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Pyruvate Dehydrogenase Activity in Group N Streptococci

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Abstract

Pyruvate dehydrogenase activity was detected in whole cells but not in cell-free extracts of *Streptococcus lactis*. However, the three component enzymes (pyruvate decarboxylase, lipoate acetyltransferase and lipoyl dehydrogenase) of the pyruvate dehydrogenase complex were identified in the cell-free extracts. Whole cells of the three species of group N streptococci formed acetoin and diacetyl only after the pathway forming acetate had become saturated. *S. lactis* subsp. *diacetylactis* DRC2 formed more acetoin and diacetyl and less acetate from pyruvate than did *S. lactis* C10. Strains C10 and DRC2 were able to form acetoin via α-acetolactate or diacetyl and to convert acetoin to butane-2,3-diol. *S. cremoris* HP was able to form acetoin only via α-acetolactate and could not convert acetoin to butane-2,3-diol.

Introduction

Pyruvic acid is a key intermediate in the intermediary metabolism of carbohydrates, amino acids (alanine, serine and aspartic acid) and citrate by group N streptococci and can be converted by these organisms into a variety of end-products such as lactic acid, acetic acid, acetaldehyde, ethanol, acetoin, diacetyl and butane-2,3-diol.

The formation of lactic acid from pyruvic acid involves only a single enzyme, lactate dehydrogenase, and is therefore a relatively simple system to study. By contrast, the formation of the other products listed above involves many enzymes including one or more of the component enzymes of the pyruvate dehydrogenase system.

The presence of a pyruvate dehydrogenase system in group N streptococci has been assumed on the basis that cells growing in the absence of acetate had an absolute requirement for lipoic acid and that resting cells metabolized pyruvate to acetate, acetoin and diacetyl (Anders and Jago 1970). The formation of the latter two compounds is dependent on the presence of the acetaldehyde–thiamine pyrophosphate (TPP) complex which is an intermediate in the pyruvate dehydrogenase system.

The pyruvate dehydrogenase system has been studied in some detail in *Escherichia coli* and a number of methods have been established to assay both the intact system as well as the three component enzymes of the system.

The intact pyruvate dehydrogenase system can be assayed in whole cells and cell-free extracts by measuring oxygen utilization or pyruvate dismutation. The component enzymes of the pyruvate dehydrogenase system (pyruvate decarboxylase, lipoate acetyltransferase and lipoyl dehydrogenase) can only be assayed using model

reactions. Pyruvate decarboxylase (EC 4.1.1.1) catalyses the initial decarboxylation of pyruvate to form an active acetaldehyde–TPP complex, 2-hydroxyethylthiamine pyrophosphate, which then reacts with the lipoic acid covalently bound to the second enzyme in the system, lipoate acetyltransferase (EC 2.3.1.12). In the model assay system for pyruvate decarboxylase, ferricyanide replaces the protein-bound lipoyl moiety and the reaction is followed by the production of ferrocyanide (Hager 1953; Koike and Reed 1960).

Lipoate acetyltransferase catalyses the transfer of the acetyl group from the acetaldehyde–TPP complex to CoA via the lipoyl moiety bound to the enzyme. This enzyme is routinely assayed in the reverse direction and the acetyl-CoA required for the reaction is generated from acetyl phosphate by the enzyme phosphate acetyltransferase (EC 2.3.1.8). The reaction is followed by the disappearance of acetyl phosphate.

Lipoyl dehydrogenase (EC 1.6.4.3) is a flavoprotein which catalyses the oxidation of the dihydrolipoyl moiety on the lipoate acetyltransferase with the concomitant production of reduced NAD. The reaction is coupled to lactate dehydrogenase (EC 1.1.1.27) and followed by measuring the disappearance of thiol groups as reduced lipoic acid is converted to its oxidized form.

This paper examines the pyruvate dehydrogenase system in *Streptococcus lactis* and the formation of some of the end-products which arise from the operation of this pathway.

