MICROBIOTA*

The experiments to be described were undertaken to determine the degree of participation of each of the pathways of propionate production in the mixed population of the bovine rumen. This was accomplished by examining the degree of randomization of C^{14} from lactate-C^{14} and glucose-C^{14} into carbon atoms 2 and 3 of propionate. In addition the selective effect upon the distribution of pathways (or distribution of species) exerted by the degree of carbohydrate availability in the diet was determined.

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*A substantial proportion of these results have been presented at the 56th Annual Meeting of the American Dairy Science Association, Madison, Wisconsin, June 11-15, 1961, and published in The Journal of Bacteriology, Vol. 83, pp. 907-913, 1962.

METHODS AND MATERIALS

Bacteriological. Cows fitted with the screw cap, plastic fistula plugs described by Hentschl et al. (112) were used as sources of rumen microflora. The experimental rations were fed for at least two weeks prior to sampling. For incubation with lactate-C^{14} and glucose-C^{14}, samples were strained through 2 layers of cheesecloth and used directly. For the enzyme experiments, after a preliminary centrifugation at 2,000 x g to remove feed particles, the cells were collected by centrifugation at 18,000 x g for 30 minutes, washed twice, and resuspended in 0.02 M potassium phosphate buffer, pH 7.4. Cell-free extracts were prepared by exposing cell suspensions to vibration in a 10 KC sonic oscillator for 35 minutes. The suspensions were centrifuged at 18,000 x g for 10 minutes to remove cellular debris. The yellow-green extracts were stored at -15C until used.

Enzymatic. Succinic dehydrogenase activity was measured in both directions by the manometric and reduced dye methods of Singer and Kearney (113). The reduced dye employed was safranine. Acetokinase and phosphotransacetylase were estimated as described by Rose (114) and Stadtman (115). Coenzyme A transphorase activity was estimated by incubating, in a reaction volume of 1 ml, 10 μmoles of acetyl phosphate, 0.01 μmole coenzyme A, 50 μmoles of potassium lactate, excess phosphotransacetylase (10 units) and cell-free extract. After 5 minutes of incubation at 38 C, neutral hydroxylamine (100 μmoles) was added and the sample allowed to stand at room temperature for an additional 10 minutes. The protein was precipitated with 25 ml. of 95% ethanol. The samples were then taken to dryness on a steam bath, the hydroxamates extracted into ethanol and spotted on paper. The chromatograms were

developed with butanol:H_2O (100:18), the spots located with a ferric chloride spray, eluted with a five per cent solution of ferric chloride in 3 N HCl, and the amount of lactyl hydroxamate determined (116). The assay was linear with enzyme concentration when the amount of lactyl hydroxamate formed during the incubation did not exceed 1 μmole. One unit was defined as the amount of coenzyme A transphorase which would form 1 μmole of lactyl hydroxamate under the conditions of the assay.

Radiochemical. Radioactivity measurements were carried out in the Packard Tricarb Liquid Scintillometer. Two scintillation systems were employed; the first (toluene system) was made up of 0.4% PPO (2,5-diphenyloxazole) and 0.005% POPOP (1,4-bis-2-[5-phenyloxazolyl]benzene) in toluene; the second (gel system) consisted of 5 grams of PPO, 50 mgrams of αNPO (alphanaphthylphenyloxazole), 80 grams of napthalene and 40 grams of cab-o-sil dissolved in 385 ml each of dioxane and xylene and 230 ml ethanol. The distribution of label in the volatile fatty acids and lactic acid was determined by adding 2 ml aliquots of the respective fractions eluted from a celite column (117) to 15 ml. of toluene scintillation fluid. Prior to degradation, acetic and propionic acids recovered from the column were further purified by 11 volume distillation. After addition of carrier, the acids were degraded by the Schmidt-azide reaction according to the method of Phares (118) in the apparatus described by Krichevsky and Wood (119). The following modifications in the degradation procedure were employed: (a) the ethylamine recovered from the degradation of propionate was oxidized to acetate and then purified by celite chromatography, (b) the carbon dioxide released in the degradations was collected in 10 ml of 0.25 M Primene 81-R in methanol (120) or 0.25 M NaOH and counted directly, (c) methylamine HCl derived from the degradation was counted directly in 8 ml ethanol plus 12 ml toluene scintillation fluid or 0.5 ml aliquots were counted in 15 ml of the

gel system. From degradation of acetate-1-C^{14}, acetate-2-C^{14}, propionate-2-C^{14}, and propionate-3-C^{14}, the cross-contamination between carbons were determined to be less than 1%. The specific activities of the single labeled carbon of these acids agreed within ±2% with the measured specific activities of the starting acids. Since the distribution of C^{14} within a given sample was the only point of interest, the yields during purification prior to degradation were not ascertained. Thus, the specific activities reported do not reflect the specific activities of the acids in the incubation mixture, but the specific activities of the respective samples at the start of the degradation.

Chemicals. Lactate-2-C^{14}, lactate-3-C^{14}, acetate-1-C^{14}, acetate-2-C^{14}, propionate-2-C^{14}, and propionate-3-C^{14} were purchased from Volk Radiochemical Company. Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were purchased from New England Nuclear Corp. Pantethine was kindly supplied by Dr. O. D. Bird of the Park Davis Company. Coenzyme A was purchased from Pabst Laboratories. Primene 81-R, kindly donated by Rohm and Haas Company, was vacuum distilled prior to use. Propionyl pantetheine was prepared by the anhydride method (121). Prior to use, the ester was purified by chromatography on Whatman 3 MM paper in butanol-water (100:18). Acyl hydroxamates were synthesized and chromatographed as described by Stadtman and Barker (116). Hydroxamates of acetate, propionate, lactate, and acrylate, used as standards, were prepared from ethyl acetate, ethyl propionate, ethyl lactate and the mixed anhydride of ethyl carbonate and acrylate, respectively.

RESULTS

Stoichiometry of lactate utilization: The diets indicated in Table 1 were selected so that relative to each other microbial activity would produce a graded increase in carbohydrate and/or lactate availability in the rumen. Previous studies (7) indicated that this increased availability would cause a decrease in the molar ratios of acetate to propionate in rumen fluid. In order to determine the amounts of acetate and propionate formed from lactate by the populations selected by these diets, 20 ml of strained rumen fluid were removed from the rumen 3 hours after feeding. The samples were added to 5 ml of 0.05 M phosphate buffer, pH 7.0 containing 1 μcurie of either sodium lactate-2-C^{14} or lactate-3-C^{14} and sufficient unlabeled sodium lactate to bring the final concentration to 2 μmoles per ml (specific activity of 0.02 μcurie/μmole). A sample of 3 ml was removed at zero time and samples of 11 ml each were removed after 45 and 90 minutes incubation at 39 C under a nitrogen atmosphere. The fermentation was stopped by the addition of several drops of 50% H_2SO_4. The percentages of the lactate remaining after 45 minutes of incubation were, respectively, 32, 51, 56, 24 and 18 in flasks A through E. The molar ratios of acetate to propionate in the rumen fluid at zero time are presented in the first column of Table 2. The production of acetate and propionate from lactate-C^{14} as determined by amount of radioactivity in the isolated acetate and propionate appears in column 2. The latter data are expressed as total counts in acetate divided by total counts in propionate. Although the ratios expressing acetate and propionate production in general are considerably higher than the molar ratios, the same trend toward decreasing ratio with increasing dietary carbohydrate availability is evident. High concentrations of lactate decrease the

Table 1. Diets of cows used as sources of rumen fluid.

Cow	Diet
A	Alfalfa pellets (18)*
B	Alfalfa hay (20)
C	Grain (14), alfalfa hay (2)
D	Cooked grain (10), grain (4), alfalfa hay (2)
E	Grass silage (65), lactic acid (1)

*Numbers indicate lb./day.

Table 2. Ratios of acetate to propionate in rumen fluid.

Diet	Acetate/propionate ratio	
	μmole/μmole[a]	dpm/dpm[b]
A	3.1	10.0
B	3.1	10.8
C	2.5	9.1
D	2.5	5.5
E	2.2	2.5

[a]Determined at zero time.

[b]After 45 min. of incubation with labeled lactate. Average of values obtained with lactate-2-C^{14} and lactate-3-C^{14}; dpm = disintegrations per min.

isotope ratios (Table 3). The data presented in Figure 4 show the effect increasing concentrations of lactate have upon the rate of lactate utilization. The maximum rates of lactate disappearance observed for the five samples ranged from 0.05 to 0.09 μmoles/min/ml.

Table 3. Effect of lactate concentration upon the ratios of acetate to propionate formed from lactate-C^{14}.

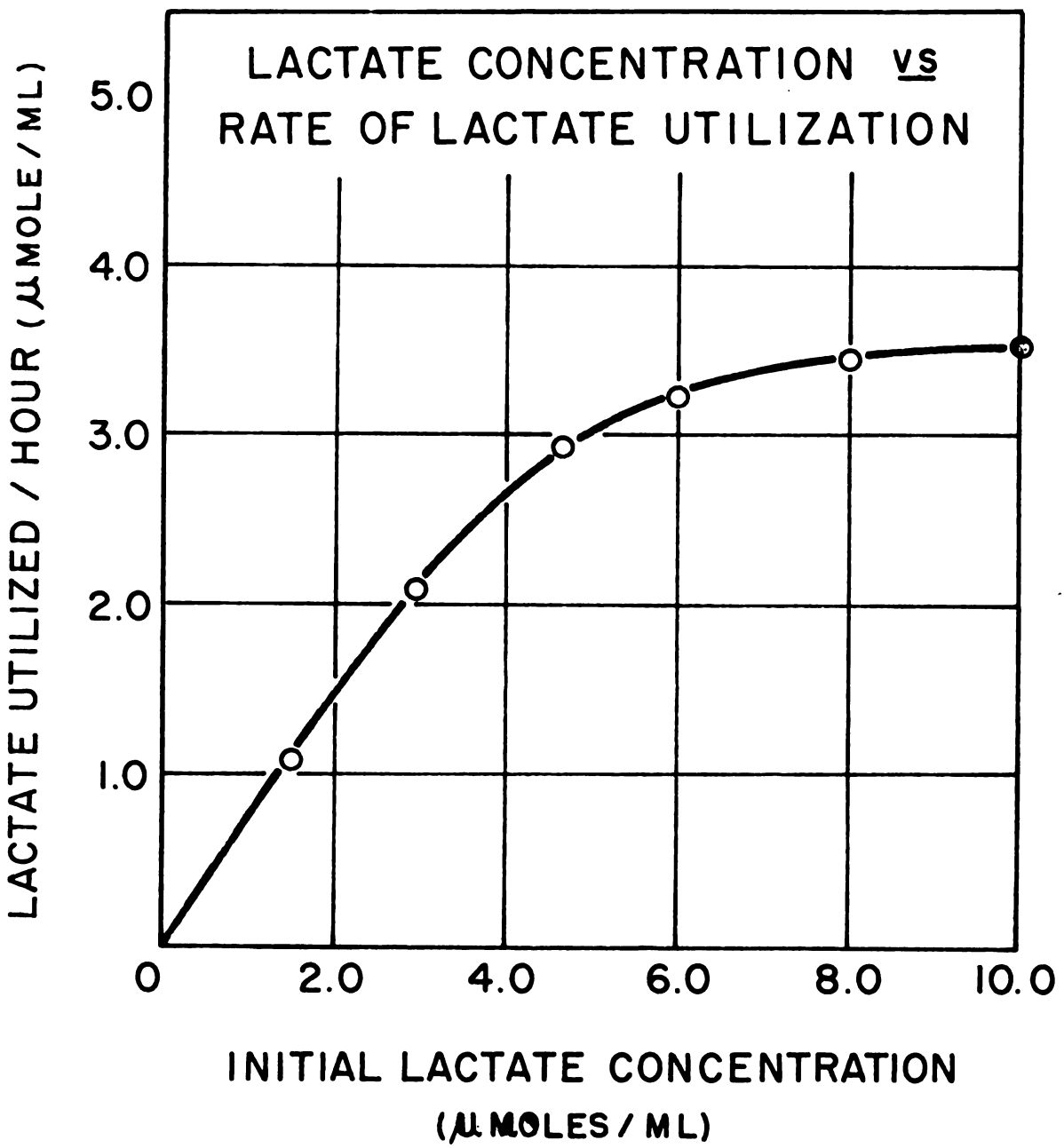
Diet	Lactate Added μmoles	Acetate/Propionate Ratio μmole/μmole	Acetate/Propionate Ratio dpm/dpm
B	0	4.2[a]	17.3[b]
	4	4.2	10.5
C	0	3.3	7.1
	4	3.3	5.5

[a]Determined at zero time.

