PURIFICATION OF TYPE L PYRUVATE KINASE BY HYDROPHOBIC AND AFFINITY CHROMATOGRAPHY TECHNIQUES

By

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Bachelor of Science

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1973

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
December, 1975

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ACKNOWLEDGEMENTS

The author extends special appreciation to his major professor, Dr. R. E. Koeppe, for his guidance and advice. Appreciation is also expressed to Dr. H. O. Spivey and Dr. R. C. Essenberg for their help as advisory committee members.

Special thanks to Mrs. Connie Grizzle for her assistance and to Dr. Jack B. Robinson for assistance and guidance during these studies.

Deep gratitude and affection is expressed to my wife, Ingrid, for the laborious job of typing this thesis and for her patience, encouragement and understanding.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADP	- adenosine 5'-diphosphate
AMP	- adenosine 5'-phosphate
ATP	- adenosine 5'-triphosphate
Cyclic AMP	- adenosine 3':5'-cyclic phosphate
EDTA	- ethylenediaminetetra-acetate
NADH	- nicotinamide-adenine dinucleotide (reduced)
$NADP^+$	- nicotinamide-adenine dinucleotide phosphate
PEP	- phosphoenol pyruvate
Tris	- tris(hydroxymethyl)aminomethane
nd	- not determined

CHAPTER I

INTRODUCTION

In 1934, Lohmann and Meyerhof (1) and Parnas et al. (2) reported a previously unrecognized intermediate in the conversion of 3 phosphoglycerate to pyruvate in the presence of adenine nucleotides and Mg^{++}. In their classical work, Lohmann and Meyerhof were successful in the isolation and determination of the structure of phosphoenol pyruvate, but noted the adenine nucloeotides only as a coenzyme concluding that the degradation of phosphoenol pyruvate gave orthophosphate and pyruvate (1). Parnas et al. (2) concluded from their observation that creatine was the acceptor of the phosphoryl group from phosphoenol pyruvate (3).

In July of the next year H. Lehman, C. Lufwak - Mann & T. Mann, and D. M. Needham & W. E. Van Heyningen independently produced evidence for the phosphorylation of AMP by PEP to give ATP (4,5,6). However, the lower initial rate of phosphorylation of AMP as compared to ADP (7) and recognition of ADP as the phosphoryl acceptor of 1,3 diphosphoglycerate (8) suggested ADP as the more likely acceptor of PEP's phosphoryl group. In addition the discovery of adenylate kinase gave a reasonable explanation for AMP phosphorylation. Finally, ADP won as the phosphoryl group acceptor from PEP by default when purified pyruvate kinase failed to phosphorylate AMP (9).

The first purification of pyruvate kinase is credited to Negelein (10) but his protocols and manuscripts related to the work were lost at

the end of W. W. II in the mass confusion. In spite of this misfortune, the enzyme was purified from rabbit muscle (10,11,12), from human muscle (13) and from cat muscle (14). As acceptable methods for purification were devised and the muscle enzyme was purified from different sources, other biochemical properties were being explored. K^+ as well as other monovalent cations were found to be activators and ATP an inhibitor. Molecular weight, apparent equilibrium constants and other properties were also determined.

In 1965 three laboratories, Krebs and Eggleston (15), Weber et al. (16), and Tanaka et al. (17) showed that pyruvate kinase of liver was under dietary and hormonal regulation. Tanaka et al. (18) noting that in contrast skeletal muscle was not regulated in this manner proceeded with a series of experiments using zone electrophoresis and immunological methods to show that there existed at least two types of pyruvate kinase which he dubbed M (muscle) and L (liver) types based on antibody response.

It should be mentioned that there is some controversy about nomenclature for the isozymes of pyruvate kinase. The nomenclature used herein is not in accordance with IUPAC-IUB but, agrees with the choice of Strandholm, Cardenas and Dyson (19).

Two years later in 1967, Tanaka et al. (20) purified to crystalline form the M and L isozymes of pyruvate kinase from rat liver. Also presented in the paper were kinetic studies, additional data on dietary and hormonal effects, further immunological studies and distribution of the two types of pyruvate kinase in other organs. In the same year following a lead from Hess et al. (21) who published evidence for fructose 1,6-diphosphate activation of yeast pyruvate kinase and Taylor & Bailey's

(22) work showing fructose 1,6-diphosphate activation only in pyruvate kinase extracted from liver, Tanaka et al. (23) investigated fructose 1,6-diphosphate effects on purified type L enzyme. The studies by Tanaka's group verified the activation of type L enzyme from liver by fructose 1,6-diphosphate, but type L enzyme from erythrocytes did not show activation by fructose 1,6-diphosphate. Equally important was the observation that fructose 1,6-diphosphate could significantly reverse the previously shown inhibition of activity by ATP.

