Sokolovsky, M., and Vallee, B. L. (1966), *Biochemistry 5*, 3574.
Sokolovsky, M., and Vallee, B. L. (1967), *Biochemistry 6*, 700.
Spackman, D. H. (1967), *Methods Enzymol. 11*, 3.
Sri Ram, J., Bier, M., and Maurer, P. (1962), *Advan. Enzymol. 24*, 105.
Sri Ram, J., Terminiello, L., Bier, M., and Nord, F. F. (1954), *Arch. Biochem. Biophys. 52*, 464.
Steyermark, A. (1961), Quantitative Organic Microanalysis, 2nd ed, Academic, New York, N. Y.
Tabachnick, M., and Sobotka, H. (1959), *J. Biol. Chem. 234*, 1726.
Walsh, K., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Whitaker, J. R., and Bender, M. L. (1965), *J. Amer. Chem. Soc. 87*, 2728.
Zollinger, H. (1961), Azo and Diazo Chemistry, Interscience, New York, N. Y.

Synthetic Homologs of Phosphoenolpyruvate and Specificity of Pyruvate Kinase*

A. E. Woods, J. M. O'Bryan, P. T. K. Mui, and R. D. Crowder

ABSTRACT: Seven homologs of phosphoenolpyruvate were synthesized and characterized. These homologs were synthesized from the corresponding β-bromo-α-keto acids using the Perkow reaction. All of the homologs were evaluated for their abilities to serve as substrates or inhibitors for pyruvate kinase (EC 2.7.1.40). Results of the kinetic studies indicated that none of the seven homologs of P-enolpyruvate will serve as a substrate for rabbit muscle pyruvate kinase. However, P-enol-α-ketobutyrate and P-enol-α-ketovalerate act as competitive inhibitors for the pyruvate kinase–P-enolpyruvate reaction. The other five homologs did not inhibit the reaction of the enzyme. The inhibition constants, K_i, for P-enol-α-ketobutyrate and P-enol-α-ketovalerate were determined by the method of M. Dixon (*Biochem. J. 55*, 170 (1953)). P-enol-α-ketobutyrate had a K_i of 6.5×10^{-5} M, while the K_i of P-enol-α-ketovalerate was 10.7×10^{-4} M. The homologs with six or more carbons or the homolog with two methyl groups on the β-carbon (P-enol-α-ketoisovalerate) do not act as substrates or inhibitors, thus indicating that pyruvate kinase is quite selective for its substrate.

Phosphoenolpyruvate is the only reported compound that is capable of phosphorylating ADP or other nucleoside diphosphates by the action of pyruvate kinase (EC 2.7.1.40). Furthermore, no work has been reported in which enolic phosphates have been examined for their possible activity as substrates or inhibitors of pyruvate kinase. However, Mildvan *et al.* (1967) showed that fluorophosphate inhibits the pyruvate kinase reaction by specifically competing with the P-enolpyruvate and not with ADP. Previous work of Mildvan and Cohn (1965, 1966) indicated that a ternary complex of P-enolpyruvate, Mn^{2+}, ADP, and enzyme was operative. This complex was proposed based on data from kinetic, conformational, and nuclear magnetic resonance studies. Examination of the proposed complex would lead one to suspect the possibility of steric specificity with respect to the site at which the enolic phosphate binds.

The present work reported herein was undertaken to elucidate the specifity of pyruvate kinase using synthetic homologs of P-enolpyruvate.

Clark and Kirby (1964) have developed a convenient method for the synthesis of P-enolpyruvate in which bromopyruvic acid is converted into the P-enolic dimethyl ester by the Perkow reaction using trimethyl phosphite. The methyl groups are removed in aqueous cyclohexylamine to yield the monocyclohexylammonium salt of P-enolpyruvate. It appeared feasible that if β-brominated α-keto acids other than pyruvate (*e.g.*, α-ketobutyric, etc.) were used, a similar reaction would yield corresponding homologs of P-enolpyruvate. The synthesis of β-bromo-α-ketobutyric acid by Sprinson and Chargaff (1946) indicated that the bromination of α-keto acids with a catalytic amount of H_2SO_4 would yield a brominated α-keto acid with a single bromine on C-3 (β-carbon). In this study we have also developed methods of synthesis for the β bromination of several α-keto acids along with the subsequent conversion of these acids into homologs of P-enolpyruvate.

