Succinic Thiokinase

I. PURIFICATION OF THE ENZYME FROM PIG HEART*

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Succinic thiokinase was found by Kaufman et al. (2, 3) and by Hift et al. (4) to be responsible for the substrate level phosphorylation reaction accompanying the oxidation of α-ketoglutarate. The enzyme has been variously termed phosphorylation ("P") enzyme (3, 4), GDP phosphorylation enzyme (5), succinic thiokinase (6-8), succinyl-CoA synthetase (9, 10), and succinate:CoA ligase (GDP) (11). This enzyme catalyzes the reaction

\[ \text{Nucleoside diphosphate} + \text{P} + \text{succinyl-CoA} \xrightleftharpoons{\text{Mg}^{++}} \text{nucleoside triphosphate} + \text{succinate} + \text{CoA} \]

The specificity of mammalian enzyme preparations for either guanosine or inosine di- and triphosphates was demonstrated by Sanadi et al. (5, 12-14). The enzyme from spinach studied by Kaufman and Alivisatos (15) and that from Escherichia coli studied by Smith et al. (9, 16) were found to be specific for adenine nucleotides.

This laboratory became interested in mammalian succinic thiokinase as a potential site of the carcinostatic action of guanine analogues such as 8-azaguanine. After the development of rapid and sensitive assay methods and of a suitable procedure for purification of the enzyme, it was decided to perform an extensive study of the enzyme itself in an attempt to elucidate its reaction mechanism.

The first report of this series is concerned primarily with the development of assay methods, the purification of the enzyme from pig heart, and the substrate activity of 8-azaguanine nucleotides. A preliminary report of this work and those described in the following publication of this series has been presented (1).

EXPERIMENTAL PROCEDURE

Materials

DEAE-cellulose (Cellex D) was purchased from the Bio-Rad Laboratories. GDP, GTP, and CoA were obtained from the Pabst Laboratories. AzaGDP† and azaGTP were generously supplied by the Cancer Chemotherapy National Service Center. The concentrations of 8-azaguanine nucleotides determined by use of the molar absorptivity of 10.5 \( \times 10^3 \) at 256 nm (17, 18), and by an enzymic method described below, in which the total absorbance changes were measured in the presence of an excess of other substrates, were in good agreement (+3%). Pig hearts were the gift of Oscar Mayer and Company. Pyruvic kinase (254 micromolar units per mg), lactic dehydrogenase (300 micromolar units per mg), phosphoenolpyruvate (trisodium salt), and DPNH were purchased from the California Corporation for Biochemical Research.

Commercial preparations of GDP were purified by ion exchange chromatography. Not more than 100 mg of GDP were applied to a DEAE-cellulose column (bicarbonate form, 2.7 \( \times \) 11 cm). The chromatogram was developed with a linear gradient of triethylammonium bicarbonate (0 to 0.4 M over 1300 ml). There were seven peaks which contained ultraviolet-absorbing material; GDP was found in the major peak which emerged at the concentration of eluent of about 0.18 M. The fractions containing GDP were pooled and evaporated under reduced pressure, with the recovery of about 75% of 252 nm-absorbing material as the triethylammonium salt of GDP. GTP was purified in a similar manner except that a linear gradient of 0.1 to 0.5 M was employed. A typical chromatogram of GTP is shown in Fig. 1. The triethylammonium salts of the nucleotides obtained by evaporation of the eluate were used without further treatment, and were found to be satisfactory for the purpose of this study.

The attempts to purify CoA by a similar method were unsuccessful because of the difficulty involved in keeping the compound in the reduced form. The commercial preparation of CoA was used without further purification. By means of assay with succinic thiokinase, it was found that the stated purity of 75% was approximately correct. To minimize the oxidation of CoA, solutions were prepared freshly for each experiment from stock solutions maintained in small aliquots at -20°C. In studies in which concentrations of CoA were critical, the solutions were used within 2 hours after dilution from the stock solution.

