UTILIZATION OF CIBACRON BLUE 3G-A SEPHAROSE 6B IN THE ISOLATION AND ENRICHMENT OF PYRUVATE, PHOSPHATE DIKINASE, ALANINE DEHYDROGENASE, AND PHOSPHOENOLPYRUVATE CARBOXYLASE FROM MITOMYCIN-PRODUCING *STREPTOMYCES VERTICILLATUS*

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Pyruvate, phosphate dikinase (E.C. 2.7.9.1) has been demonstrated in mitomycin-producing *Streptomyces verticillatus*. Low levels of activity are detectable in noninduced cultures; however, in alanine or pyruvate containing media the enzyme activity is induced 20 to 40 fold. Assays for the dikinase are subject to very large interferences if pyruvate kinase and adenylate kinase are present together, or if alanine dehydrogenase is present. Chromatography of dialyzed cell-free extracts of induced *S. verticillatus* on Cibacron Blue 3G-A Sepharose 6B affords a clean separation of the dikinase from these interfering enzymes which are present in this organism. While the dikinase is eluted in the early fractions, pyruvate kinase and alanine deh drogenase are retained on the column. The dikinase is inhibited by free Cibacron 3G-A Blue and therefore its non-binding to the dye Sepharose column is presently unexplained. Dikinase activity has been detected in two of four strains of *Streptomyces* tested and it was found to be absent in rifamycin-producing *Nocardia mediterranei*. Lactate dehydrogenase (E.C. 1.1.1.27) activity could not be detected in *S. verticillatus*, but alanine dehydrogenase (E.C. 1.4.1.1) has been purified 75 fold in a single step by elution with 0.25 mM NADH from Cibacron Blue 3G-A Sepharose 6B. Further studies have shown the presence of phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) in extracts of *S. verticillatus*. This acetyl coenzyme A activated enzyme readily binds to the dye columns and can be specifically eluted with acetyl-coenzyme A. It presumably interacts with the dye on the column by way of its allosteric site.

The investigations reported in this paper were carried out in connection with studies on the biosynthesis of the mitomycin antibiotics in *Streptomyces verticillatus* (HORNEMANN, U.: Biosynthesis of the mitomycins. *In* J. W. CORCORAN (*ed.*), Antibiotics. Vol. 4, Biosynthesis, Springer, New York, in press). The biosynthetic studies have demonstrated that C_1 and C_2 of pyruvic acid are specifically incorporated into C_6 and C_{8a}, respectively[16], and that C_3 of pyruvic acid most likely labels C_5 of these antibiotics[15]. It was suggested that the incorporation of pyruvate may occur *via* phosphoenolpyruvate (PEP) and 3, 4-dideoxy-4-aminoheptulosonic acid-7-phosphate, a hypothetical branch point metabolite of the shikimic acid pathway[18], which could act as a precursor for an as yet not well defined *meta*-C-C_6-N unit (C_7N unit)[15] as outlined in Fig. 1. A prerequisite for this pathway would be that *S. verticillatus* possesses enzymes to effect the conversion of pyruvic acid into phosphoenolpyruvic acid, therefore, this organism was studied with regard to relevant pyruvate metabolizing enzymes. Other antibiotics from actinomycetales, including rifamycin[35], geldanamycin[11] and pactamycin[34] appear to contain similar *meta*-C-C_6-N units which are presumably also derived from either 3,4-dideoxy-4-aminoheptulosonic acid-7-phosphate or an early intermediate of the shikimic acid pathway[9,11,34,35]. Pyruvate was an inefficient

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Fig. 1. Utilization of pyruvate in mitomycin biosynthesis in *S. verticillatus*.
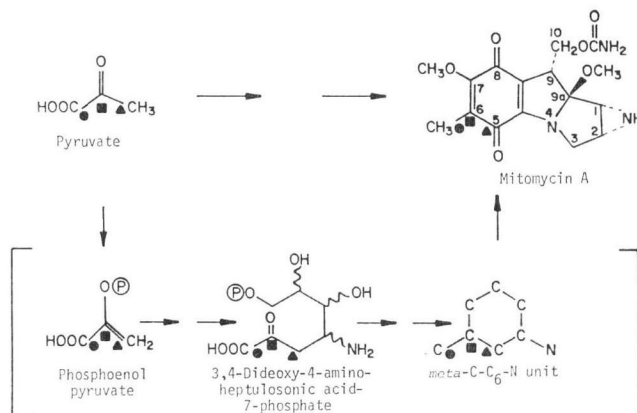
precursor in rifamycin-producing *Nocardia mediterranei* [18], and its metabolism was therefore studied in this organism as well.

The conversion of pyruvate to phosphoenolpyruvate could occur directly through the reversal of pyruvate kinase, or by either pyruvate, phosphate dikinase or phosphoenolpyruvate synthetase. Alternatively, the conversion could be accomplished indirectly by the combined reactions of pyruvate carboxylase or malic enzyme and malic dehydrogenase with one of the phosphoenolpyruvate carboxy kinases. These possibilities are outlined in Fig. 2. RAFALSKI and RACZYNSKA-BOJANOWSKA[26] using an isotope assay provided suggestive evidence for the presence of pyruvate, phosphate dikinase in *Streptomyces noursei* var. *polifungi*, and following this lead we have obtained direct evidence that the dikinase is present in *S. verticillatus*.

Fig. 2. Pathways and enzymes capable of converting pyruvate into phosphoenolpyruvate.
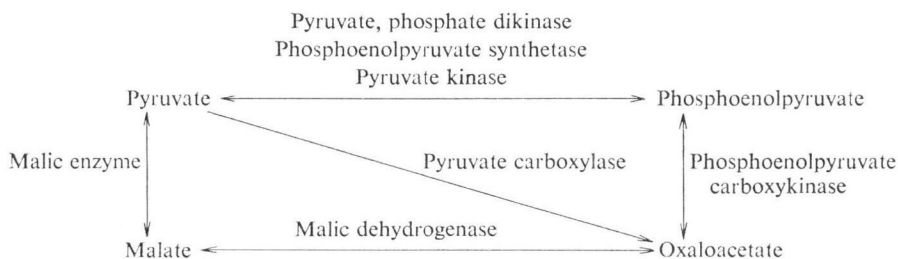
The demonstration of the presence of the dikinase in *S. verticillatus* was greatly facilitated by the utilization of Cibacron Blue 3G-A Sepharose 6B (CBS) affinity chromatography[31]. This method enabled us to remove in a single step pyruvate kinase, adenylate kinase and alanine dehydrogenase which severely interfere with assays for the dikinase. This affinity method was also used to survey three other strains of *Streptomyces* and *N. mediterranei* for the presence of dikinase activity. In addition Cibacron Blue affinity chromatography allowed the isolation and partial purification of alanine dehydrogenase and of phosphoenolpyruvate carboxylase from *S. verticillatus*. The latter can be used advantageously in a coupled assay for the dikinase.