Materials and Methods

Materials. Bromopyruvic, α-ketobutyric, α-ketovaleric, α-ketocaproic, α-ketoisovaleric, and α-ketoisocaproic acids were purchased from Sigma Chemical Co. The α-ketocaprylic acid was obtained from Pfaltz and Bauer Inc. α-Ketodecanoic acid was supplied by Aldrich Chemical Co. The α-ketoisovaleric acid was also synthesized according to the method of Ramage and Simonsen (1935). Trimethyl phosphite was purchased from J. T. Baker Chemical Co. and Eastman

* From the Department of Chemistry, Middle Tennessee State University, Murfreesboro, Tennessee. *Received January 19, 1970.* This work was supported in part by a grant from the Faculty Research Committee, Middle Tennessee State University.

Organic Chemical Co. Cyclohexylamine was also purchased from Sigma Chemical Co. All chemicals were the best grade available from the various sources. Since the free acids were known to decompose, initial synthetic reactions were carried out using α-keto acids freshly prepared from their sodium salts. Commercially available free α-keto acids gave results comparable with the freshly prepared α-keto acids; thus, the preparation of the free acid from the salt was discontinued.

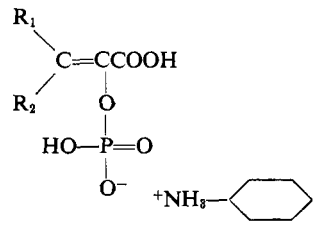
Synthesis of β-Bromo-α-keto Acids. The procedure of Clark and Kirby (1964) for synthesizing the phosphoenol homologs required β-bromo derivatives of the α-keto acids. The procedure used to prepare these acids combined the methods of Ward (1923) and Sprinson and Chargaff (1946) with some modifications. The α-keto acid was reacted (50°) by the dropwise addition of a molar equivalent of bromine along with an additional 5% excess bromine. The bromine had previously been dried by washing with concentrated H_2SO_4. Dry nitrogen was passed through the reaction mixture which aided in mixing and removal of the HBr that formed during the reaction. Two hours after the addition of the bromine, the nitrogen flow was stopped and the viscous liquid product was stored under vacuum over KOH pellets. The yield of brominated acid was increased by adding 5% excess bromine. The brominated α-keto acids were purified by recrystallization from benzene, chloroform, or carbon tetrachloride.

Synthesis of Phosphoenol Homologs. The purified brominated α-keto acids were then reacted with a molar equivalent of trimethyl phosphite (dissolved in dry ether). The reaction mixture was allowed to stand at 25° for at least 10 min, followed by removal of the ether by vacuum on a rotary evaporator. The resulting viscous liquid was reacted with a molar equivalent of cyclohexylamine (dissolved in water). The mixture was maintained at 25° for 3 days followed by the removal of the water on a rotary evaporator. The resulting crystalline mass was dried under vacuum over KOH pellets. Recrystallization was performed by dissolving in a minimum amount of methanol followed by the addition of an equal volume of dry ether. Crystallization occurred within a few minutes. The mixture was allowed to stand at 0° overnight. Subsequent recrystallizations were carried out in an identical manner. Final drying was accomplished under vacuum over phosphorous pentoxide.

Analytical Procedures. Nuclear magnetic resonance and infrared spectra of the brominated α-keto acids were used to verify their structures. The nuclear magnetic resonance spectra were determined using a Varian T-60 nuclear magnetic resonance spectrometer. The brominated acids were dissolved in CCl_4 or $CDCl_3$ and Me_4Si was used as in internal standard. The infrared spectra were run in a Beckman IR-20 spectrophotometer as KBr pellets. The molar absorptivities (ε) of the phosphoenolic homologs were determined in a Beckman DU-2 spectrophotometer. Kinetic studies were performed using a Gilford 2400 spectrophotometer. Melting points were determined in both a Mettler FP-1 melting point apparatus and a Mel-Temp melting point apparatus. A purified standard sample of benzoic acid melted from 122–123°. Elemental analyses were performed by Galbraith Laboratories Inc. (Knoxville, Tenn.).

