Synthesis of Pyruvate Carboxylase from its Apoenzyme and (+)-Biotin in *Bacillus stearothermophilus*

PURIFICATION AND PROPERTIES OF THE APOENZYME AND THE HOLOENZYME SYNTHETASE

By J. J. CAZZULO,* T. K. SUNDARAM, SUSAN N. DILKS AND H. L. KORNBERG

*Department of Biochemistry, School of Biological Sciences,
University of Leicester, Leicester LE1 7RH, U.K.*

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1. Methods are described for the assay and purification of pyruvate apocarboxylase and pyruvate holocarboxylase synthetase from biotin-deficient *Bacillus stearothermophilus*. 2. Pyruvate apocarboxylase was obtained 200-fold purified and in a nearly homogeneous state; it closely resembled the holoenzyme of the thermophile in fractionation properties, electrophoretic mobility and molecular weight (estimated to be 350 000 by gel filtration). 3. Pyruvate holocarboxylase synthetase, purified more than 50-fold, was estimated to have a molecular weight of approx. 40 000. 4. The conversion of the purified apoenzyme into the holoenzyme required the presence of the synthetase, ATP ($K_m 3.3 \times 10^{-7}$ M), (+)-biotin ($K_m 7.5 \times 10^{-8}$ M) and Mg^{2+} ; it differed from the conversions effected by systems forming other carboxylases in mesophilic organisms in also requiring the presence of acetyl-CoA.

The final step in the biosynthesis of biotin-containing carboxylases is the attachment of (+)-biotin by a peptide bond to an ϵ -amino group of lysine on the apoenzyme (Kosow & Lane, 1962). This reaction, catalysed by holocarboxylase synthetase, has been demonstrated *in vitro* to effect the formation of propionyl-CoA carboxylase (Kosow & Lane, 1961; Siegel, Foote, Christner & Coon, 1963), β -methylcrotonyl-CoA carboxylase (Höpner & Knappe, 1965), acetyl-CoA carboxylase (Lynen & Rominger, 1963) and methylmalonyl-CoA-oxaloacetate transcarboxylase (Lane, Young & Lynen, 1964). *In vivo*, it has been shown that active pyruvate carboxylase is produced upon administration of (+)-biotin to biotin-deficient rats (Deodhar & Mistry, 1969).

We have demonstrated (Cazzulo, Sundaram & Kornberg, 1969) that *Bacillus stearothermophilus*, when grown under conditions of biotin insufficiency, produces pyruvate apocarboxylase and the holoenzyme synthetase and that the cells rapidly effect the conversion of the apoenzyme into active enzyme when biotin is added. The same conversion can be achieved *in vitro* with cell-free preparations; one of the notable features of this system is the requirement for acetyl-CoA as an allosteric effector. Such

a requirement for an allosteric activator has not been observed in the formation of the other biotin-containing carboxylases, although in other respects the reaction mechanism seems similar in all these systems.

We report here the resolution of the *B. stearothermophilus* system into the apocarboxylase [apoenzyme of pyruvate-CO₂ ligase (ADP); EC 6.4.1.1] and the pyruvate holocarboxylase synthetase [(+)-biotin-pyruvate apocarboxylase ligase (AMP); EC 6.3.4.-], the isolation of these two proteins in a high degree of purity, and some properties of this system.

MATERIALS AND METHODS

Reagents used

NADH, ATP, ADP, AMP, GTP, ITP, UTP, CTP, sodium pyruvate, tris base, (+)-biotin and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; malate dehydrogenase (crystalline) and CoA were from Boehringer (London) Ltd., London W.5, U.K.; protamine sulphate was from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; and lysozyme, deoxyribonuclease and (NH₄)₂SO₄ (specially low in heavy metals) were from British Drug Houses Ltd., Poole, Dorset, U.K. All other chemicals used were analytical reagents. Acetyl-CoA was prepared from CoA and acetic anhydride by the method of Stadtman (1957).