Experimental Procedures

Cultivation of Organisms:

Streptomyces verticillatus (ATCC 13495) was grown at 24°C in 100 ml shake culture in medium I consisting of (g/liter); Trypticase (BBL) 10.0, glycerol 10.0, glucose 5.0, NH_4HPO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.25, NaCl 2.0, trace element solution 1.0, and, differently from KIRSCH and KORSHALLA[19], Phytone (BBL) 5.0 was used instead of soya peptone. Cultures were propagated by inoculating medium I with 2 ml of a 3 or 4 day old culture. Good growth was reached after 4 days; however, the amount of mitomycin formed was negligible. Maintenance of cultures in a non-active state can be accomplished by storing 2-ml portions of a culture which has been made 20% in glycerol in sterile vials at −20°C. For antibiotic production cells grown in medium I were aseptically filtered and replaced into 100 ml of a synthetic production medium (medium II) consisting of (g/liter); mannose 5.0, $CaCO_3$ 5.0, $(NH_4)_2SO_4$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.2, KH_2PO_4 0.2, $FeSO_4 \cdot 7H_2O$ 0.1, and trace element solution 2.0 which yielded approximately 4 mg of mitomycins A and B after shaking for 4 days at 24°C.

Media used for the induction studies were prepared by substituting 5 g/liter L-alanine (2 g/liter are also effective) or 5 g/liter filtration sterilized pyruvate for mannose in medium II. Induction by pyruvate involved transferring cells grown in medium II for 24~72 hours to pyruvate-containing medium 8~16 hours before enzyme extraction. Induction by alanine involved transferring cells grown for 3~5 days in medium I directly to alanine-containing media for one day. Antibiotic production in induced cultures was reduced to approximately 50% of normal. *Nocardia mediterranei* (ATCC 13685), *Streptomyces achromogenes* var. *streptozoticus* (NRRL 3125), *Streptomyces murayamaensis* and *Streptomyces* species MK90 (ATCC 31017) were grown in medium I for 2~6 days depending on the strain used, and the mycelia were aseptically transferred into alanine-containing medium II for 1~3 days for possible induction of pyruvate, phosphate dikinase.

Enzyme Preparations

Pyruvate, Phosphate Dikinase and Alanine Dehydrogenase: The initial extraction and purification steps were carried out at 25°C. Mycelia from 3~6 flasks containing approximately 9~18 g wet weight of cells were harvested by vacuum filtration, and rinsed twice with 30 mM Tris, 1 mM EDTA buffer at pH 6.8 (buffer I), slurried in 25~50 ml buffer I and disrupted in a French pressure cell at approximately 10,000 psi. Cell debris was removed by centrifugation at $20,000 \times g$ for 20 minutes at 18~20°C. The cell-free extract was dialyzed for 2~3 hours against two changes of buffer I at half strength. The enzyme solution was then passed through a CBS column (2.2 × 50 cm) at a flow rate of 1 ml per minute.

Pyruvate, Phosphate Dikinase: This enzyme was eluted in the unbound fractions and was concentrated by ammonium sulfate precipitation at 35~60% saturation. The pellet was then dissolved in buffer I containing 0.5 M sucrose or glucose. Further steps were then carried out at 4°C and the enzyme was stored at −20°C.

Alanine Dehydrogenase: This enzyme was eluted together with other bound enzymes by passing buffer I containing 0.12 M KCl through the column. Further purification was achieved by binding the dialyzed enzyme to a small column (0.8 × 26 cm) of CBS which was then washed with buffer I containing 0.25 mM NADH.

PEP Carboxylase: This enzyme was isolated from *S. verticillatus* cells grown in medium I. Mycelia were harvested by vacuum filtration, rinsed with buffer I containing 0.7 mM 2-mercaptoethanol, slurried in the same buffer and passed through a French pressure cell at 5°C at 10,000~15,000 psi. Cell debris was removed by centrifugation at $20,000 \times g$ for 30 minutes at 5°C and the enzyme solution was dialyzed for three hours against three changes of 2-mercaptoethanol containing buffer I. The enzyme was then passed through a CBS column (3.2 × 25 cm) and it was eluted with 1 liter of a 0~1.2 M linear KCl gradient in 7-ml fractions. The enzyme was collected in fractions 56 through 75. The active fractions were dialyzed against 3 changes of buffer I containing 2-mercaptoethanol and the enzyme precipitated with ammonium sulfate at a saturation of 40~50% at 0°C. The pellet was dissolved in buffer I and ammonium sulfate was removed by dialysis against buffer I. The resulting enzyme solution was then passed through a 1.2 × 22 cm column of CBS and the activity was eluted with 22 ml of 0.5 mM acetylcoenzyme A (CoASAc).

Enzyme Assays

Pyruvate, Phosphate Dikinase: The enzyme was assayed by five different methods, a lactate de-
hydrogenase coupled assay, a hexokinase-glucose-6-phosphate dehydrogenase coupled assay and 2,4-
dinitrophenylhydrazine (DNPH) dependent assays, all measuring the enzyme in the direction of pyruvate
formation, and by a PEP carboxylase coupled assay and a 3-deoxy-D-arabinoheptulosonic acid-7-phos-
phate (DAHP) synthetase coupled assay both measuring the enzyme in the direction of PEP formation.

The lactate dehydrogenase coupled assay was employed routinely in fractions obtained after CBS
column chromatography from induced cultures. The assay mixture contained per 1.25 ml 50 μmol
imidazole, pH 7.0, 10 μmol MgCl$_2$, 16 μmol NH$_4$Cl, 0.13 μmol NADH, 0.8 μmol PPi, 1 μmol PEP,
1 μmol AMP, and 20 units of lactate dehydrogenase. The activity of the enzyme was measured by the
decrease in absorbance at 340 nm.