Kinetic Studies. The kinetic studies were performed using the method of Pon and Bondar (1967). The concentrations of the components of the assay mixture were: ADP, 1.22×10^{-3} M; $MgCl_2$, 4.0×10^{-2} M; KCl, 7.5×10^{-2} M; Tris-HCl buffer, 0.05 M (pH 7.5); pyruvate kinase (Sigma Chemical Co.), 0.8 μg/ml of reaction mixture. Each of the homologs were substituted for P-enolpyruvate in a range of concentration from 5×10^{-4} to 2.5×10^{-3} M. No measurable change in absorbance was observed. A further test of the activity of each of the homologs was performed. In this procedure P-enolpyruvate was varied from 5.6×10^{-4} to 2.6×10^{-3} M for each reaction with the respective homolog held at 1×10^{-3} M. No apparent velocity changes were observed for the reactions except those containing P-enol-α-ketobutyrate and P-enol-α-ketovalerate. The other homologs did not appear to inhibit or activate the normal P-enolpyruvate–pyruvate kinase reaction. However, a more detailed kinetic study was performed on the inhibitory homologs and those that had no apparent activating or inhibiting characteristics toward pyruvate kinase. The Dixon (1953) plots shown in Figures 3 and 4 were determined on the two inhibitory homologs (P-enol-α-ketobutyrate and P-enol-α-ketovalerate). Michaelis constants were determined for P-enolpyruvate and also P-enolpyruvate with the individual noninhibitory homologs added. The ADP concentration was held at 5.96×10^{-4} M while the P-enolpyruvate was varied from 1.38×10^{-4} M to 11.33×10^{-4} M. The individual homologs were held at 8×10^{-4} M.

CHART I



Compound[a]	Name
A, R_1 = H; R_2 = H	P-enolpyruvate
B, R_1 = H; R_2 = methyl	P-enol-α-ketobutyrate
C, R_1 = H; R_2 = ethyl	P-enol-α-ketovalerate
D, R_1 = H; R_2 = propyl	P-enol-α-ketocaproate
E, R_1 = H; R_2 = pentyl	P-enol-α-ketocaprylate
F, R_1 = H; R_2 = heptyl	P-enol-α-ketodecanoate
G, R_1 = H; R_2 = isopropyl	P-enol-α-ketoisocaproate
H, R_1 = methyl; R_2 = methyl	P-enol-α-ketoisovalerate

[a] R_1 and R_2 may be reversed since *cis–trans* relationships are not established.

Results

Analytical Data. The nuclear magnetic resonance spectra verified that the α-keto acids were monobrominated on the β-carbon. These conclusions were based on the most salient features of the spectra; namely, the β-carbon proton absorption peak(s) (with the exception of β-bromo-α-ketoisovaleric acid) and the carboxyl proton peak along with the proper spectra for the other protons. The integral ratio of the β-carbon proton to the carboxyl proton was used as an indication of the purity of the brominated α-keto acid. Multiple substitution or lack of substitution would have given integral ratios different from the 1:1 ratio that was observed in all of the spectra. The carboxyl proton was found in the region of $\tau = -0.90$ to -2.50 ppm and appeared as a singlet. The

TABLE I: Elemental Analyses and Physical Constants of P-enolpyruvate and Homologs of P-enolpyruvate.

Compd[a]	Calcd (%)				Found (%)				ϵ (10^{-3})[b]	mp (°C)[c]
	C	H	N	P	C	H	N	P		
A	40.45	6.78	5.24	11.59	40.63	6.71	5.16	11.64	2.93	144–146[d]
B	42.70	7.16	4.98	11.01	42.68	7.07	4.89	10.80	3.65	139–140.5
C	44.74	7.51	4.74	10.48	45.05	7.63	4.72	10.30	4.46	133–134
D	46.60	7.82	4.53	10.01	46.86	7.97	4.67	10.24	5.18	132–133
E	49.85	8.37	4.15	9.18	49.73	8.43	4.33	8.96	4.43	142–143
F	52.59	8.83	3.83	8.48	52.68	8.87	3.81	8.41	5.53	140–141.5
G	46.60	7.82	4.53	10.01	46.88	8.02	4.65	9.87	4.63	140–141
H	44.74	7.51	4.74	10.48	44.57	7.60	4.83	10.42	3.40	141–142