[b]After 45 min. of incubation with labeled lactate. dpm = disintegrations per min.

Pathways contributing to lactate utilization. Acetate and propionate isolated from the incubation mixtures after 45 and 90 minutes were purified and degraded as outlined in Figure 5. The distribution of radioactivity within these acids is presented in Tables 4, 5, and 6. The data in Table 4 show that the acetate formed from lactate-2-C^{14} or -3-C^{14} was, respectively, carboxyl- and methyl- labeled as should be expected. This indicates that randomization between carbon atoms 2 and 3 of lactate during incubation does not occur to the extent that it does in the experiments with propionibacteria described by Leaver, Wood and Stjernholm (38). It was necessary in these experiments to utilize acetate as a measure of lactate randomization because the levels of

Figure 4. Lactate concentration vs. rate of lactate utilization. The curve presented was obtained from average values determined for each of the five diets A through E. Incubation mixtures containing 10 ml of rumen fluid, five ml of 0.02 M potassium phosphate buffer, pH 7.0, and the indicated amount of potassium lactate were gassed with nitrogen and incubated at 37 C for 45 minutes. The samples were deproteinized with trichloroacetic acid (5%) and the remaining lactate determined.


LACTATE CONCENTRATION VS
RATE OF LACTATE UTILIZATION
LACTATE UTILIZED / HOUR (µMOLE / ML)
5.0
4.0
3.0
2.0
1.0
0
2.0
4.0
6.0
8.0
10.0
INITIAL LACTATE CONCENTRATION
(µMOLES / ML)

SAMPLE PREPARATION AND DEGRADATION

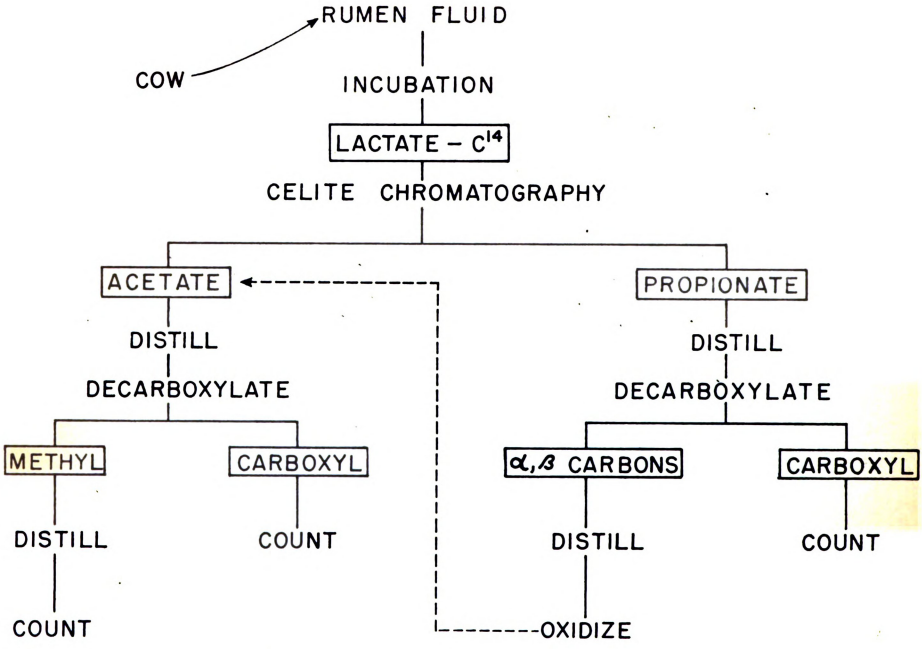


Table 4. Distribution of label in acetate from lactate-2-C^{14} and lactate-3-C^{14}.[a]

Diet	Specific Activity					
	Lactate-2-C^{14}			Lactate-3-C^{14}		
	Total	C_1	C_2	Total	C_1	C_2
A	1,828	1,787	24	1,287	41	1,228
B	--	--	--	1,552	34	1,541
C	1,216	1,283	77	1,299	36	1,183
D	1,244	1,104	20	1,340	17	--
E	--	--	--	1,789	14	1,780

[a]Values reported were obtained on samples isolated after 90 min of incubation and are expressed as dpm/μmole.

lactate remaining after incubation were too low to permit isolation and degradation. Tables 5 and 6 show the radioactivity appearing in the individual carbon atoms of propionate formed from lactate-2-C^{14} and -3-C^{14}, respectively. Calculations of the contribution of the non-randomizing (acrylate) pathway shown in Table 5 were based on the following equation for lactate-2-C^{14}:

$$\% \text{ nonrandomizing pathway} = \frac{\text{sp. act. of carbon 2 minus sp. act. of carbon 3}}{\text{sp. act. carbon 2}} \times 100$$

Similar calculations were employed for lactate-3-C^{14}. No corrections for the small randomization of lactate label were applied. The calculations presented in Table 5 show that the acrylate pathway contribution increases with increasing carbohydrate availability from 73% to 94%. Results obtained with lactate-3-C^{14} (Table 6) are consistent with these observations (70% to 87%).

Table 5. Distribution of label in propionate from lactate-2-C^{14}.

Diet	Specific activity[a]								% Acrylate pathway	
	Total[b]		C_1		C_2		C_3			
	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.
A	33	51	2.1	4.1	23.4	36.0	6.4	9.8	73	73
B	17	38	1.6	3.2	11.3	28.6	2.6	7.2	77	75
C	46	24	3.3	2.8	38.2	18.3	5.7	3.1	85	83
D	59	28	4.7	4.2	47.8	23.1	4.7	3.5	90	85
E	55	132	7.1	9.1	44.2	107	2.9	7.0	93	94

[a] dpm/μmole.

[b] determined after addition of carrier.

Table 6. Distribution of label in propionate from lactate-3-C^{14}.

Diet	Specific activity[a]								% Acrylate Pathway	
	Total[b]		C_1		C_2		C_3			
	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.	45 min.	90 min.
A	63	57	1.8	1.8	11.8	12.6	47.0	42.3	75	70
B	67	291	1.9	7.1	12.2	52.0	54.0	226.0	77	77
C	57	178	2.3	9.2	7.3	29.4	47.0	138.0	85	79
D	34	62	6.1	2.3	2.9	10.0	21.6	50.4	87	80
E	63	234	8.2	4.7	---	27.1	---	195.0	---	86

[a] dpm/μmole

[b] determined after addition of carrier.

Pathways contributing to glucose utilization. The amounts of acetate and propionate formed from glucose by the populations selected by three diets (hay, hay plus grain, and high grain) were determined. Twenty ml samples of strained rumen fluid were added to 5 ml of 0.05M phosphate buffer (pH 7.0) containing 1 μcurie of either glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} and sufficient unlabeled glucose to bring the final concentration to 0.5%. A sample of 3 ml was removed at zero time and the remainder incubated for 45 minutes at 39C under a nitrogen atmosphere. The fermentation was stopped with H_2SO_4. Glucose utilization was virtually complete in all cases. The molar and isotope ratios of acetate to propionate are presented in Table 7. Degradation of the acetate (Table 8) indicated that glucose degradation occurs via the Emden-Meyerhof glycolytic pathway and that very little randomization of label occurred during the incubation.

The results obtained from degradation of the propionate formed from glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} are presented in Table 9. Calculations of the contribution of the nonrandomizing (acrylate) pathway were based on the following equation for glucose-1-C^{14} and glucose-6-C^{14}:

$$\% \text{ nonrandomizing pathway} = \frac{\text{sp. act. of carbon 3 minus sp. act. of carbon 2}}{\text{sp. act. of carbon 3}} \times 100$$

Similar calculations were employed for glucose-2-C^{14}. No corrections for randomization were employed. The calculations show that the "acrylate" pathway contributes up to 40% of the propionate formed.

Enzymes for propionate formation in cell-free extracts of rumen fluid. In support of the isotope experiments, cell-free extracts of bovine rumen microflora were prepared by sonic oscillation and tested for the ability to catalyze certain reactions of the succinate and acrylate pathways to

Table 7. Ratios of acetate to propionate in rumen fluid.

Diet	Acetate/Propionate Ratio	
	μmole/μmole[a]	dpm/dpm[b]
Hay (20)[c]	4.0	3.1
Hay (10), grain (8)	2.6	1.4
Hay (2), grain (14)	3.2	1.8

[a]Determined at zero time.

[b]After 45 min. of incubation with labeled glucose. Average of values obtained with glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14}; dpm = disintegrations per min.

[c]Numbers indicate pounds per day.

Table 8. Distribution of label in acetate from glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14}.[a]

Diet	Glucose-1-C^{14}		Glucose-2-C^{14}		Glucose-6-C^{14}	
	C_1	C_2	C_1	C_2	C_1	C_2
Hay	25	720	800	73	56	625
Hay, grain	25	500	760	11	30	600
Hay, grain	7	1,000	990	17	6	845

[a]Values expressed as dpm/μmole.

Table 9. Distribution of label in propionate from glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14}.

Diet	Substrate	Specific Activity[a]				% Acrylate pathway
		Total[b]	C_1	C_2	C_3	
Hay	G-1-C^{14}	87.0	2.7	42.0	44.5	0.6
Hay + grain		86.0	2.8	33.8	52.0	34.5
High grain		42.0	0.7	14.7	26.3	45.8
Hay	G-2-C^{14}	100.5	17.0	42.5	43.5	1
Hay + grain		115.5	6.8	64.5	41.5	36.0
High grain		138.0	1.7	84.0	50.5	40.5
Hay	G-6-C^{14}	77.5	1.7	33.2	37.2	1
Hay + grain		114.0	1.7	39.5	68.0	41.0
High grain		32.0	0.2	11.2	18.4	39.0

[a] dpm/μmole.

[b] after addition of carrier (1mmole).

propionate. Table 10 gives the specific activities found for succinic dehydrogenase, acetokinase, phosphotransacetylase and coenzyme A transphorase. Succinic dehydrogenase activity compares quite favorably with the activity found in V. alcalescens used as a positive control. The high rate of fumarate reduction observed in the control organism was not observed in the extracts of the rumen microflora. Acetokinase, phosphotransacetylase and coenzyme A transphorase were very active indicating that the activating system for organic acids is present.

To demonstrate further the existence of the acrylate pathway, extracts were incubated with propionyl pantetheine and propionate-2-C^{14}.

Table 10. Specific activities* of several enzymes present in rumen fluid extracts.

Enzyme	Source of Extract			
	Rumen (grain)	Rumen (hay)	Peptostreptococcus	V. alcalescens
Acetokinase	0.72	0.75	2.5	---
Phosphotransacetylase	3.4	3.5	10.6	---
CoA transphorase	1.0	0.81	1.8	---
Succinic dehydrogenase				
succinate → fumarate	0.20	0.20	---	0.19
fumarate → succinate	0.28	0.32	---	6.3

*Units/mg protein under standard assay conditions for the respective enzymes.

After 5 min. of incubation, 1 ml of 2M hydroxylamine was added and allowed to react. The samples were chromatographed on paper and the hydroxamates located by spraying with ferric chloride; radioactivity was located on duplicate chromatograms by counting 1 cm strips immersed in 15 ml of scintillation fluid. Figure 6 shows that 4 radioactive peaks were obtained. The peaks corresponded in mobility to known acetyl, propionyl, lactyl, and acrylyl hydroxamates. The spots located with the ferric chloride corresponded to the radioactive peaks.