Succinyl-CoA was synthesized by a modification of the method of Simon and Shemin (19). Approximately 20 to 100 mg of CoA were dissolved in 20 ml of cold water. The concentrations of CoA solutions were estimated from the absorbance of small aliquots diluted in pH 7.0 buffer; it was assumed that 75% of the 260 nm-absorbing material was reduced CoA and that the molar absorptivity of CoA at 260 nm and at pH 7.0 was the same as that of AMP (15.4 \( \times 10^3 \)). A 5-fold molar excess of succinic...
anhydride was added at ice bath temperature, and the mixture was shaken frequently. The pH of the solution was maintained at neutrality by the addition of potassium bicarbonate. After 30 to 60 minutes, the succinyl-CoA was purified by ion exchange chromatography on a column of DEAE-cellulose (formate form) with sodium formate buffer as the eluting agent. Elution patterns of the crude and purified preparations are shown in Fig. 2. Since succinyl-CoA is rapidly hydrolyzed at alkaline pH, triethylammonium bicarbonate could not be used as an eluting agent.

Calcium phosphate gel was prepared according to the method of Tsuboi and Hudson (20).

Methods

Measurements of Enzyme Activity—The three methods described below were used. The direction of reaction from left to right in Equation 1 will be designated as the forward, and from right to left, as the reverse.

Rate of Reverse Reaction—A direct spectrophotometric method was used. The principle of this method is similar to that of the spectrophotometric assay of Kaufman and Alivisatos (15), and is based on the measurement of the increase in absorbance at 235 µm which is due to the formation of the thioester bond of succinyl-CoA. A Zeiss spectrophotometer, model MH Q11, equipped with a thermospacer, a Gilford optical density converter, and a Barber-Colman recorder, was used. Water was circulated through the thermospacer from a 30° bath. In order to minimize extraneous absorbance at 235 µm, reducing agents such as cysteine or glutathione were not added to the reaction mixture.

The above method was used as the standard assay during purification of the enzyme. The standard reaction mixture in a 1-ml cuvette having a 1.0-cm light path contained, in addition to enzyme, the following components: succinate, 50 mM (adjusted to pH 7.4 to give a final Tris concentration of approximately 110 mM), MgCl₂, 10 mM, GTP, 0.1 mM, and CoA, 0.1 mM. The cuvette containing all the components (0.9 ml) except the enzyme was preincubated for 2 to 3 minutes in the cuvette holder within the thermospacer of the spectrophotometer. The reaction was started by the addition of 0.1 ml of enzyme solution at ice bath temperature.

The velocity was proportional to the amount of enzyme when the latter was properly diluted so that the rate did not exceed a change in absorbance of 0.040 per minute. The enzyme was usually diluted in 0.2 M Tris-acetate buffer, pH 7.4. Triethylammonium bicarbonate, Cl⁻, acetate, Tris, Na⁺, and K⁺ did not affect the reaction velocity at a concentration of 0.1 M.

Unit of Succinic Thiokinase Activity—One unit is defined as that amount of enzyme which gives an absorbance increase of 1.000 per minute at 235 µm under the conditions of the standard assay. One unit of enzyme defined in this manner corresponds to 1 Kaufman hydroxamate unit (21) with a discrepancy of less than 10%.

Forward Reaction—In a manner similar to that described above, the decrease in absorbance at 235 µm due to the disappearance of the thioester bond of succinyl-CoA was recorded to follow the forward reaction. A typical reaction mixture contained the following components: potassium phosphate, pH 7.4, 50 mM; MgCl₂, 10 mM; succinyl-CoA, 0.1 mM; GDP, 0.1 mM; and enough enzyme to give an absorbance decrease of 0.010 to 0.050 per minute. Since succinyl-CoA is hydrolyzed slowly at neutral pH, the true enzymic reaction rates were estimated by subtracting the rate of nonenzymic hydrolysis from the observed velocities. The reaction rates obtained under these conditions are approximately 30% greater than those obtained in the reverse.
direction under the conditions of the standard assay for the same amount of enzyme.