In 1969, H. Carminatti, E. Rozengurt & L. Jimenez de Asua (24) published a paper in which they described the successful elution of type L pyruvate kinase from CM cellulose by its allosteric effectors. The enzyme was purified 30 fold with 60% recovery. Also, this procedure provided an alternative to the Tanaka et al. (20) separation of the two types of liver enzyme. The type L was removed with an effector; the type M was not, but could be removed with 120 mM Tris-maleate.

The following year, G. E. J. Stall et al. (25) and K. G. Blume et al. (26) used the Haeckel et al. (27) technique (with some modification) for the purification of human erythrocyte pyruvate kinase. This involved the reversible binding of Blue Dextran to the type L pyruvate kinase. This serendipidous event was then used to purify the enzyme by passing the Blue Dextran-enzyme complex over a Sephadex G-200 column. After disassociating the complex the enzyme and Blue Dextran were again passed through the G-200 column. A purification of approximately ten to thirty thousand (cumulative) fold was achieved.

In 1972, another method for human erythrocyte pyruvate kinase purification was reported by C. Chern, M. B. Rittenberg and J. A. Black (28) using the method of Carminatti et al. (24).

In the next year J. Cardenas & R. Dyson (29,30,31) published three excellent papers on purification of bovine skeletal muscle and liver pyruvate kinase and hybridization studies of the two isozymes. The purification of type L followed closely the scheme of Tanaka et al. (20) but included the use of a Sephadex G-200 column in the culminating step.

Finally, at the University of Uppsala in Sweden, 1974, Engström et al. (32) devised yet another method for type L purification and presented evidence which suggested that the type L enzyme may be phosphorylated by (^{35}P) ATP and cyclic AMP stimulated protein kinase. The authors suggest that type L pyruvate kinase belongs to the group of enzymes whose activity is regulated by phosphorylation-dephosphorylation reactions.

The purification scheme presented by Engström et al. (32) is somewhat different from previous attempts. Advantage was taken of pyruvate kinase stability to lower pH and allosteric effectors were used to retain the enzyme on a DEAE-cellulose column under two sets of conditions. Also, Hydroxylapatite was used to gain significant purification.

Historically, the purification of type L pyruvate kinase has been plagued with the problem of poor yield, tediousness of the process and instability of the enzyme. Moreover, there have been several reports (22,33,34,35) of changes in enzymatic activity and/or susceptibility to modifiers.

Due to the extensive research being done in this area a simplified procedure with better yields and stability would be a significant contribution to researchers. For these reasons the present study was undertaken.

CHAPTER II

MATERIALS AND METHODS

Materials

Agarose-Hexane-AMP (adenine N^6-amino linkage), Agarose-Hexane-ATP (ribose hydroxl linkage) and Agarose-Hexane-AMP (ribose hydroxl linkage) were from P. L. Biochemicals, Inc. 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane were supplied by Aldrich. $MgSO_4$, $(NH_4)_2SO_4$, Tris, and Na_2EDTA were obtained from Schwartz/Mann. Cyanogen Bromide activated Sepharose 4B, Sepharose 4B-200, PEP (monopotassium salt), ADP (sodium salt grade I), D-fructose 1,6-diphosphate (tetra sodium salt), β-mercaptoethanol, NADH and $NADP^+$ were obtained from Sigma Chemical Co.

Methods

Enzyme Assay: Two methods were used to assay for pyruvate kinase activity. For determination of specific activity the 2,4-dinitrophenylhydrazone method was employed. This assay procedure was performed according to the method of Tanaka et al. (20) with the exception that 2,4-dinitrophenylhydrazine was 0.0125% in 2N HCl and optical density was measured at 440 nm. The former modification gave greater sensitivity and the latter gave a more acceptable standard curve.

In the second assay procedure, the enzyme system was coupled with lactate dehydrogenase, and the oxidation of NADH was followed at 340 nm in a Hitachi Perkin-Elmer recording spectrophotometer fitted with a 124-

801 scale expander (10). This method was used both for following enzyme elution from columns and for kinetic studies.

