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Role of Pyruvate and *S*-Adenosylmethionine in Activating the Pyruvate Formate-Lyase of *Escherichia coli*[1]

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The pyruvate formate-lyase activity of extracts of *Escherichia coli* is stimulated and the dilution effect is abolished by the addition of pyruvate to the extract. The activity can be purified fourfold from pyruvate-supplemented extracts by isoelectric precipitation under anaerobic conditions. The activity of extracts not supplemented with pyruvate has been separated into two fractions by treatment with protamine sulfate—fraction PS, the soluble portion, and fraction N, an extract of the precipitate formed upon the addition of protamine sulfate. After treatment of these fractions with charcoal, pyruvate formate-lyase activity is stimulated by the addition of *S*-adenosylmethionine. When sodium pyruvate is added to the crude extract before the fractionation, fraction PS has full enzymatic activity and is not stimulated by fraction N or by *S*-adenosylmethionine. Incubation of the inactive fractions with pyruvate and *S*-adenosylmethionine in the absence of other substrates similarly results in a highly active preparation, not subject to the "dilution effect" obtained when the fractions are added separately to the assay. These observations suggest that the component in the protamine supernatant fraction is activated by the other fraction and that *S*-adenosylmethionine and pyruvate are required for the activation reaction. The activating factor present in the protamine precipitate fraction may be further purified by heating for 10 min at 100 C under H_2 atmosphere. The yield of this factor from crude extract is not affected by activation of the pyruvate formate-lyase of the extract, indicating that the factor acts catalytically. The requirement for pyruvate is only partially satisfied by α-ketobutyrate and not at all by other α-keto acids, acetyl phosphate, or adenosine triphosphate. The rate of activation is maximal at 0.01 M sodium pyruvate and 3×10^{-4} M *S*-adenosylmethionine; it is linearly dependent on the amount of activating factor added. The rate of activation is the same when the activation reaction is initiated by addition of any of the four required components, indicating that no slow step of activation can be carried out by any three of the components. A similar pyruvate formate-lyase system was found in extracts of the methionine/B_{12} autotroph 113-3, grown with methionine supplement, indicating that vitamin B_{12} derivatives do not participate in the system.

The pyruvate formate-lyase activity discovered by Kalnitsky and Werkman (10) is stimulated by a variety of known cofactors, cell extracts, and incubation procedures, even when using unfractionated cell extracts. [Traditional terms for the pyruvate-lyase activity do not indicate that formate is a product of the "pyruvate clastic reaction," in contrast to the "phosphoroclastic" or "pyruvate clastic" systems of clostridia, which produce CO_2. The term "phosphoroclastic" is a misnomer, since acetyl coenzyme A (CoA) is the initial product (5), and phosphate enters only at the phosphotransacetylase reaction. We have, therefore, tentatively used the term "pyruvate formate-lyase."] Lipmann and Tuttle (13) demonstrated that maximal rates of pyruvate dissimilation by extracts of *Escherichia coli* were obtained only after prolonged incubation of extracts under the assay conditions. The induction

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period was abolished by the addition of boiled rat liver preparation (13). The pyruvate-formate exchange activities of *E. coli* (G. D. Novelli, H. Gest, and L. O. Krampitz, Bacteriol. Proc., p. 97, 1954), *Clostridium butyricum* (17), and *Micrococcus lactilyticus* (15, 17) show disproportionately low activity with low amounts of extract, a phenomenon termed "dilution effect." The *E. coli* system has also been reported to be stimulated by boiled extract of yeast (1, 24), and both the *E. coli* and *M. lactilyticus* systems are stimulated by a heated (65 C) extract of these bacteria (1, 16) as well as by preparations derived from bacteria that do not carry out the pyruvate-formate exchange reaction (16; Novelli et al., Bacteriol. Proc., p. 97, 1954). More recently, a requirement for three protein factors and a low molecular weight cofactor has been reported for both exchange and overall reactions with *E. coli* (G. G. Midwinter, H. Nakayama, and L. O. Krampitz, Federation Proc., p. 531, 1965), and Knappe et al. (8) have found two protein factors and *S*-adenosylmethionine to be required.

In contrast to the results of Midwinter et al. (Federation Proc., p. 531, 1965) and Knappe et al. (8), we did not observe resolution of the system into two fractions, nor did we observe a requirement for a low molecular weight cofactor, other than coenzyme A (CoA), for the overall reaction, even after isoelectric precipitation of the enzyme and filtration through Sephadex G-25. It seemed possible that the difference observed might result from the fact that we routinely add sodium pyruvate to extracts because this stimulates activity and abolishes the "dilution effect." Consequently, we sought to reproduce the results of Knappe et al. (8) and to study the effect of the addition of pyruvate to the extracts.

MATERIALS AND METHODS

Materials. Acetyl phosphate dilithium salt, *S*-adenosyl-L-methionine iodide (SAM), adenosine triphosphate (ATP) disodium salt, and diphosphopyridine nucleotide (DPN) were obtained from Sigma Chemical Co., St. Louis, Mo. Lithium hydroxypyruvate (recrystallized from hot water), sodium pyruvate, sodium α-ketovalerate, dimethyl-β-propiothetin chloride, and *S*-methylmethionine chloride were products of Nutritional Biochemicals Corp., Cleveland, Ohio. Vitamin B_{12} (cyanocobalamin) was a product of Merck & Co., Inc., Rahway, N.J. ^{14}C-sodium formate, diphosphothiamine, 1,4-dithiothreitol (DTT), and vitamin K_5 (4-amino-2-methyl-1-naphthol-chloride) were obtained from Calbiochem, Los Angeles, Calif. CoA was obtained from Boehringer & Soehne, Mannheim, Germany, and from Calbiochem. Protamine sulfate was obtained from Eli Lilly & Co., Indianapolis, Ind. Spermine tetrahydrochloride was purchased from Mann Research Laboratories, New York, N.Y. Sodium α-ketobutyrate and α-ketoglutaric acid were generous gifts of E. E. Snell. H. A. Barker and Robert Ronzio generously provided Factor B (cobinamide) and acetoin. Other chemicals were purchased from usual commercial sources; complex materials for bacterial culture were products of Difco Laboratories. Thiol-Sephadexes were prepared by the method of Eldjarn and Jellum (6).

Enzymes. Lactate dehydrogenase (EC 1.1.1.27) was purchased from Boehringer & Soehne. Formyltetrahydrofolate synthase (EC 6.3.4.3) for formate assays was purified from *C. cylindrosporum* or *C. acidi-urici* (21). Phosphotransacetylase (EC 2.3.1.8) was purified from extracts of *C. butyricum* or *E. coli* by procedures to be described elsewhere.

Bacterial strains. E. coli E-26 [Werkman strain (10)] was obtained originally from the American Type Culture Collection (11097); except where noted, these studies utilized extracts of this strain. We are grateful to Charles Yanofsky for the wild-type K_{12} strain of *E. coli* and to H. A. Barker for the methionine/B_{12} auxotrophic strain 113-3.