Formation of GDP—This was determined by coupling the succinic thiokinase reaction to pyruvic kinase and lactic dehydrogenase reactions. This assay method was used when the measurement of succinyl-CoA formation was not feasible, as in the presence of arsenate or hydroxylamine. The absorbance decrease due to the disappearance of DPNH was measured at 340 \text{nm}.

A typical assay mixture contained, in addition to succinic thiokinase, the following components: Tris-acetate buffer, pH 7.4, 100 mM; KCl, 100 mM; MgCl₂, 10 mM; phosphoenolpyruvate (trisodium salt), 1.5 mM; DPNH, 0.2 mM; succinate, 1.0 mM; GTP, 0.1 mM; CoA, 0.1 mM; and non-rate-limiting amounts of pyruvic kinase and lactic dehydrogenase. A 1-mL cuvette, containing all components except succinic thiokinase, was preincubated for 2 minutes in a 30° water bath and for another 2 minutes in the cuvette holder within the thermostaper of the Zeiss spectrophotometer. The reaction was started by the addition of 0.1 mL of succinic thiokinase solution at ice bath temperature.

Statistical Analyses of Kinetic Data—The computer program developed by Cleland (22) was used.

Protein Concentrations—The biuret procedure of Gornall, Bardawill, and David was used (23) unless stated otherwise. When interfering materials were present, the biuret procedure was performed after the protein was precipitated with 10% trichloroacetic acid.

RESULTS

Purification of Enzyme

All procedures were carried out at 0–5°, unless stated otherwise. The pig hearts were packed in ice after removal from the carcasses, and the enzyme isolation was undertaken within a few hours after the death of the animals. A series of preliminary studies revealed that greater amounts of enzyme can be extracted from fresh heart than from frozen heart, and that freezing and thawing causes a considerable loss of activity. An enzyme preparation at any stage of purification after the initial extraction can be stored without loss of activity as a suspension in 80% saturated ammonium sulfate solution3 at approximately 0–5°.

A typical procedure for the preparation of the enzyme is described below.

Step 1: Preparation of Crude Extract—Eighteen fresh pig hearts were freed of fat and connective tissue, minced with a meat grinder, and washed in a plastic pail with large quantities of cold distilled water until the washings were almost colorless. The washed mince was transferred into a cheesecloth bag, and as much water as possible was pressed out by hand. The washed tissue, amounting to 4,000 g, was divided into 100-g batches. Each batch was homogenized for 3 minutes with 300 ml of 0.01 M K₂HPO₄ in a high-speed Waring blender. The homogenate was then centrifuged for 20 minutes at 10,000 × g, and the residue was discarded. The combined supernatant fluids were filtered through several layers of cheesecloth. The total volume was 9,940 mL. The specific activity and the protein content were 0.63 unit per mg and 8.14 mg per ml, respectively (Fraction I).

Step 2: Removal of Impurities by Lowering pH—Fraction I was adjusted to pH 5.2 by slowly adding 0.1 N acetic acid. During the addition, the solution was stirred constantly with a magnetic stirrer, and the pH was continuously measured with a Beckman model G pH meter. The suspension was centrifuged at 10,000 × g for 15 minutes, and the clear, reddish colored supernatant fluid (Fraction II) was collected. The volume was 9,900 ml. The specific activity was 0.77 unit per mg, and the protein content was 4.8 mg per ml.

Step 3: Adsorption of Succinic Thiokinase on Calcium Phosphate Gel—To each liter of Fraction I were added 50 ml of 1.0 M potassium phosphate buffer, pH 7.4. Then 2.5 liters of calcium phosphate gel (45 g, dry weight) were added, and the mixture was stirred for 30 minutes and allowed to settle overnight. The bulk of the clear supernatant fluid was siphoned off, and the gel was collected by centrifugation at 10,000 × g for 5 minutes. The enzyme was eluted from the gel by three successive treatments with 30 ml of 10% saturated ammonium sulfate in 0.1 M potassium phosphate buffer (pH 7.4 before addition of ammonium sulfate) per g of gel, dry weight. The volume, specific activity, and protein concentration were 4,550 ml, 2.3 units per mg, and 2.4 mg per ml, respectively (Fraction III).