The hexokinase-glucose-6-phosphate dehydrogenase coupled assay was used with enzyme obtained
after CBS column chromatography from non-induced cultures essentially as described by BENZIMAN[1]
except that the NADPH formed was measured by its fluorescence at 460 nm after excitation with UV
light at 360 nm to increase sensitivity.

DNPH assays were used to locate the enzyme activity in column eluate fractions as well as in the
dye inhibition assays. Reaction mixtures for column eluates contained in 0.5 ml 25 μmol imidazole,
pH 7.0, 10 μmol NH$_4$Cl, 1 μmol PPi, 1 μmol PEP, 0.5 μmol AMP, 10 μmol MgCl$_2$ and 0.1 ml of column
fractions which were incubated for 20 minutes at 30°C; 0.2 ml of the reaction mixture was then pipetted
into a tube containing 0.3 ml of 0.1% DNPH in 2 N HCl and 0.5 ml H$_2$O. After ten minutes, 1.5 ml of
10% NaOH was added and the optical density (OD) measured at 445 nm. The dye inhibition assay was
an adaptation of an assay by MILNER et al[24]. Assay mixtures contained in 2.1 ml 72 μmol imidazole
pH 7.0, 24 μmol NH$_4$Cl, 4.8 μmol PPi, 2.8 μmol PEP, 14 μmol MgCl$_2$, and enzyme obtained by passage
of a crude extract through a 1.2 × 50 cm CBS column as well as varying concentrations of AMP and
Cibacron Blue 3G-A. The reaction mixtures were incubated for 25 minutes at 25°C and each was stop-
ped by addition of 0.5 ml of a reagent mixture containing 0.2 ml H$_2$O, and 0.3 ml of 0.1% DNPH in
2 N HCl; 0.5 ml of 2 N HCl was then added and the absorbance was measured at 390 nm and a correc-
tion for the absorbance of the dye which absorbed in a linear manner over the concentration range used,
was made. The dye absorbs less in basic solutions, however, in the presence of Mg^{2+} ions, it precipi-
tates from the reaction mixture causing erroneous absorbance measurements.

The PEP carboxylase coupled assays were carried out in 1.45 ml of solution containing 109 μmol
imidazole pH 7.8, 21 μmol MgCl$_2$, 40 μmol NH$_4$Cl, 58 μmol NaHCO$_3$, 0.22 μmol NADH, 2.0 μmol
ATP, 3 μmol pyruvate, 5.2 μmol phosphate, 0.05 units of PEP carboxylase and 10 units of malic acid
dehydrogenase. The decrease in absorbance at 340 nm was observed in a one-cm cuvette.

The DAHP synthetase coupled assays were only used in the initial studies and were carried out in a
1.6-ml volume containing 49 μmol triethylamine pH 7.8, 4.8 μmol ATP, 6.4 μmol pyruvate, 9.6 μmol
MgCl$_2$, 19.2 μmol phosphate, 1.1 μmol erythrose-4-phosphate and 0.5 units of partially purified phenyl-
alanine isozyme of DAHP synthetase from Escherichia coli. The reaction was stopped at various times by
adding 0.5 ml of the reaction mixture to 0.3 ml 10% trichloroacetic acid. The DAHP formed was then
determined chemically[10]. Because the DAHP synthetase was provided in phosphate buffer containing
2 mM PEP it was dialyzed 2 hours with one change against triethylamine buffer containing 2 mM PEP to
remove most of the phosphate, then it was dialyzed thirty minutes against PEP free buffer immediately
prior to use in the assay.

Alanine Dehydrogenase: Column fractions were assayed by incubating 50 μl of each fraction in
0.5 ml of a reaction mixture containing at pH 7.8, 25 μmol sodium phosphate, 10 μmol NH$_4$Cl, 0.2 μmol
pyruvate and 0.25 μmol of NADH. After 20 minutes of incubation at 25°C, 0.2 ml of the reaction mix-
ture was added to another tube containing 0.8 ml H$_2$O and 0.33 ml of DNPH solution (0.1 M in 2 N
HCl). After 10 minutes, 1.5 ml of 10% NaOH was added, and after another 10 minutes, the absorbance
at 445 nm was measured. The absence of color (removal of pyruvate) is equated with the presence of the
enzyme in the respective fraction. The activity of the enzyme was measured by the change in absorbance
at 340 nm in a reaction mixture containing 118 μmol sodium phosphate pH 7.8, 3.5 μmol pyruvate, 0.18
μmol NADH, and 43 μmol NH$_4$Cl per 1.2 ml of solution, to which was added 20～50 μl of enzyme.

The reverse reaction of alanine dehydrogenase was assayed by measuring the formation of pyruvate from alanine with DNPH. The reaction mixture contained in one ml 2.5 μmol L-alanine, 0.5 μmol NAD$^+$, 50 μmol sodium carbonate pH 9.3, and enzyme. At various times of incubation, 0.2 ml of the mixture was assayed for pyruvate as in the assay for column fractions above, except that the enzyme gives a positive color response in this assay.

Chromatography of the products of alanine dehydrogenase reaction mixtures in methanol - H$_2$O - pyridine (80: 20: 4), ethanol - H$_2$O - (4.5 N)NH$_4$OH (100: 20: 4.5), and methanol - H$_2$O - pyridine (60: 20: 20) all showed the enzyme-dependent formation of a ninhydrin spot that migrated with the same Rf as standard alanine on thin-layer silica plates (Rf alanine: 0.47, 0.27 and 0.62, respectively). A reverse reaction mixture was stopped by reaction with DNPH and the hydrazine derivative formed was extracted with chloroform. Chromatography showed that the major spot had the same Rf as a derivative made with pyruvate when run on a silica thin-layer plate in a solvent system containing methanol - benzene - n-butanol - H$_2$O(4: 2: 2: 2), Rf=0.83.

PEP Carboxylase: Assay of column fractions: A 50-μl sample of fractions was incubated twenty minutes at 25°C in 0.2 ml of a mixture containing 18 μmol imidazole pH 7.8, 2 μmol MgCl$_2$, 0.4 μmol PEP and 4 μmol KHCO$_3$; 0.1 ml of this mixture was then assayed for oxaloacetate by the DNPH method as used in the assay of column fractions of alanine dehydrogenase.

Spectrophotometric assay: The decrease in absorbance at 340 nm in an assay mixture containing in 2.3 ml 200 μmol Tris pH 8.5, 4.6 μmol PEP, 0.21 μmol NADH, 30 μmol NaHCO$_3$, 30 μmol MgCl$_2$ and 50 units of malic dehydrogenase was measured with time.