Materials and Methods

Bacteria

S. lactis strain C10, *S. lactis* subsp. *diacetylactis* strain DRC2, *S. cremoris* strain HP and *Escherichia coli* (Crookes strain ATCC 8739–6) were used in this investigation. The group N organisms were obtained from the CSIRO Dairy Research Laboratory, Highett, Vic. and were maintained by daily subculture in sterile skim milk [10% (w/v) solution of skim-milk powder autoclaved at 121°C for 10 min] and grown in quantity in a protein-digest (TYL) broth (pH 6·5) containing tryptone (Oxoid), 30 g; yeast extract (Difco), 10 g; lactose, 30 g (except where specified); KH_2PO_4, 5 g; and Lab-Lemco powder (Oxoid), 2 g dissolved in 1 litre of distilled water. The carbohydrate was autoclaved separately before adding to the growth medium. The cells were grown at 30°C for 16 h and the pH of the growth medium was maintained at 6·3 by the automatic addition of 10 M NaOH.

E. coli was maintained by daily subculture in a medium (M_1) containing per litre: tryptone (Oxoid), 10 g; yeast extract (Difco), 10 g; KH_2PO_4, 5 g; and glucose, 3 g; and grown in quantity in a medium (M_2) consisting of NH_4Cl, 2 g; glucose, 4 g; sodium glutamate, 2·5 g; yeast extract (Difco), 5 mg; KH_2PO_4, 1·5 g; Na_2HPO_4, 13·5 g; $MgSO_4.7H_2O$, 200 mg; $CaCl_2$, 10 mg; and $FeSO_4.7H_2O$, 500 μg; in a total volume of 1 litre. The phosphate, glutamate and glucose were each autoclaved separately before adding to the autoclaved salt solution. When cells were grown in quantity, 950 ml of M_2 was inoculated with 50 ml of an 8-h culture of M_1 and incubated with vigorous shaking at 30°C for 16 h.

Cells were harvested from the growth medium by centrifugation at 10 000 *g* and 4°C for 10 min, washed once in 0·9% (w/v) NaCl and resuspended in the same medium. The concentration of *S. lactis* C10 cells was determined by reference to a standard curve which related dry weight with absorbance at 650 nm. The concentration of *E. coli* cells was determined by measuring the amount of protein in cell suspensions.

Cell-free extracts were prepared from whole cells by extruding a bacterial suspension [1 g (wet wt) of cells per 3 ml buffer (20 mM KH_2PO_4, pH 7·0)] through a French pressure cell (French and Milner 1955; Vanderheiden *et al.* 1970) at a constant pressure of 154 MPa. Unbroken cells and cellular debris were removed by centrifugation at 35 000 *g* and 4°C for 30 min.

Respiratory Studies

The amount of oxygen utilized and CO_2 evolved by whole cells and cell-free extracts was estimated manometrically as described by Umbreit *et al.* (1957). All incubations were carried out at 30°C in single side-arm Warburg flasks. The reaction mixture (3·0 ml) contained: 100 mM Na_2HPO_4 (adjusted to pH 5·5 with 0·3 M succinic acid or HCl); 1·6 mM $MgCl_2$; 33·3 mM sodium pyruvate, and either 10 mg (dry wt) of *S. lactis* C10 whole cells, 6·9 mg (protein) of *E. coli* whole cells, or 0·4–10·0 mg (protein) of cell-free extract. The reaction was initiated by the addition of sodium pyruvate. Manometric experiments were carried out in duplicate using a single batch of cells and the results reported are mean values. Manometric experiments are generally accurate to ±5%.

Assay of Enzyme Activities

Pyruvate dismutation, pyruvate decarboxylase and *lipoate acetyltransferase* were assayed as described by Reed and Willms (1966).

Lipoyl dehydrogenase was assayed by the method of Hagar and Gunsalus (1953) except that residual dihydrolipoic acid was measured by the method of Habeeb (1972).

NADH dehydrogenase (EC 1.6.99.3). The reaction mixture (3·0 ml) contained 66 mM KH_2PO_4 (pH 6·5); 0·13 mM NADH and dialysed cell-free extract. The reaction was started by the addition of the cell-free extract and followed by measuring the change in absorbance at 340 nm.