A demonstration of the reverse reaction was accomplished by incubating the cell-free extracts with lactate-2-C^{14}, ATP, and acetate. The hydroxamates were prepared and chromatographed. A distinct radioactive peak corresponding to acrylyl hydroxamate was observed and the $FeCl_3$ spray located spots corresponding to acrylyl, acetyl, lactyl, and propionyl hydroxamates. The radioactivity in the areas of the latter three acids could not be resolved into discrete peaks presumably because

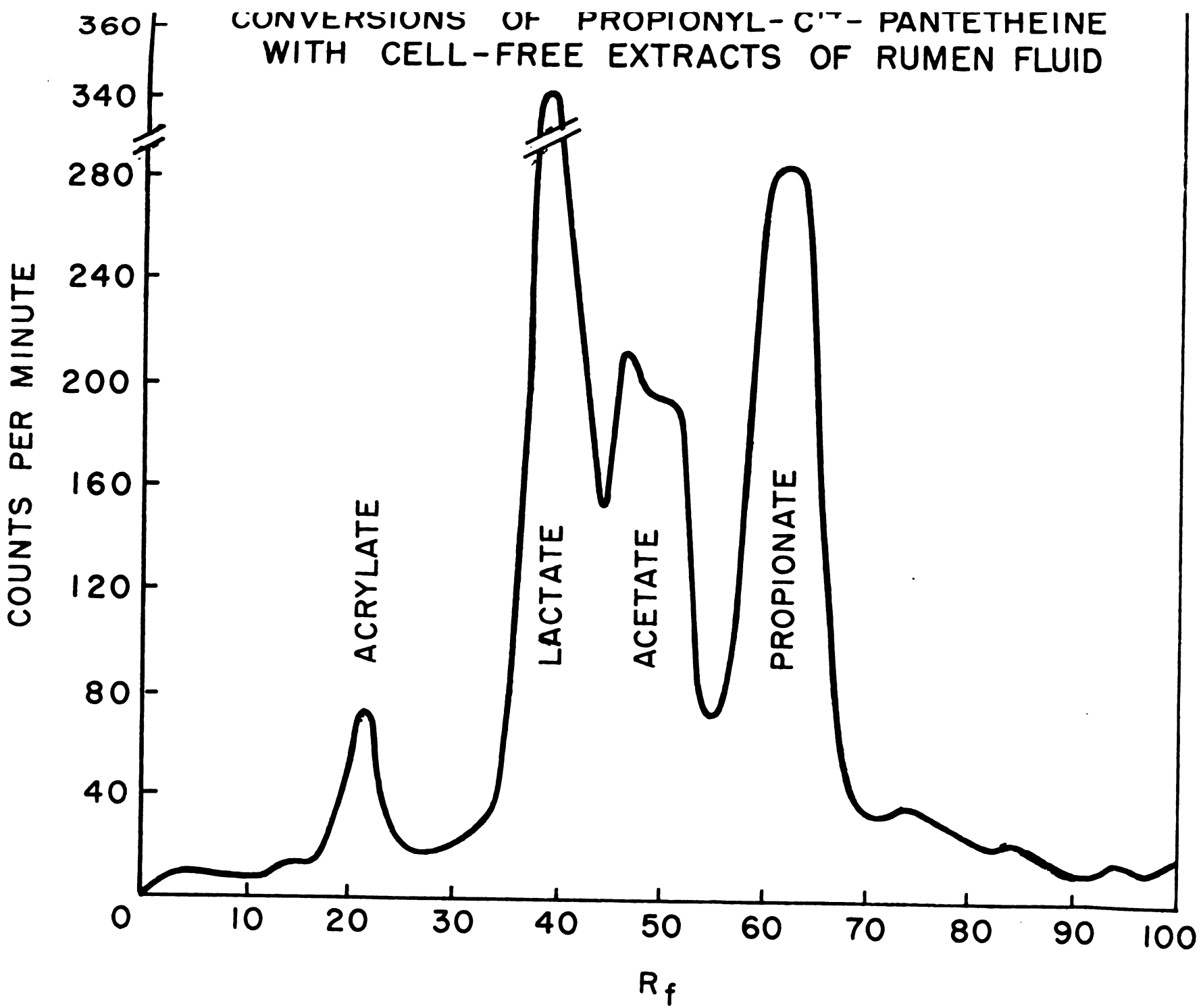

CONVERSIONS OF PROPIONYL-C¹⁴- PANTETHEINE
WITH CELL-FREE EXTRACTS OF RUMEN FLUID
COUNTS PER MINUTE
360
340
280
240
200
160
120
80
40
0
ACRYLATE
LACTATE
ACETATE
PROPIONATE
10
20
30
40
50
60
70
80
90
100
Rf

FIGURE 6

the activity of the acetate and lactate peaks was too high. However, a distinct radioactive area for propionyl hydroxamate was found.

DISCUSSION

The isotope experiments establish the fact that two mechanisms of propionate formation exist in the natural ecological system of the bovine rumen. It is interesting to note that the lesser known, non-randomizing route, predominates in lactate utilization and performs a significant role in glucose utilization. Opposite situations prevail with lactate and glucose as substrates, in that the dicarboxylic acid route plays a minor role in lactate metabolism and a major role in glucose metabolism.

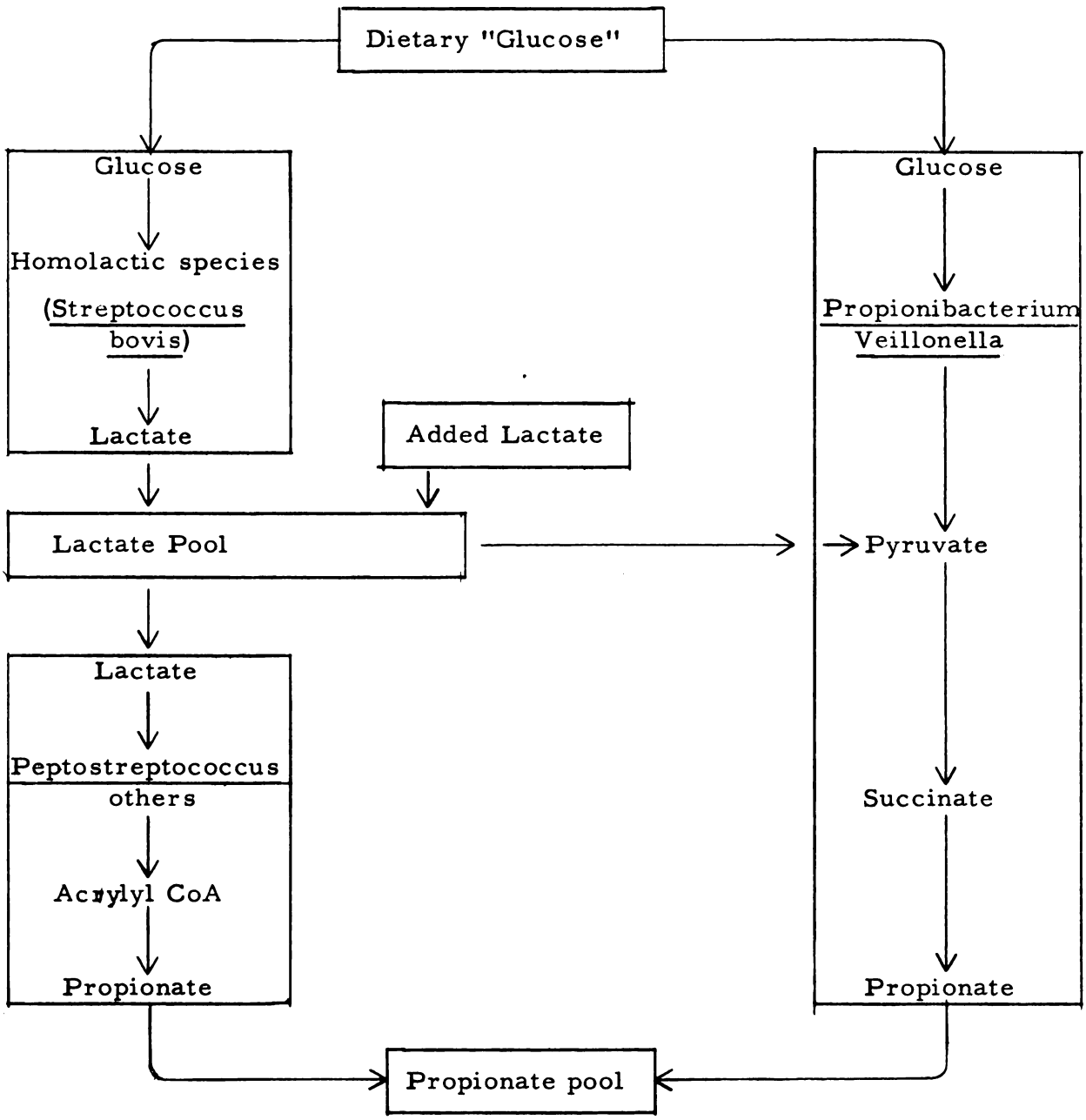
The feeding regimes were selected to present varied amounts of available carbohydrate on the assumption that higher carbohydrate availability promotes the growth of organisms which produce higher levels of propionate (7). The molar and isotope ratios of acetate to propionate reported are consistent with the conclusion that as the carbohydrate or lactate availability of the diet increases, a higher proportion of propionate is produced. Apparently under conditions of increasing substrate availability organisms possessing the nonrandomizing pathway are favored because the contribution of this pathway increases with the grain content of the diet. This conclusion is supported by the studies carried out on five diets with lactate-C^{14} and on three diets with glucose-C^{14}.

The contrast between the glucose-C^{14} experiments wherein the molar and isotope ratios of acetate to propionate agree and the lactate-C^{14} experiments where there is a sharp discrepancy between the molar and isotope ratios, suggests that lactate does not equilibrate with the three-carbon intermediates arising from hexose fermentation. Consideration of this observation and the fact that the pathways of propionate formation differ quantitatively with respect to the two substrates

and with diet led to the concept presented in Figure 7. Two routes of glucose fermentation are indicated: the first involves a direct conversion of glucose to propionate and acetate by a single organism such as a propionibacterium; the second requires the participation of two types of organisms--a lactate former (Streptococcus bovis) and a lactate utilizer such as P. elsdenii. If diets high in starch promote the growth of homolactic species such as S. bovis and the increased lactate formed by these organisms in turn selectively increases the population of lactate fermenters--a supposition amply supported by direct evidence (7, 9, 18)--the apparent result would be a relative increase in the participation of the two step sequence. Further, if the lactate utilizers selected convert lactate to propionate via acrylate, and lactate does not equilibrate with other 3-carbon intermediates arising from glucose the expected result would be an increase in the contribution of the acrylate pathway. These effects have been clearly demonstrated and are consistent with the postulate presented in Figure 7. Lack of knowledge of the many types of organisms isolated from lactate enrichment cultures precludes critical examination of the suggestion that the bulk of these organisms convert lactate to propionate via the nonrandomizing pathway. However, under the conditions employed in the experiments with uniquely labeled lactate the acrylate route was predominant.

It should be recognized that the experimental conditions were not strictly parallel to the conditions existing in the rumen: i.e., less CO_2 tension and a higher pH (7.0 experimental vs. 6.2-6.8 for rumen fluid). The degree to which these differences influence the distribution of pathways is not known. The dicarboxylic acid pathway--long considered to require a CO_2 fixation step--is now known to involve a transcarboxylation between methylmalonyl CoA and pyruvate (66). Hence the dependence of this route upon exogenous CO_2, although unknown, is probably not very high. Similarly, the pH differential could favor the

Figure 7. Routes of Conversion to Propionate in Rumen Fluid.



acrylate route if the pH optima of the two systems differed in a way so as to favor the acrylate route at pH 7.0. The effect of this shift in pH is not known.

Ample evidence is available to indicate that the dicarboxylic acid cycle operates in the rumen (7). The nonrandomizing pathway has been partially characterized in C. propionicum and P. elsdenii (27, 93). The isotopic, enzymatic and chromatographic data obtained have consequently been interpreted against this background, without unequivocal proof that the same reaction sequences occur. The incubation of cell-free extracts with (a) propionyl-pantethine and propionate-C^{14}, and (b) lactate-2-C^{14}, ATP and acetate, yielded results consistent with the routes proposed for C. propionicum and P. elsdenii.

Measurement of succinic dehydrogenase, acetokinase, phosphotransacetylase and coenzyme A transphorase activity in cell-free extracts of rumen microflora selected on several diets indicated no discernible trend. Errors due to sampling and cell preparation were relatively large, and attempts to correlate the activity of these enzymes with diet were therefore not feasible.