Pyruvate Kinase: The DNPH assay for pyruvate kinase in column fractions was carried out in 0.35 ml of solution containing 21 μmol of imidazole pH 7.0, 4.3 μmol MgCl$_2$, 0.9 μmol PEP, 0.9 μmol ADP, 24 μmol KCl, and 50 μl of the fraction being assayed. After a twenty-minute incubation at 37°C the pyruvate in 0.2 ml of the mixture was determined by the NaOH procedure used in the assay of fractions for dikinase, which measures the absorbance of the hydrazine derivative at 445 nm.

Published methods were used to assay for the presence of malic enzyme[17], malic dehydrogenase[37], pyruvate carboxylase[28,29] PEP synthetase (6, methods 1 and 2), PEP carboxytransphosphorylase (20, method B), adenylate kinase[4] and for an additional assay of pyruvate kinase[32].

For all enzymes 1 unit of enzyme activity is defined as that quantity of enzyme which catalyzes the formation of 1 μmol of product in 1 minute as measured under the assay conditions described. Specific activity is expressed as milliunits per mg of protein. Protein was determined by the method of LOWRY et al.[21] using bovine serum albumin as standard, or by the method of BRADFORD[3] using gamma globulin as standard.

Electrophoresis: Electrophoresis of PEP-carboxylase was carried out in 6% polyacrylamide gels in buffer containing 20 μM Tris base, 1 μM EDTA, and 1 μM MgCl$_2$ which was titrated to pH 7.5 with citric acid, the final concentration of which is approximately 4 μM. The 0.7 × 12.5 cm gels were run for approximately two hours at 2 mA per gel tube; bromophenol blue was used as a marker. Protein was stained with commassie blue, and then the gels were destained in 7% acetic acid in a magnetically stirred destainer. Activity gels were incubated in a solution containing 1 μM PEP, 10 μM NaHCO$_3$, 10 mM MgCl$_2$, and 20 mM Tris at pH 7.8 for 20 minutes. The formation of free phosphate was then determined by incubating the gel in a solution containing 2.5% ammonium molybdate in 2 N H$_2$SO$_4$ which is also 1.5% in ascorbic acid after the molybdate is dissolved. A dark precipitate is formed where free phosphate is present[12].

Materials

Strains of microorganisms were obtained from the American Type Culture Collection, Rockville, MD. Trypticase and Phytone were purchased from Baltimore Biological Laboratories, Baltimore, MD. Imidazole and DNPH were products of Eastman, Rochester, NY, PEP carboxylase from wheat, and erythrose-4-phosphate were purchased from Boehringer Mannheim Corporation, Indianapolis, IN. Salt free lactate dehydrogenase and all other enzymes, Sepharose Cl-6B and all other substrates, cofactors and biochemical reagents used were obtained from Sigma, St. Louis, MO. Merck TLC plates were purchased from Brinckmann, Chicago,IL and materials for gel electrophoresis, the Electrophoresis set

150A and destainer 172A were acquired from Biorad, Richmond, CA. CBS was prepared essentially by the procedure of EASTERDAY and EASTERDAY[7]. This procedure is reported to yield a preparation containing approximately 2 μmol of dye per ml. Acetylcoenzyme A was prepared chemically from coenzyme A by the acid anhydride method of STADTMAN[30].

Results

Demonstration of Pyruvate, Phosphate Dikinase Activity

Pyruvate, phosphate dikinase catalyzes the reaction shown in equation (1).   We observed

$$(1) \quad Pyruvate+ATP+Pi \leftrightarrow PEP+PPi+AMP$$

this activity initially using a crude enzyme preparation from *S. verticillatus* grown on pyruvate supplemented production medium and a coupled assay employing DAHP synthetase (equation 2, Fig. 3).

Preliminary reports of this work have been presented (K. L. REDMAN & U. HORNEMANN, 1976. Abstr., Amer. Soc. Pharmacognosy Meet. Cable, Wisconsin, Lloydia 39: 480~481, and K. L. REDMAN & U. HORNEMANN, 1978. Abstr., Joint Central-Great Lakes Regional Meet., Amer. Chem. Soc., Indianapolis, Indiana, May 24~26, BIOL-16). This assay, although it is fairly specific for the crude enzyme is cumbersome since the DAHP formed can be determined only discontinuously. Furthermore, this assay suffers from the

Fig. 3. DAHP synthetase coupled assay of dikinase.
Formation of PEP by dikinase was coupled to DAHP synthetase. DAHP is measured colorimetrically as described in the experimental procedures. The symbols are: Complete reaction mix ▲, Pyruvate blank ○, ATP blank ●, Phosphate blank □. Low amounts of Pi in the ATP and E-4-P solutions along with any Pi which was not dialyzed completely from the DAHP synthetase which was supplied in Pi buffer are probably the reason for the low dependence on phosphate in this assay.

$$(2) \quad PEP+E-4-P \rightarrow DAHP+Pi$$

pronounced instability of the *E. coli* DAHP synthetase used which prevails when the stabilizing agent PEP is removed by dialysis as is necessary before the enzyme can be used to measure PEP formation.
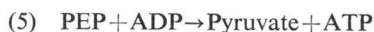
Attempts to measure the dikinase in crude extracts in the direction of pyruvate formation by the lactate dehydrogenase coupled assay (equation 3) or

$$(3) \quad Pyruvate+NADH \leftrightarrow Lactate+NAD$$

by the PEP carboxylase-malate dehydrogenase coupled forward assay (equations 4a and 4b) were subject to large interferences.   The presence in the
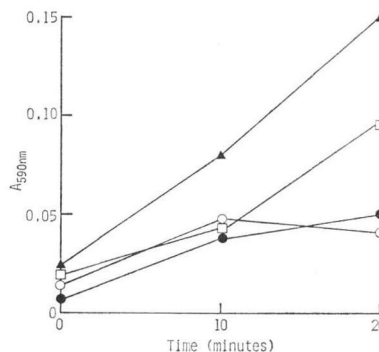
$$(4a) \quad PEP+CO_2+ATP \rightarrow Oxaloacetate+ADP$$

$$(4b) \quad Oxaloacetate+NADH \rightarrow Malate+NAD$$

S. verticillatus extracts of pyruvate kinase (equation 5) in concert with

$$(5) \quad PEP+ADP \rightarrow Pyruvate+ATP$$

adenylate kinase and any ATP generating enzyme such as the dikinase rendered the lactate dehydrogenase coupled assay very imprecise.   In addition the combined presence of ammonium chloride

(required as a dikinase activator) and of alanine dehydrogenase (equation 6) caused severe difficulties with the PEP carboxylase-malate dehydrogenase coupled assay since this enzyme

$$(6) \quad Pyruvate + NH_3 + NADH \leftrightarrow Alanine + NAD$$

competed with malate dehydrogenase for NADH.   It was therefore imperative to remove these enzymes before the dikinase could be readily assayed.   However, ammonium sulfate fractionation proved to be impractical due to difficulties in locating the activity in fractions and DEAE-Sephadex chromatography afforded a highly unstable enzyme and thus could not be used to remove the interfering enzymes.