Diacetyl reductase (EC 1.1.1.5). The reaction mixture (2·0 ml) contained 66 mM KH_2PO_4 (pH 6·5); 0·13 mM NADH; 4·6 mM diacetyl and dialysed cell-free extract. The reaction was carried out under anaerobic conditions in a Thunberg cuvette to overcome endogenous NADH dehydrogenase activity. The contents of the cuvette were sparged with N_2 and then twice evacuated and flushed with N_2 to ensure anaerobic conditions. The reaction was started by the addition of diacetyl from the side-arm and followed by measuring the change in absorbance at 340 nm.

D(−)-*Butanediol Dehydrogenase* (EC 1.1.1.4)

Acetoin reduction. The reaction mixture and conditions were as described for diacetyl reductase except that 1·3 mM acetoin replaced the diacetyl.

Butane-2,3-diol oxidation. The reaction mixture (3 ml) contained 66 mM KH_2PO_4 (pH 7·0) or 66 mM glycine (adjusted to pH 10 with 1 M NaOH); 0·13 mM NAD; 2·3 mM butane-2,3-diol and dialysed cell-free extracts. The reaction was carried out as described for diacetyl reductase.

Estimation of the Products of Pyruvate Metabolism

Acetate was measured as volatile acid as described by Anders and Jago (1970) except that a 10-ml portion of the steam distillate was titrated to pH 7·0 with 5 mM NaOH.

Separation of acetoin, diacetyl and butane-2,3-diol by salting-out chromatography. Acetoin, diacetyl and butane-2,3-diol were separated by salting-out chromatography as described by Speckman and Collins (1968).

A portion (1 ml) of the solution to be analysed was applied to the Dowex-1 (sulfate form) column (1·5 by 45 cm) and eluted with 0·5 M $(NH_4)_2SO_4$ at a flow rate of 0·5 ml/min and 1·3-ml fractions collected. Column eluates were assayed for radioactivity or for acetoin, diacetyl and butane-2,3-diol. The radioactivity in each fraction was determined by counting 0·1-ml samples in 10 ml of a scintillation mixture containing 4 g 2,5-diphenyloxazole (PPO) and 0·1 g of 1,4-bis(4-methyl-5-phenyloxazol-2-yl) benzene (dimethyl POPOP) per litre of a toluene–ethanol–formic acid mixture (51 : 47 : 2 by vol.). The efficiency of counting, as determined by the channels ratio method, was constant for all samples.

Acetoin and diacetyl were estimated by the method of Westerfield (1945). *Butane-2,3-diol* was estimated by the method of Keen and Walker (1973). *Pyruvate* was estimated by the enzymic method of Von Korff (1969).

Estimation of Protein

The protein in cell-free extracts was estimated by the method of Lowry *et al.* (1951). The protein in whole cells was estimated by the method of Lowry *et al.* (1951) as modified by Herbert *et al.* (1971).

Results and Discussion

Since the pyruvate dehydrogenase system was assayed by model systems which had been developed with *E. coli*, this organism was included in the present studies as a control.

As shown in Table 1, oxygen was utilized by whole cells and cell-free extracts of *E. coli* and by whole cells of *S. lactis* C10 when the organisms were incubated with pyruvate. However, oxygen was not utilized by cell-free extracts of *S. lactis* C10. In all cases, CO_2 was produced (Table 1) which suggested that, at least, the pyruvate decarboxylase component of the pyruvate dehydrogenase system was operative in cell-free extracts of *S. lactis* C10.

Table 1. Pyruvate dehydrogenase activity in *S. lactis* C10 and *E. coli*

Details of incubation mixture given in Materials and Methods. All incubations were carried out at 30°C for 120 min. Specific activities expressed as μmoles CO_2 evolved or O_2 utilized per 2 h per milligram of cells or protein

Organism	Preparation	Oxygen utilized	CO_2 evolved
S. lactis	Whole cells	0·6	3·6
	Cell-free extracts	<0·02	1·0
E. coli	Whole cells	7·1	12·6
	Cell-free extracts	1·4	3·2

When pyruvate dehydrogenase activity was assayed by the pyruvate dismutation reaction, activity was detected in cell-free extracts prepared from *E. coli*, but not in cell-free extracts prepared from *S. lactis* C10.