SUMMARY

Rumen microflora enriched on diets calculated to present increasing carbohydrate or lactate availability were employed to determine the contribution of the randomizing (succinate) and nonrandomizing (acrylate) routes to propionate with lactate-2-C^{14}, lactate-3-C^{14}, glucose-1-C^{14}, glucose-2-C^{14} and glucose-6-C^{14} as substrates. On the lactate substrates the propionate was labeled as though 70-90% was formed via the nonrandomizing route, while on the glucose substrates the contribution of this pathway ranged from 0-40%. The contribution of the nonrandomizing pathway was highest on diets containing high levels of readily available carbohydrate or lactate or both. Evidence for the presence of succinic dehydrogenase, acetokinase, phosphotransacetylase and coenzyme A transphorase was obtained with cell-free extracts. Propionate-C^{14} and lactate-C^{14} were converted by extracts to the activated derivatives of acrylate, lactate, propionate and acetate.

PART II

LACTATE METABOLISM BY PEPTOSTREPTOCOCCUS ELSDENII

The primary aim of the investigations reported in this section was to provide unequivocal proof for existence of the direct reductive or acrylate pathway. This was accomplished by identifying, purifying and partially characterizing the enzymes of the pathway.

METHODS AND MATERIALS

Bacteriological. Peptostreptococcus elsdenii, strain B-159, kindly provided by Dr. M. P. Bryant and employed throughout these studies, was grown on the corn steep liquor (Staley) sodium lactate medium described by Ladd and Walker (93). Cells, grown in deep culture at 38 C, were collected in a Sharples centrifuge and washed twice in 0.02 M phosphate buffer, pH 7.5, prior to disruption. Cell-free extracts were prepared by exposing thick cell suspensions to sonic oscillation (Raytheon 10 KC) for 5 to 10 minutes. The suspensions were centrifuged at 18,000 x g for 15 minutes to remove cellular debris. Extracts were stored at -14 C.

Chemicals. Lactate-1-C^{14}, lactate-2-C^{14} and propionate-3-C^{14} were purchased from Volk Radiochemical Company. Safranine, cytochrome c, 2,6 dichlorophenol indophenol (DCP), methylene blue (MB), 2,3,5 triphenyl tetrazolium·HCl(TTZ), diphosphopyridine dinucleotide (DPN), and 3-p-nitrophenyl-2-p-iodophenyl tetrazolium chloride (INT) were commercial preparations. Pantetheine (Pa), purchased from the California Corporation for Biochemical Research, was reduced with potassium borohydride. Coenzyme A (CoA) was purchased from Pabst Laboratories. Thiol esters of acetate, propionate, butyrate and acrylate were prepared by the anhydride method (121). Pantetheine esters were purified prior to use by extraction with ethyl acetate and chromatography on acid washed Whatman 3 MM paper in water-saturated butanol. Typical spectra for acetyl CoA, propionyl Pa and acrylyl Pa are presented in Figure 8. Lactyl CoA was prepared from the mixed anhydride (94) of lactate and ethyl formate by the thiol ester exchange method

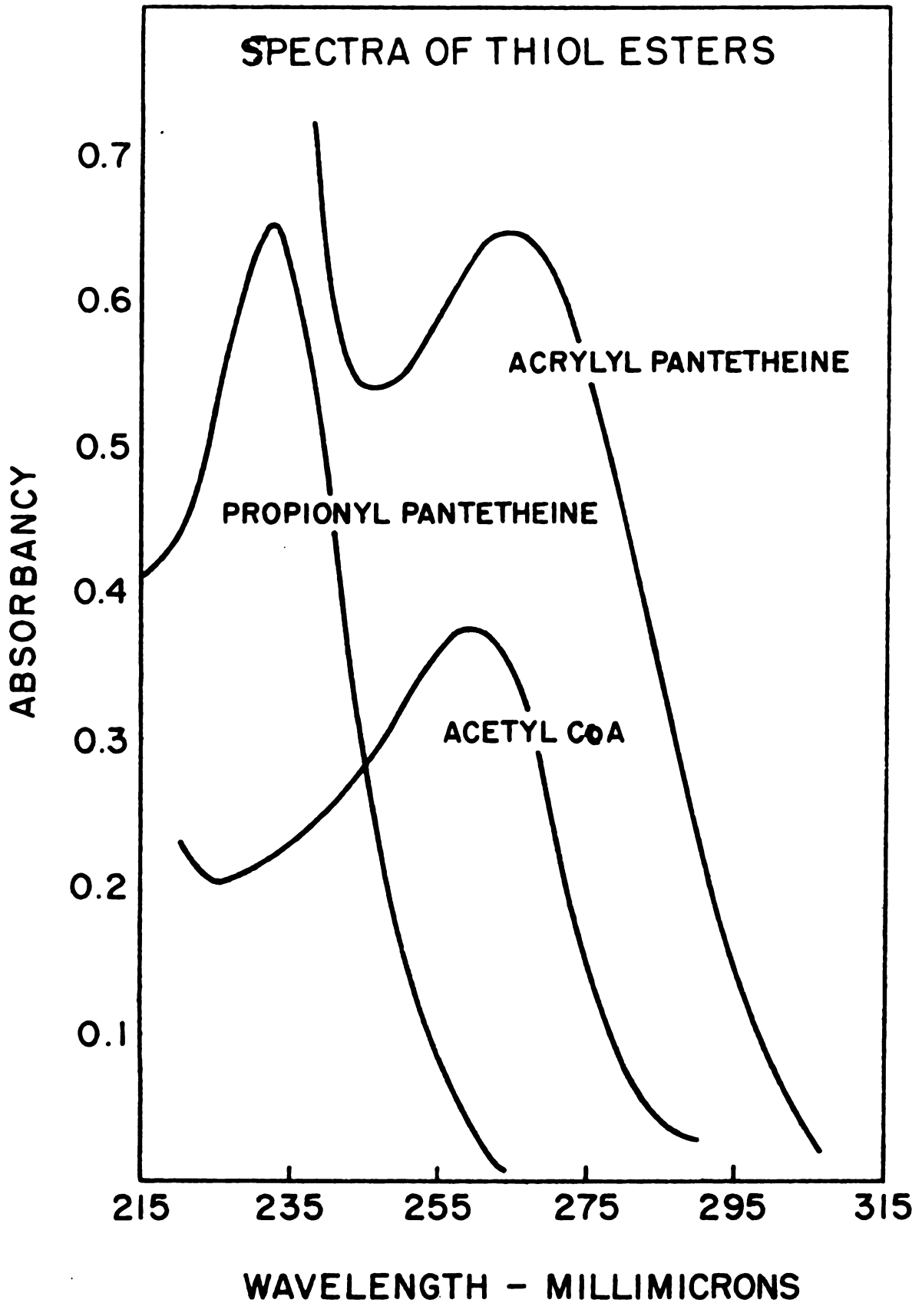

SPECTRA OF THIOL ESTERS
ABSORBANCY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ACRYLYL PANTETHEINE
PROPIONYL PANTETHEINE
ACETYL CoA
215
235
255
275
295
315
WAVELENGTH - MILLIMICRONS

FIGURE 8

described by Wieland (122). Lactyl CoA and acrylyl CoA were prepared enzymatically by incubating acetyl CoA (10 μmoles) with potassium lactate or acrylate (100 μmoles) and purified CoA transphorase (100 units) for 15 minutes at 37 C. The protein was removed by alcohol precipitation and the product purified by chromatography on acid-washed Whatman 3 MM paper in ethanol, sodium acetate (50:50) as described by Stadtman (121). In several cases the esters synthesized by this method were used without prior purification by paper chromatography. All thiol esters were assayed by the hydroxylamine method (121) and tested for purity by hydroxamate chromatography (116). Coenzyme A esters were assayed enzymatically by arsenolysis with purified phosphotransacetylase and CoA transphorase (121). Acetic, propionic, butyric, acrylic and lactic acids were identified by hydroxamate chromatography in two solvent systems according to the method of Seubert (123). Lactic and acrylic acids were separated prior to identification either by column chromatography (117) or by distillation.

Standard techniques were employed for the isolation and identification of pyruvate (124-126): samples were deproteinized with metaphosphoric acid, reacted with dinitrophenylhydrazine for five minutes, and the hydrazone purified for chromatography by extraction serially into xylene, 1 M sodium carbonate, and chloroform: ethanol (80:20), respectively. The purified hydrazones were separated by paper chromatography in methanol:benzene:n-butanol:H_2O (4:2:2:2).

Spectrophotometric measurements were carried out with a Beckman DU monochromator fitted with the cuvette positioning and automatic recording equipment described by Wood and Gilford (127, 128). Protein was determined by the Lowry method (129).

Radioactivity was determined by the procedures outlined in Part I.

Enzymatic. Several enzymes were identified, purified and characterized in the course of this work. Since each enzyme required the application of different techniques, the methods employed will be described separately.

Phosphotransacetylase was identified by the methods described by Stadtman (115). The arsenolysis assay, preferred by earlier workers (115), was employed in some of the studies, but was later discarded because large amounts of enzyme were required, reproducibility was poor and the assay was not linear with enzyme concentration. The preferred assay system involved measurement of the rate of formation of acetyl CoA (XXIV) at 232 mμ.

$$\text{acetyl phosphate} + \text{CoASH} \rightleftharpoons \text{acetyl CoA} + \text{Pi} \qquad \text{(XXIV)}$$

The incubation mixture (0.20 ml) contained 25 μmoles of Tris buffer, pH 8.0, 0.05 μmoles of CoA, 5 μmoles of acetyl phosphate and enzyme. One unit was defined as the amount of enzyme required to form 1.0 μmole of acetyl CoA per minute under standard assay conditions.

Phosphotransacetylase was purified 92-fold (Table 11). The initial heat treatment at 55 C for six minutes served to coagulate residual cellular debris and some protein. The nucleic acids were removed by adjusting the protein content of the extract to approximately 25 mg/ml adding 0.20 volumes of a 2% protamine sulfate solution, and removing the precipitate by centrifugation. Solid ammonium sulfate was added to the protamine sulfate supernatant to 45% saturation (calculated at 0 C, (130)). The precipitate, containing the enzyme, was resuspended in 0.05 M phosphate buffer, pH 7.5. The enzyme was then precipitated with ethanol at -15 C. After the addition of an equal volume of ethanol and centrifugation to remove the precipitate, the enzyme was precipitated by adding a second volume of ethanol. The enzyme was resuspended in phosphate buffer and treated with a saturated solution of

Table 11. Phosphotransacetylase purification.

Step[a]	Total Units	Sp. Act.	Fold
Crude	89,000	19.8	
Heat	66,500	31.1	1.6
Protamine	67,000	34.2	1.7
Ammonium sulfate I	65,000	87.0	5.8
Ethanol	58,500	265	13.4
Ammonium sulfate II	51,500	680	34.3
Heat	50,000	1,820	92.0

[a]See text for details.

ammonium sulfate, pH 8.2. The ammonium sulfate concentration was increased in a stepwise manner until all the activity was recovered. The preparations having the highest specific activities were combined, the protein concentration adjusted to 10 mg per ml, and heated at 58 C for three minutes. The preparations were stored frozen overnight at -14 C, thawed and centrifuged. The purified enzyme was very stable when stored at -14 C. No measurable contaminants were present.

Coenzyme A transphorase was assayed by the method of Stadtman (115) (XXV, XXVIII).

butyryl CoA + acetate $\longrightarrow$ acetyl CoA + butyrate (XXV)

acetyl CoA + arsenate $\longrightarrow$ acetyl arsenate + CoASH (XXVIII)

The only change involved a ten-fold decrease in volume. The 0.25 ml incubation mixture contained 7.5 μmoles of potassium acetate, 50 μmoles potassium arsenate, pH 8.0, 1.0 unit of phosphotransacetylase, and CoA transphorase. The assay is not linear with time, however, if initial

rates are considered, the velocity is linear with enzyme concentration when the rate does not exceed four times the endogenous rate due to phosphotransacetylase. One unit of enzyme converts 1 μmole per minute under standard assay conditions.