[a] A, P-enolpyruvate; B, P-enol-α-ketobutyrate; C, P-enol-α-ketovalerate; D, P-enol-α-ketocaproate; E, P-enol-α-ketocaprylate; F, P-enol-α-ketodecanoate; G, P-enol-α-ketoisocaproate; H, P-enol-α-ketoisovalerate. [b] Determined in 0.05 M Tris-HCl buffer at pH 7.5 (230 nm). [c] Uncorrected (purified benzoic acid standard melted at 122–123°). [d] Decomposes.

β-carbon proton appeared in the region near τ = 5.0 ppm. This proton (β-carbon) was a quartet for β-bromo-α-ketobutyric acid, a triplet for β-bromo-α-ketovaleric acid, a triplet for β-bromo-α-ketocaproic acid, a triplet for β-bromo-α-ketocaprylic acid and a triplet for β-bromo-α-ketodecanoic acid. Since β-bromo-α-ketoisovaleric had no β-carbon proton, the absence of the peak was the important feature. β-Bromo-α-ketoisocaproic acid revealed a peak for the β-carbon proton as a doublet. In each of the spectra the overall integral ratios for all of the protons of the compounds were as predicted from their structures. The brominated acids were subsequently converted into the phosphoenolic forms.

The structures of P-enolpyruvate and the seven homologs are shown in Chart I. Table I presents the elemental analyses of the seven homologs of P-enolpyruvate and synthesized P-enolpyruvate (monocyclohexylammonium salts) also presented in Table I are the molar absorptivities and melting points of P-enolpyruvate and the seven homologs. Although they are not shown, the majority of the α-keto acids have molar absorptivities near 6.5 $\times$ 10^2 cm^{-1} M^{-1}. Shown in Figure 1 are the ultraviolet absorption spectra of six of the homologs and P-enolpyruvate in 0.05 M Tris-HCl buffer (pH 7.5). The ultraviolet spectra of P-enol-α-ketoisovalerate and P-enol-α-ketoisocaproate are not presented in Figure 1 although their molar absorptivities are given in Table I. Presented in Figure 2 are the infrared spectra of P-enolpyruvate and six of the homologs. The infrared spectrum

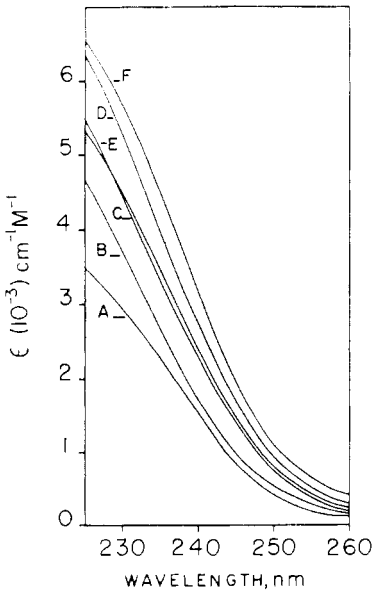


FIGURE 1: Ultraviolet absorption spectra of P-enolpyruvate and homologs of P-enolpyruvate: in 0.05 M Tris-HCl (pH 7.5, 29°) (see Chart I for explanation).