Since the assay of pyruvate dismutation involved the measurement of acetyl phosphate as the end-product, it seemed possible that the absence of any activity in cell-free extracts of *S. lactis* C10 could have been due to the rapid conversion of acetyl phosphate to acetate by acetate kinase (EC 2.7.2.1). A cell-free extract prepared from whole cells of *S. lactis* C10 was therefore dialysed against 150 vol. of Tris–HCl (pH 8·16) for 18 h to remove ADP and magnesium, the cofactors necessary for the acetate kinase reaction. However, no pyruvate dismutation was detected using the dialysed cell-free extracts.

To confirm that the utilization of oxygen by whole cells of *S. lactis* C10 and the dismutation of pyruvate by cell-free extracts of *E. coli* was, in fact, due to the pyruvate dehydrogenase system [and not due to an alternative system such as pyruvate oxidase (EC 1.2.3.3)], the effect of arsenite, an inhibitor of the pyruvate dehydrogenase system, was investigated. Arsenite, which inhibits the lipoate acetyltransferase enzyme of the pyruvate dehydrogenase complex (by combining with the thiol groups of lipoic acid), was shown to almost completely inhibit pyruvate dismutation in cell-free extracts of *E. coli* and the utilization of oxygen by whole cells of *S. lactis* C10 (Table 2).

The presence of the pyruvate dehydrogenase system in *S. lactis* was conclusively established by the identification of the three component enzymes of the complex

(pyruvate decarboxylase, lipoate acetyltransferase and lipoyl dehydrogenase) in cell-free extracts (Table 3). Pyruvate decarboxylase was found to be much less stable

Table 2. Effect of sodium arsenite on the metabolism of pyruvate by *S. lactis* C10 and *E. coli*

Oxygen utilization was measured in an incubation mixture (3·0 ml) which contained: 100 mM KH_2PO_4 (adjusted to pH 5·5 with NaOH); 1·6 mM $MgCl_2$; 33 μM TPP; 20 mM sodium pyruvate and 10 mg (dry wt) of *S. lactis* C10 cells. The reaction was initiated by the addition of sodium pyruvate and the mixtures were incubated at 30°C for 120 min. A cell-free extract (1·2 mg protein) of *E. coli* was used in the pyruvate dismutation assay

Sodium arsenite concn (mM)	Oxygen utilized by *S. lactis* (μmoles)	Acetyl phosphate formed by *E. coli* (μmoles)
Nil	7·76	3·15
1·0	0·79	0·04
2·5	0·82	

in *S. lactis* C10 than in *E. coli* and therefore was assayed immediately after the preparation of the cell-free extract. The activity of the *S. lactis* C10 pyruvate decarboxylase was one-third that of the *E. coli* enzyme while the specific activity of lipoate acetyltransferase in *S. lactis* C10 was six times the specific activity of the

Table 3. Component enzymes of the pyruvate dehydrogenase system in *E. coli* and *S. lactis* C10

Cell-free extracts were prepared from whole cells and the enzymes assayed as described under Materials and Methods. The values in parentheses represent the range of activities obtained in six replicate experiments

Organism	Specific activity of: Pyruvate decarboxylase[A]	Lipoate acetyl-transferase[B]	Lipoyl dehydrogenase[C]
S. lactis	1·74 (1·41–2·04)	4·73 (3·80–5·52)	0·24 (0·09–0·43)
E. coli	5·49 (4·52–6·45)	0·78 (0·58–0·89)	1·50 (0·45–2·11)

[A] As μmoles ferrocyanide produced per hour per milligram protein.
[B] As μmoles S-acetylhydrolipoate produced per hour per milligram protein.
[C] As μmoles dihydrolipoic acid utilized per hour per milligram protein.

E. coli enzyme (Table 3). Lipoyl dehydrogenase was present at very low levels of activity in cell-free extracts of both *E. coli* and *S. lactis* C10.

Since the component enzymes of the pyruvate dehydrogenase system could be detected in cell-free extracts of *S. lactis* C10, the inability to assay the pyruvate dehydrogenase system as a whole was not due to the removal of the complex with the cell debris during the preparation of the cell-free extracts. It is possible that the pyruvate dehydrogenase system is linked to the plasma membrane by weak, non-covalent forces and that this linkage is readily broken by any disruption of the plasma membrane. Alternatively, the complex itself may be readily dissociated when the intracellular environment is altered by the removal of the cell wall and plasma membrane.