It appeared to be attractive to explore the possible binding of the dikinase to CBS columns[31] to achieve the desired purification.   Unexpectedly the dikinase did not bind at either pH 6 or pH 7, while pyruvate kinase, alanine dehydrogenase and presumably adenylate kinase were cleanly removed from well dialyzed cell-free extracts, especially by long dye columns.   Thus the non-binding of the dikinase affords a convenient method for the removal of enzymes that interfere with dikinase assays.   This is clearly demonstrated in Tables 1 and 2 which show lactate dehydrogenase and PEP carboxylase-malate dehydrogenase coupled assays, respectively, for the dikinase before and after passage through a 50-cm long dye column of a well dialyzed extract.   The column length is of great importance in achieving adequate separations.   While dye Sepharose columns of the dimensions 2.2×50 cm clearly remove all interfering activities, columns $10 \sim 15$ cm in length even if the column volume is the same, yield non-specific activity amounting to 10% or more of the AMP- and PPi-dependent dikinase activity in the lactate dehydrogenase coupled assay (Table 1).   Adequate dialysis is clearly important to achieve a good separation of the dikinase from interfering activities, presumably because nucleotides or nucleotide-containing compounds such as NAD and NADH would otherwise compete with the column bound dye for the nucleotide binding site on the respective enzymes.   It is possible that the more efficient longer dye Sepharose columns have a "dialysis" effect which enable them to outperform the shorter dye columns. It was noted when a dikinase preparation originally obtained on a short dye column was chromatographed on a long plain Sepharose 6B column that the dikinase appeared in a tailing protein peak as shown in Fig. 4 thus affording a seven-fold purification of this enzyme.   Pyruvate kinase activity was measured in this same experiment and as can be seen from its activity profile (Fig. 4, ●—●—●) it comes through

Table 1. Analysis of different preparations of the dikinase using the lactate dehydrogenase coupled assay.

Values in A are the ranges most commonly seen with crude dialyzed enzyme. Preparation B was run on a short CBS column (10 cm) and then passed over a 44 cm column of Sepharose 6B. C shows the range of values seen for enzyme prepared by the dikinase procedure outlined in the experimental procedures.

Enzyme preparation	Dikinase activity*				
	Complete	PEP blank	AMP blank	PPi blank	MgCl₂ blank
A	100	$4 \sim 8$	$24 \sim 31$	$44 \sim 65$	—
B	100	8	17	18	9
C	100	$1.8 \sim 2$	$2 \sim 4$	$2 \sim 5$	—

* Activity is expressed as % of the complete assay mixture.

Table 2. Analysis of different preparations of the dikinase using the PEP-carboxylase coupled assay.

Enzyme preparation A was treated by ammonium sulfate precipitation ($45 \sim 60$% saturation) and run on a 1.6×47 cm column of Sepharose 6B. Enzyme B was prepared by the dikinase procedure given in the experimental procedures section using a CBS column. The NH_4^+ dependence of the interfering alanine dehydrogenase could be demonstrated even in crude enzyme preparations.

Enzyme preparation	Dikinase activity*				
	Complete	Pyruvate blank	ATP blank	Phosphate blank	NH₄⁺ blank
A	100	2.5	114	107	1
B	100	4	5	9	—

* Activity is expressed as % of the complete assay mixture.

Fig. 4. Elution of dikinase from Sepharose 6B.

The dikinase (□—□) as measured by the DNPH assay is held back somewhat by the column and appears in the second protein peak (○—○ $A_{280 nm}$). Apparently the dikinase is not completely separated from pyruvate kinase (●—●) that is present. Pyruvate kinase was measured by the DNPH assay. The elution profile of the dikinase is the same as that seen with CBS columns of similar size.
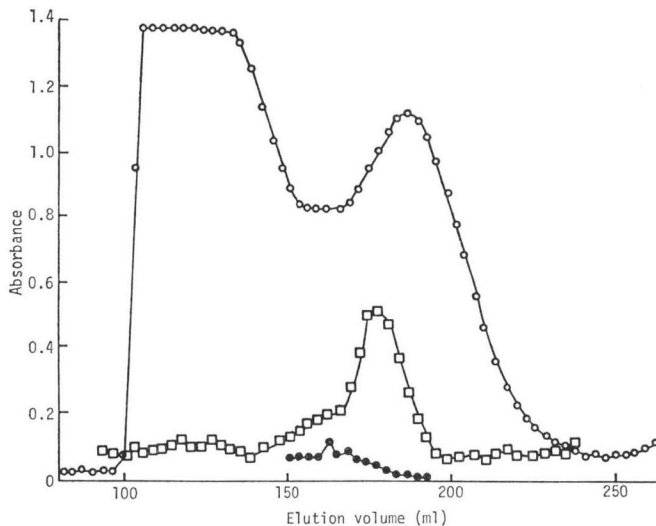
the plain Sepharose 6B column at a position near that of the dikinase. Evidently the short dye Sepharose column used in the preceding step did not bind all of the pyruvate kinase as would have a longer CBS column. The loading capacity of the long dye Sepharose column was approximately 1.2 mg protein per ml gel. The binding of pyruvate kinase to the Blue Sepharose column, which can be eluted with 1.2 M KCl, has been reported previously[22] for the enzyme from human liver.