Coenzyme A transphorase has been purified 87-fold (Table 12). The initial heat and protamine steps employed were the same as those described for phosphotransacetylase. Ammonium sulfate steps I and II were the same, the protein precipitating between 55 and 70% saturation was collected in both cases. The ethanol precipitation was not very reproducible and was run in a stepwise fashion, only the fractions containing high specific activity were retained. This step was carried out at -15 C. The terminal heat treatment involved holding the preparation at 65 C for four minutes. The purified enzyme was stored in 0.02 M phosphate buffer, pH 7.5, at -14 C. The heat treatment inactivated all the residual phosphotransacetylase.

D-Lactic dehydrogenase was identified by the methods described by Nygaard (51). The standard assay employed during purification of D-lactic dehydrogenase involved measurement of the rate of 2,6-dichlorophenol indophenol (DCP) reduction at 600 mμ (XXIX).

$$\text{DL lactate} + \text{dye} \longrightarrow \text{pyruvate} + \text{reduced dye} \qquad \text{(XXIX)}$$

The assay mixture contained 30 μmoles of phosphate buffer, pH 7.0, 0.002 μmoles of dye, 5.0 μmoles of D-lactate, pH 7.0, and enzyme. The final volume was 0.25 ml. One unit was defined as the amount of enzyme causing an absorbancy change of 1.0 in five minutes at 600 mμ under standard conditions.

Lactic dehydrogenase was purified 25 fold (Table 13). In order to avoid precipitation of the enzyme during protamine treatment, the salt concentration of the extract was adjusted to 0.2 M with solid

Table 12. Coenzyme A transphorase purification.

Step[a]	Total Units	Sp. Act.	Fold
Crude	48,000	10.7	
Heat	48,000	22.4	2.1
Protamine	48,000	24.6	2.3
Ammonium sulfate I	46,500	60.8	5.7
Ethanol			
Ammonium sulfate II	28,800	266	24.8
Heat	25,000	930	87.0

[a]See text for details.

Table 13. Lactic dehydrogenase purification.

Step[a]	Total Units	Sp. Act.	Fold
Crude	1,140,000	490	
Protamine sulfate	1,100,000	510	1.1
Ammonium sulfate I	1,030,000	795	1.6
Calcium phosphate gel	500,000	3,920	8.0
Ammonium sulfate II	324,000	12,000	24.5

[a]See text for details.

ammonium sulfate and then 0.2 volumes of 2% protamine sulfate was added. The first ammonium sulfate step involved collection of the protein precipitated between 60% and 80% of saturation and the second between 55% and 65% of saturation (obtained with solid ammonium sulfate, 130). The calcium phosphate gel absorbtion was run directly on the resuspended ammonium sulfate precipitate. The enzyme preparation was treated with increasing aliquots of the gel suspension until adsorption occurred (usually at a gel/protein ratio of 0.1), and the several batches of gel containing the bulk of the activity were combined. The gel was washed with water and the enzyme was eluted with 0.5 M phosphate buffer, pH 7.5. The purified enzyme was yellow-green in color, relatively unstable during storage and was contaminated with diaphorase and acyl CoA dehydrogenase.

Acyl CoA dehydrogenase was purified 23-fold (Table 14) by a) precipitation with protamine sulfate (0.2 volume of a 4% solution) and resuspension in 0.05 M ammonium sulfate, b) stepwise absorption on alumina C γ gel and elution with 0.5 M phosphate buffer, pH 7.5, and c) precipitation with ammonium sulfate (65 to 75% saturation). The purified enzyme was stable in 0.2 M phosphate buffer, pH 7.0, but very unstable at low salt concentrations.

The standard assay procedure employed was the same as the one employed for lactic dehydrogenase except that butyryl CoA was the substrate (XXX).

$$\text{butyryl CoA} + \text{dye} \longrightarrow \text{crotonyl CoA} + \text{reduced dye} \qquad \text{(XXX)}$$

The same definition for one unit of activity was employed.

Diaphorase activity was very high in crude extracts (Table 15). Since this enzyme was employed in assays for other enzymes, the primary aim during fractionation was removal of contaminating activities, not

Table 14. Acyl coenzyme A dehydrogenase purification.

Step[a]	Total Units	Sp. Act.	Fold
Crude	55,000	33.5	
Protamine sulfate	52,000	99.0	3.0
Alumina C_γ gel	32,600	168.0	5.0
Ammonium sulfate	12,000	775	23

[a]See text for details.

Table 15. Diaphorase purification.

Step[a]	Total Units	Sp. Act.	Fold
Crude	640	0.21	
Protamine	510	0.36	1.7
Ammonium sulfate I	340	0.50	2.4
Ethanol	260	1.10	5.2
Ammonium sulfate II	185	3.10	14.6

[a]See text for details.

purification. With the exception of the protamine step, which was the same as that described for lactic dehydrogenase, all of the methods employed during fractionation were run in the stepwise manner described earlier and only the fractions containing high diaphorase activity and low contaminating activity were retained; hence, the recovery was very poor but the final preparation was entirely free of measurable contaminants. The standard 2,6 dichlorophenol assay procedure described by Mahler et al. (131) was employed. The definition of unit and specific activity reported are the same as those described for lactic dehydrogenase and acyl CoA dehydrogenase.

RESULTS

Demonstration of lactyl CoA dehydrase. The enzyme of particular interest in these studies was lactyl CoA dehydrase because, unlike the other enzymes of the pathway, this dehydrase had not been demonstrated in an organism capable of converting lactate to propionate via a non-randomizing route (94). Vagelos et al. (100) reported an irreversible acrylyl CoA hydrase in extracts of a pseudomonad and in pigeon breast muscle. Presumably, this enzyme could not function in propionate formation.

Early evidence, indicating that lactyl CoA dehydrase was present in crude extracts of the organism, was obtained as described in Part I. Lactyl-C^{14}-CoA or propionyl-C^{14}-CoA was incubated with crude extracts, the thiol esters converted to hydroxamates, separated by chromotography, and counted. The results, presented in Figure 9, curve I, show that lactyl-C^{14}-CoA is converted to the activated derivatives, presumably the CoA esters, of acetate, acrylate, propionate, and β-hydroxypropionate, suggesting that the following reactions occur:

lactyl-C^{14}-CoA $\rightleftharpoons$ acrylyl-C^{14}-CoA (XI)

acrylyl-C^{14}-CoA $\rightleftharpoons$ propionyl-C^{14}-CoA (XIII)

acrylyl-C^{14}-CoA $\rightleftharpoons$ β-hydroxypropionyl CoA (XVI)

lactyl-C^{14}-CoA $\rightleftharpoons$ lactate-C^{14} + CoA (XXXI)

lactate-C^{14} $\rightleftharpoons$ pyruvate-C^{14} (XXIX)

pyruvate-C^{14} + CoASH $\longrightarrow$ acetyl-CoA + CO_2 (XXXII)

Although highly suggestive, these data do not constitute proof for the existence of the dehydrase. The desired proof required purification of the dehydrase and isolation of the products. Toward this end a spectrophotometric assay system based upon the measurement of lactic acid formation was devised as follows:

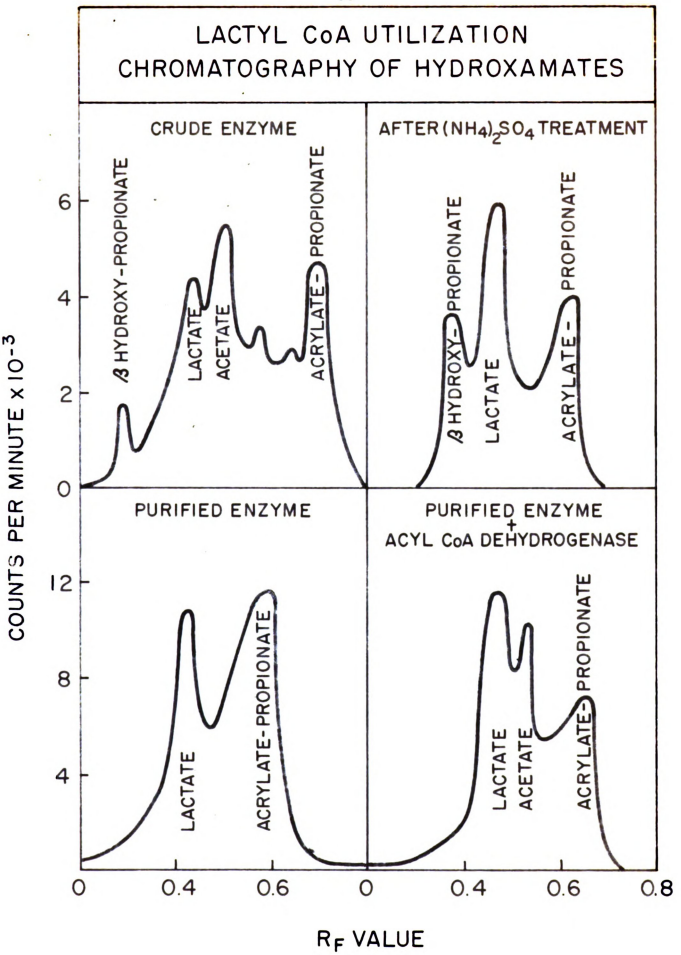
Figure 9. Lactyl CoA utilization: chromatography of hydroxamates.

Curve I -- crude enzyme

Curve II -- after $(NH_4)_2SO_4$ treatment

Curve III -- purified enzyme

Curve IV -- purified enzyme plus acyl CoA dehydrogenase


LACTYL CoA UTILIZATION
CHROMATOGRAPHY OF HYDROXAMATES
CRUDE ENZYME
β HYDROXY-PROPIONATE
LACTATE
ACETATE
ACRYLATE-PROPIONATE
AFTER (NH4)2SO4 TREATMENT
β HYDROXY-PROPIONATE
LACTATE
ACRYLATE-PROPIONATE
PURIFIED ENZYME
LACTATE
ACRYLATE-PROPIONATE
PURIFIED ENZYME + ACYL CoA DEHYDROGENASE
LACTATE
ACETATE
ACRYLATE-PROPIONATE
COUNTS PER MINUTE x 10-3
0
2
4
6
8
12
0
0.4
0.6
0
0.4
0.6
0.8
RF VALUE

acrylyl CoA ⟶ lactyl CoA (XII)

lactyl CoA + acetate ⟶ lactate + acetyl CoA (XXV)

lactate + DPN ⟶ pyruvate + DPNH (XXXIII)

DPNH + dye ⟶ DPN + reduced dye (XXXIV)

The incubation mixture (0.3 ml) contained 15 μmoles of tris-acetate buffer, pH 8.0, 0.0004% INT, 1.0 μmole of DPN, 0.15 μmole of acrylyl CoA, 0.02 ml of muscle lactic dehydrogenase (1 to 10 dilution of Worthington slurry of crystals), 10 units each of diaphorase and CoA transphorase and varying amounts of lactyl CoA dehydrase. The rate of INT reduction was followed at 485 mμ. This procedure could not be employed to assay crude extracts because of lactate contamination, therefore, an alternate assay based on hydroxamate chromatography (as described above) was employed to locate the activity in an ammonium sulfate fraction (60-90% of saturation), and this preparation was used to standardize the spectrophotometric assay. The data presented in Figure 10 show that substrate, DPN, dehydrase and lactic dehydrogenase are required for dye reduction. Neither acrylyl Pa, acetyl CoA nor potassium acrylate substituted for acrylyl CoA, however, acetyl CoA and potassium acrylate in combination did partially replace acrylyl CoA. The flavin-linked D-lactic dehydrogenase isolated from the extracts (Table 13) did not substitute for the muscle L-lactic dehydrogenase. Hence, it appears that lactyl CoA dehydrase is specific for L-lactate. The assay was linear with respect to enzyme concentration only at very low rates (Figure 11) but is sufficiently accurate for purification studies.

It was found that freshly prepared diaphorase, which was free of phosphoroclastic enzymes (Table 15), contained appreciable amounts of lactyl CoA dehydrase. This preparation was employed in experiments designed to accumulate sufficient pyruvate in the assay mixture for isolation, and to demonstrate the interconversion of lactyl CoA and acrylyl CoA.

Figure 10. Lactyl CoA dehydrase: requirements of the assay system. Different components of the assay system as described in the text were omitted as indicated.

