PYRUVIC ACID METABOLISM

A Factor Required for Oxidation by Streptococcus faecalis

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The metabolism of pyruvic acid by lactic acid bacteria occurs through several pathways whose mechanisms and cofactors are for the most part unknown. The growth factor requirements of these organisms suggest the possibility of determining the cofactor requirements by adjusting the nutritional levels for maximum function (Bellamy and Gunsalus, 1944).

Among the enterococci, *Streptococcus faecalis*, strain 10C1, has been shown to use several organic acids as energy sources. The metabolism of citric and malic acids is adaptive (Campbell, Bellamy, and Gunsalus, 1943), whereas the metabolism of pyruvate is constitutive, although the amount of enzyme is altered somewhat by the growth conditions (Campbell, Bellamy, and Gunsalus, 1943; Gunsalus and Campbell, 1944). The growth conditions that favor the greatest rate of pyruvate oxidation and dismutation have been described by Miller (1942) and Miller *et al.* (1947).

Lipmann (1939, 1944) studied a stable pyruvic acid oxidation system of *Lactobacillus delbrueckii* (Davis strain) in some detail and found at least 5 components to be required: the enzyme protein, flavine adenine dinucleotide, inorganic phosphate, cocarboxylase, and magnesium. Manganese or cobalt could replace the latter. For pyruvate dismutation a sixth component, riboflavin, was required as a link to the lactic dehydrogenase. Inorganic phosphate was taken up during the reaction and appeared as acetyl phosphate, from which the high energy phosphate could be transferred to the adenylic acid system.

Dorfman *et al.* (1942) and McElroy and Dorfman (1948) observed that cell suspensions of *Proteus morganii* oxidize pyruvate slowly and that the rate could be stimulated by the addition of pantothenate. Later Stumpf (1945) isolated and purified a pyruvic acid oxidase from *Proteus vulgaris*, which was shown to require the enzyme protein, cocarboxylase, and magnesium. Neither an inorganic phosphate nor a pantothenate requirement was demonstrated. Pilgrim *et al.* (1942) had also shown that liver homogenates of pantothenate- or biotin-deficient rats were deficient in ability to oxidize pyruvate.

Lipmann and his associates (Novelli and Lipmann, 1947) have shown that pantothenate functions in acetylation. Thus the site of action in pyruvate metabolism appears to be beyond the oxidation reaction, specifically in the disposition of the acetate. On the basis of preliminary experiments with *Proteus morganii* McElroy and Dorfman (1948) have suggested that pantothenate is involved in the removal of acetylmethylcarbinol.

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In the present study, a synthetic medium has been obtained for the growth of *Streptococcus faecalis*, strain 10C1, with a very active pyruvate oxidase system. With the synthetic medium devised (O'Kane and Gunsalus, 1947) the enzyme occurs in the apoenzyme form and may be activated by a substance (or substances) present in yeast extract. Dried cell preparations as well as cell suspensions are activated, thus constituting another case of apoenzyme formation during cell growth (Bellamy and Gunsalus, 1945).

METHODS

Culture. *Streptococcus faecalis*, strain 10C1, from the departmental collection was stored in stock agar deeps at 5 C and transferred every 6 months. Inoculum was prepared by transferring the culture into a medium composed of 1 per cent each of tryptone and yeast extract, 0.5 per cent K_2HPO_4, and 0.1 per cent glucose. After 4 hours' incubation at 37 C, transfer was made to a second tube, which after 8- to 10-hour incubation was diluted with 20 parts of sterile distilled water and used at the rate of 0.05 ml per 10 ml of medium. If the cultures were transferred at 12-hour intervals for more than 72 hours, the growth rate in the synthetic medium decreased; therefore the culture was removed from stock every other day.

Cell suspensions. Cell suspensions were prepared from 12-hour cultures as suggested by Miller (1942)—8- to 14- hour cells gave satisfactory rates of pyruvate oxidation. The cells were harvested by centrifugation, washed once with $\frac{1}{3}$ the growth volume of M/30 phosphate buffer, pH 6.5, and resuspended in $\frac{1}{10}$ the growth volume of the same buffer.