Growth of cells and preparation of extracts. E. coli E-26 was routinely grown on 0.4% peptone–0.4% beef extract–0.2% yeast extract–0.2% NaCl, as originally described (10), in the 14-liter glass tanks of a New Brunswick fermentor, each tank containing 11.3 liters of medium. Each tank was inoculated with a 300-ml shake flask of overnight culture, incubated at 37 C, and aerated at a rate of 700 ml/min. Growth under fully anaerobic conditions did not increase the pyruvate formate-lyase activity in extracts, although hydrogenase and formic dehydrogenase, induced under these conditions (18), increased 50- to 100-fold. Growth at high aeration (12 liters per min) decreased the pyruvate formate-lyase activity by a factor of three; addition of 1% glucose to the medium doubled activity. In some studies, we used a glucose-salts medium supplemented, when indicated, with DL-methionine (250 μmoles/liter) or vitamin B_{12} (1 μg/liter). Growth of cultures was followed by determination of the optical density at 660 nm in a Coleman Junior spectrophotometer. When the culture was essentially stationary (increase less than 0.01 optical density units per hr, generally after 18 to 24 hr), cells were harvested with a Sharples continuous-flow centrifuge. The cell yield under these conditions was typically 2.2 g (wet weight) of cells per liter. They were washed once with distilled water and suspended in 0.05 M potassium phosphate (*p*H 7.0) or 0.05 M sodium dimethylglutarate (DMG), pH 6.8, with 1.5 ml of buffer per g (wet weight) of cells. Occasionally, the buffers were supplemented with 20 mM DTT and 2×10^{-5} mM Factor B and/or 10 mM sodium pyruvate and 2 mM sodium formate. Extracts were prepared by treating 50- to 75-ml samples of the cell suspension in a Raytheon 10-kc sonic oscillator for 10 min. The cell debris was removed by centrifugation for 20 min at 96,000 $\times$ *g* in a Beckman/Spinco preparative ultracentrifuge. The supernatant fraction, referred to as crude extract or S, was stored at -20 C in filled polypropylene tubes capped with rubber

serum stoppers so that extract, removed by means of a hypodermic syringe, could be replaced by an N_2 or H_2 atmosphere.

Enzyme assays. Pyruvate formate-lyase was assayed both by exchange of ^{14}C-formate with the carboxyl group of pyruvate and by measurement of the overall reaction (formation of acetyl phosphate and/or formate). The assay mixture for the exchange reaction (total volume 1.0 ml) contained 50 mM potassium phosphate buffer, *p*H 7.6; 50 mM sodium pyruvate; 10 mM ^{14}C-HCOONa, containing 5,000 counts/min; 5 mM DTT; and 2×10^{-5} mM Factor B. For the overall reaction, the sodium formate was omitted and 0.125 mM CoA and 10 Bergmeyer units (3) of *E. coli* phosphotransacetylase or 2 units of the *C. butyricum* enzyme were added. In some cases, the overall assay conditions of Knappe et al. (8) were used. The assay mixture (1 ml) contained: 75 mM potassium phosphate (*p*H 7.5), 30 mM sodium pyruvate, 10 mM $MgCl_2$, 6 mM $Fe(NH_4)_2(SO_4)_2$, 0.4 mM diphosphothiamine, 0.2 mM CoA, and 10 units of *E. coli* phosphotransacetylase. Use of this assay was obligatory with extracts of *E. coli* K-12, whose pyruvate formate-lyase activity, unlike that of strains E-26 and 113-3, appeared to be inhibited almost completely by the concentration of DTT used in the standard assay. The activity of cell extracts of strains E-26 and 113-3 was about twice as great under our standard conditions as under those of Knappe et al. (8).

In all cases, the assay tubes (10×75 mm) containing all components except the pyruvate formate-lyase were stoppered with assemblies consisting of a silicone rubber gasket (Wilkens Instrument and Research Co., Walnut Creek, Calif.) held in a polyethylene adaptor. Nitrogen gas (Matheson Co., Inc., Rutherford, N.J.; prepurified grade) was passed through a long hypodermic needle inserted through the gasket to the bottom of the tube; a short needle also inserted through the gasket allowed escape of gas. After 10 to 15 min of bubbling with N_2, the needles were removed; the enzyme was injected by means of a Hamilton microsyringe, and the tubes were placed in a bath (37 C). After incubation for 30 min, the tubes were removed from the bath and unstoppered. The exchange reaction was stopped by addition of 0.1 ml of 20% perchloric acid, and pyruvate was isolated as the 2,4-dinitrophenylhydrazone and counted in a Nuclear-Chicago gas-flow low-background counter, as described previously (20). The overall reaction was stopped by addition of 0.5 ml of neutralized hydroxylamine and acetyl hydroxamate determined according to Lipmann and Tuttle (14); in some cases a sample (usually 20 μliters) was removed prior to addition of hydroxylamine and used for determination of formate with formyltetrahydrofolate synthetase (19). Production of acetyl phosphate and formate and disappearance of pyruvate [measured by a modification of the method of Friedemann and Haugen (7)] were approximately stoichiometric (the initial pyruvate concentration was reduced to 15 mM for these measurements). Under the above conditions, Michaelis-Menten constants in the exchange reaction were 3.4×10^{-4} M for pyruvate and 0.017 M for formate; Michaelis-Menten constants in the overall reaction were 1.2×10^{-3} M for pyruvate and 2.4×10^{-5} M for CoA. When acetyl hydroxamate precursor and formate had accumulated to a level of 3 mM, further production was inhibited, due to the unfavorable equilibrium constant of the phosphotransacetylase reaction and consequent lowering of the CoA concentration by accumulation of acetyl-CoA. Therefore, when high levels of enzyme were used (as when the reaction was followed through 30 min), phosphate buffer was replaced by the same concentration of arsenate and formate was determined. Under these conditions, the K_m for CoA was 8×10^{-6} M.

To correct for mechanical losses during isolation and washing, incorporation of ^{14}C-formate into pyruvate was determined as counts per minute per milligram of isolated dinitrophenylhydrazone, with correction for the varying self-absorption of various amounts of the precipitate. One unit of exchange activity incorporates 1 μmole of formate per 30 min at 37 C (28.6 counts per min per mg of dinitrophenylhydrazone. A unit of overall activity forms 1 μmole of acetyl phosphate or formate in 30 min.

Noncontribution of pyruvate dehydrogenase to acetyl phosphate formation. If acetyl phosphate formation is to be considered a valid assay for pyruvate formate-lyase, it is necessary to exclude contribution of acetyl phosphate by the pyruvate dehydrogenase complex. For this reason, the crude extract was also assayed under conditions described by Reed et al. (22) for the assay of pyruvate dehydrogenase. Conditions for this assay differ from those described for the assay of pyruvate formate-lyase in two ways: the presence of added DPN and lactate dehydrogenase, and relatively aerobic conditions (6.4×10^{-3} M cysteine, under air). The formation of acetyl phosphate under the conditions of the pyruvate dehydrogenase assay was about one-tenth of the amount formed under the conditions used for the determination of pyruvate formate-lyase activity (Table 1). As indicated by the absence of formate formation, pyruvate formate-lyase is not active when DTT, Factor B, and N_2 atmosphere are omitted. Pyruvate dehydrogenase is not significantly active in the absence of DPN and lactate dehydrogenase, since no significant quantity of acetyl phosphate was formed in the absence of DPN, lactate dehydrogenase, DTT, and Factor B. Therefore, the latter system does not contribute to acetyl phosphate formation under the assay conditions for pyruvate formate-lyase used in this investigation.