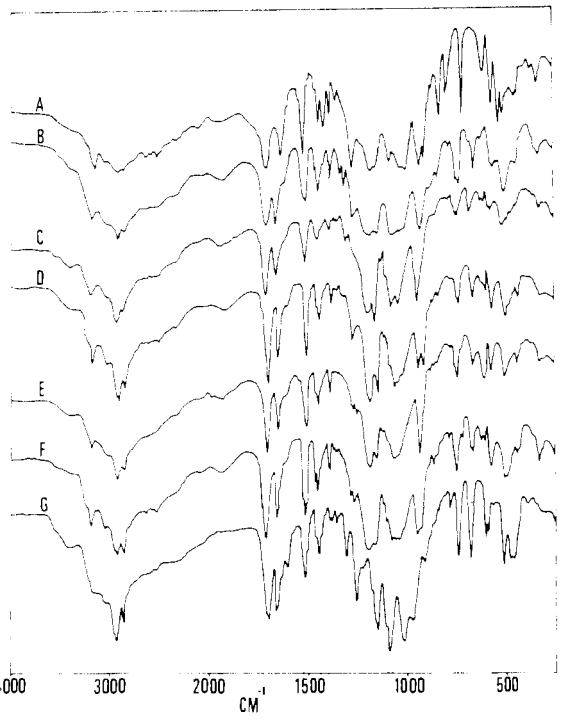


FIGURE 2: Infrared absorption spectra of P-enolpyruvate and homologs of P-enolpyruvate (see Chart I for explanation).

of P-enol-α-ketoisovalerate is not shown in Figure 2; however, it is quite similar to the other spectra.

Kinetic Studies. The homologs and the synthetic P-enolpyruvate were evaluated for their abilities to serve as substrates for pyruvate kinase according to the method of Pon and Bondar (1967). Since the phosphoenolic forms of the α-keto acids had molar absorptivities much higher than the hydrolysis product (α-keto acid), enzyme activity could be evaluated in a manner identical with that described by Pon and Bondar (1967). The synthesized P-enolpyruvate showed activity equivalent to that of an authentic sample (Nutritional Biochemical Corp.). None of the homologs showed substrate activity when substituted for P-enolpyruvate at the same concentrations. Raising or lowering their concentrations did not produce a measurable activity. These statements are best supported by the Michaelis constants, K_m, obtained for the reactions. P-enolpyruvate, varied over the range of concentration from 1.38×10^{-4} to 11.33×10^{-4} M with ADP held at 5.96×10^{-4} M, gave a K_m of $7.0 \pm 0.3 \times 10^{-5}$ M. Under identical conditions but with the respective homolog added at a concentration of 8×10^{-4} M to each of the reaction mixtures, the K_m observed for each of the reactions was well within the confidence limits of the determination. These Michaelis constants ranged from 6.8×10^{-5} to 7.1×10^{-5} M for P-enolpyruvate with the noninhibitory homologs added. This would indicate that these homologs are neither activators nor inhibitors. P-enol-α-ketobutyrate and P-enol-α-ketovalerate were shown to be very effective competitive inhibitors as shown by the Dixon plots in Figures 3 and 4. The inhibition constant, K_i, for the P-enol-α-ketobutyrate was 6.5×10^{-5} M, while the K_i for the P-enol-α-ketovalerate was 10.7×10^{-4} M. The homologs were examined for traces of calcium, magnesium, manganese, sodium, and potassium by atomic absorption spectrophotometry. Negligible quantities were found in solutions of the homologs.

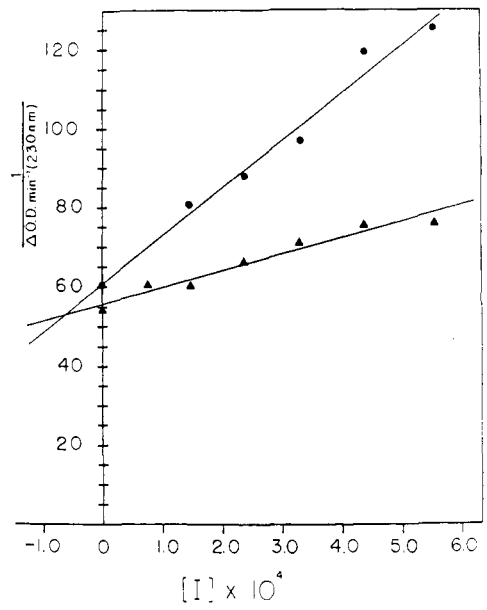


FIGURE 3: Dixon plot of P-enol-α-ketobutyrate inhibition of pyruvate kinase. Lower: P-enolpyruvate concn, 0.57 mM (closed circles); higher: P-enolpyruvate concentration, 1.45 mM (closed triangles); ADP, 0.9 mM; $MgCl_2$, 10 mM; 29.5° other conditions are listed in Materials and Methods.