The fact that the intact pyruvate dehydrogenase system could not be detected in cell-free extracts of *S. lactis* C10 implies that the structural organization of the complex differs quite markedly from the intact pyruvate dehydrogenase system in *E. coli*, which could be readily measured in cell-free extracts.

In *S. faecalis*, an organism closely related metabolically to *S. lactis*, the pyruvate dehydrogenase system has been shown to resemble the mammalian complex rather than the *E. coli* complex in size and appearance (Reed 1974). However, the pyruvate dehydrogenase system in *S. faecalis*, like the enzyme from *E. coli*, can be assayed by the pyruvate dismutation reaction in cell-free extracts.

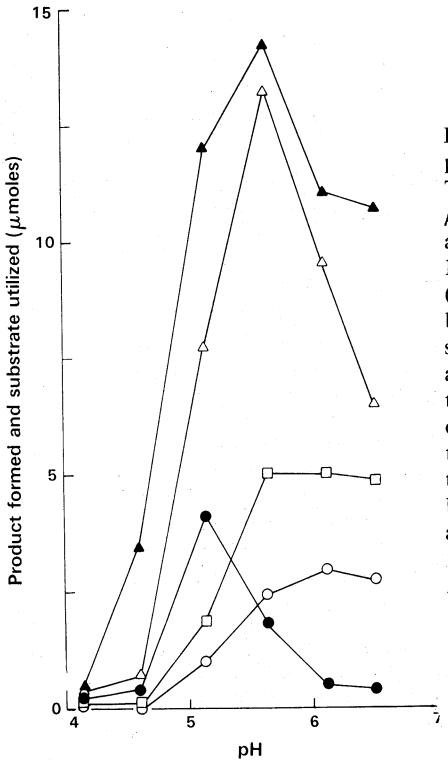


Fig. 1. Effect of pH on the metabolism of pyruvate by whole cells of *S. lactis* C10. The incubation mixture (3 ml) contained (in μmoles): Na_2HPO_4, 300 (adjusted to the appropriate pH with 0·3 M succinic acid); $MgCl_2$, 5; sodium pyruvate, 60; and 10 mg (dry wt) of cells. Reactions were initiated by the addition of sodium pyruvate from the side-arm of a Warburg flask and were allowed to proceed for 210 min at 30°C. At the end of the incubation period the pH of each incubation mixture was measured and the value for CO_2 evolution corrected for the amount of CO_2 retained in solution as bicarbonate. The analytical procedures used are indicated in the Materials and Methods. ○ Oxygen uptake. ● Acetoin plus diacetyl formed. △ CO_2 evolved. ▲ Pyruvate utilized. □ Acetate formed.

Pyruvate Metabolism via the Pyruvate Dehydrogenase System

The utilization of pyruvate by whole cells of *S. lactis* C10 was studied over the pH range 4·0–7·0. As shown in Fig. 1, the pyruvate utilized and the CO_2 evolved

followed a similar pattern over the entire pH range and were maximal at about pH 5·5. This value probably represents a balance between the optimum pH of enzymes utilizing pyruvate and the pH at which exogenously supplied pyruvate passes most easily into the cell. In the absence of an active transport mechanism, pyruvate would pass more easily into the cell at pH 5·5 than at pH 6·5 because more pyruvate would exist in the uncharged non-dissociated form at the lower pH. The concentration of succinate in the buffer increased as the pH decreased and it was thought this may compete with pyruvate for diffusion into the cell. However, similar results were obtained when chloride replaced succinate as the counter ion.

The uptake of oxygen and formation of acetate were constant between pH 6·5 and 5·5 in spite of the increase in the amount of pyruvate utilized at the lower pH. Approximately 2 μmoles of acetate were formed per μmole of oxygen utilized. Following the saturation of the acetate-forming pathway, acetoin and diacetyl formation increased as the pH decreased. At external pH values below 5·5 acetate production appeared to be inhibited, while acetoin and diacetyl formation was inhibited at external pH values below 4·5.