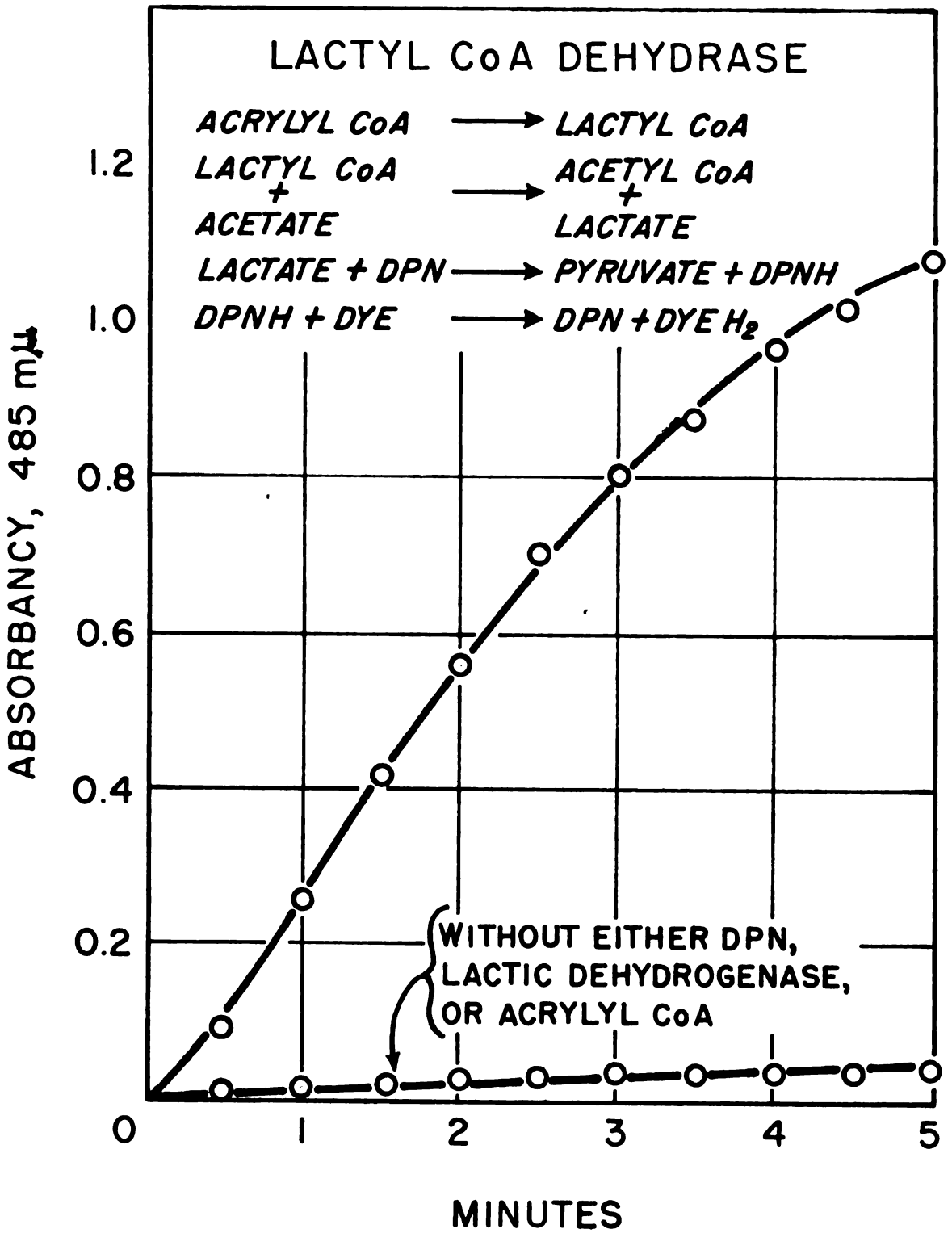
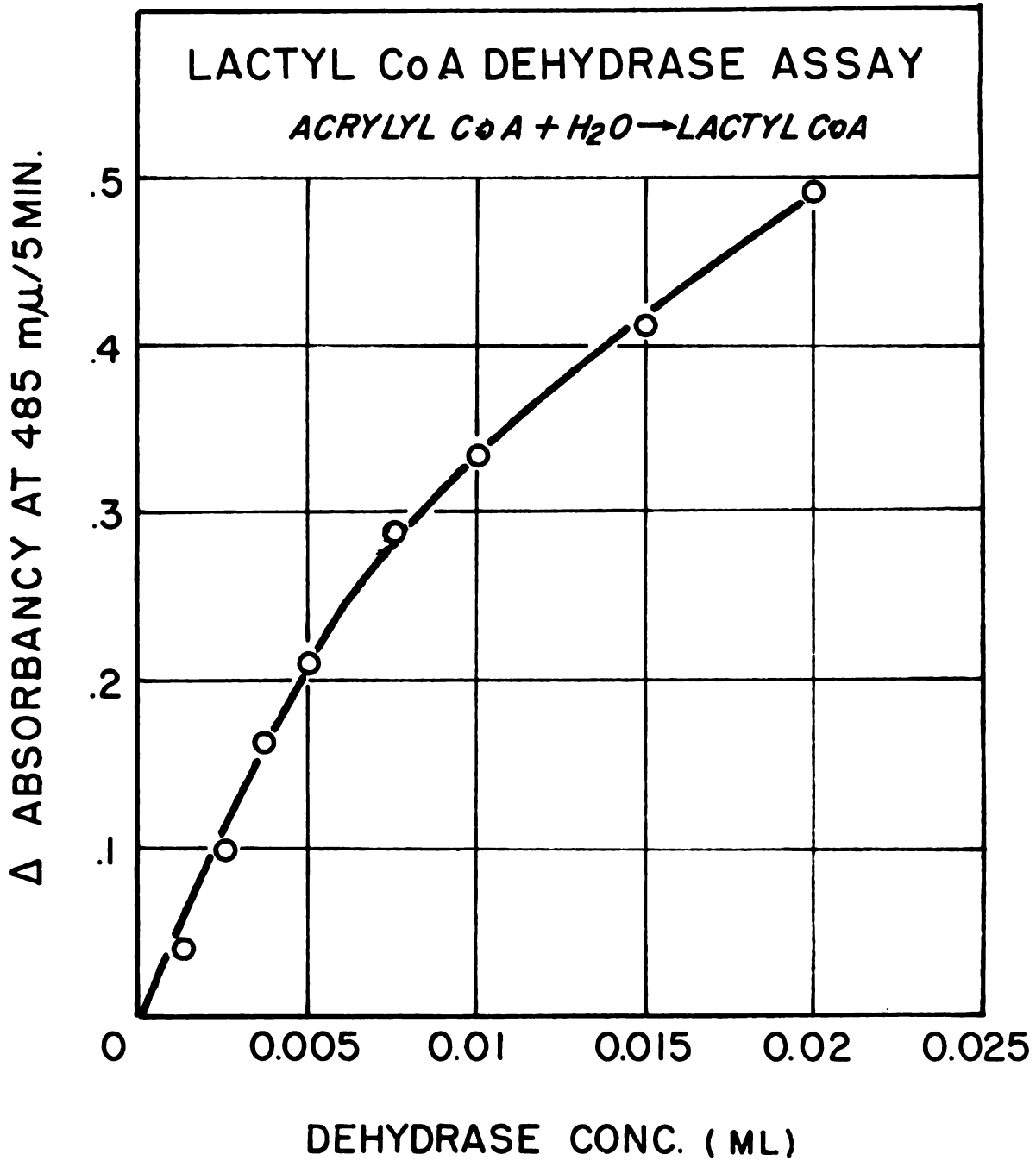

LACTYL CoA DEHYDRASE
ACRYLYL CoA → LACTYL CoA
LACTYL CoA + ACETATE → ACETYL CoA + LACTATE
LACTATE + DPN → PYRUVATE + DPNH
DPNH + DYE → DPN + DYE H2
ABSORBANCY, 485 mμ
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
MINUTES
WITHOUT EITHER DPN, LACTIC DEHYDROGENASE, OR ACRYLYL CoA

Figure 11. Lactyl CoA dehydrase assay: rate vs. enzyme concentration. The standard assay system described in the text was employed.


LACTYL CoA DEHYDRASE ASSAY
ACRYLYL CoA + H2O → LACTYL CoA
Δ ABSORBANCY AT 485 mμ/5 MIN.
.5
.4
.3
.2
.1
0
0.005
0.01
0.015
0.02
0.025
DEHYDRASE CONC. (ML)

Pyruvate was isolated from incubation mixtures as its dinitrophenylhydrazone and identified by paper chromatography. The incubation mixture (2 ml) contained 250 μmoles of Tris·HCl buffer, pH 8.0, 0.5 μmole of DPN, 0.1 ml of muscle lactic dehydrogenase diluted 1 to 10, 0.005% INT, dehydrase and either two μmoles of acrylyl CoA or two μmoles of acetyl CoA plus 50 μmoles of potassium acrylate. The reaction mixtures were incubated at 39°C for 15 minutes. The samples were reacted with 2,4-dinitrophenyhydrazine and the hydrazones were purified and separated on paper chromatograms. Spots corresponding in mobility to dinitrophenylhydrazones prepared from standard pyruvate solutions were observed when either acrylyl CoA or acetyl CoA plus potassium acrylate served as the substrate. No spots were observed when either substrate, enzyme or lactic dehydrogenase was omitted from the reaction mixture. The spots were more intense with acrylyl CoA as substrate. A further check on the identity of the phenylhydrazone spots was accomplished by adding sodium lactate-3-C^{14} (0.01μcuries) to one duplicate containing the complete reaction mixture. The pyruvate-C^{14} dinitrophenylhydrazones formed in the reaction mixture chromatographed identically to the spots observed in a duplicate without lactate-C^{14} added (Figure 12).

The pH optimum of the assay is 8.0. Versene is slightly stimulatory in some cases. The reaction is inhibited by divalent cations, protamine sulfate, both sodium and potassium phosphate and potassium acrylate.

Several experiments were carried out to demonstrate the conversion of lactyl CoA to acrylyl CoA. The first method involved incubation of the dehydrase with lactyl-C^{14}-CoA, reaction with hydroxylamine, paper chromatography and counting the chromatogram. The results presented in Figure 9, curve III, show that acrylyl-C^{14}-CoA was formed. The second method employed acyl CoA dehydrogenase and reduced safranine as a linking system to determine acrylyl CoA formation.

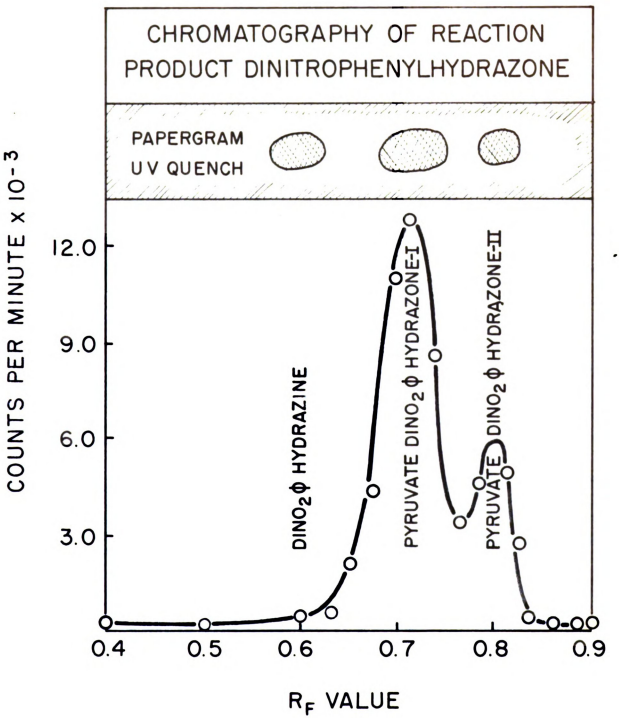

CHROMATOGRAPHY OF REACTION
PRODUCT DINITROPHENYLHYDRAZONE
PAPERGRAM
UV QUENCH
COUNTS PER MINUTE x 10-3
12.0
9.0
6.0
3.0
0.4
0.5
0.6
0.7
0.8
0.9
RF VALUE
DINO2 φ HYDRAZINE
PYRUVATE DINO2 φ HYDRAZONE-I
PYRUVATE DINO2 φ HYDRAZONE-II

FIGURE 12

Attempts to employ the system in a continuous assay for reduced safranine oxidation were unsuccessful.

$$\text{lactyl CoA} \longrightarrow \text{acrylyl CoA} \qquad \text{(XI)}$$

$$\text{acrylyl CoA} + \text{leucosafranine} \longrightarrow \text{propionyl CoA} + \text{safranine} \qquad \text{(XIII)}$$

However, when lactyl-C^{14}-CoA was preincubated with lactyl CoA dehydrase prior to the addition of acyl CoA dehydrogenase, oxidation of the reduced dye occurred (Figure 13). Isolation of the hydroxamates formed in this reaction mixture after the addition of hydroxylamine indicated that acetyl-C^{14}-CoA and either acrylyl-C^{14}-CoA or propionyl-C^{14}-CoA or both were formed (Figure 9, curve IV). Apparently the purified acyl-CoA dehydrogenase preparation also contained the phosphoroclastic enzymes.