Discussion

Evidence has been presented (Davidson, 1959; Klenow and Anderson, 1957; Plowman and Krall, 1965) that pyruvate kinase will function with several nucleoside diphosphates, *e.g.*, ADP, GDP, IDP, and 2′-deoxyadenosine diphosphate. Rose (1960) has presented evidence that other α-keto acids do not bind to the enzyme in a manner identical with pyruvate but apparently no direct evidence has been presented that established the specificity of pyruvate kinase toward homologs of P-enolpyruvate.

Examination of the structures of the homologs of P-enolpyruvate reveals an alkyl group attached to the β-carbon. Consideration of an enzyme–substrate complex as proposed by Mildvan and Cohn (1966) would give credence to steric hindrance effect by the alkyl group. This is complicated somewhat by the possibility of *cis–trans* isomers existing in the homologs, *i.e.*, the alkyl group on the β-carbon could be either *cis* or *trans* to the carboxyl group with the exception of P-enol-α-ketoisovalerate. If the substrate homologs were exclusively *trans* to the carboxyl group, they should be sterically more appropriate than those that were *cis*, if the model proposed by Mildvan and Cohn (1966) is indeed correct. Work is presently underway in an attempt to resolve the *cis–trans* forms of the compounds. Since the homologs other than P-enol-α-ketobutyrate and P-enol-α-ketovalerate did not inhibit the normal reaction of the conversion of P-enolpyruvate into pyruvate, as evidenced by the Michaelis constants, it would appear that these homologs are either chemically or stereochemically inappropriate to allow them to bind at the active site of pyruvate kinase. The model proposed by Mildvan and Cohn (1966) considers two binding sites on the enzyme. The carboxylate is proposed to bind at an unidentified site and the vinyl carbon is protonated by an uniden-

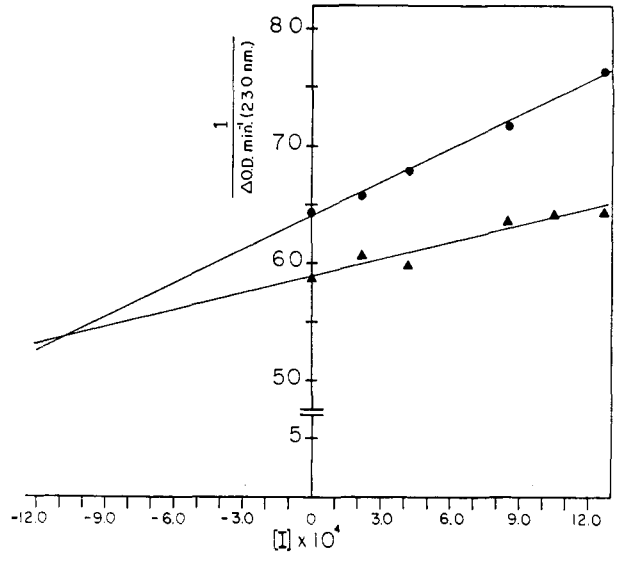


FIGURE 4: Dixon plot of P-enol-α-ketovalerate inhibition of pyruvate kinase. Lower: P-enolpyruvate concentration, 0.4 mM (closed circles); higher: P-enolpyruvate concentration, 0.79 mM (closed triangles); ADP, 0.51 mM; $MgCl_2$, 10 mM; 29.5°; other conditions are listed in Materials and Methods.

tified proton donor (see also Rose, 1960) during the phosphoryl transfer from the P-enolpyruvate to ADP. Since the four- and five-carbon homologs act as effective competitive inhibitors and the homologs with greater than five carbons have no apparent inhibitory characteristics, it would appear that the enzyme is capable of distinguishing quite specifically between them, thus, the active site must be quite restrictive. Also, the increased effectiveness of the four-carbon homolog compared with the five-carbon homolog also would appear to support the extreme steric selectivity of pyruvate kinase. In light of these observations, one might postulate that the methyl or the ethyl group attached to the vinyl carbon prevents the attachment of these homologs to the unidentified proton donor site, but does not prevent the attachment of the carboxylate group to the other substrate site (or possibly *vice versa*). The homologs having greater than five carbons and the dimethyl homolog apparently do not bind to the enzyme since no observed effect on the activity of the enzyme was noted. One must, therefore, conclude that the most plausible explanation for the different behavior of the homologs in enzymatic reactions must be stereochemical.