* Present address: Catedra de Química Biológica, Facultad de Ciencias Bioquímicas, Universidad Nacional de Rosario, Suipacha 570, Rosario, Argentina.

Sephadex G-200 was obtained from Pharmacia, Uppsala, Sweden; DEAE-cellulose (DE 52, microgranular, pre-swollen) from Whatman Ltd., Maidstone, Kent, U.K., and hydroxyapatite was prepared by the method of Tiselius, Hjerten & Levin (1956).

Analytical procedures

The protein content of crude bacterial extracts was determined as described by Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as standard; in Steps 2 to 5 of the purification procedure (Table 1), protein was determined by the spectrophotometric method of Warburg & Christian (1941). Acetyl-CoA was determined with hydroxylamine and dithiobis-(2-nitrobenzoic acid) by the procedure of Srere, Brazil & Gonen (1963) as described by Canovas & Kornberg (1966).

Polyacrylamide-gel electrophoresis. The procedure of Ornstein & Davis (1961) was employed with the acrylamide concentration at 6% (w/v). After electrophoresis, the gels were stained with Amido Black, as described by these authors, and then destained in several changes of 7% (v/v) acetic acid.

Analytical ultracentrifugation. The sedimentation behaviour of purified apocarboxylase was studied in a MSE analytical ultracentrifuge equipped with schlieren optics. The protein sample was contained in a 5 mm single-sector quartz cell, and the material was centrifuged at 23°C and 60 400 rev./min.

Assay of pyruvate holocarboxylase synthetase. This was assayed by a two-step procedure. In the first (step A) the synthetase catalysed the conversion of apo- into holoenzyme; in the second (step B) the quantities of holocarboxylase thus formed were measured.

Step A. The reaction mixture contained, in a total volume of 0.3 ml: tris-HCl buffer, pH 7.6, 15 μ mol; $MgCl_2$, 0.5 μ mol; ATP, 0.125 μ mol; (+)-biotin, 0.07 μ mol; acetyl-CoA, 0.1 μ mol; pyruvate apocarboxylase (specific activity 10 units/mg of protein or higher), 0.5 unit (see later); and synthetase, up to 0.012 unit. After incubation for 15 min at 45°C, the reaction was terminated by cooling the mixture to 0°C and adding 0.01 ml of 0.1 M-EDTA, pH 6.8. A portion, usually 0.2 ml, was then assayed for pyruvate carboxylase activity as described under Step B. Appropriate controls were set up to correct for the presence of any synthetase or pyruvate holocarboxylase in the apoenzyme preparation used as substrate.

Step B. The pyruvate holocarboxylase synthesized in Step A was assayed by the procedure of Cazzulo, Sundaram & Kornberg (1970a). The assay mixture contained, in a total volume of 3 ml: tris-HCl buffer, pH 8.0, 300 μ mol; $MgCl_2$, 15 μ mol; $KHCO_3$, 150 μ mol; sodium pyruvate, 10 μ mol; NADH, 0.65 μ mol; acetyl-CoA, 0.1 μ mol; ATP, 5 μ mol; malate dehydrogenase, 1 unit; and a portion (usually 0.2 ml) of the reaction mixture of Step A. The assay, initiated by addition of ATP, was performed at 45°C and involved the monitoring, in a Unicam SP.1800 spectrophotometer, of the decrease in E_{340} concomitant with the oxidation of NADH as the oxaloacetate formed in the pyruvate carboxylase reaction was reduced to malate. One unit of pyruvate holocarboxylase is defined as the amount causing the oxidation of 1 μ mol of NADH/min under these conditions of assay. One unit of pyruvate holocarboxylase synthetase is defined as the amount cata-

lysing the synthesis of 1.0 unit of pyruvate holocarboxylase/min under the conditions described in Step A. Specific activities are expressed as units per mg of protein.