The standard assay mixture for column monitoring was as follows: 50 mM Tris (hydroxymethyl)-aminomethane 50 mM K^+ and 5 mM β-mercaptoethanol (pH 7.5) final volume 2 ml (20). PEP, ADP and NADH were 0.6, 1.5 and 0.14 mM respectively, and 10 units of lactate dehydrogenase were added per assay. Temperature for these studies was 25°C.

Composition of the assay mixture for kinetic studies was as follows: 50 mM phosphate, 90 mM K^+ and 5 mM β-mercaptoethanol (pH 7.5) final volume 2 ml (36). Concentrations of PEP, ADP, NADH and lactate dehydrogenase were the same as for column monitoring. Temperature for these studies was 37°C.

Protein Estimations During Purification: The method of Lowry et al. was used. In some cases the modified method was used for 5 to 25 μg of protein monitored at 750 nm (37). Blanks with appropriate buffer were run for each sample.

Synthesis of ω-aminoalkyl-agarose: Essentially the method of Shaltiel and Er-el (38) was employed. Sepharose 4B was suspended in three times its volume of 1:1 dimethylformamide and 0.1 M $NaHCO_3$ buffer. The pH was adjusted to 10.5. Added to this solution was 1 gram of CNBr for every 10 grams (wet weight) of Sepharose 4B. The temperature was maintained at 22°C by the addition of ice chips and the pH was held between 10.5 and 11 by the addition of 6N NaOH. The reaction was terminated by filtration of the gel, which was washed with several volumes of cold deionized water.

When CNBr-activated-Sepharose 4B from Sigma was used, an appropriate amount of freeze-dried gel was weighed out (1 gram freeze-dried

material gave a final volume of approximately 3.5 ml). The dried gel was transferred to a glass fritted funnel and washed with several additions of 1 mM HCl solution (200 ml per gram dried gel). After each addition of HCl solution the supernatant was removed by filtration. Immediately after washing the hydrated gel was treated with α,ω-diaminoalkane.

The activated gel (purchased or prepared) was suspended in twice its volume of cold 100 mM $NaHCO_3$, pH 9. The α,ω-diaminoalkane to be coupled was dissolved in 100 mM $NaNCO_3$, pH 9. 4 moles of α,ω-diaminoalkane were added per mole of CNBr used for activation. The moles of CNBr used by Sigma for the activation of their Sepharose 4B was given in the Axen et al. (39) publication. The final volume of the α,ω-diaminoalkane solution was equal to the volume of the gel solution. The two solutions were mixed and stirred very gently for 24 hours in the cold room. Finally, the gel was washed with (100 ml each) water, 100 mM $NaHCO_3$, 0.05 N NaOH, water, 0.1 M Acetic acid, and water.

CHAPTER III

EXPERIMENTAL PROCEDURE

Purification Procedure

Holtzman rats were maintained on standard laboratory chow and water *ad libitum*. 3 or 4 rats were decapitated using a guillotine, the livers excised and placed in a cold H-buffer consisting of 5 mM Tris, pH 7.4, 150 mM KCl, 5 mM $MgSO_4$, 1 mM EDTA and 5 mM β-mercaptoethanol. The livers were weighed and transferred to a pre-cooled Eberbach, semi-micro, stainless steel blender with 2 ml H-buffer per gram liver. All purification steps were performed at 0-4°C unless otherwise stated. The livers were homogenized twice for one minute, with adequate cooling between intervals. The homogenate was decanted into a beaker and allowed to stand for approximately 30 minutes. The foam which had risen to the top was spooned off and the remaining homogenate was centrifuged at 56,000g for one hour. The centrifugation was performed using a Type 30 head in a refrigerated Beckman Spinco model L preparative ultracentrifuge.

The supernatant solutions were carefully decanted, pooled and slowly brought to 25% saturation with 13.8 grams of powdered ammonium sulfate per 100 ml (if necessary the pH was adjusted to 7.4 with 2N NH_4OH). After standing for twenty minutes the solution was centrifuged at 10,000g for 30 minutes in a refrigerated Servall RC-2B with a SS-34 head or a Servall SS-1 tabletop centrifuge in the cold room. The super-

natant was saved and slowly increased from 25% to 45% saturation by the addition of 11.9 grams of powdered ammonium sulfate per 100 ml (when necessary the pH was adjusted as mentioned before). The mixture was allowed to stand for 30 minutes before centrifugation as described above.