Properties of the Dikinase

Crude dikinase preparations lose nearly all activity when stored frozen or at 4°C overnight, but most of the activity remains when the enzyme is kept at 25°C. The activity does not seem to be stabilized by pyruvate, PPi, PEP or glycerol at 10 or 30% but sucrose or glucose at high concentrations (0.5 ~ 1 M) render the enzyme stable at 4°C and to freezing. Fructose stabilizes the enzyme less efficiently than glucose and sucrose, and all these carbohydrate supplements were found to be less effective at 25°C than at low temperature. As a result of these observations all extraction procedures up to and including the ammonium sulfate precipitation step were carried out at 25°C. However, once the pellet obtained after the ammonium sulfate precipitation step was dissolved in the sucrose or glucose containing buffer it was kept frozen or at 4°C.
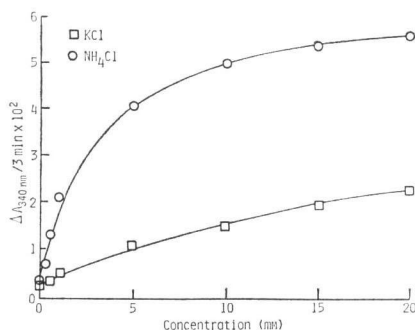
The lactate dehydrogenase coupled assay of dikinase activity (Table 1) shows that the reverse reaction is dependent on PEP, AMP and PPi. Addition of Pi to these assays did not support activity. In the forward assay, equations (4a) and (4b) are dependent on pyruvate, ATP, and Pi (Table 2). The dependence on PPi in the reverse reaction and Pi in the forward reaction show that the enzyme being measured is pyruvate, phosphate dikinase and not PEP synthetase, which has been reported in *E. coli*[5] and which catalyzes the reaction shown in equation 7.

(7)   Pyruvate+ATP→PEP+Pi+AMP

Another property which distinguishes pyruvate, phosphate dikinase and PEP synthetase is a mono-

Fig. 5. Dependence of dikinase activity on mono-valent cations.

The activity was measured in the standard lactate dehydrogenase coupled assay using salt-free lyophilized lactate dehydrogenase. Sodium ions were present in the assay mixture, introduced with substrate salts. The dikinase was desalted on a 22-cm column of Sephadex G-25 which was equilibrated with glucose-free buffer and the enzyme was used immediately.

valent cation dependence of the dikinase[8]. All dikinases[2,23] except that of *Entamoeba histolytica*[27] have been demonstrated to have a monovalent cation dependence. The dikinases from plants[14] also require a monovalent cation but in this case Na^+ is active, which is inactive with the bacterial dikinases. PEP synthetase from *E. coli* does not have a monovalent ion requirement[5]. The enzyme from *S. verticillatus* has a monovalent cation requirement for activity as shown in Fig. 5. NH_4^+ ions are more effective than K^+ ions, and Na^+ ions give little or no activity. The apparent *Km* for NH_4^+ ions is approximately 2 mM which corresponds well with *Km* values for NH_4^+ of other dikinases[2,5,8,14,23,27]. The dikinase from *S. verticillatus* also has a divalent metal requirement (Mg^{2+}) as do dikinases and PEP synthetase from other sources. Divalent ions other than magnesium were not tested for activity.

A twenty to forty fold induction of the dikinase occurs when *S. verticillatus* is cultured in pyruvate- or alanine-containing medium II as compared to the activity present when the organism is grown in the mannose-containing medium II. The degree of induction is difficult to determine accurately due to a very low level of activity present in noninduced cells. Subcultures started freshly from an ATCC lyophilized culture were not induced by pyruvate in several experiments, while subcultures that have been propagated by flask to flask transfer in medium I for approximately one month can be induced by pyruvate after replacement into pyruvate-supplemented medium II. Alanine induced activity in either culture line and it was therefore used routinely for enzyme induction. After approximately 5 months of subculture a new ATCC line was again tested with pyruvate and induction was noted. In order to verify that the low level of enzyme in noninduced cells was dikinase, cell-free extracts were prepared from mycelia grown on medium II by the normal procedure. The enzyme obtained after the ammonium sulfate precipitation was then dialyzed and passed over a second small CBS column to remove any remaining interfering activity. The enzyme was not active enough to be measured by an assay other than by the hexokinase-glucose-6-phosphate dehydrogenase-coupled assay. Fig. 6 shows the assay results. The enzyme being measured is clearly demonstrated to be dikinase by virtue of the dependence of the activity on PEP, AMP, and PPi, and by the inability of Pi to support activity.

It is curious that the dikinase which clearly uses ATP and AMP and which would therefore be expected to bind to CBS columns like most other kinases, does in fact not bind to these columns. In order to determine if the free Cibacron Blue 3G-A dye was able to interact with the enzyme, an inhibition assay was performed with various concentrations of AMP and dye and the data obtained are shown in Fig. 7. The activity had to be measured by the DNPH assay of pyruvate formed since coupling enzymes would be inhibited by the dye. Fig. 7 shows that the dikinase is inhibited by the free Cibacron Blue dye and the results suggest uncompetitive inhibition although some curving of the lines is noted especial-

Fig. 6. Fluorescence assay of noninduced dikinase activity.

Enzyme from cells grown in medium II was extracted by the normal dikinase procedure. The ammonium sulfate concentrate was then dialyzed to remove salt and run on a second CBS column (1.5×22 cm) to lower the background activity as much as possible. The activity of the preparation was measured in a hexokinase glucose-6-phosphate dehydrogenase coupled assay
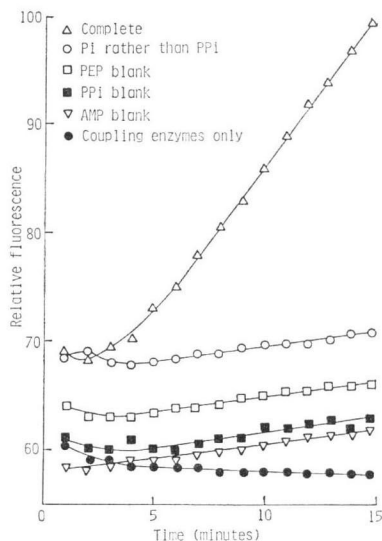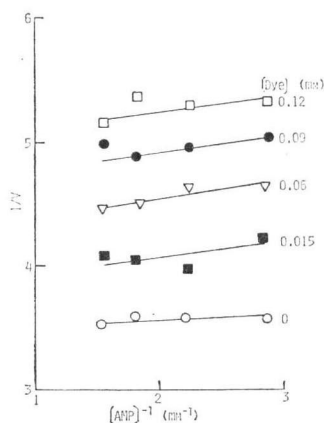

Fig. 7. Inhibition of the dikinase by free Cibacron Blue 3G-A.