It is recognized that the amount of apoenzyme (0.5 unit) chosen for Step A of the assay procedure was not sufficient to saturate the synthetase (Fig. 1), but prohibitively large amounts of the apoenzyme would be required to work under saturating conditions. The present procedure was regarded as satisfactory, since the rates obtained were linear for at least 30 min and stoichiometric with respect to the amounts (up to 0.012 unit) of synthetase added (Fig. 2, lower curve). Similar characteristics have been observed in the transcarboxylase system by Lane *et al.* (1964).

Assay of pyruvate apocarboxylase. The procedure used was the same as for the assay of the synthetase, but with the following modifications designed to ensure the complete conversion of the apoenzyme into holoenzyme. The amount of apocarboxylase in the assay sample was adjusted to be no greater than 0.16 unit, the incubation period in Step A was 30 min, and an adequate excess of synthetase (established experimentally) was used. As shown in Fig. 3, 0.025 unit of synthetase was sufficient to effect the quantitative conversion of 0.16 unit of apocarboxylase into active enzyme. Fig. 2 (upper curve) shows that with 0.032 unit of synthetase, the holoenzyme synthesized was stoichiometric with the amount (up to 0.2 unit) of apoenzyme added. One unit of pyruvate apocarboxylase is defined as the amount which, upon complete conversion, yields 1 unit of pyruvate holocarboxylase.

In some experiments (Figs. 4–6), the apoenzyme and synthetase activities are expressed directly in terms of the change in E_{340} obtained in Step B of the assay procedure.

Fig. 1. Pyruvate holocarboxylase synthetase activity as a function of pyruvate apocarboxylase concentration. A small amount of synthetase (5.9 μ g; from step 5S) was used and the concentration of apocarboxylase was varied as indicated. Other conditions were as described in the text for the assay of synthetase.

Fig. 2. Stoichiometry of pyruvate carboxylase formation with respect to synthetase and apocarboxylase. ▲, Amount of apocarboxylase was fixed at 0.5 unit and the amount of synthetase was varied; other details were as described for the assay of synthetase; ●, amount of apocarboxylase was varied and the amount of synthetase, adequate to effect the complete conversion of up to 0.2 unit of apoenzyme to holoenzyme, was fixed at 0.032 unit; other details were as described for the assay of apocarboxylase.

Fig. 3. Titration of pyruvate apocarboxylase. A small amount of pyruvate apocarboxylase (14.8 μ g; 0.16 unit) was used and the synthetase concentration was varied as indicated. Other conditions were as described for the assay of apocarboxylase.

Purification of the pyruvate apocarboxylase and holocarboxylase synthetase

Organism used. The strain of *B. stearothermophilus* used in this study was obtained from Professor N. Grossowicz, Hebrew University-Hadassah Medical School, Jerusalem,

Israel. This is a prototrophic strain (Epstein & Grossowicz, 1969) which grows on a defined salts medium with succinate as the sole carbon source and without any added biotin, and under these conditions produces the apocarboxylase and the synthetase (Cazzulo *et al.* 1969). The organism was maintained on Oxoid nutrient agar slopes and subcultured at monthly intervals.

Growth of the organism. Cultures (500 ml) were grown in 2 litre Erlenmeyer flasks at 55°C with shaking on salts medium containing 50 mM-sodium succinate and no added biotin (Sundaram, Cazzulo & Kornberg, 1969). Growth was followed by measuring the E_{680} of the culture in cuvettes of 1 cm light-path. An extinction of 1 under these conditions corresponds to a dry weight of cells of about 0.33 mg/ml of culture. The cultures were grown to a cell density of about 0.5 mg dry wt./ml; the cells were harvested by centrifugation at 5°C, washed with 50 mM-tris-HCl buffer, pH 7.6, containing 1 mM-EDTA and 150 mM-KCl, and stored frozen until used. In the later stages of this work, a batch of 600 g (wet wt.) of cells, grown under these conditions, was kindly supplied by the Microbiological Research Establishment, Porton Down, Wilts., U.K.