Solutions of vitamins, accessory substances, and acid-hydrolyzed vitamin-free casein were prepared and stored as suggested by Williams and coworkers (1941). Enzyme-hydrolyzed casein (strepogenin), shown by Wright and Skeggs (1944) to stimulate the growth of several strains of streptococci including 10C1, was prepared as suggested by Roberts and Snell (1946).

The rate of oxidation expressed as Q_{O_2} (N)—microliters of oxygen taken up per hour per mg bacterial nitrogen—is based on the linear portion of the curve for the oxygen uptake, taken at 10-minute intervals for 40 minutes after the addition of substrates. The quantity of cells was estimated turbidimetrically from a previously calibrated curve.

Attempts to replace the yeast extract tryptone medium of Miller (1942) with a synthetic medium, such as that of Bellamy and Gunsalus (1945), resulted in poorer growth and cells nearly devoid of pyruvate oxidase. It was shortly found, however, that the addition of yeast extract to the Warburg cups would stimulate pyruvate oxidation by the cells from the synthetic medium. In order to obtain rapid pyruvate oxidation upon stimulation, several adjustments in the composition of the medium were necessary.

The most suitable medium found for the production of pyruvate oxidase apoenzyme is shown in table 1. The more pertinent alterations necessary to produce good growth from the synthetic medium for tyrosine apoenzyme production were higher purine and pyrimidine levels and the addition of strepogenin. The tyrosine decarboxylase medium was devised for *Streptococcus faecalis*, strain

TABLE 1

Medium for the production of pyruvate oxidase apoenzyme containing cells

INGREDIENT	QUANTITY PER 100 ML
Acid-hydrolyzed casein	1.0 g
K_2HPO_4	0.5 g
Glucose	0.3 g
Strepogenin	1,500.0 mg
Tryptophan	10.0 mg
Cystine	20.0 mg
Sodium thioglycolate	10.0 mg
Adenine sulfate	2.5 mg
Guanine hydrochloride	2.5 mg
Uracil	2.5 mg
Salts B	0.5 ml
Nicotinic acid	500.0 μg
Riboflavin	100.0 μg
Calcium pantothenate	100.0 μg
Pyridoxine hydrochloride	100.0 μg
Thiamine hydrochloride	100.0 μg
Folic acid	1.0 μg
Biotin	0.1 μg
pH	7.0–7.3

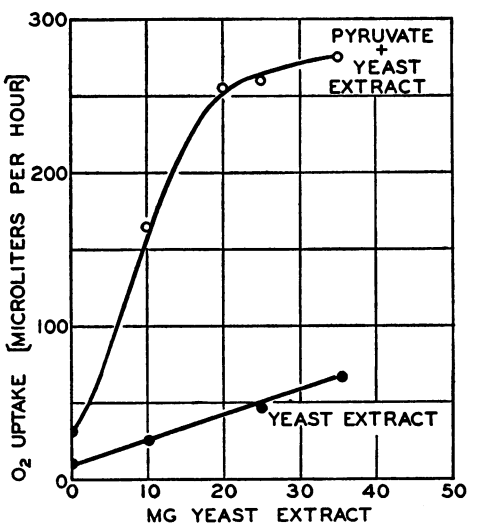

Figure 1. Activation of pyruvate oxidase apoenzyme by yeast extract. Per Warburg cup: 0.5 ml cell suspension (≈ 0.2 mg bacterial N); 2.0 ml M/20 phosphate buffer pH 6.5. Side arm: 0.2 ml M/10 pyruvate; water or factors to 0.5 ml.