Fractionation of extracts. Crude cell extracts were fractionated by a slight modification of the procedure of Knappe et al. (8). A sample of extract (5 to 8 ml), in a 10-ml polypropylene centrifuge tube under N_2 or H_2 atmosphere, stoppered with a rubber serum cap, was adjusted to *p*H 6.0 with 8 M HCOONa buffer (*p*H 3.5) through the use of bromocresol purple as an external indicator. A solution of protamine sulfate, containing 10 or 25 mg of solution per ml in 0.05 M potassium phosphate or potassium DMG buffer at *p*H 6.0, was added to give a concentration of 0.2 mg of protamine sulfate per mg of protein, with DTT and Factor B added if the extract contained them. After

TABLE 1. *Comparison of activities of pyruvate dehydrogenase complex and pyruvate formate-lyase in extract of E. coli E-26*[a]

Assay system	Activity (units/mg)	
	Acetyl phosphate formation	Formate formation
Complete pyruvate dehydrogenase system	2.26	0
Complete minus DPN, lactate dehydrogenase	0.15	0
Complete minus CoA, phosphotransacetylase	0.34	0
Complete pyruvate formate-lyase system	24.1	21.7
Complete, aerobic	11.0	9.05
Complete, aerobic, cysteine replacing DTT + Factor B	0.18	0
Complete, aerobic, cysteine, + DPN + lactate dehydrogenase	4.4	0

[a] The assay system (1.0 ml) for pyruvate dehydrogenase complex (22) contained the following components (mM): potassium phosphate buffer, *p*H 7.0, 100; sodium pyruvate, 50; cysteine·HCl, 6.4; CoA, 0.1; DPN, 0.23; diphosphothiamine, 0.2; $MgCl_2$, 4; lactate dehydrogenase, 1 unit; *E. coli* phosphotransacetylase, 4 units; atmosphere air. Crude extract, S, containing 32.7 mg of protein/ml, was assayed under these conditions and under the standard assay conditions for pyruvate formate-lyase, incubating both assay mixtures for 30 min at 37 C. In changing the pyruvate formate-lyase assay system, stoppers were omitted ("aerobic") and cysteine·HCl, DPN, and lactate dehydrogenase were added in the concentrations used in the pyruvate dehydrogenase system.

15 min at 0 C, the mixture was centrifuged for 10 min at 39,000 $\times g$. The supernatant fraction (fraction PS) was removed and stored under N_2; the precipitate was taken up with two-fifths of the original extract volume of 0.2 M potassium phosphate buffer (*p*H 7.0) with DTT and Factor B added if the original extract contained them. After 10 min at 0 C, this mixture was centrifuged for 10 min at 39,000 $\times$ *g*; this supernatant fraction (fraction N) was set aside under N_2 or H_2. Both fractions were routinely stored in liquid nitrogen, in which they were stable for months.

In a few early experiments, the exact procedure of Knappe et al. (8) was followed; it differed from the above in that 0.23 mg (rather than 0.2 mg) of protamine sulfate was added per mg of protein of the extract, and the protamine precipitate was eluted with 0.1 M potassium phosphate, *p*H 7.0 (rather than 0.2 M). In the former case, the activity of fraction PS was sometimes considerably reduced, in the latter case, less activation activity was recovered in fraction N. Potassium DMG buffer (0.2 M) was also tried as an eluant of the protamine precipitate; little activation activity was eluted unless the DMG was supplemented with 0.2 M K_2SO_4, and in this case the activity was less stable to heat than in phosphate buffer.

For further purification of the activating factor, fraction N was placed in a Pyrex centrifuge tube under N_2 or H_2 atmosphere; the tube was placed in a bath (100 C) for 10 min, removed, cooled to 0 C, and centrifuged for 30 min at 30,000 $\times$ *g*. The supernatant fraction is "heated fraction N."

After addition of sodium pyruvate (10 mM) to crude extract, some purification of the activated enzyme could be achieved by treatment with reagents precipitating nucleic acids and isoelectric precipitation of the activity. In a typical experiment, spermine·4HCl (30 mg in 0.3 ml of sodium DMG buffer, supplemented with DTT, Factor B, sodium pyruvate, and sodium formate, as described above) was added to 10 ml of crude extract in a centrifuge tube under N_2 and the solutions were mixed by inversion. After 15 min, the mixture was centrifuged for 15 min at 39,000 $\times$ *g* and the supernatant fraction was transferred by means of a hypodermic syringe to a second tube with N_2 atmosphere. Small samples of 8 M HCOONa buffer (*p*H 3.5) were added by microsyringe with mixing by inversion until the *p*H had been lowered to 5.1. Chlorphenol red was used as an external indicator. After 15 min, the mixture was centrifuged for 20 min at 39,000 $\times$ *g*, the precipitate was discarded, and the supernatant solution was transferred to a third tube. The *p*H was adjusted to 4.5 (bromocresol green as external indicator). After 15 min, it was centrifuged for 10 min at 39,000 $\times$ *g* and the supernatant solution was removed and replaced with N_2. The precipitate was taken up in 1.5 ml of sodium DMG buffer (*p*H 6.8), supplemented with DTT, Factor B, sodium pyruvate, and sodium formate, as described above, and brought to *p*H 7 with concentrated NH_4OH ("*p*H 4.5 precipitate"). The purification is summarized in Table 2. Similar results were achieved with equal amounts (by weight) of protamine sulfate in place of spermine·4HCl. $MnCl_2$ ($\frac{1}{20}$ volume of 1.0 M) gave poor recovery in the isoelectric precipitation. When the *p*H 4.5 precipitate was taken up under air, only 7% of the initial activity was recovered, indicating the extreme lability of the enzyme to air oxidation. Considerable difficulty was experienced in repeating the acid precipitation step until it was found that fresh unfrozen extracts must be used. The nonactivated enzyme in fraction PS (pyruvate not added to the extract) could not be purified successfully by this procedure.

Attempts to purify the enzyme further by using gel adsorption, diethylaminoethyl-cellulose chromatography, and solvent precipitation were unsuccessful. Some fractionation was achieved by gel filtration with Sephadex G-150 (containing about 25% thiol-Sephadex G-150), and the exchange and overall activities chromatographed together in this procedure; however, 65% of the activity of the active fractions was lost in 4 hr at 0 C or in 24 hr at −196 C, even when stored under N_2 atmosphere. The *p*H 4.5 precipitate could, however, be filtered through thiolated (6)

TABLE 2. *Purification of pyruvate formate-lyase*

Fraction	Protein (mg/ml)	Acetyl phosphate formation			Exchange activity		
		Units/mg	Total units	Yield (%)	Units/mg	Total units	Yield (%)
Crude extract, 10.1 ml	41.9	33.2	14,050	100	4.8	2,080	100
Spermine supernatant fluid, 9.9 ml	38.9	41.1	15,840	113	7.8	2,980	143
*p*H 4.5 precipitate, 2.25 ml	41.7	113.2	10,625	76	21.8	2,040	98

Sephadex G-25 without loss of activity if collected under N_2 atmosphere.

Charcoal treatment. Acid-washed charcoal (50 mg; Norite) was placed in a 3-ml polypropylene centrifuge tube. The tube, capped with a serum stopper, was evacuated and filled three times with H_2 by means of hypodermic needles. Fraction PS (1.0 ml) or 0.5 ml each of fractions PS and N were injected and mixed with the charcoal. After 10 min at 0 C, the mixture was centrifuged for 5 min at 39,000 × *g*; the supernatant fraction was removed by means of a hypodermic syringe and stored under N_2 or H_2.