Step 1: preparation of cell-free extract. Cells (42 g) were suspended in 350 ml of the tris-HCl-EDTA-KCl mixture and lysozyme (60 μ g/ml) was added. After incubation at 37°C for 30 min, an equal volume of buffer without KCl was added. The mixture was centrifuged at 25000g for 30 min at 5°C and the precipitate was discarded. All subsequent operations were performed at 5°C and all buffers contained 1 mM-EDTA, unless otherwise specified.

Step 2: treatment with protamine sulphate. To the supernatant solution (640 ml, containing 2.8 g of protein) was added with constant stirring 25 ml of an aq. 2% (w/v) solution of protamine sulphate. The mixture was left for 10 min, then centrifuged at 25000g for 40 min, and the precipitated material was discarded.

Step 3: ammonium sulphate fractionation. To the supernatant solution (640 ml) was added with constant stirring 279 g of $(\text{NH}_4)_2\text{SO}_4$. The mixture, 65% saturated with respect to $(\text{NH}_4)_2\text{SO}_4$, was left for 2 h and centrifuged at 25000g for 30 min. The precipitate was then extracted successively with solutions, adjusted to pH 7, containing 1 mM-EDTA and decreasing concentrations of $(\text{NH}_4)_2\text{SO}_4$ [27.6, 27.0, 26.0, 23.3 and 21.6% (w/v)]. For each extraction, the precipitate was homogenized and suspended in 40 ml of the appropriate $(\text{NH}_4)_2\text{SO}_4$ solution and the suspension was stirred slowly for 10 min and centrifuged. The initial extraction with the 27.6% solution was performed twice and both extracts were discarded. The other four extracts, which contained apocarboxylase and holoenzyme synthetase activities, were pooled and saturated $(\text{NH}_4)_2\text{SO}_4$ solution, pH 7, was added to make the mixture 65% saturated. The precipitated proteins were collected by centrifugation. (Occasionally the 27.0% extract contained very little activity and could also be discarded.) The extraction step usually achieves some fractionation of the apoenzyme and the synthetase, the 26.0% extract being richer in the synthetase and the 21.6% fraction concentrating the apoenzyme. However, since step 4 effects a clean separation of the two proteins, all the active extracts were combined. The precipitated

protein from the active fractions was dissolved in 20 ml of 50 mM-tris-HCl buffer, pH 7.6, and this solution was incubated with 425 μg of deoxyribonuclease for 30 min at 30°C: this treatment generally lowers the viscosity of the protein solution and improves the results of step 4. Saturated $(\text{NH}_4)_2\text{SO}_4$ solution (40 ml) was then added and the precipitated protein was collected by centrifugation.

Step 4: resolution of the pyruvate apocarboxylase and the holocarboxylase synthetase by gel filtration through Sephadex G-200. The proteins from step 3 (175 mg) were dissolved in 8 ml of 0.1 M-tris-HCl buffer, pH 7.6, containing 0.4 M- $(\text{NH}_4)_2\text{SO}_4$. The solution was applied to the top of a column of Sephadex G-200 (gel bed 2.5 cm $\times$ 40 cm), which was eluted with the same buffer mixture at a flow rate of 18 ml/h. Fractions (2 ml) were collected and assayed for protein, apoenzyme and synthetase. As shown in Fig. 4, this step effected a clean separation of the two proteins. Fractions 39–50, which contained most of the apoenzyme, and fractions 73–86, which contained the synthetase, were separately pooled and treated with $(\text{NH}_4)_2\text{SO}_4$ to 65% saturation. With larger batches of cells, 1 g of protein has been passed through a column (4 cm $\times$ 90 cm) with equally successful results.

Step 5A: chromatography of pyruvate apocarboxylase on hydroxyapatite. The apoenzyme fraction obtained in step 4 was dissolved in 4 ml of 10 mM-potassium phosphate buffer, pH 7, containing 0.1 mM-EDTA and the solution was dialysed against 2 litres of the same buffer. All buffers used in this step and in step 5S contained 0.1 mM-EDTA. The dialysed sample (containing 22.3 mg of protein) was applied to the top of a column (1.5 cm $\times$

Fig. 4. Resolution of pyruvate apocarboxylase and holocarboxylase synthetase by gel filtration through Sephadex G-200. For experimental details see the text. ●, E_{280} (protein); □, pyruvate apocarboxylase activity; ○, holocarboxylase synthetase activity.