R, which did not require strepogenin. In order to obtain the maximum apopyruvate oxidase content, levels of riboflavin, nicotinic acid, and pyridoxine were increased above those required for maximum growth, as were levels of sodium

acetate and of strepogenin. The relationship of these factors to the enzyme production is not clear beyond the previous suggestions of Lipmann (1939) that flavin is involved in pyruvate oxidation. The effect of the nicotinic acid level may be general, since it was also observed with tyrosine apoenzyme production (Bellamy and Gunsalus, 1944). The effect of pyridoxine may well be on amino acid formation (Umbreit, Wood, and Gunsalus, 1946; Lichstein, Gunsalus, and Umbreit, 1945) and protein synthesis (Stoerk and Eisen, 1946).

The stimulation of pyruvate oxidase apoenzyme by yeast extract is shown in figure 1. The rate of pyruvate oxidation by cells from the synthetic medium, here shown as 30 μl per hour, varies from one batch to another, apparently depending upon the completeness of removal of the pyruvate oxidase factor from the strepogenin preparation. Most samples of the medium yield cells in-

TABLE 2

Stimulation of pyruvate dismutation by yeast extract factor

Conditions: 0.2 mg bacterial nitrogen per cup. 2.0 ml M/30 phosphate buffer, pH 6.0. 0.2 ml M/10 pyruvate + additions as indicated tipped at 0 time. Atmosphere—N_2.

	μL CO_2 PER HOUR
Endogenous, cells alone	8
Pyruvate, 20 μM	8
Pyruvate + yeast extract, 20 mg	128
Yeast extract, 20 mg	12
Pyruvate + fraction C_{12} (potency 28) 0.5 mg	65
C_{12} (potency 28) 0.5 mg	5
Pyruvate + fraction 19-11 (potency 200) 75 μg	55
Fraction 19-11 (potency 200) 75 μg	5

capable of oxidizing pyruvate. The cells used in the experiment indicated showed 10-fold stimulation in activity upon the addition of yeast extract, with a maximum of about 300 μl per hour by the cells from 5 ml of medium. With yeast extract as the starting material, partial purification of the stimulant has been accomplished. These fractions were used in several experiments because they are free of an oxidizable material present in yeast extract (figure 1).

The pyruvate oxidase can, as shown by Lipmann (1939) and others, be linked via lactic dehydrogenase with a second molecule of pyruvate to yield lactic and acetic acids and CO_2. If the stimulatory effect of the yeast extract were more or less directly on the pyruvate oxidase, the cells containing the apoenzyme should also show a decreased rate of dismutation that could be stimulated by yeast extract. As shown in table 2, the deficient cells, which were unable to dismutate pyruvate, were activated by yeast extract or by the partially purified fractions. Cells from the synthetic medium are not devoid of oxidative power since they oxidize glucose rapidly and are not further stimulated by yeast extract. How-

ever, the pyruvate oxidation rate approaches that of glucose only in the presence of yeast extract (table 3).

In attempts to analyze the mechanism of the stimulatory action, deficient cells were incubated with yeast extract and both the solution and the cells assayed for the factor. It was found that some of the factor was stored by the cells since they exhibited an increased rate of pyruvate oxidation (table 4). Incubation of cells with pyruvate and yeast extract, under conditions that allowed pyruvate oxidation, resulted in the removal of nearly all of the stimulant from the supernatant and in a still greater rate of pyruvate oxidation by the cells. Not all the

TABLE 3

The specificity of the yeast extract stimulation for pyruvate oxidation

SUBSTRATE	Q_{O_2} (N)
Pyruvate (20 μM)	30
Glucose (20 μM)	580
Yeast extract (20 mg)	70
Pyruvate + yeast extract	480

TABLE 4

Absorption of the factor by cell suspensions

	Q_{O_2} (N)	Q_{O_2} (N) ON PYRUVATE* AFTER 30-MINUTE OXIDATION	
		Cells	Supernatant
Cells + pyruvate	65	28	65
Cells + yeast extract	25	125	230
Cells + yeast extract + pyruvate	340	165	80

* Experiment as in table 3; then the cells were collected by centrifugation and resuspended in buffer and pyruvate; supernatant was returned to Warburg cup and new cells were added.

activity removed from solution, however, is found in the cells; this may be due to the destruction of the factor by hydrogen peroxide formed from pyruvate oxidation.