Assay of activation activity. The factor responsible for activation of pyruvate formate-lyase ("activation factor") was assayed as follows. Fraction PS (50 μliters), 1 μmole of sodium pyruvate, and 0.02 μmole of SAM were injected into a glass tube (6 × 50 mm) under H_2 atmosphere (serum stoppered). When less than 50 μliters of fraction N was to be added, sufficient buffer (0.2 M potassium phosphate, *p*H 7.0, 0.02 M in DTT and 2×10^{-8} M in Factor B) was added so that the volume (after addition of fraction N) was 0.103 ml. The fraction PS used in these experiments was a standard preparation stored in liquid nitrogen. Its activity without activation was 0 to 50 units per ml; its activity after full activation was 2,000 to 2,500 units per ml. Fraction N was added to initiate activation; the tubes were incubated at 25 C; and, at suitable times after addition of fraction N (usually 1, 2, 4 min), samples were removed by means of a microsyringe and injected into standard assay tubes for measurement of pyruvate formate-lyase activity. A unit of activating factor activity ("activation unit") is defined as that amount required to activate 50 μliters of fraction PS at a rate of 20 acetyl phosphate formation units per min at 25 C. Thus, if 25 μliters of fraction N activated 50 μliters of fraction PS at a rate of 150 acetyl phosphate formation units per min, fraction N had an activity of 300 activation units per ml.

The potential activity of fraction PS was assayed by preincubating a 50-μliter sample for 10 min under the same conditions used for assay of the activating factor, using 20 to 25 activation units of fraction N, and adding samples of the incubation mixture to standard assay tubes for measurement of pyruvate formate-lyase activity. "Incubation-1," in Fig. 6 and 8, refers to the incubation described in the above paragraphs.

Other procedures. Protein was routinely estimated by the phenol method of Sutherland et al. (25) as modified in this laboratory (21). Buffers for enzyme purification were bubbled with N_2 or H_2 before use and stored under rubber serum stoppers for withdrawal by hypodermic syringe. Buffers for preparation of cell extracts were brought to a boil, then cooled while being bubbled with N_2 or H_2; labile components (sodium pyruvate, DTT) were added after cooling to 0 C. In Sephadex G-25 filtration of the crude extract, collection of eluate was begun at the first appearance of cloudy, colored proteinaceous material.

RESULTS

Effect of adding pyruvate and formate to extract. Crude extracts prepared with unsupplemented DMG buffer showed a disproportionately low activity with low amounts of extract ("dilution effect") and were largely or completely inactivated by passage through Sephadex G-25. The effect of the substrates on the stabilization of the enzyme activity was therefore examined. As shown in Fig. 1, addition of pyruvate to extract increased pyruvate formate-lyase activity considerably with abolition of the "dilution effect," but did not protect against loss of activity on passage through Sephadex G-25; formate did not stimulate the activity, but did protect the activity against loss on passage through Sephadex. Phosphate, acetate, carbonate, and formamide were unable to substitute for formate in protection. This protective effect was maximal with 2 mM formate. Consequently, many extracts were prepared with buffer 10 mM in sodium pyruvate and 2 mM in sodium formate.

Formate could be replaced by 1 mM vitamin K_5 or 40 mM DTT plus 2×10^{-5} mM Factor B. Formate did not protect effectively after particulate material had been removed by high-speed centrifugation (4 hr at 140,000 × *g*). This suggests that a component of the particulate material is responsible for the protective effect of formate, rather than a direct action of formate itself on pyruvate formate-lyase. A requirement for a particulate factor for the pyruvate formate-lyase reaction has been reported (G. G. Midwinter, H. Nakayama, and L. O. Krampitz, Federation Proc., p. 531, 1965). We found no requirement for a particulate factor in assay except under aerobic conditions (tube stopper and N_2 bubbling omitted). It therefore seems

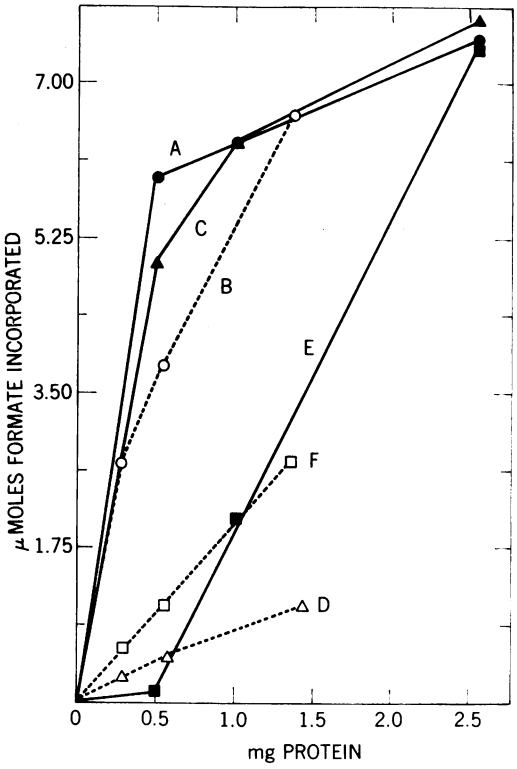

FIG. 1. *Effect of pyruvate and formate on activity and stability of pyruvate formate-lyase in crude extracts. Samples of crude extract, S, 56.5 mg of protein/ml, prepared with unsupplemented 0.02 M sodium DMG buffer (pH 6.8) were made 0.005 M in glutathione and 0.01 M in HCOONa and/or sodium pyruvate, as indicated. Portions (2.5 ml) of each sample were filtered through a Sephadex G-25 column equilibrated with 0.1 M sodium DMG, pH 6.8–0.005 M glutathione, supplemented with pyruvate and/or formate as appropriate; 2.5 ml of eluate was collected for each sample. Untreated (but supplemented) extracts (solid lines) and the Sephadex filtrates (dashed lines) were assayed by exchange. A (●) and B (○), pyruvate and formate added; C (▲) and D (△), pyruvate alone added; E (■) and F(□), formate alone added.*

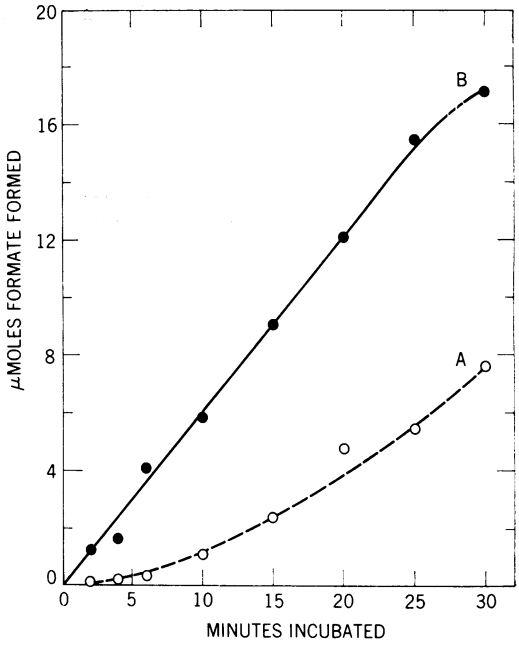

FIG. 2. *Effect of pyruvate activation on course of the pyruvate formate-lyase reaction. The crude extract (30 μliters containing 23 mg of protein) with and without supplementation with sodium pyruvate, was injected into assay tubes. Potassium arsenate, at 0.05 M, pH 6.8, replaced potassium phosphate in the assay. At the indicated times, the reaction was stopped and the formate concentration of diluted samples of the reaction mixture was measured. (A) Unsupplemented extract; (B) extract 0.01 M in sodium pyruvate.*

likely that the above effect of formate and the reported requirement for a particulate factor may be explained by action of a particulate formate dehydrogenase on the formate present as a substrate in the exchange assay to maintain anaerobic conditions.

With unsupplemented crude extract, the rate of acetyl phosphate production increased during the assay incubation (Fig. 2). Prior addition of pyruvate to the crude extract rendered the preparation capable of producing acetyl phosphate at a high, constant rate from the beginning of incubation.