The activity of the dikinase was measured at various inhibitor and AMP concentrations by the acidic DNPH assay. V is expressed as ΔA 390 nm/30 min.

Table 3. Survey of several actinomycetales for presence of pyruvate, phosphate dikinase.

Crude enzyme from alanine induced cultures was assayed by the DNPH assay. This assay often has very poor PPi blanks, therefore, formation of the pyruvate DNPH adduct does not confirm the presence of dikinase. Crude enzyme preparations of *Streptomyces* species positive in the DNPH assay and *N. mediterranei* were run on CBS columns and the column fractions, including the salt eluted material, were assayed by the DNPH assay. If activity was noted in the fractions, it was then confirmed to be dikinase by the lactate dehydrogenase coupled assay.

Organism	Dikinase measured by		Lactate dehydrogenase coupled assay
	DNPH assay of		
	Crude enzyme	Column fractions	
S. verticillatus	+	+	+
Streptomyces species MK 90	−		
S. murayamaensis	+	+	+
S. achromogenes var. *streptozoticus*	+	−	
N. mediterranei		−	

ly at lower AMP concentrations. Thus it is apparent that the dikinase possesses a binding site for the free dye.

A survey of three additional strains of *Streptomyces* and of *N. mediterranei* for the presence of an inducible dikinase revealed (Table 3) that only *S. murayamaensis* besides *S. verticillatus* contained this enzyme, while the other two *Streptomyces* strains and the *Nocardia* did not show this activity when investigated using the lactate dehydrogenase coupled dikinase assay.

Alanine Dehydrogenase

Alanine dehydrogenase was readily identified as the enzyme causing interferences in the PEP carboxylase, malate dehydrogenase coupled assay of the dikinase when the dependence of the interfering activity on NH_4^+ ion was shown. Chromatographic analysis of alanine dehydrogenase reaction mixtures in three solvent systems has revealed that alanine is the product in the forward reaction (equation 6) and that pyruvate, identified as the DNPH derivative, is the product in the reverse reaction, respectively. Alanine dehydrogenase activity was detected in both alanine or

Table 4. Reverse assay of alanine dehydrogenase. The formation of pyruvate from alanine was assayed by the DNPH method outlined in the experimental section.

Incubation time (min.)	Absorbance at 445 nm
0	0.017
1	0.064
2	0.090
3	0.119
10	0.183
10 (NAD^+ blank)	0.000
10 (Boiled enzyme)	0.009
10 (L-Alanine blank)	0.008
10 (D-Alanine)	0.000

Table 5. Purification of crude alanine dehydrogenase on a CBS column.

	Total activity (units)	Total protein (mg)	Specific activity (milli-units/ mg/min)	Yield (%)	Purification factor
Crude extract	1.32	26.0	50.7	100	1
Column eluate*	0.92	0.24	3830	70	75

* Only the most active fraction was assayed.

Fig. 8. Chromatography of alanine dehydrogenase on a CBS column.

A portion of the bound enzymes from a dikinase isolation experiment after elution with 1.2 M KCl and subsequent dialysis was applied to a 0.8×26 cm column of CBS. The column was washed with 50 ml 1 mM NADH and 2 ml fractions were collected. The column was subsequently washed with 1 M KCl. Protein was measured in each fraction by measuring the UV absorption at 280 nm (□—□). While most of the absorption is clearly due to NADH, the peak at fractions 45 and 46 is due to protein being eluted by KCl. This is correlated by the fact that the color of these two fractions was brownish yellow, while all other fractions were colorless. An assay dependent on the disappearance of pyruvate (see enzyme assays) was used to determine alanine dehydrogenase activity in all fractions (○—○). It is apparent that NADH elutes the enzyme and that KCl is able to remove a further small amount that remained after the NADH elution step.
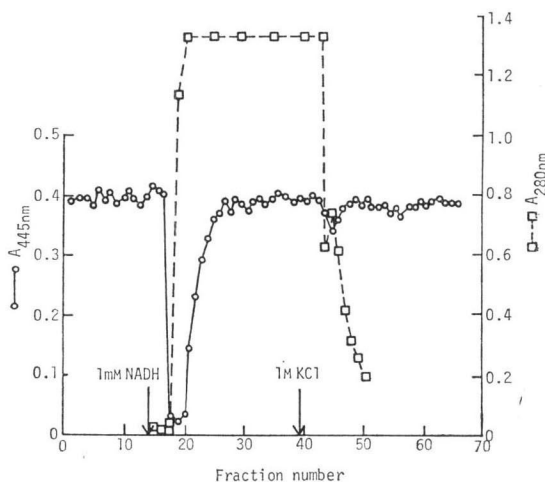

pyruvate induced and in noninduced cultures. It is of interest to note that all pyruvate and NADH dependent enzyme activity in any extract from S. verticillatus investigated, independent of age and method of growth of the cultures, was always dependent on ammonium ion. Therefore this activity must be due to alanine dehydrogenase and cannot be due to lactate dehydrogenase. Thus it appears that lactate dehydrogenase is not present in S. verticillatus or is present in exceedingly low concentration.

The enzyme is specific for pyruvate and NADH. It has no measurable activity with α-ketoglutarate, and NADPH only supports maximally 3% of the activity that the enzyme exhibits with NADH. In the reverse assay the enzyme was active with L-alanine but not D-alanine (Table 4). The elution of alanine dehydrogenase from a CBS column is shown in Fig. 8. Elution by low concentrations of NADH results in a 75-fold purification when crude enzyme is applied to the column (Table 5) and an eleven fold purification when the Cibacron Blue-bound fraction from a dikinase preparation is used as the starting material.

PEP Carboxylase

The three-step purification of PEP carboxylase outlined in the experimental procedures section and

Table 6. Purification of PEP carboxylase.