Fig. 5. Chromatography of apocarboxylase on hydroxyapatite. For experimental details see the text. ●, E_{280} ; ○, apocarboxylase activity.

Fig. 6. Chromatography of synthetase on DEAE-cellulose. For experimental details see the text. ●, E_{280} ; ▲, synthetase activity.

2.6 cm) of hydroxyapatite equilibrated with 10 mM-potassium phosphate buffer, pH 7. The column was washed with the same buffer and then eluted successively with

0.09, 0.10, 0.14 and 0.20 M-potassium phosphate buffer, pH 7. Fractions (2 ml) were collected at a flow rate of about 7 ml/h and assayed for protein and apocarboxylase. As

shown in Fig. 5, the 0.14M buffer released the apoenzyme, which was then concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ added to 70% saturation.

Step 5S: chromatography of synthetase on DEAE-cellulose. The synthetase fraction obtained in step 4 was dissolved in 5ml of 10mM-potassium phosphate buffer, pH7, and dialysed against 2 litres of the same buffer. The dialysed solution, which contained 40mg of protein, was applied to a column of DEAE-cellulose (1.5cm $\times$ 9.2cm) equilibrated with 10mM-potassium phosphate buffer, pH7. After washing with the same buffer, the column was eluted successively with 0.06, 0.08 and 0.2M-potassium phosphate buffer, pH7. Fractions (5ml) were collected at a flow rate of about 35ml/h. As shown in Fig. 6, the 0.08M buffer released most of the synthetase, which was then concentrated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ added to 70% saturation.

Chromatography of the synthetase on hydroxyapatite. The synthetase preparation obtained in step 5S can be further purified by adsorption on a column of hydroxyapatite in 10mM-potassium phosphate buffer, pH7. A considerable amount of inactive protein can be eluted from the column with 0.07M buffer before the enzyme is released with 0.14M-potassium phosphate buffer, pH7. This step, which achieves an approximately twofold purification, was not included in the standard purification procedure (Table 1) since it produced the enzyme in low yield and in a still inhomogeneous state.

RESULTS

The results of a typical purification experiment are summarized in Table 1. The apoenzyme was purified about 200-fold with a yield of 19% and the synthetase was purified more than 50-fold with a yield of 7%. Synthetase preparations with considerably better specific activity (1–1.3units/mg) and in higher yield have been obtained from the batch of cells supplied by the Microbiological Research Establishment and have been further purified to a specific activity of 2.3 by hydroxyapatite chromatography.

Purity of the preparations. The apoenzyme obtained in step 5A was nearly homogeneous as judged by polyacrylamide-gel electrophoresis (Fig. 7a). In addition to the main band of apoenzyme, a very faint band was usually observed, which was estimated to account for less than 5% of the total protein. Active pyruvate carboxylase, purified by the method of Cazzulo *et al.* (1970a) to near homogeneity had a mobility similar to that of the apoenzyme (Fig. 7b); when the apoenzyme and the holoenzyme were run in mixture, a single major protein band was obtained (Fig. 7c). The sedimentation behaviour of the apocarboxylase preparation in the ultracentrifuge confirmed its state of virtual homogeneity; as shown in Fig. 8, a single protein peak was observed.

Polyacrylamide-gel electrophoresis showed the synthetase preparation from step 5S to be still not homogeneous.