Fractionation of extracts. Extract to which pyruvate has not been added can be fractionated with protamine sulfate to yield two fractions, both of which are required for pyruvate formate-lyase activity (Table 3, experiment 1). The addition of SAM to the assay has little effect unless fractions are treated with charcoal. Treatment with charcoal results in loss of virtually all activity, but activity can be recovered upon addition of *S*-adenosylmethionine to the assay system. Similar results were obtained with extracts of *E. coli* E-26 grown on synthetic medium and with *E. coli* K-12 grown on either synthetic or peptone medium.

This experiment confirms the results of Knappe et al. (8) and differs from our previous results, in which no requirement could be demonstrated for more than one protein factor or for any low molecular weight cofactor other than CoA, Mn^{++}, or diphosphothiamine. It seemed possible that this difference was due to our practice of adding sodium pyruvate to extracts.

TABLE 3. *Fractionation of extracts with protamine sulfate: effect of pyruvate*[a]

Expt	Fraction	Volume (ml)	Protein (mg/ml)	Activity (formate formation)			
				Without SAM		With SAM	
				Units/mg	Total units	Units/mg	Total units
1. Extract prepared with no additions	Extract	5.0	33.0	4.6	756	5.3	868
	PS	8.2	15.5	1.46	185	1.9	239
	N	2.0	9.5	0	0	0.6	11
	PS + N			4.25	540	5.8	733
	Charcoal treated PS + N	7.2	9.6	1.6	110	9.5	657
2. Extract prepared with added pyruvate	Extract	5.0	33.0	9.0	1,510	6.9	1,143
	PS	8.4	14.9	12.6	1,361	10.8	1,163
	N	1.95	8.7	2.3	39	1.95	33
	PS + N			8.7	932	13.5	1,449
	Charcoal treated PS + N	7.8	9.4	6.6	486	7.2	524

[a] The crude extract of *E. coli* E-26 cells grown on peptone medium was prepared in 0.05 M potassium phosphate buffer, *p*H 7.0. To one 5.0-ml sample of extract (expt 2) was added 50 μliters of 1 M sodium pyruvate. Fractions PS and N were then prepared from the untreated and pyruvate-treated samples by the exact procedure of Knappe et al. (8). Samples of the fractions obtained were combined and treated with charcoal. Pyruvate formate-lyase activity was assayed under the conditions of Knappe et al. (8), with or without addition of SAM (2×10^{-4} M). Activity in each fraction was calculated from the results obtained with the largest amount of the fraction used, since a pronounced "dilution effect" was displayed. When both fractions PS and N were added to the assay, results are expressed as units per milligram of protein of fraction PS. For the charcoal-treated samples, the volume and total units are expressed as if all of fraction PS had been used; units per milligram of protein was based on the observed protein concentration of the charcoal-treated fraction containing fraction N as well as PS.

The effect of the addition of pyruvate to the extract before fractionation was therefore examined (Table 3, experiment 2). The requirements for SAM and fraction N were abolished by this procedure. Furthermore, when the fractions prepared from extract not supplemented with pyruvate were first incubated together in the presence of pyruvate, the increase of activity during assay was abolished (Fig. 3, A). When the fractions were preincubated together in the absence of added pyruvate, there was an intermediate curve of increase of activity during the assay (Fig. 3, B), corresponding to the linear formation of product by a small amount of activated enzyme, plus activation of inactive enzyme during the assay (Fig. 3, C).

Further evidence that the pyruvate-activated enzyme no longer requires low molecular weight cofactors was suggested by the observation that, when crude extracts are filtered through Sephadex G-25, the pyruvate formate-lyase activity of the protein fraction is stimulated by the salt fraction; however, this requirement was abolished by addition of pyruvate and formate to the extract and to the buffer with which the column was equilibrated (Table 4). As previously demonstrated, pyruvate is the factor responsible for activation and formate protects against oxidative inactivation during the procedure.

Activation reaction. These results suggested that the "dilution effect" and the observed increase of activity during the assay of extracts not supplemented with pyruvate result from activation of an inactive form of pyruvate formate-lyase, in an enzymatic reaction for which pyruvate, SAM, and the factor in fraction N are required. Addition of pyruvate to the extract, where all other components are present in high concentration, would result in rapid activation to a high level of activity. Small amounts of extract would now show high activity without a "dilution effect" or autocatalytic activation (cf. Fig. 3, curve A).

The possible function of pyruvate as an energy source for formation of ATP used in the adenosylation of methionine was considered. However, the effect of ATP, with or without methionine, was slight compared to that of pyruvate, and SAM had even less effect (Fig. 4). In another experiment, higher concentrations of ATP and SAM were without any significant effect. It was therefore suggested that there is a specific requirement for pyruvate in the activation of pyruvate formate-lyase.

When the enzyme was activated in the crude

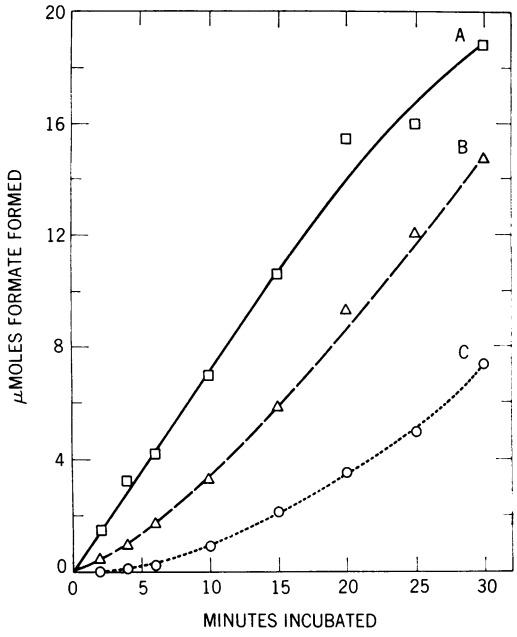

FIG. 3. *Effect of preincubation of fractions on the pyruvate formate-lyase reaction. Fraction PS (29.1 mg of protein per ml) and heated fraction N (4.5 mg of protein per ml) were prepared as described in Materials and Methods. Assay tubes were incubated at 37 C for the indicated time after adding enzyme. Potassium arsenate, at 0.05 M, pH 6.8, replaced phosphate; 0.2 mM SAM was also added to the standard mixture. (A) Equal volumes of fractions PS and N were first incubated for 15 min at 25 C with 10 mM sodium pyruvate and 0.2 mM SAM, under H_2 atmosphere, and samples equivalent to 25 μliters of each fraction were added to tubes. (B) Equal volumes of fractions PS and N were preincubated for 30 min at 25 C with 0.2 mM SAM and samples equivalent to 25 μliters of each fraction were added to the assay tubes. (C) Fractions PS and N (25 μliters each) were injected into assay tubes without prior incubation together. Points shown on curve B were determined experimentally; the curve is based on points derived by adding the observed values of C to a linear curve of 0.245 μmole of formate formed per min.*

extract by addition of pyruvate, no requirement for fraction N was seen after fractionation (Table 3), indicating that the active component in this fraction is required only for the activation reaction and can be described as an activating factor. The reaction occurring in the presence of this factor was linear with time after a short lag (Fig. 5); the lag was often not present at all. Therefore, the activating factor could be assayed by the rate of the activating reaction under these conditions. The rate of activation derived from the slope of the lines shown in Fig. 5, and corrected for the slight activation observed in the absence of added fraction N, is proportional to the amount of fraction N added. With 50 μliters of fraction PS, the rate was directly proportional to the amount of fraction N added up to about 15 activation units; however, decrease in the amount of fraction PS added resulted in a sharper decrease in the rate of activation (Fig. 6). The final activity of the preincubation depended only on the amount of fraction PS, being independent of the amount of fraction N added.