	Total activity (units)	Total protein (mg)	Specific activity (milli-units/ mg/min)	Yield (%)	Purifi-cation factor
Crude extract	45.9	778	59	100	1
40~ 50% Am-monium sulfate cut	—	—	1,490	—	25
Column eluate	13.1	4.5	2,910	28	49

summarized in Table 6 gives an enzyme of suffi-cient purity for use as a coupling enzyme in the dikinase forward assay (equation 4) since the ac-tivity eluted with CoASAc from the CBS column (Fig. 9) was completely free of alanine dehydro-genase activity. The first column fraction con-tained NADH oxidase activity and was therefore not included when the active fractions were com-bined. The calculated degree of 48-fold purifica-tion for the PEP carboxylase was unexpectedly low, probably due to a loss of activity and not due to the presence of other proteins since gel elec-trophoresis revealed only three minor bands and a major band of which the latter carried all the activity. PEP carboxylase activity was seen equally well in cells grown in medium I and medium II. It was readily possible to purify the enzyme using small CBS columns and thus to save CoASAc.

Fig. 9. Elution of PEP carboxylase from a CBS column.

PEP carboxylase was prepared as outlined in the experimental procedures. The specific elution of the enzyme by CoASAc from the second column is shown. This procedure allows the utilization of a minimum amount of CoASAc. The column was eluted with buffer I and 2-mercaptoethanol at a flow rate of 25 ml/hour and fractions of 3.5 ml were collected. Addition of 22 ml of 0.5 mM CoASAc eluted the majority of the enzyme and a further small amount of the enzyme along with the bulk of the protein was eluted in fraction 21 when 1.2 M KCl was passed through the column. The enzyme activity was measured by assaying oxaloacetate production with DNPH.
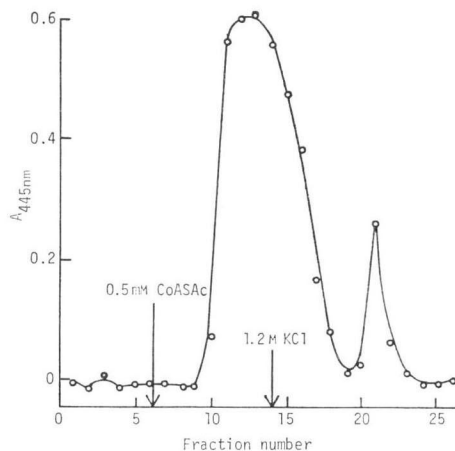

Discussion

The evidence provided in this paper, especially the results presented in Tables 1 and 2 established conclusively that *S. verticillatus* contains pyruvate, phosphate dikinase. This enzyme appears to be similar in many of its characteristics to dikinases isolated from other bacteria and its cold lability, dependence on monovalent cations and its inducibility are important criteria in this comparison. The pronounced induction seen on pyruvate and alanine containing media suggests that this enzyme may fulfill a gluconeogenic role in this organism as in others. Why pyruvate can induce the dikinase in cul-tures that have been propagated by several transfers but not in cultures started from a lyophilized ino-culum is not understood.

The isolation and partial purification of the dikinase were greatly facilitated by the discovery that this enzyme does not bind to CBS columns, while a number of other kinases and dehydrogenases which interfere with facile continuous assays of the enzyme, are readily bound to such columns. Previously the enzyme was isolated in microorganisms such as *B. symbiosus*[25] and *Entamoeba histolytica*[27] which lack pyruvate kinase, one of the principal sources of interference in the lactate dehydrogenase coupled dikinase assay, or the enzyme was isolated following cumbersome discontinuous assays. Assuming

that the dikinases from other microorganisms have properties similar to the *Streptomyces* enzyme CBS chromatography should be of general utility for their detection and purification.   The reason for the inability of the dikinase to bind to CBS columns is not known.   Clearly the dikinase has a binding site for the free dye as shown by the fact that the free dye inhibits the enzyme (Fig. 7).   This figure suggests uncompetitive inhibition for which several explanations may exist if it is assumed that the *Streptomyces* enzyme is similar in its mechanism of catalysis and in its subunit structure to the dikinase from *Bacteroides symbiosus* which was studied in detail by Wood and co-workers[25,86].   Further investigations will be required to interpret the nature of the uncompetitive inhibition and to decide if factors other than steric interference are responsible for the nonbinding.

The presence in *S. verticillatus* of the pyruvate-inducible dikinase, the likely absence of PEP synthetase (equation 7) and the likely irreversibility of pyruvate kinase suggest that the dikinase route, if not other routes, is available for the generation of phosphoenolpyruvate for possible use in mitomycin biosynthesis.   Concerning the indirect pathways for the generation of phosphoenolpyruvate from pyruvate (Fig. 2) we can eliminate the first pathway from consideration since we could not detect pyruvate carboxylase activity in *S. verticillatus* under a variety of conditions.   Presumably this enzyme is lacking in *Streptomyces* in general since it was also reported that *Streptomyces ambofaciens* lacks this activity[33].   Our results concerning the second indirect pathway are inconclusive due to difficulties in identifying malate-dehydrogenase unambiguously in our preliminary studies.   The distribution of the dikinase in *Streptomyces* species appears to be random (Table 3) and it is of interest to note that *N. mediterranei*, which is unable to utilize pyruvate efficiently for rifamycin biosynthesis appears to lack this enzyme.

Alanine-dehydrogenase has apparently neither previously been described in *Streptomyces* nor has its binding to Cibacron Blue 3G-A Sepharose 6B columns been reported.   Its tight binding and the specific elution by low concentrations of NADH permitted both its clean separation from the dikinase and its facile purification.

Phosphoenolpyruvate carboxylase has to our knowledge also not previously been shown to interact with Cibacron Blue 3G-A Sepharose 6B columns.   This CoASAc activated enzyme is assumed to interact with the blue dye through its allosteric CoASAc binding site since it has neither nucleotide substrates nor cofactor requirements.   Cibacron Blue 3G-A has been shown in studies with molecular models to be capable of assuming conformations very similar to coenzyme A, and it was predicted by Stellwagen[81] that interactions of enzymes possessing coenzyme A binding sites with Cibacron Blue 3G-A would be found.   Our report presumably constitutes the first instance where this interaction has been exploited for enzyme purification.   A specific interaction between this enzyme and the column material is indicated by the fact that 0.4 M KCl was required for elution while only 0.5 mM CoASAc was sufficient to elute the enzyme from the column.   The enzyme obtained in this manner was completely free of alanine-dehydrogenase activity and therefore was useful as a coupling enzyme in the forward coupled assay of the dikinase.

Added in Proof

The occurrence of alanine dehydrogenase in *Streptmyces clavaligerus* and its partial purification have recently been described by Y. Aharonowitz and C. B. Friedrich: Arch. Microbiol. 125: 137~142, 1980.

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