Table 4. Metabolism of sodium pyruvate by whole cells of *S. lactis* C10 and *S. lactis* subsp. *diacetylactis* DRC2

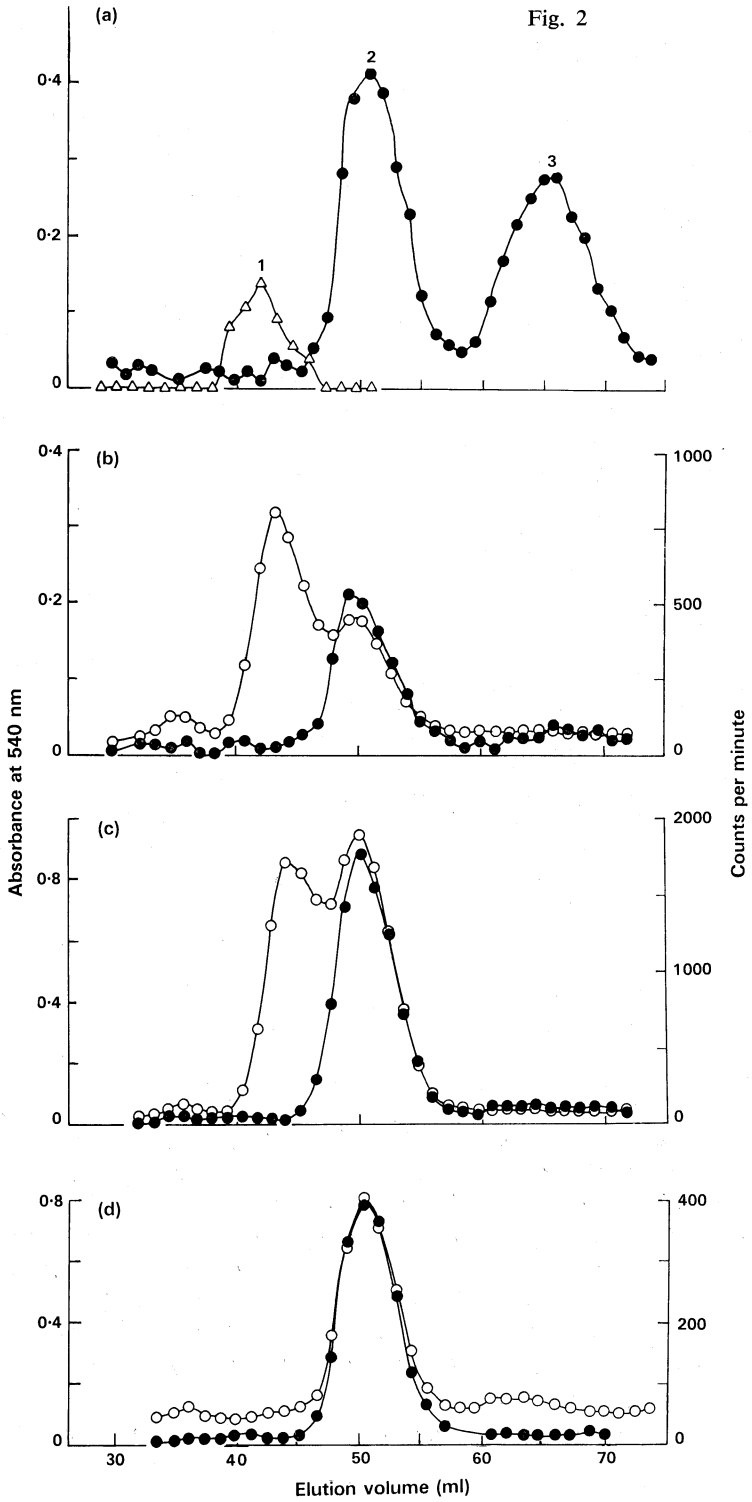
The incubation mixture (3 ml) contained: 100 mM Na_2HPO_4 (adjusted to pH 5·5 with 0·3 M succinic acid); 1·6 mM $MgCl_2$; sodium pyruvate at the concentrations indicated, and 10 mg (dry wt) of cells. Reactions were carried out in Warburg flasks for 180 min at 30°C. Analytical procedures are described under Methods