TABLE 4. *Effect of supplementation with pyruvate and formate on requirement for salt fraction after Sephadex filtration*[a]

Sephadex column supplements	Salt fraction	Protein	Exchange activity
		mg/ml	*units/mg*
None	−	41.7	0.
None	+	41.7	3.6
Pyruvate+ formate	−	39.0	7.8
Pyruvate+ formate	+	39.0	7.2

[a] Samples (2 ml) of the crude extract (50.8 mg of protein per ml) were filtered through a Sephadex G-25 column (1.2 × 10 cm) equilibrated with either 0.01 M sodium DMG buffer (*p*H 6.8) 0.005 M in glutathione, or this buffer supplemented with 0.01 M sodiumpyruvate and 0.01 M sodium formate. Fractions following the void volume were collected (five fractions of 0.88 ml each), and 0.1-ml samples were used in the determination of the pyruvate formate-lyase exchange activity with and without the addition of 0.1 ml of the "salt fraction." This fraction was obtained from the filtration without the addition of pyruvate or formate in the volume eluting from 6.1 to 7.4 ml after the void volume. It contained 0.9 mg of protein per ml. Results are for the activity of fraction 2 of each filtration; similar results were obtained with fractions 1 through 4. Fraction 5 showed little activity with or without addition of the salt fraction.

Purification of the activating factor. The activating factor was found to be reasonably stable to heating for 10 min at 100 C under a hydrogen atmosphere; longer periods of heating caused considerable loss of activity (75% in 30 min at 100 C). The procedure described in Materials and Methods was therefore routinely used for purification of the activating factor, yielding a preparation about 7.5-fold purified over the crude extract (Table 5). No further purification has been attained; the factor apparently precipitates between 60 and 90% saturation in $(NH_4)_2SO_4$, but recovery was poor, even under H_2 atmosphere. When the material precipitated by saturation of the heated fraction N with $(NH_4)_2SO_4$ was filtered through Sephadex G-25, the activating factor was

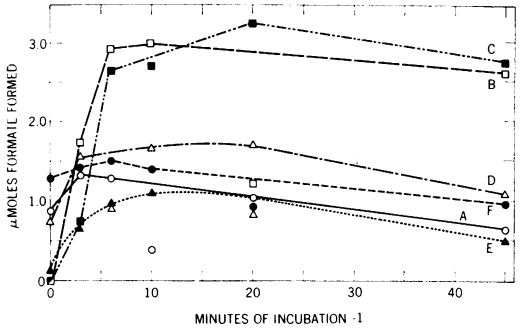

FIG. 4. *Effect of various compounds in activation of pyruvate formate-lyase. Samples (1.0 ml) of crude extract (33.0 mg of protein per ml) were placed in tubes under H_2 atmosphere. Additions were made as indicated below; the tubes were incubated at 25 C. At the indicated times, 10-μliter samples were removed and injected into standard assay tubes. Acetyl phosphate formation, also determined, was similar to the formate formation shown. Additions were: (A) none; (B) 10 μmoles of sodium pyruvate; (C) 10 μmoles of sodium pyruvate + 1 μmole of L-methionine; (D) 1 μmole of ATP; (E) 2 μmoles of ATP + 1 μmole of L-methionine; (F) 0.2 μmole of SAM.*

recovered almost fully in the fraction excluded from the gel pores, suggesting that it is macromolecular in nature.

The addition of pyruvate to the extract before fractionation did not affect the yield of activating factor (Table 6), indicating that activation is not a stoichiometric addition of the activating factor to the inactive pyruvate formate-lyase (unless it is in such large excess over the inactive enzyme that the decrease in concentration of activation factor is negligible).

Specific requirements of the activation reaction. The rate of activation was maximal at 0.01 M sodium pyruvate and 3×10^{-4} M SAM (Fig. 7). The data do not permit accurate calculation of Michaelis-Menten constants, especially since slight activation did occur in the absence of added pyruvate or SAM, presumably due to the presence of small amounts of both of these compounds even in charcoal-treated fraction PS; rates were half-maximal at 8×10^{-5} M added SAM and 0.025 M sodium pyruvate.

The requirement for pyruvate is quite specific (Table 7). Among the various analogues of pyruvate, only α-ketobutyrate is active. It is about 35% as effective as pyruvate. α-Ketovalerate, which is a substrate for the active enzyme, is not active in the activation reaction. In a similar experiment, no activity was found when SAM was replaced by DL-methionine, dimethyl acetothetin, dimethyl-β-propiothetin, or *S*-methylmethionine; *S*-adenosylethionine and

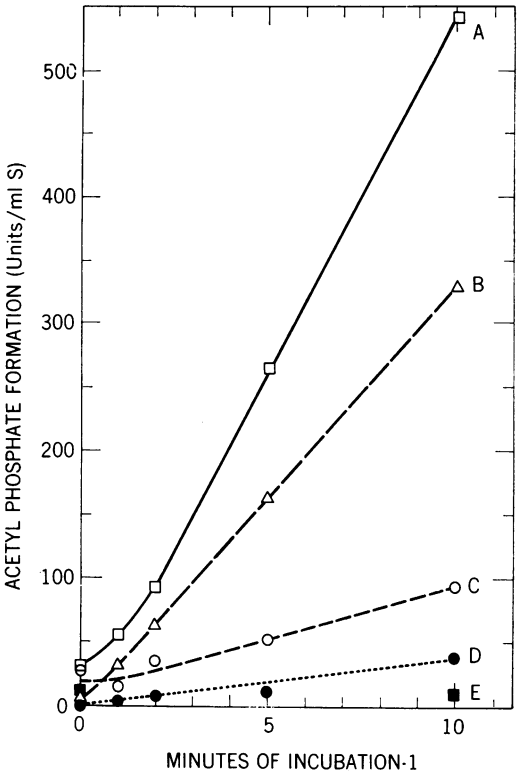

FIG. 5. *Kinetics of the activation reaction. Preincubations contained fraction PS (1,229 units per ml when fully activated, 14.2 mg of protein per ml), sodium pyruvate and SAM as described for assay of activating factor, and 0.25 M potassium phosphate buffer, pH 7.0, 0.02 M in DTT and 2×10^{-8} M in Factor B, to give a total volume of 0.103 ml after addition of fraction N. Fraction N (21.4 mg of protein per ml) was added, the tubes were incubated at 25 C, and samples were taken for assay of pyruvate formate-lyase activity. (A) 50 μliters of fraction N added; (B) 30 μliters of fraction N added; (C) 10 μliters of fraction N; (D) no fraction N; (E) fraction PS omitted, 50 μliters of fraction N added.*

S-adenosylhomocysteine were not available for comparison with SAM.