The accumulation of the factor in the cells may indicate a coenzyme function, the greater absorption during pyruvate oxidation suggesting a possible energy requirement for the use of the factor. The removal of the activator from solution by the cells serves to differentiate this substance from the amino acid stimulation of pyruvate oxidation by pneumococci as demonstrated by Bernheim and Bernheim (1943).

Several compounds of known physiological activity were tested either by incorporation in the growth medium or by addition to cell suspensions (table 5). Those added to the growth medium without effect included choline, inositol,

thymine, xanthine, fumarate, citrate, and oleic acid. No evidence of stimulation was found by either procedure with the compounds tested. Since neither amino acids, yeast ash, nor known factors replace the yeast extract, it is suggested that an unknown factor is involved.

TABLE 5

Known substances tested for pyruvate oxidase stimulation with resting cells

	mg/cup*
DL-Leucine	0.6
DL-Phenylalanine	0.6
DL-Methionine	0.6
Cysteine	1.0
Glutathione	1.0
Acid-hydrolyzed casein	30.0
Wright's strepogenin	15.0
Yeast ash	≈100 mg yeast extract
Folic acid	0.03
SLR factor	0.003
Pyridoxal	0.01
Mixture of vitamins†	
Mixtures of these vitamins with each omitted in turn	
Coenzyme A preparation‡	0.50
Coenzyme A preparation hydrolyzed§	0.50

* Incubated at 37 C with cells 20 minutes before addition of pyruvate.

† Contained 6 μg each of pyridoxine, thiamine, riboflavin, nicotinic acid, pantothenic acid, folic acid, and 60 mμg biotin.

‡ Kindly supplied by Dr. N. O. Kaplan, 25 to 50 per cent pure.

§ Fifteen minutes at 120 C in N/2 H_2SO_4.

DISCUSSION

The observation of more specific nutritive requirements for pyruvate oxidation than for growth, and the stimulation of the pyruvate oxidation rate of cell suspensions, appears to constitute another case of the formation of an apoenzyme during growth. Similar cases are the formation of tyrosine apodecarboxylase (Bellamy and Gunsalus, 1945) and apotransaminase (Lichstein, Gunsalus, and Umbreit, 1945).

The activation of pyruvate oxidation by resting cells, and dried cell preparations, with yeast extract though not with the known substances tested indicates that an unidentified factor may be involved. The coenzyme nature of the activating agent is suggested by its removal from solution and storage in the cells during pyruvate oxidation. It is, however, possible that the factor is an active form of one of the known factors that the cells are unable to synthesize. The mechanism of action must await the identification of the active agent(s) and a study of the enzyme system involved.

Since several pyruvate oxidation enzymes have been reported (Lipmann, 1939; Still, 1941; Hills, 1943; Stumpf, 1945), speculation as to the mechanism of the action would be premature.

SUMMARY

With strain 10C1 of *Streptococcus faecalis*, the nutritive requirements for pyruvate oxidation and dismutation were found to be more specific than those for growth. Cells harvested from synthetic media that support good growth contain pyruvate oxidase in the apoenzyme form, as indicated by the activation of oxidation by cell suspensions and dried cell preparations.

Yeast extract contains a substance (or substances), apparently not identical with known accessory factors, that is required for pyruvate oxidation. This is demonstrated by the inability of cell suspensions to oxidize pyruvate unless they are grown in the presence of yeast extract, or unless yeast extract is added to the cell suspensions. The active principle is taken up by the cells during pyruvate oxidation and is retained, thus indicating the possibility of a coenzyme nature.

The oxidative deficiency is not general since the deficient cells oxidize glucose rapidly.

A synthetic medium has been obtained that supports good growth and highly active pyruvate oxidase apoenzyme production.

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