References

Clark, V. M., and Kirby, A. J. (1964), *Biochem. Prepn. 11*, 101.
Davidson, E. A. (1959), *Biochim. Biophys. Acta 33*, 238.
Dixon, M. (1953), *Biochem. J. 55*, 170.
Klenow, H., and Anderson, B. (1957), *Biochim. Biophys. Acta 23*, 92.
Mildvan, A. S., and Cohn, M. (1965), *J. Biol. Chem. 240*, 238.
Mildvan, A. S., and Cohn, M. (1966), *J. Biol. Chem. 241*, 1178.
Mildvan, A. S., Leigh, J. S., and Cohn, M. (1967), *Biochemistry 6*, 1805.
Plowman, K. M., and Krall, A. R. (1965), *Biochemistry 4*, 2809.
Pon, N. G., and Bondar, R. J. L. (1967), *Anal. Biochem. 19*, 272.
Ramage, G. R., and Simonsen, J. L. (1935), *J. Chem. Soc.*, 532.
Rose, I. A. (1960), *J. Biol. Chem. 235*, 1170.
Sprinson, D. B., and Chargaff, E. (1946), *J. Biol. Chem. 164*, 417.
Ward, C. F. (1923), *J. Chem. Soc. 123*, 2207.

Subunits, Composition, and Related Properties of Succinyl Coenzyme A Synthetase*

Claus Leitzmann,† Jang-Yen Wu, and P. D. Boyer

ABSTRACT: Succinyl coenzyme A synthetase from *Escherichia coli* (mol wt *ca.* 146,000), both phosphorylated or nonphosphorylated, was dissociated into subunits by exposure to dilute *p*-mercuribenzoate or sodium dodecyl sulfate solutions, to concentrated urea or guanidine hydrochloride solutions, or by alkylation with iodoacetamide or succinylation with succinic anhydride in urea solutions. Considerable aggregation tendency was noted. Subunits were characterized by sucrose density gradient, gel filtration, column chromatography, gel electrophoresis, and ultracentrifugal analyses. Alkylation in urea gave subunits of about 0.5 and succinylated subunits of about 0.25 of the original size. Sedimentation equilibrium measurements indicated possible presence of smaller fragments after both alkylation and succinylation. Complete amino acid analyses are given; tryptophan at approximately eight residues per mole is present in the smallest amount. The results are consistent with presence of at least four subunits of approximately equal size and with similar or possibly identical composition of the phosphorylated and nonphosphorylated subunits. Tests with possible modifiers of catalytic activity showed no prominent effects other than those indicative of competition with substrates. Some improvements in enzyme preparation and a more sensitive catalytic assay are reported. A lack of correlation between capacity for phosphoenzyme formation and catalytic activity of different preparations is confirmed.

Succinyl-CoA synthetase from *Escherichia coli* has been reported to have a molecular weight of approximately 160,000 (Gibson *et al.*, 1967) and of 141,000 (Ramaley *et al.*, 1967). Presence of subunits thus seems probable. Evidence for dissociation of the enzyme by *p*-mercuribenzoate has been mentioned (Ramaley *et al.*, 1967), and merthiolate treatment has been shown to give subunits as measured by immunodiffusion (Grinnell *et al.*, 1969). The enzyme can be phosphorylated by more than one and approaching two phosphoryl groups per mole (Moyer *et al.*, 1967; Grinnell and Nishimura, 1969), suggesting that at least two and possibly more subunits may be present.

The present paper presents some modifications in enzyme preparation, the amino acid composition of the enzyme, and evidence for dissociation of the enzyme into at least four subunits. A considerable tendency for aggregation

* Supported in part by Grant GM-11094 from the Institute of General Medical Sciences, U. S. Public Health Service. *Received November 13, 1969.*

† Postdoctoral Fellow of the Institute of General Medical Sciences, U. S. Public Health Service.