Table 1. Resolution and purification of pyruvate apocarboxylase and holocarboxylase synthetase from *B. stearothermophilus*

Step	Fractionation procedure	Volume (ml)	Total protein (mg)	Apocarboxylase			Synthetase		
				Total activity (units)	Sp. activity (units/mg of protein)	Yield	Total activity (units)	Sp. activity (units/mg of protein)	Yield
1	Crude extract	640	2800	586	0.20	100	27.3	0.01	100
2	Protamine sulphate precipitation	640	2240	694	0.31	118	24.3	0.01	88
3	Ammonium sulphate fractionation	18	174	520	2.75	88	20.3	0.11	73
4	Sephadex G-200 filtration	7.2 7.5	22.3 —	286 —	12.8 —	49 —	— 5.7	— 0.14	— 20
5A	Hydroxyapatite chromatography	22	2	113	38.9	19	—	—	—
5S	DEAE-cellulose chromatography	30	3.7	—	—	—	1.81	0.50	52

Fig. 7. Disc electrophoresis of apocarboxylase and holocarboxylase in polyacrylamide gel. Details are given in the text. (a) pyruvate apocarboxylase (26.5 μ g); (b) pyruvate holocarboxylase (15 μ g); (c) apocarboxylase (26.5 μ g) + holocarboxylase (15 μ g).

Fig. 8. Sedimentation of pyruvate apocarboxylase in the ultracentrifuge. Purified apocarboxylase in 50 mM-tris-HCl buffer, pH 7.6, 1 mM-EDTA, at a protein concentration of 3.1 mg/ml was used. (a) 200 s, (b) 400 s and (c) 800 s, after the attainment of top speed (60 400 rev./min). Sedimentation of protein is from right to left. Other details are in the text.

Both the apocarboxylase and the synthetase preparations were devoid of acetyl-CoA-hydrolysing activity and suitable for kinetic studies involving acetyl-CoA. The preparations were tested for this activity by measuring the E_{412} of a reaction mixture

identical with that in step A of the synthetase assay procedure after the addition of 0.6 μ mol of dithiobis-(2-nitrobenzoic acid).

Molecular weights of the proteins. An estimate of the molecular weights of the apocarboxylase and the synthetase was made by the technique of gel filtration through Sephadex G-200 as described by Andrews (1965). A plot of elution volume against log (molecular weight), constructed with proteins of known molecular weights, yielded a value of 350 000–400 000 for the apocarboxylase and a value of 40 000 for the synthetase (Table 2). A separate experiment, in which a less pure preparation from step 3 was used, yielded the same value for the synthetase. The molecular weight of the holoenzyme, estimated by this procedure, does not appear to differ significantly from that of the apoenzyme (Table 2).

Some properties of the holoenzyme synthetase system. The synthesis of pyruvate holocarboxylase requires (+)-biotin, ATP, a divalent cation (Mg^{2+} or Mn^{2+} is equally effective), apoenzyme and synthetase (Cazzulo *et al.* 1969). There is, in addition, an almost complete requirement for acetyl-CoA, as less than 10% of the rate of holoenzyme synthesis is observed in the absence of this activator. Unlike other synthetase systems (Lane *et al.* 1964) the system derived from *B. stearothermophilus* is not stimulated by GSH.

Optimum holoenzyme synthesis occurs over a broad pH range between 6.4 and 7.4 (Fig. 9). As in other carboxylase synthetase systems, ATP is the preferred nucleoside triphosphate; the other triphosphates tested were effective to different degrees (Table 3). ADP or AMP at 0.66 mM caused about 12% inhibition (with ATP at 0.4 mM concentration). The Michaelis constant (K_m) for ATP was 3.3×10^{-7} M and that for (+)-biotin 7.5×10^{-8} M. The plot of synthetase activity against the concentration of either of these reactants was hyperbolic.