Initiation of the activation reaction by addition of any of the four required components resulted in approximately the same rate of activation (Fig. 8). If a slow step of activation could be accomplished by preincubating together any three of the components, the fourth being required only for a later, faster step, the activation would be much faster on addition of that component to the other three than when the reaction was initiated by addition of any other component. This eliminates the possibility that the activating factor acts by converting SAM or pyruvate to an

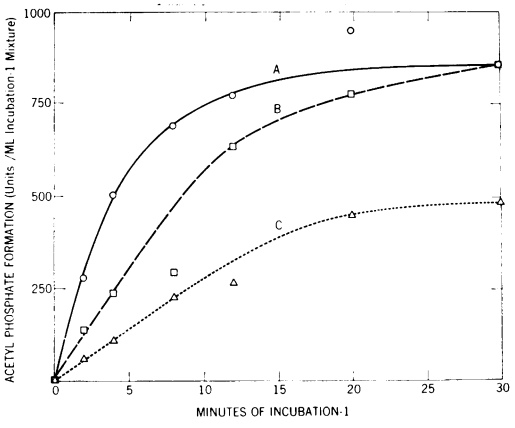

FIG. 6. *Dependence of rate of activation and final level of activity on amount of fractions PS and N. Preincubation A contained 50 μliters of fraction PS (1.45 mg of protein) and 30 μliters of heated fraction N (0.09 mg of protein); B contained 50 μliters of fraction PS and 15 μliters of fraction N; C contained 25 μliters of fraction PS and 30 μliters of fraction N. Results are reported as units of acetyl phosphate formation activity per milliliter of preincubation mixture, rather than per milliliter of fraction PS, since the amount of the latter was varied.*

active cofactor, unless the conversion reaction requires a factor in fraction PS.

Noninvolvement of a vitamin B_{12} derivative in the pyruvate formate-lyase system. SAM is required as a cofactor by the B_{12}-dependent methionine synthetase (5-methyl-tetrahydrofolate-homocysteine methyltransferase) of mammalian liver (J. H. Mangum and K. C. Scrimgeour, Federation Proc., p. 242, 1962) and *E. coli* (23, 26). Although the role of SAM in methyl transfer is not understood, it seemed of interest to determine whether a B_{12} derivative could be a component of the pyruvate formate-lyase system. *E. coli* normally does not synthesize vitamin B_{12} (26); when grown on minimal medium, it utilizes a different, non-B_{12}-dependent enzyme for methionine biosynthesis. Presence of pyruvate formate-lyase in cells grown on minimal medium therefore suggests that no B_{12} derivative is involved; to make this conclusion more certain, extracts of the methionine/B_{12} auxotrophic strain 113-3, grown on minimal medium supplemented with methionine, were assayed for pyruvate formate-lyase. The pyruvate formate-lyase activity in cell extracts of *E. coli* 113-3, although low, was more than half that in extracts of *E. coli* E-26 grown under the same conditions (Table 8). These extracts could be fractionated with protamine sulfate to give two fractions, both required for pyruvate formate-lyase activity, exactly as was the case with strains E-26 and K-12. Little pyruvate formate-lyase activity could be recovered after treatment of the fractions with charcoal, but some stimulation by SAM was apparent. It was therefore concluded that the pyruvate formate-lyase system synthesized by strain 113-3 in absence of vitamin B_{12} was similar to that formed by strain E-26, and thus it seems very unlikely that vitamin B_{12} is involved in the system.

DISCUSSION

A requirement for two or more protein factors for an enzymatic reaction may be explained according to one of three general models.

(i) Two or more proteins, not individually able to catalyze the reaction, combine stoichiometrically and reversibly to yield the active enzyme. Enzymes whose active form is an association of subunits are formal cases of this; examples of combination of readily separable, nonidentical proteins are seen in the glutamate mutase (2) and mesaconase (4) systems. The dissociation of a protein inhibitor, such as trypsin inhibitor from trypsin, is an example of activation by the reversal of combination.

(ii) One factor acts catalytically upon an inactive form of the other, converting it to an active form. Frequently, this type of activation is addition of a cofactor to the apoenzyme, as in formation of holo-pyruvate dehydrogenase (pyruvate-lipoate oxidoreductase, EC 1.2.4.1) from the apoenzyme in presence of lipoate, ATP, and two *Streptococcus faecalis* fractions (22), and

TABLE 5. *Purification of the activating factor*

Fraction	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Activation units		
				Units/mg	Total units	Recovered (%)
Crude extract	36.4	46.1	719	15.6	26,170	100
Protamine eluate (fraction N)	15.4	20.3	673	33.2	10,360	40
Heated fraction N	13.4	4.5	516	115	6,910	26

TABLE 6. *Effect of pyruvate on fractionation of the activating factor*[a]

Fraction	Volume (ml)	Protein (mg/ml)	Activation activity	
			Units/ml	Units/mg of protein
N-A	3.05	28.3	272	9.6
N-B	3.2	25.7	269	10.4

[a] A 7.5-ml sample (A) of freshly prepared crude extract was made 0.01 M in sodium pyruvate, another (B) was not supplemented. Both were treated with protamine sulfate (0.2 mg per mg of protein), and the precipitates were eluted with 0.2 M phosphate (*p*H 7.0), 0.02 M in DTT and 2×10^{-8} M in Factor B, to yield fractions N (fraction N-A from crude extract sample A, N-B from sample B). The pyruvate formate-lyase activities of fractions PS, derived from samples A and B, were 453 and 65 overall units per ml, respectively.

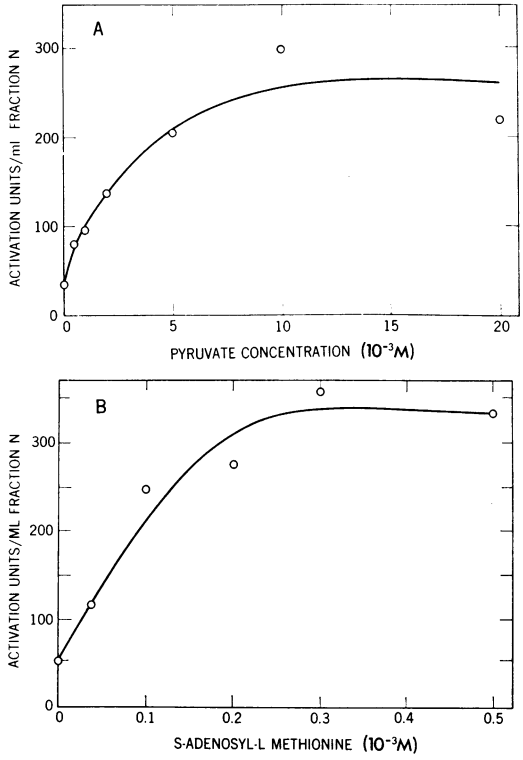

FIG. 7. *Dependence of the rate of activation on pyruvate and SAM concentration. Preincubations were as described for assay of the activating factor, except for variation of sodium pyruvate or SAM as indicated. Fraction PS (29.1 mg of protein per ml) had been treated with charcoal. A 50 μliter amount of heated fraction N (0.23 mg of protein) was used. Results are expressed as activation units per milliliter of heated fraction N.*

TABLE 7. *Activity of pyruvate analogues as substrates for pyruvate formate-lyase and in the activation reaction*

Compound	Activity	
	Formate formation[a] (units/mg of protein)	Activation[b] (activation units/ml of fraction N)
None	0	24
Lithium hydroxypyruvate	0.2	0
Acetoin		3
Lithium acetyl phosphate		25
Sodium α-ketoglutarate	2.0	20
Sodium α-ketovalerate	4.9	23
Sodium α-ketobutyrate	7.1	189
Sodium pyruvate	16.8	514

[a] Indicated analogues (0.05 M) replaced pyruvate in the standard assay. The enzyme was a *p*H 4.5 precipitate containing 16.8 mg of protein per ml. It was filtered through a thiol-Sephadex G-25 column equilibrated with 0.05 M sodium DMG (*p*H 6.8), 0.02 M in DTT and 2×10^{-8} M in Factor B. The activities shown are the averages observed using 2, 4, and 6 μliters of the enzyme solution.