Step 4: Ammonium Sulfate Fractionation—Fraction III was subjected to ammonium sulfate fractionation between 50 and 75% saturation. After each addition of the appropriate amount of solid ammonium sulfate, the mixture was kept in the cold for several hours and was then centrifuged at 10,000 × g for 30 minutes. The 50 to 75% precipitate was dissolved in 0.05 M potassium phosphate buffer, pH 7.0 (Fraction IV). The volume, specific activity, and protein content were 50 ml, 5.7 units per mg, and 87.8 mg per ml, respectively.

Step 5: Heat Treatment and Second Ammonium Sulfate Fractionation—Fraction IV was dialyzed overnight against three changes of 4 liters each of 0.05 M potassium phosphate buffer, pH 7.0 (Fraction IV). After dialysis, the volume of the enzyme solution was 80 ml. The temperature of the solution was quickly brought to 52° by immersing and swirling the container in a 90° water bath. As soon as the temperature reached 52°, the flask was transferred to a 52° water bath and held for 3 minutes without agitation. Then 50 ml of ice-cold, 100% saturated ammonium sulfate solution were added rapidly to the enzyme solution and mixed by swirling. The mixture was kept in an ice bath for about 1 hour, after which time it was centrifuged at 20,000 × g for 60 minutes. The supernatant fluid was clear and yellow, with a slight reddish tinge. Solid ammonium sulfate was added to bring the supernatant solution to 70% saturation, and the precipitate obtained by centrifugation was dissolved in a minimal amount of 0.25 M Tris-acetate, pH 7.4 (Fraction V). Fraction V had a volume of 12.7 ml, and contained 98 mg of protein per ml and 15.8 units of succinic thiokinase per mg of protein. A large quantity of crystals formed in the solution during the next 12 hours. Most of the succinic thiokinase activity was found in the mother liquor. The identity of this crystalline protein has not been determined. Similar crystals

\[ w = \frac{0.515(S_1 - S_2)V_1}{1 - 0.272(S_2)} \]

where \( w \) is grams of ammonium sulfate; \( S_1 \) and \( S_2 \), the initial and final saturation; and \( V_1 \), the initial volume in milliliters.
have been obtained on several occasions at comparable stages of purity (1).

**Step 6: DEAE-cellulose Column Chromatography**—The supernatant solution after the removal of the crystals by centrifugation was dialyzed overnight against 4 liters of 0.05 M potassium phosphate, pH 7.0, followed by a further dialysis against 2 liters of 0.01 M triethylammonium bicarbonate for 2 hours. The protein solution was added to a DEAE-cellulose column (bicarbonate form, 2.7 X 16 cm) which was equilibrated with 0.01 M triethylammonium bicarbonate. The column was washed with 200 ml of 0.01 M triethylammonium bicarbonate, then eluted with a linear gradient of triethylammonium bicarbonate from 0.01 to 0.2 M in a total volume of 500 ml. Fractions of 10 ml were collected, and those containing the bulk of succinic thiokinase were pooled. The resulting dilute enzyme solution (230 ml) was concentrated in two steps. Calcium phosphate gel (30 ml, approximately 500 mg of solid) was added, after which the solution was stirred for 30 minutes and allowed to settle overnight. The gel was collected in a small plastic centrifuge tube and eluted four times with 2-ml portions of 10% saturated ammonium sulfate in 0.2 M ammonium sulfate (final 80% saturation). The mixture was allowed to stand overnight; the precipitate was collected by centrifugation and then dissolved in a minimal quantity of 0.2 M ammonium bicarbonate, then eluted from the gel four times with 0.75-ml portions of 10% saturated ammonium sulfate in 0.2 M ammonium bicarbonate, pH 6.5, and dialyzed against the same buffer for 2 hours. The volume of the dialyzed solution (Fraction VI) was 5.1 ml, the specific activity was 25.2 units per mg, and the protein concentration was 26.2 mg per ml.