DISCUSSION

To the best of our knowledge, the present studies represent the only pyruvate apocarboxylase-synthetase system wherein the apoenzyme and the holoenzyme synthetase have been separated from each other and clearly characterized. The strain of *B. stearothermophilus* used here is readily rendered biotin-deficient; cells grown under the conditions described are rich in the apocarboxylase and holoenzyme synthetase, and contain very little holoenzyme. Since it is derived from a thermophile, the protein components of the system that form the holoenzyme are relatively stable and can operate at high temperatures, which further enhance its activity. A similar system present in *Saccharomyces cerevisiae* is much more labile and less active

Table 2. *Molecular weights of synthetase, apocarboxylase and holocarboxylase determined by gel filtration through Sephadex G-200*

The sample contained the proteins listed in a total volume of 2 ml. The buffer used for elution of the column (2.5 cm × 40 cm) was 0.05 M-tris-HCl, pH 7.6, containing 1 mM-EDTA and 0.4 M-(NH₄)₂SO₄. Fractions (2 ml) were collected at a flow rate of 13.2 ml/h and assayed for protein and the enzyme activities. Proteins other than apocarboxylase, holocarboxylase and synthetase were standards and were determined as described by Andrews (1965).

Protein	Amount (mg)	Elution volume (ml)	Estimated molecular weight
Cytochrome <i>c</i>	2	166	12 400
Synthetase	0.96	138	40 000
Ovalbumin	10	136	45 000
Malate dehydrogenase	0.1	127	55 000–70 000
Bovine serum albumin	8	124	65 000–70 000
Pyruvate apocarboxylase	1.42	86	350 000
Pyruvate holocarboxylase	1.3	84	380 000
Thyroglobulin	4	76	670 000

Fig. 9. Synthetase activity at different pH values. Imidazole-HCl (●) or tris-HCl buffer (▲) was used to vary the pH of the reaction mixture in step A (see 'Assay of pyruvate holocarboxylase synthetase'). The incubation in this step was performed at 30°C, a temperature close to that at which the pH was measured. Other conditions were as for the synthetase assay described in the text.

(Sundaram, Cazzulo & Kornberg, 1971b). The apocarboxylase behaves similarly to the holoenzyme during the various fractionation procedures, filtration through Sephadex G-200 (Cazzulo, Sundaram & Kornberg, 1970b) and polyacrylamide-gel electrophoresis, and the two proteins seem not to differ greatly in molecular weight. This suggests that the attachment of biotin to the apoenzyme is not accompanied by aggregation or disaggregation of the protein. In molecular size, the thermophile pyruvate carboxylase protein rather resembles the mesophilic pyruvate carboxylases that have been similarly characterized.

Table 3. *Effectiveness of different nucleoside triphosphates in pyruvate holocarboxylase synthesis*

All the nucleotides were tested at a concentration of 0.33 mM, in the presence of 75 μM-acetyl-CoA, with 8.8 μg (0.0095 unit) of synthetase and 38.8 μg (0.58 unit) of pyruvate apocarboxylase. Other conditions were as described in the Materials and Methods section.

Nucleoside triphosphate	Pyruvate holocarboxylase synthesized	
	(unit)	(% relative to ATP)
ATP	0.162	100
GTP	0.067	41
ITP	0.026	16
UTP	0.016	10
CTP	0.008	5

The holoenzyme synthetase is a much smaller protein. No information is available on the molecular size of the other carboxylase synthetases. Studies on these (mesophilic) synthetase systems indicate that the synthetase is an enzyme of no great species-specificity. We have, however, observed little cross-reaction between the *B. stearothermophilus* and the *S. cerevisiae* apocarboxylase-synthetase systems (Sundaram *et al.* 1971b).

The synthesis of active pyruvate carboxylase from the apoenzyme and biotin in the thermophile resembles the formation of other biotin-containing carboxylases in its requirements (Lane *et al.* 1964), in the low *K_m* values for ATP and biotin and, mechanistically, in the intermediate formation of biotinyl adenylate as the 'active' form of the vitamin (Cazzulo *et al.* 1970b). The effect on this synthesis of the allosteric effectors acetyl-CoA (positive) and L-aspartate (negative), which has thus far been observed only in the *B. stearothermophilus* and *S. cerevisiae* systems, is described in the following paper (Sundaram, Cazzulo & Kornberg, 1971a).

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