Theoretically, when acetyl CoA, lactate-C^{14}, CoA transphorase, and lactyl CoA dehydrase are incubated together the following reactions would occur:

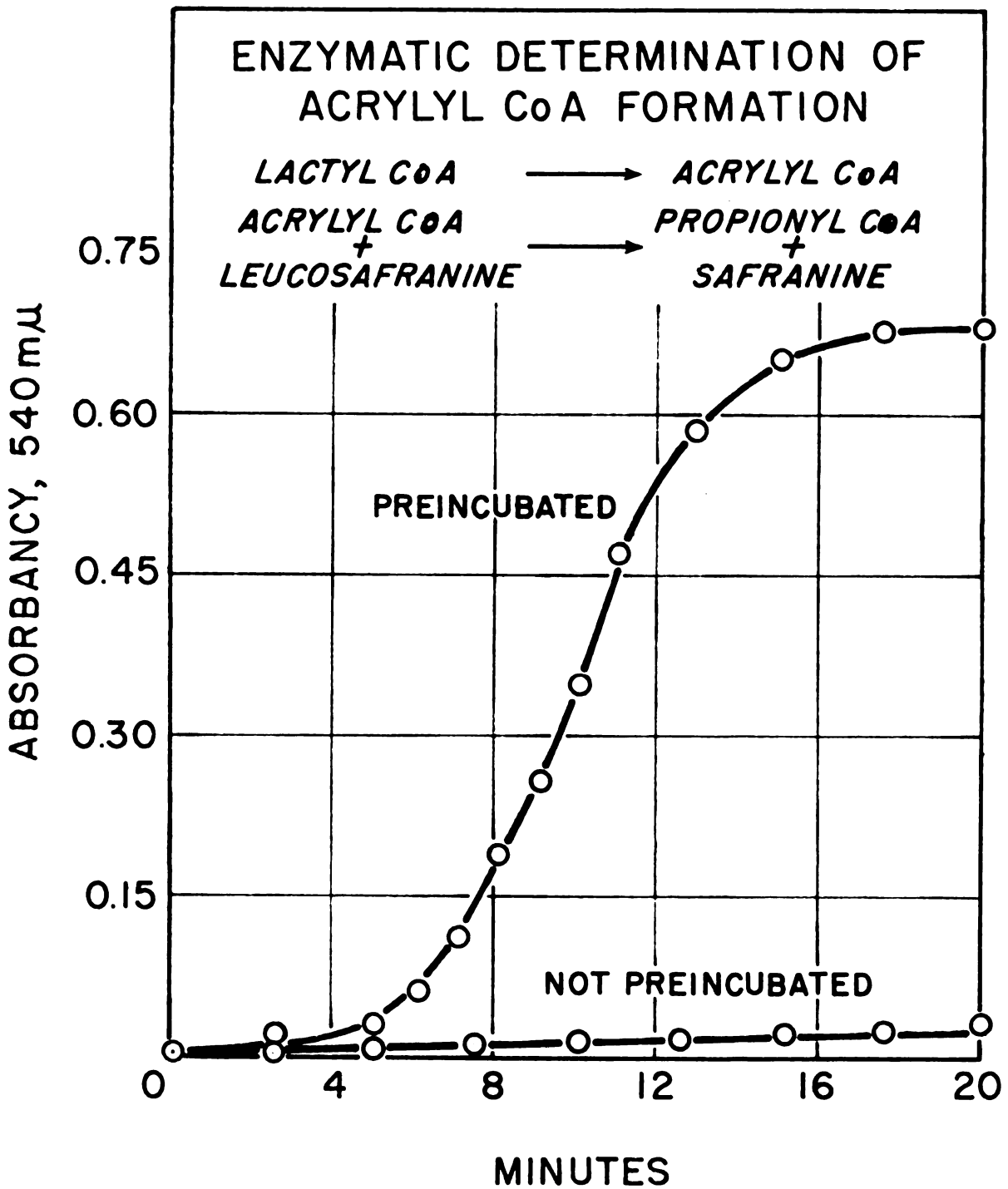
$$\text{lactyl-}C^{14}\text{-CoA} \longrightarrow \text{acrylyl-}C^{14}\text{-CoA} + H_2O \qquad \text{(XI)}$$

$$\text{acrylyl-}C^{14}\text{-CoA} + \text{acetate} \longrightarrow \text{acrylate-}C^{14} + \text{acetyl CoA} \qquad \text{(XXV)}$$

$$\text{acetyl-CoA} + \text{lactate-}C^{14} \longrightarrow \text{lactyl-}C^{14}\text{-CoA} + \text{acetate} \qquad \text{(XXV)}$$

This should result in a net synthesis of acrylate-C^{14} through continuous regeneration of substrate. Less than stoichiometric amounts of CoASH are needed because of recycling. Reaction mixtures contained 250 μmoles of Tris·HCl buffer, pH 8.0, 150 μmoles of potassium lactate, 0.5 μcuries of sodium lactate, 10 μmoles of acetyl phosphate, 50 units each of phosphotransacetylase and CoA transphorase, 1.0 μmole of lactyl-C^{14}-CoA and dehydrase. Incubation periods ranging from five to thirty minutes were employed. Five to ten per cent of the original substrate was converted to acrylate-C^{14}. Apparently, the CoA moiety was not recycled back to form new lactyl-C^{14}-CoA.

Figure 13. Enzymatic determination of acrylyl CoA formation. The incubation mixtures consisted of two parts. The first contained 100 μmoles of Tris·HCl buffer, pH 8.0, 3 μmoles of lactyl-C^{14}-CoA (sp. act. approximately 2,000 dpm per μmole), and 0.1 ml of lactyl CoA dehydrase (equivalent activity in standard assay was 100.0 optical density units per 5 minutes) in a total volume of 1.5 ml. The second part, the analytical system, contained 0.015% safranine and 500 units of acyl CoA dehydrogenase in a total volume of 1.1 ml. The analytical system was added at zero time or after the preincubation period, as indicated. The reactions were run in Thunberg tubes which were flushed with nitrogen prior to adding 0.4 ml of sodium hydrosulfite (0.12% sol'n) to reduce the safranine. The products were reacted with hydroxylamine, separated on paper, and counted (see Figure 9, curve 4).


ENZYMATIC DETERMINATION OF
ACRYLYL CoA FORMATION
LACTYL CoA → ACRYLYL CoA
ACRYLYL CoA + LEUCOSAFRANINE → PROPIONYL CoA + SAFRANINE
PREINCUBATED
NOT PREINCUBATED
ABSORBANCY, 540mμ
0.75
0.60
0.45
0.30
0.15
0
4
8
12
16
20
MINUTES

Lactyl CoA dehydrase has been purified 15 fold through the application of the following techniques: Protamine precipitation and elution with either 0.1M ammonium sulfate or 0.1M potassium phosphate buffer, pH 7.5; adsorption on calcium phosphate gel and elution with 0.1M potassium phosphate buffer, pH 7.5; precipitation with ammonium sulfate (50-70% saturation); adsorption on hydroxyapatite gel and elution with 0.5M potassium phosphate buffer, pH 7.5; and precipitation with ammonium sulfate (55-75% saturation). The yields during purification are in the range of 10-15 per cent. Sometimes the protamine step was omitted.

Lactyl-CoA dehydrase is relatively stable in crude extracts but rapidly loses activity during purification. Attempts to stabilize the purified enzyme with a variety of buffers over a pH range of 6.0-8.0, reducing agents, AMP, ADP, lactate, acrylate, boiled crude extracts, and bovine serum albumin have been unsuccessful.

Purified preparations of the enzyme react slowly with acrylyl Pa and the reaction cannot be followed spectrophotometrically at 263 mμ because the rates hardly exceed the rate of spontaneous degradation of this substrate. However, lactyl hydroxamate was isolated from a reaction mixture containing five μmoles of acrylyl Pa, 25 μmoles of tris acetate buffer pH 8.0, and enzyme.

Characterization of phosphotransacetylase and CoA transphorase. Phosphotransacetylase and CoA transphorase were identified, purified and partially characterized in extracts of the organism. Phosphotransacetylase is specific for the CoA moiety, its pH optimum is 8.0 and it is strongly inhibited by sodium ions. Propionyl CoA and butyryl CoA will substitute for acetyl CoA in the arsenolysis assay to the extent of 18% and 1.5%, respectively, of the rates observed with acetyl CoA.

$$\text{acetyl CoA} + \text{arsenate} \longrightarrow \text{acetyl arsenate} + \text{CoASH} \quad \text{(XXIV)}$$

$$\text{acetyl arsenate} \longrightarrow \text{arsenate} + \text{acetate} \quad \text{(XXXV)}$$

Coenzyme A transphorase uses formate, propionate, butyrate, lactate, α-ketobutyrate, α-hydroxybutyrate and acrylate but not succinate as acceptors (Table 16) according to the following reaction:

$$\text{acetyl CoA} + X \longrightarrow X\text{-CoA} + \text{acetate} \quad \text{(XXV)}$$

Coenzyme A transphorase is specific for the CoA moiety (Table 16), and its pH optimum lies between 8.0 and 8.5.

Characterization of several electron transport enzymes. Three enzymes concerned with electron transport in this organism have been identified, partially purified and characterized: acyl CoA dehydrogenase (Table 14), lactic dehydrogenase (Table 13), and diaphorase (Table 15). All three enzymes readily reduce a number of dyes, have no cofactor requirement, and possess many characteristics in common with comparable enzymes reported by earlier workers (51-53). Data pertaining to the dye specificity of these dehydrogenases is summarized in Table 17. The standard assay procedure employed in these studies contained 25 μmoles of phosphate buffer, sufficient substrate to attain maximum velocity, partially purified enzyme (not the most active preparations in all cases) and the indicated amount of dye. The final volume of the incubation mixtures was adjusted to 0.25 ml and the reaction started by addition of enzyme. The same enzyme preparations were employed for all the determinations.

The purified lactic dehydrogenase is specific for D-lactate and requires no cofactors. The pH optimum of this enzyme is 7.0. Attempts to reverse the reaction, i.e., reduce pyruvate, with either DPNH or reduced safranine have been unsuccessful. No other enzymes possessing lactic dehydrogenase activity appear to be present in the crude extracts.

Table 16. Specificity of CoA transphorase.[a]

Acceptor	Donor	Products Formed
DL lactate	acetyl-PO_4, CoASH	lactyl hydroxamate
DL lactate	acetyl-CoA (2μmoles)	lactyl CoA (1.6 μmoles)
DL lactate	acetyl-Pa (5 μmoles)	none
propionate	acetyl-PO_4, CoASH	propionyl hydroxamate
butyrate	acetyl-PO_4, CoASH	butyryl hydroxamate
acrylate	acetyl-PO_4, CoASH	acrylyl hydroxamate
acrylate	acetyl CoA (2 μmoles)	acrylyl CoA (1.3 μmoles)
succinate	acetyl-PO_4, CoASH	none
formate	acetyl-PO_4, CoASH	formyl hydroxamate
α-ketobutyrate	acetyl-PO_4, CoASH	α-ketobutyryl hydroxamate
α-hydroxybutyrate	acetyl-PO_4, CoASH	α-hydroxybutyryl hydroxamate

[a]The assay systems employed contained 50 μmoles of Tris buffer, pH 8.0, 50 μmoles of the acceptor, 1 unit of purified CoA transphorase, 1.0 μmoles of reduced glutathione and either 1 unit of phosphotransacetylase, 10 μmoles of acetyl-PO_4, 0.1 μmoles of CoASH and 25 μmoles of neutral hydroxylamine or acetyl CoA or acrylyl Pa, as indicated. The hydroxamates were identified by paper chromography and the CoA esters were assayed enzymatically.