**Step 7: Carboxymethyl Cellulose Column Chromatography**—Fraction VI was chromatographed on a carboxymethyl cellulose column (2.7 X 12 cm) equilibrated with 0.01 M Tris-acetate, pH 6.5, containing 1 X 10^{-6} M MgCl₂. The enzyme was eluted from the column with a linear gradient of Tris-acetate, pH 6.5, from 0.01 to 0.2 M, while the MgCl₂ concentration was kept constant at 1 X 10^{-6} M. (In the absence of MgCl₂ the enzyme was eluted at a much lower concentration of the eluent and the separation of proteins was poor.) The chromatogram is shown in Fig. 3. Also shown in the figure is the relative activity of CoA transferase (25), which was one of the protein impurities contained in Fraction VI. The three 10-ml fractions containing the highest activity were pooled and treated with 2.5 ml of calcium phosphate gel (4.1 mg of solid). The enzyme was eluted from the gel four times with 0.75-ml portions of 10% saturated ammonium sulfate in 0.2 M Tris-acetate, pH 7.4. The enzyme in the pooled eluates was precipitated by the addition of saturated ammonium sulfate solution to bring the mixture to 80% saturation. The mixture was allowed to stand overnight; the precipitate was collected by centrifugation and dissolved in a minimal quantity of 0.2 M Tris-acetate, pH 7.4. The volume of the colorless, clear solution was 0.8 ml (Fraction VI). The protein concentration estimated by the method of Warburg and Christian (26) was 4.6 mg per ml. The specific activity was 230 units per mg, which is the highest ever obtained for this enzyme from a mammalian source. The preparation at this stage of purity catalyzes the formation of about 6,000 moles each of succinyl-CoA, GDP, and orthophosphate from succinate, pH 7.4. Results of several similar purifications are summarized in Table I.

![Fig. 3. Purification of succinic thiokinase by chromatography on a CM-cellulose column. Details of the procedure are described in the text.](http://www.jbc.org/)

**Substrate Activities of AzaGDP and AzaGTP**

AzaGDP and azaGTP replace GDP and GTP, respectively, in the succinic thiokinase reaction. The extrapolated V_max values obtained with these analogue-containing nucleotides are comparable to those obtained with the corresponding guanine nucleotides. However, the apparent K_m of azaGDP was 1.11 (±0.16) X 10^{-4} M, or about 40 times higher than that of GDP, 2.85 (±1.18) X 10^{-4} M, obtained under the same experimental conditions.

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific activity (units/mg)</th>
<th>Yield</th>
<th>No. of preparations included</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract (0.01 M K₂HPO₄)</td>
<td>0.46 ± 0.15*</td>
<td>100 ± 34.5%†</td>
<td>16</td>
</tr>
<tr>
<td>II. pH 5.2 supernatant</td>
<td>0.76 ± 0.14</td>
<td>82.1 ± 6.1%</td>
<td>8</td>
</tr>
<tr>
<td>III. Calcium phosphate</td>
<td>2.4 ± 0.6</td>
<td>52.1 ± 14.1%</td>
<td>8</td>
</tr>
<tr>
<td>IV. Ammonium sulfate</td>
<td>5.5 ± 0.6</td>
<td>34.4 ± 9.5%</td>
<td>8</td>
</tr>
<tr>
<td>V. Heat (52°C, 3 minutes) and ammonium sulfate (50 to 70%)</td>
<td>10.6 ± 4.5</td>
<td>95.0 ± 9%</td>
<td>6</td>
</tr>
<tr>
<td>VI. DEAE-cellulose (bicarbonate) column eluate</td>
<td>28.9 ± 8.7</td>
<td>6.8 ± 1.0%</td>
<td>5</td>
</tr>
<tr>
<td>VII. CM-cellulose-Tris⁺⁺Mg⁺⁺ column eluate</td>
<td>230.0</td>
<td>1.6</td>
<td>1</td>
</tr>
</tbody>
</table>