[b] Preincubations were as described for assay of the activation factor, except that the indicated compounds, 0.01 M, replaced pyruvate. SAM was 3×10^{-4} M rather than 2×10^{-4} M. A 25-μliter amount of heated fraction N (0.115 mg of protein) was used; rates of activation are expressed as activation units per milliliter of heated fraction N.

formation of holo-propionyl carboxylase [EC 6.4.1.3 (9)], holo-oxalacetate transcarboxylase [methylmalonyl-CoA-pyruvate carboxyltransferase, EC 2.1.3.1 (11, 12)], and presumably other biotinyl enzymes in presence of biotin, ATP, and another enzyme. Activation of trypsin and chymotrypsin by cleavage of a peptide bond of the zymogen is an example of activation by subtraction.

(iii) The two factors catalyze separate reactions of low molecular weight compounds; the enzymatic reaction under consideration may occur in two distinct and separable steps, or one enzyme may act by the formation of a cofactor for the other enzyme.

We tentatively assign the requirement for two factors for the pyruvate formate-lyase reaction to the second class, where an inactive form of the enzyme pyruvate formate-lyase is converted to an active form by the action of an enzyme fraction N in the presence of pyruvate and SAM. It seems unlikely that the activation reaction represents reversible stoichiometric combination of two proteins, since the activated enzyme is stable to

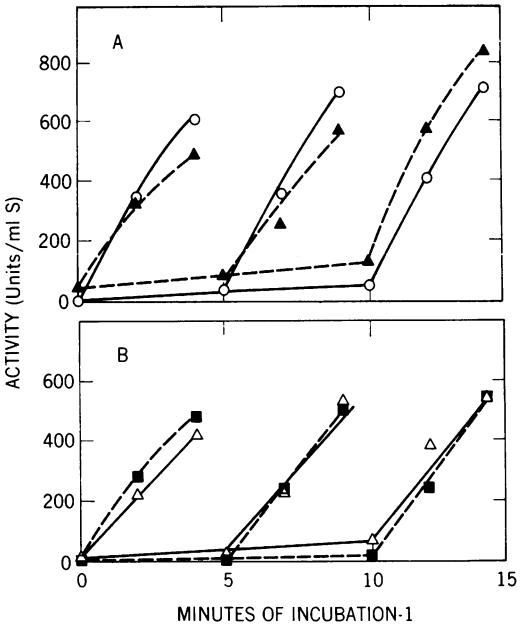

FIG. 8. *Preincubation of incomplete mixtures. All preincubations finally contained: charcoal-treated fraction PS, 50 μliters (1.45 mg of protein); heated fraction N, 25 μliters (0.11 mg of protein); sodium pyruvate, 1 μmole; SAM, 0.03 μmole; and 0.05 M sodium DMG buffer, pH 6.8, 0.02 M in DTT, 2 × 10^{-8} M in Factor B, to a final volume of 0.100 ml, under H_2 atmosphere. Tubes 1 to 3 initially lacked pyruvate, but contained the other three components; tubes 4 to 6 initially lacked SAM, but contained the other three components; tubes 7 to 9 lacked fraction PS, and tubes 10 to 12 lacked fraction N. At time-zero (as soon as possible after addition of the third component), the fourth component was injected into the first tube of each series, starting the activation reaction; a sample was immediately withdrawn and injected into a standard assay tube for measurement of pyruvate formate-lyase activity, and all preincubation tubes were incubated at 25 C. At 2 and 4 min after injection of the fourth component, further samples were withdrawn from the first tubes for measurement of pyruvate formate-lyase activity. After 5 min at 25 C, the fourth component was added to the second tube in each series, and samples were drawn from these tubes after 0, 2, and 4 min for assay of pyruvate formate-lyase activity. After 10 min at 25 C, the procedure was repeated with the third tube of each series. The pyruvate formate-lyase activity, as units per milliliter of fraction PS, of each preincubation tube at each point is shown; the nearly horizontal lines at the bottom represent slight activation in absence of the fourth component, the diagonal lines represent activation after addition of the fourth component. (A) ○, activation initiated by addition of pyruvate; ▲, activation initiated by addition of SAM. (B) △, activation initiated by addition of fraction N; ■, activation initiated by addition of fraction PS.*

dilution, to fractionation with protamine sulfate (Table 3), to Sephadex filtration, and to isoelectric precipitation, although occasionally considerable activity lost in the last procedure could be regenerated by addition of heated fraction N, pyruvate, and SAM. It is to be emphasized that the inactivated enzyme resulting from most procedures of protein fractionation is not reactivated by this procedure. Indeed, the nonactivated enzyme appears to be even more labile to fractionation procedures than the activated enzyme, since it could not be further purified by isoelectric precipitation.

Further evidence against a stoichiometric combination of two protein factors is seen in Table 6: the amount of activating factor in crude extract was not decreased by activation of the enzyme.

The experiment described in Fig. 8 is evidence against the third model: if the activating factor were required only for formation of an active cofactor, preincubation together of fraction N, pyruvate, and SAM should result in full activity immediately on addition of fraction PS, unless some component of fraction PS, other than the inactive pyruvate formate-lyase, is also required for formation of an active cofactor. However, the active enzyme may be precipitated at *p*H 4.5 and filtered through Sephadex G-25 without showing a requirement for any cofactor, other than CoA for the overall reaction. Also, no further requirements appear after treatment of fraction PS with charcoal.

We therefore conclude that the activation reaction is a modification of the inactive enzyme to yield the active enzyme, and that this modification reaction requires the activating factor in fraction N, pyruvate, and SAM. The nature of the activation reaction remains unknown. It is not even certain that the activating factor is a protein, and an enzyme. However, it seems likely that this factor is a protein and it may be identical to the protein of low molecular weight and considerable stability at *p*H 4.0 reported to be a component of the pyruvate formate-lyase system of Midwinter et al. (Federation Proc., p. 531, 1965). In consideration of the heat stability of the activation factor of *E. coli*, it is likely that such a factor is also an active component of the stimulatory heated extract preparations from *M. lactilyticus*, *C. butyricum*, *C. pasteurianum*, and *Peptococcus aerogenes* (16); however, these preparations may also contain SAM, which is probably the stimulatory component of rat liver and yeast (1, 13, 24).

The most likely role for SAM, by analogy to its possible role in methionine synthesis, would be

TABLE 8. *Pyruvate formate-lyase activity in extracts of E. coli E-26 and 113-3*[a]

Strain	Supplement to medium	Protein (mg/ml)	Activity			
			Acetyl phosphate formation		Formate formation	
			Units/ml	Units/mg	Units/ml	Units/mg
E-26	Methionine	38.4	186	4.8	180	4.7
E-26	Vitamin B_{12}	48.1	166	3.45	175	3.64
113-3	Methionine	30.9	101	3.3	103	3.3
113-3	Vitamin B_{12}	35.7	70.5	2.0	68	1.9

[a] Cells were grown on a synthetic 0.5% glucose-salts medium supplemented with either DL-methionine (250 μmoles/liter) or vitamin B_{12} (1 μg/liter). Extracts were prepared with 0.05 M sodium DMG buffer (*p*H 6.8), 0.002 M in vitamin K_5; samples of the extract were made 0.01 M in sodium pyruvate and 2×10^{-4} M in SAM and assayed for pyruvate formate-lyase activity.

methylation of a group on the enzyme; however, no obvious role for methylation appears in the pyruvate formate-lyase reaction. Similarly, there is no obvious role for pyruvate in the activation reaction; the failure of α-ketovalerate, which is a substrate of the active enzyme, to act in the activation reaction (Table 7), suggests that the site for pyruvate in the activation reaction is not the same as its site as substrate of the active enzyme.

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