The precipitated enzyme was then gently mixed with 35 ml (packed volume) of Celite (40) which had been washed extensively with H-buffer saturated to 45% with ammonium sulfate. A column, 1.5 X 25 cm, was packed with 5 ml of washed Celite; onto this the Celite-protein mixture was poured and allowed to pack by gravity. This resulted in a column with a total bed volume of 40 ml, 35 ml Celite-protein on top of 5 ml pure Celite. After packing a linear gradient of 60 ml 45% ammonium sulfate saturated H-buffer and 60 ml 20% ammonium sulfate saturated H-buffer was applied. A pressure head of one meter of buffer was required to keep the flow rate at 0.5 ml/min. Fractions of 4 ml volume were collected and those containing activity were pooled.

In earlier experiments the Celite method differed in the following way. The Celite was washed with H-buffer saturated to 45% with ammonium sulfate and dried with suction. 12 ml (pack volume) were then mixed with the 45% ammonium sulfate enzyme pellet which had been resuspended in a minimal volume of H-buffer saturated to 45% with ammonium sulfate. The mixture was then poured into a column (1 X 16 cm) and allowed to pack by gravity or by pumping action with a Beckman solution metering pump. A linear gradient described before was used to elute the enzyme. There were two drawbacks to this method 1) in some experiments the enzyme bled from the column in the first one or two 4 ml fractions reducing yield, and 2) the flow rate was exceedingly slow

even with the help of the Beckman metering pump. However, the purification achieved was equivalent in both methods. Because elution volume of the enzyme (in both techniques) was variable, when necessary the Celite effluent was concentrated to 35 ml by the collodion-bag technique (Bulletin No. 145; Schleicher and Schuell, Keene, N. H., USA).

The enzyme was sometimes dialyzed against ten times its volume of H-buffer and then against the same volume of 1065 buffer consisting of 10 mM phosphate, pH 6.5, 18 mM K^+, 5 mM $MgSO_4$, and 5 mM β-mercaptoethanol. This procedure required at least 12 hours. A Sephadex G-25 column was also employed. The enzyme solution was passed through a Sephadex G-25 column (250 ml bed volume) equilibrated with 10 mM phosphate, pH 6.5, 18 mM K^+, 5 mM β-mercaptoehtanol. The column void volume was about 65 ml at a flow rate of 3 ml/minute. The enzyme eluted just behind the void volume and was diluted approximately two fold.

The enzyme solution was immediately applied to an ω-aminoalkly-agarose column (10 ml bed volume). While several hydrophobic columns with differing hydrocarbon "arms" were used, the methods employed for enzyme application, washing and enzyme elution were the same for each. The ω-aminoalkyl-agarose columns were synthesized by the method of Shaltiel and Er-el (38). The columns were equilibrated with 1065 buffer. During the application of enzyme solution the elution of unabsorbed protein was followed by the absorbance at 280 nm. When the enzyme application was complete the column was washed until the effluent absorbance was approximately equal to that of the 1065 buffer blank. When the enzyme was retained on the column a linear gradient of 50 ml 1065 buffer and 50 ml 50 mM phosphate, pH 6.5, 90 mM K^+, 5 mM $MgSO_4$ and 5 mM β-mercaptoethanol was effective in elution. The flow was variable

(0.25 ml - 1 ml/min.) but 3 ml fractions were collected and fractions with activity were pooled.

This enzyme solution was then dialyzed twice against ten times its volume of 1065 buffer for 8 to 12 hours.

The dialyzed enzyme was then applied to an N^6-(6-aminohexyl)-AMP-Sepharose column (5 ml bed volume) equilibrated with 1065 buffer. Unbound protein was washed from the column with 1065 buffer and the elution of protein was monitored as before. The enzyme was eluted with 50 ml 1065 buffer and 50 ml 100 mM phosphate, pH 7.5, 5 mM $MgSO_4$, 180 mM K^+ and 5 mM β-mercaptoethanol. The gradient was linear and again flow rate was somewhat variable (0.5 ml - 1 ml/min.). Two ml fractions were collected and those with activity pooled. To enhance stability the enzyme was concentrated by the collodion-bag technique to a volume of 1 ml.

CHAPTER IV

RESULTS AND DISCUSSION

Antibodies for M type isozyme were not available so that the homogenate assays reflect the presence of both isozymes. Also, some difficulty was encountered with the homogenate assay using the 2,4 dinitrophenylhydrazine technique. The blank for this assay consistently gave an inordinately high absorbance. Accuracy was, therefore, somewhat suspect.