* Standard deviation given in units per mg.
† Standard deviation given as percentage.
‡ Equivalent to 899 ± 310 units per 100 g of tissue.
conditions (Fig. 4). A similar experiment with azaGTP and GTP (Fig. 5) revealed that the apparent $K_m$ values for GTP and azaGTP are $1.0 \times 10^{-5}$ and $2.1 \times 10^{-4}$ M, respectively. Although the $V_{\text{max}}$ obtained with azaGTP was approximately 60% of the $V_{\text{max}}$ with GTP, this apparent difference may have been due to the experimental errors inherent in measurements of slow reaction rates.

**FIG. 4.** Plot of reciprocal of initial reaction velocity ($v$) versus reciprocal of substrate concentration ($S$) (millimolar). The variable substrate was either GDP or azaGDP as indicated. Held constant were potassium phosphate, pH 7.4, 50 mM; MgCl$_2$, 10 mM; and succinyl-CoA, 0.1 mM. Velocities are expressed as the decrease in absorbance at 235 mp per minute. The kinetic parameters ($\pm$ standard error) for GDP and azaGDP are: apparent $K_m$, $0.0029 \pm 0.0012$ and $0.111 \pm 0.166$ mM; vertical intercepts ($1/A$ min$^{-1}$), $37.9 \pm 1.2$ and $34.1 \pm 2.9$; with 5 and 7 degrees of freedom, respectively.

**FIG. 5.** Plot of reciprocal of initial reaction velocity ($v$) versus reciprocal of substrate concentration ($S$) (millimolar). The variable substrate was either GTP or azaGTP (as indicated). Held constant were succinate (Tris salt, pH 7.4), 50 mM; MgCl$_2$, 10 mM; and CoA, 0.05 mM. Velocities are expressed as the increase of absorbance at 235 mp per minute. The kinetic parameters ($\pm$ standard error) for GTP and azaGTP are: apparent $K_m$, $0.010 \pm 0.002$ and $0.212 \pm 0.025$ mM; vertical intercepts ($1/A$ min$^{-1}$), $19.3 \pm 0.6$ and $31.1 \pm 2.3$; with 7 and 5 degrees of freedom, respectively.

**FIG. 6.** Effect of hydroxylamine on the rate of GDP formation in the succinic thiokinase reaction as a function of CoA concentration; plot of the reciprocal of velocity ($v$) versus the reciprocal of the concentration (millimolar) of CoA. The pyruvic kinase-lactic dehydrogenase coupled system was used. The components and conditions were the same as those described under “Methods” except that hydroxylamine (neutral pH) was present at a concentration of either 0, 50, or 100 mM as indicated in the figure. Velocities are expressed as the decrease in absorbance at 340 mp per minute. Kinetic parameters ($\pm$ standard error) at hydroxylamine concentrations of 0, 50, and 100 mM are: apparent $K_m$ values for CoA, $0.020 \pm 0.003$, $0.027 \pm 0.004$, and $0.041 \pm 0.004$ mM; vertical intercepts ($1/A$ min$^{-1}$), $43.8 \pm 1.3$, $51.0 \pm 1.7$, and $67.7 \pm 2.3$; slopes ($1/A$ mM$^{-1}$ min$^{-1}$), $0.87 \pm 0.09$, $1.92 \pm 0.14$, and $2.77 \pm 0.20$; with 2 degrees of freedom in each case. Approximate values for apparent inhibition constants of hydroxylamine are: $K_i$ from intercepts, $2 \times 10^{-1}$ M; $K_i$ from slopes, $5 \times 10^{-2}$ M.

**Effect of Hydroxylamine**

Hydroxylamine at the concentration (0.5 mM) used previously in the assay of succinic thiokinase is a strong inhibitor (21, 27). As is shown in the Lineweaver-Burk plots in Fig. 6, when CoA was the variable substrate the inhibition constant estimated from the slopes was $5 \times 10^{-2}$ M and that estimated from the intercepts was $2 \times 10^{-1}$ M.