Table 17. Dye reduction of several dehydrogenase systems.

Dye	Dehydrogenase and Substrate				
	Lactic	Acyl CoA		Diaphorase	"Clastic"
	D-lactate	Propionyl CoA	Butyryl CoA	DPNH	Pyruvate
Cyt C	2.50[a]	.35	2.0	26.5	25.0
2,6 DCP	1.45	3.50	4.9	157.0	140.0
INT	.65	0	0.68	45.0	50.0
INT + DPN	---	0.6	1.08	3.4	---
TTZ	.15	0	0.4	13.0	5.0
MB	2.4	0	1.4	---	10.2
Ferricyanide[b]	2.2	---	3.0	---	---
Safranine[c]	---	0.5	---	---	---

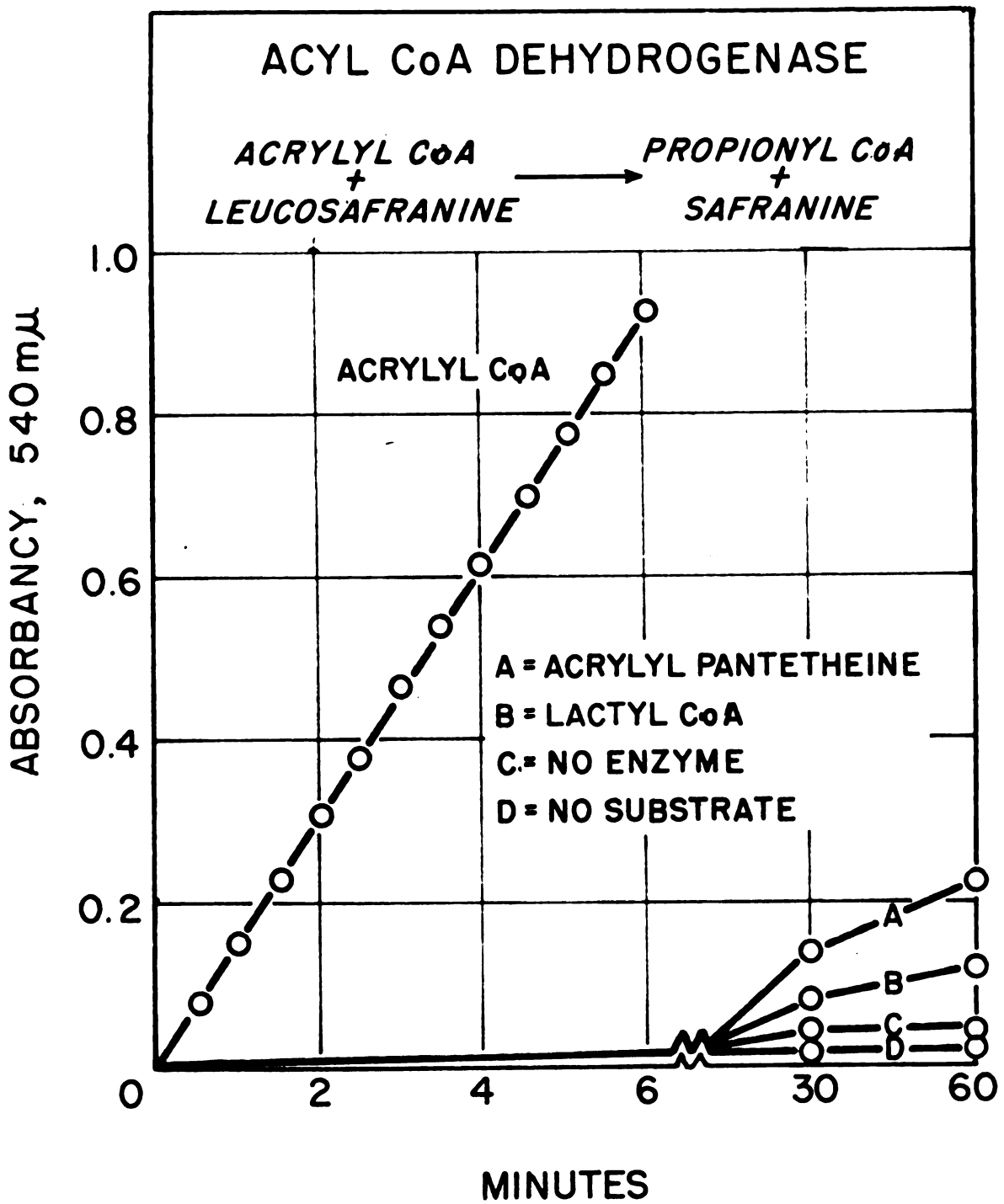
[a] Arbitrary units based upon change in absorbancy per five minutes with respective dyes per 0.1 mg of protein.

[b] Extremely inhibitory in the two cases tested.

[c] Reverse direction.

Acyl CoA dehydrogenase is readily reversible and is specific for the CoA moiety (Figure 14). It has a pH optimum of 7.5 and is unstable in solution unless the salt concentration exceeds one-tenth molar. The enzyme reduces a number of dyes quite readily when butyryl-CoA serves as the substrate but not with propionyl CoA as substrate (Table 17). The addition of DPN to the reaction mixture tends to stimulate propionyl CoA dehydrogenase activity.

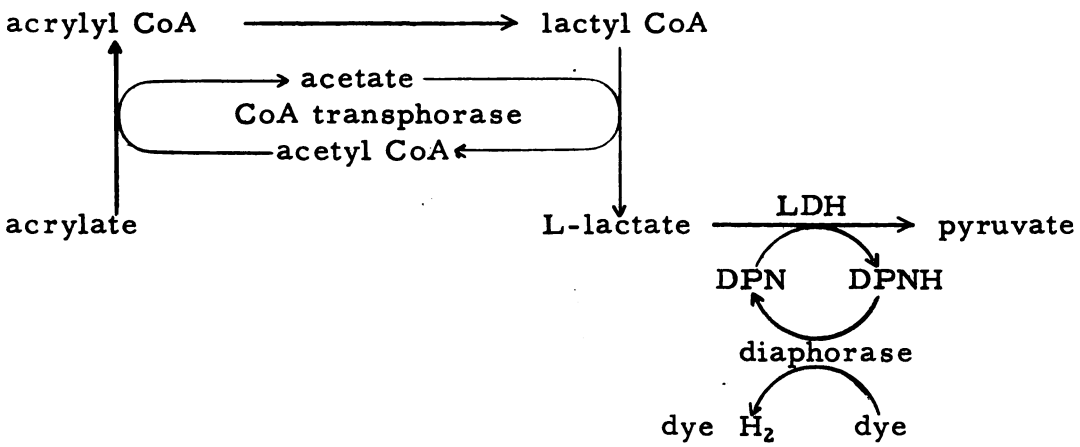
Figure 14. Acyl CoA dehydrogenase: reduction of acrylyl CoA. The incubation mixtures contained 150 μmoles potassium phosphate, pH 7.5, 50 units of acyl CoA dehydrogenase, 0.015% safranine and 2 μmoles of substrate as indicated. The reactions were run in Thunberg tubes which were gassed with nitrogen prior to reduction of the safranine with sodium hydrosulfite.


ACYL CoA DEHYDROGENASE
ACRYLYL CoA + LEUCOSAFRANINE
PROPIONYL CoA + SAFRANINE
ABSORBANCY, 540 mμ
1.0
0.8
0.6
0.4
0.2
0
ACRYLYL CoA
A = ACRYLYL PANTETHEINE
B = LACTYL CoA
C = NO ENZYME
D = NO SUBSTRATE
A
B
C
D
2
4
6
30
60
MINUTES

DISCUSSION

The direct reductive pathway (Figure 2) has been postulated for some time (85, 98), however, until now investigators have been unable to provide unequivocal proof for the existence of the pathway (94). Early workers succeeded in demonstrating all of the reactions except the dehydration mediated by lactyl CoA dehydrase (27). The primary aim of the present study, therefore, was to identify, purify and characterize this enzyme. Considerable progress towards this goal has been achieved through the development of an assay procedure for the enzyme (Figure 15).

Figure 15. Lactyl CoA dehydrase assay system.



Lactic dehydrogenase, DPN, acrylyl CoA and enzyme are required for activity. The assay is linear with enzyme concentration at low rates and the final product, pyruvate, has been isolated and identified, thereby providing proof for the conversion of acrylyl-CoA to lactyl-CoA.

The ability of the enzyme to convert lactyl-C^{14}-CoA to acrylyl-C^{14}-CoA was tested. A spot corresponding in mobility to acrylyl and/or propionyl hydroxamate has been isolated from incubation mixtures containing lactyl-C^{14}-CoA and enzyme. Attempts to devise an assay system to measure acrylyl CoA formation were partially successful. No reaction was observed when lactyl-C^{14}-CoA was incubated with the dehydrase, acyl CoA dehydrogenase and leucosafranine. However, when substrate and dehydrase were preincubated prior to the addition of acyl CoA dehydrogenase and leucosafranine, a reaction did occur as evidenced by the spectrophotometric change observed upon the addition of acyl CoA dehydrogenase and leucosafranine.

The dehydrase has been purified 15 fold but is very unstable. A method for the stabilization of the enzyme must be devised before further purification can be achieved. The conversion of lactyl CoA to acrylyl CoA has been tentatively established but further proof is required; perhaps, acrylyl CoA aminase (99) could be employed as a linking enzyme to remove the acrylyl CoA formed.

Several aspects of lactate metabolism in P. elsdenii require further investigation. The relative contributions of the acrylate pathway, the β-oxidation pathway for butyrate synthesis, and hydrogenase should be studied. Information concerning this aspect of the problem may enable future investigators to reconstruct the pathway in vitro, i.e., demonstrate a rapid conversion of lactyl CoA to propionyl CoA.

The nature of the electron transport reactions which occur concomitant with lactate and pyruvate oxidation and the reduction steps indicated above must be elucidated. Also, the possibility that these reactions are coupled to an energy trapping system is worthy of investigation (53). The identity of the enzyme(s) catalyzing the reduction of acrylyl CoA and/or crotonyl CoA has not been fully established. The observation that DPN stimulates acyl CoA dehydrogenase activity under

certain conditions should be investigated further since the possibility exists that the acrylyl CoA reduction reaction may be mediated by a DPN-linked enzyme (82).

The specificities of D-lactic dehydrogenase and L-lactyl CoA dehydrase suggest that a very interesting phenomenon may exist; wherein, the organism reduces L-lactate to propionate while D-lactate is oxidized to acetate and CO_2. The fact that P. elsdenii was first isolated from starch enrichment cultures where S. bovis was the predominant organism are inconsistent with the operation of this phenomenon, however. P. elsdenii would not grow on L-lactate (produced by S. bovis) if the only enzymes involved in lactate conversions were the D-lactic dehydrogenase and the L-lactyl CoA dehydrase. Either there is a racemase present in the organism, or a third enzyme for lactate metabolism has been overlooked.

In view of the fact that phosphotransacetylase, CoA transphorase and acyl CoA dehydrogenase have been identified and evidence presented indicating that lactyl CoA dehydrase is present, it is concluded that the direct reductive pathway proceeds as postulated in the rumen microorganism P. elsdenii.

SUMMARY

Lactyl CoA dehydrase, acyl CoA dehydrogenase, lactic dehydrogenase, diaphorase, phosphotransacetylase and CoA transphorase have been identified, partially purified and characterized in extracts of the rumen microorganism Peptostreptococcus elsdenii. On the basis of the results presented it is suggested that the conversion of lactate to propionate occurs via the CoA esters of lactate, acrylate and propionate, respectively, as postulated.

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