Organism	Sodium pyruvate added (μmoles)	Sodium pyruvate consumed (μmoles)	Oxygen uptake (μmoles)	CO_2 evolved (μmoles)	Acetoin+ diacetyl formed (μmoles)	Acetate formed (μmoles)
S. lactis C10	60	18·8	7·3	17·3	0·7	14·3
	100	41·2	8·4	35·8	2·5	18·1
	150	42·1	7·7	41·0	10·8	14·9
S. lactis subsp. *diacetylactis* DRC2	100	42·3	2·3	40·0	12·9	5·7

When whole cells of *S. lactis* C10 and *S. lactis* subsp. *diacetylactis* DRC2 were incubated with pyruvate at pH 5·5 some quantitative differences were observed in the products formed by the two organisms (Table 4). Strain DRC2 utilized less oxygen, and formed less acetate and more acetoin and diacetyl than did strain C10. This difference between the strains will be the subject of a later publication.

The effect of pyruvate concentration on the products of pyruvate metabolism is also shown in Table 4. There was no change in oxygen uptake and acetate formation when *S. lactis* C10 was incubated with increasing amounts of pyruvate. However, there were large increases in the amount of CO_2 evolved and acetoin plus diacetyl formed. As the rate of oxygen uptake and acetate formation remained constant with increasing amounts of pyruvate it appeared that this pathway was saturated even at 60 μmoles of pyruvate and that pyruvate in excess of this amount was being metabolized to acetoin and diacetyl.

Fig. 2


(a)
0·4
0·2
0
1
2
3
(b)
0·4
0·2
0
1000
500
0
(c)
0·8
0·4
0
2000
1000
0
(d)
0·8
0·4
0
400
200
0
30
40
50
60
70
Elution volume (ml)
Absorbance at 540 nm
Counts per minute

The formation of 1 mole of acetate by group N streptococci involves the oxidation of 1 mole of pyruvate and the evolution of 1 mole of CO_2, while the formation of 1 mole of acetoin or diacetyl requires 2 moles of pyruvate with the evolution of 2 moles of CO_2. A discrepancy appears in Table 4 between the amount of pyruvate consumed (or CO_2 evolved) and the amount of products (acetoin, diacetyl and acetate) formed. The fact that there was an almost 1 : 1 relationship between CO_2 evolved and pyruvate utilized suggested that most of the pyruvate was being metabolized via the pyruvate dehydrogenase system and therefore the possibility that acetoin was being converted to its reduction product butane-2,3-diol was investigated. Whole cells of *S. lactis* C10, *S. lactis* subsp. *diacetylactis* DRC2 and *S. cremoris* HP were incubated with [^{14}C]pyruvate and the products of pyruvate metabolism were separated by salting-out chromatography. As shown in Fig. 2, both strains C10 (Fig. 2*b*) and DRC2 (Fig. 2*c*) formed significant quantities of butane-2,3-diol in addition to acetoin. However, strain HP did not form any butane-2,3-diol from pyruvate (Fig. 2*d*). In all three organisms no detectable quantities of diacetyl were formed and consequently when acetoin and diacetyl were estimated together by the method of Westerfield (1945), the compound measured was, in fact, mostly acetoin. The formation of butane-2,3-diol by strains C10 and DRC2 was therefore responsible for the utilization of some of the pyruvate not accounted for in Table 4.

The specific activities of some of the enzymes involved in pyruvate metabolism by group N streptococci are shown in Table 5. When strains C10 and DRC2 were grown in TYL broth for the preparation of cell-free extracts it was found that the lactose concentration of the medium affected the enzyme activities. However, the reason for the decreased enzyme activities of cells grown in the presence of high levels (3%) of lactose was not investigated.