**Enzymic Determination of Difference in Molar Absorptivities of Succinyl-CoA and CoA**

A preliminary study indicated that upon hydrolysis of succinyl-CoA there is a maximal decrease in absorbance at 235 mp. GDP chromatographed twice on DEAE-cellulose was used as the primary standard. The concentration of GDP in the stock standard solution was determined spectrophotometrically. The molar absorptivity of GDP, $13.7 \times 10^3$ M$^{-1}$ cm$^{-1}$ at 252 mp and pH 7.0, was employed (28).

A cuvette with a 1-cm light path containing GDP and a large excess of phosphate (100 mM) and succinyl-CoA (approximately 0.1 mM) in a 0.9-ml volume was preincubated in the cuvette holder of the spectrophotometer; the slow decrease in absorbance at 235 mp due to the nonenzymic hydrolysis of succinyl-CoA was recorded. After 2 to 3 minutes of incubation, 0.1 ml of a highly active enzyme solution was added and the absorbance was continuously recorded until its change became slow and linear for 2 to 5 minutes. The correction procedure for the nonenzymic hydrolysis and the results obtained with various concentrations of GDP are shown in Fig. 7.

Since it is reasonable to assume that the equilibrium constant of the guanine nucleotide-dependent succinic thiokinase reaction is about the same as that of the adenine nucleotide-dependent reaction ($K_{eq} = 3.7$) reported by Kaufman and Alivisatos (15),...
the conversion of GDP to GTP must be virtually quantitative under the conditions of this experiment. Thus, for every mole of GDP phosphorylated, 1 mole of succinyl-CoA must be hydrolyzed. The difference of the molar absorptivities of succinyl-CoA and CoA at 235 μm and pH 7.4 calculated from the data shown in Fig. 7 is 4.0 X 10³ M⁻¹ cm⁻¹. A value of 4.5 X 10³ M⁻¹ cm⁻¹ at 232 μm and at an unspecified pH has been published.⁴

**SUMMARY**

1. Chromatographic methods for purification of guanosine di- and triphosphate and succinyl coenzyme A are presented.
2. Direct spectrophotometric methods for the measurement of the succinic thiokinase reaction from both directions are described, as is a coupled assay system (pyruvic kinase-lactic dehydrogenase) which is suitable for the study of the effects of hydroxylamine and arsenate.
3. The succinic thiokinase has been purified from fresh pig hearts about 500-fold (specific activity, 230 units per mg) over the crude extract with an activity yield of about 2%. This preparation catalyzes the formation of about 6,000 moles each of succinyl-CoA, guanosine diphosphate, and orthophosphate from succinate, guanosine triphosphate, and CoA per 100,000 g of protein per minute at 30° and at pH 7.4.
4. Hydroxylamine is highly inhibitory at the concentration (0.5 mM) employed in Kaufman’s hydroxamate method for succinic thiokinase assay. The Kᵢ value estimated from the slopes of the Lineweaver-Burk plot (1/v versus 1/[CoA]) is approximately 5 X 10⁻³ M.
5. The decrease in the molar absorptivity upon hydrolysis of succinyl-CoA has been determined by an enzymic method to be 4.0 X 10³ M⁻¹ cm⁻¹ at 235 μm and pH 7.4.
6. Succinic thiokinase does not appear to be a site of the carcinostatic action of 8-azaguanine. 8-Azaguanosine diphosphate (apparent Kᵢ, 1 X 10⁻⁴ M) replaces guanosine diphosphate (apparent Kᵢ, 3 X 10⁻⁴ M), and 8-azaguanosine triphosphate (apparent Kᵢ, 2 X 10⁻⁴ M) replaces guanosine triphosphate (apparent Kᵢ, 1 X 10⁻⁴ M). Although the apparent Kᵢ values of the analogues are about 20 to 30 times higher than those of corresponding guanine nucleotides, the Vₘₐₓ values are of similar orders of magnitude.

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