Using cell-free extracts prepared from cells grown in TYL broth containing 0·5% lactose it was possible to show that the NADH dehydrogenase/peroxidase system was much more active in *S. cremoris* HP than in either *S. lactis* C10 or *S. lactis* subsp. *diacetylactis* DRC2. The activities of diacetyl reductase and D(−)-butanediol dehydrogenase were much greater in strain DRC2 than in strain C10. However, no diacetyl reductase or D(−)-butanediol dehydrogenase activity could be detected in strain HP. The oxidation of butane-2,3-diol to acetoin by the D(−)-butanediol dehydrogenase was much greater at pH 10 than at pH 7. This suggested that the accumulation of butane-2,3-diol rather than acetoin would occur in media containing cells of strain C10 or strain DRC2, provided NADH was not limiting.

Keen and Walker (1974) found that cheese manufactured with *S. cremoris* HP as the starter organism contained very low levels of butane-2,3-diol. However, the use of *S. cremoris* AM_2 as starter organism resulted in the formation of relatively

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Fig. 2. Fractionation of C_4 compounds produced by group N streptococci from pyruvate. The incubation mixture (3 ml) contained (in μmoles): Na_2HPO_4, 300 (adjusted to pH 5·5 with 0·3 M succinic acid); $MgCl_2$, 5; [U-^{14}C]sodium pyruvate (0·033 μCi/μmole), 100; and 10 mg (dry wt) of whole cells. All incubations were carried out in Warburg flasks at 30°C for 210 min. The products were separated and assayed as indicated in Materials and Methods. (*a*) Elution profile of butane-2,3-diol (1), acetoin (2) and diacetyl (3) standards; (*b*) *S. lactis* C10; (*c*) *S. lactis* subsp. *diacetylactis* DRC2; (*d*) *S. cremoris* HP. ○ Radioactivity. ● Absorbance of acetoin and diacetyl at 540 nm. △ Absorbance of butane-2,3-diol at 540 nm.

high levels of butane-2,3-diol, which suggested that the inability to form butane-2,3-diol is not common to all strains of *S. cremoris*. It was shown in this investigation that *S. cremoris* HP does not form butane-2,3-diol because it lacks the enzyme D(−)-butanediol dehydrogenase.

Table 5. Effect of lactose concentration on some specific enzyme activities associated with pyruvate metabolism in *S. lactis* C10, *S. lactis* subsp. *diacetylactis* DRC2 and *S. cremoris* strain HP

Cell-free extracts were prepared from cells grown in TYL broth containing 0·5% or 3·0% (w/v) lactose and the enzyme assays were carried out as indicated in the Materials and Methods. The enzyme activities given in parentheses were determined at pH 7·0. Specific enzyme activities expressed as μmoles per minute per milligram protein

Lactose concn (% w/v)	NADH dehydrogenase/ peroxidase system	Diacetyl reductase	D(−)-Butanediol dehydrogenase	
			Acetoin reduction	Butane-2,3-diol oxidation
		S. lactis C10		
0·5	11·8	90·0	52·2	105·0(16·3)
3·0	4·2	10·8	4·7	11·0(Nil)
		S. lactis subsp. *diacetylactis* DRC2		
0·5	13·7	283·2	113·4	113·4(15·0)
3·0	5·3	13·2	7·1	4·9(Nil)
		S. cremoris HP		
0·5	95·4	Nil	Nil	Nil

Keen and Walker (1974) also found that the level of diacetyl in cheeses manufactured with *S. lactis* subsp. *diacetylactis* declined over a period of time to concentrations similar to those found in *S. cremoris* AM_2 and HP cheeses. This concentration remained fairly constant over a period of 1 year. The decrease in diacetyl content was attributed to the presence of diacetyl reductase. It was shown in this investigation that *S. cremoris* HP lacked the enzyme diacetyl reductase as well as the enzyme D(−)-butanediol dehydrogenase.

Although the absence of diacetyl reductase from strains of *S. lactis* and *S. cremoris* has been demonstrated by Seitz *et al.* (1963), this has not been correlated with the absence of D(−)-butanediol dehydrogenase. Bryn *et al.* (1971) have suggested that the reduction of diacetyl to acetoin and the reversible conversion of acetoin to butane-2,3-diol could be carried out by the same enzyme in *Aerobacter aerogenes*. The absence of both diacetyl reductase and D(−)-butanediol dehydrogenase in *S. cremoris* HP suggests that a similar situation could apply to this organism.

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