The methods used for the homogenization and the separation of M and L type isozymes by ammonium sulfate are well documented. The specific activities and yields presented in Table I for these methods are in good agreement with other researchers.

Some explanation should be given for the choice of homogenization buffers. Previous reports from this lab and others (32,36) have indicated pyruvate kinase to have increased stability in phosphate buffers. Phosphate would be the buffer of choice but to avoid the precipitation of $MgNH_4PO_4$ during the addition of ammonium sulfate and the use of the Celite column, a Tris buffer was used. (Results not presented herein indicated only a slight instability in Tris over short periods of time). The buffer was then changed to phosphate both for stability and convenience.

The Celite technique was found to be an effective method for achieving significant purification. When perfected, this step took

little time to accomplish and results were reproducible. It has been reported by King (40) that several parameters effect this procedure, these being the ratio in weight of protein to Celite used, the steepness of the decreasing salt gradient and control of pH. Also, the temperature may be significant.

For purification of type L pyruvate kinase the Celite to protein ratio, between 30 and 90 grams (dry weight) of Celite per gram protein, was not found to be critical. But, this ratio was important for practical reasons. The flow rate of the lower ratio was about half that of the higher ratio. The best results were obtained when the gradient had a decrease of 0.2% saturation in ammonium sulfate per milliliter. Gradients with greater change in percent saturation in ammonium sulfate per ml were tried but results were not as good. The pH was controlled at 7.4 with the temperature between 0-4°C. Figure #1 shows the peak position at 34% ammonium sulfate saturation. A specific activity as high as 2.1 has been obtained with this method.

In preparation for employing hydrophobicity in purification, four ω-aminoalkane sepharose columns (2 ml bed volume) were examined to determine which if any would retain the type L enzyme (the enzyme was suspended in 1065 buffer for these experiments). Seph-C_4NH_2, Seph-C_5-NH_2 and Seph-C_6-NH_2 successfully retained the enzyme but, Seph-C_3-NH_2 would not. The enzyme would be eluted with the buffer described in Chapter III. It should be noted that enzymes bound to alkylamine agarose of different chain lengths may be eluted by the same buffer system (41). Seph-C_4-NH_2 and Seph-C_5-NH_2 were used for further purification studies. Between 20 and 30 mg of protein per gel were added to these columns. The results given in Table II show an approximate

two fold difference in the purification achieved.

Based on these preliminary experiments, Seph-C_4-NH_2 was chosen for use in the final purification scheme. The protein loaded was 20 to 25 mg per ml gel. Purification of this step was about 6 fold providing an overall purification of 59 fold (see Table I).

An attempt will be made to explain the difference in Seph-C_4-NH_2 and Seph-C_5-NH_2 purification. Jennissen and Hielmeyer (42) have shown two ways to obtain the critical hydrophobicity needed to absorb protein to an alkylamine agarose gel. The degree of alkylamine residue substitution may be increased or the degree of substitution may be held constant and the alkylamine elongated by one or more carbon atoms. It seems unlikely that the use of α,ω-diaminoalkanes would alter this principle.

The scope of this research made it impractical to quantitatively determine the residues of α,ω-diaminoalkane bound per agarose sphere. It was not possible therefore, to determine accurately the hydrophobicity of the Seph-C_4-NH_2 or Seph-C_5-NH_2. But, considering that the coupling conditions of the α,ω-diaminoalkane for each column were the same, it is likely the Seph-C_5-NH_2 had the greater hydrophobicity. The Seph-C_5-NH_2 could then absorb additional protein not absorbed by the Seph-C_4-NH_2. This protein might then be eluted with the enzyme as indeed the results indicate.

In addition to hydrophobic forces, ionic interaction undoubtedly contributed to enzyme retention (38).

N^6-(6-aminohexyl)-AMP (containing 6.5 μmoles of AMP per ml of gel), AMP bound to the agarose through the ribose hydroxyls with a six carbon space (containing 3.7 μmoles of AMP per ml gel) and ATP bound to the

agarose through the ribose hydroxyls with a six carbon spacer (containing 7.3 μmoles of ATP per ml gel) were obtained commercially. Preliminary experiments were performed to determine which if any would be helpful with purification. The enzyme used for these studies had been purified through the Celite procedure and then desalted by dialysis against H-buffer, then buffer consisting of 5 mM phosphate pH 7.5, 159 mM K^+, 5 mM $MgSO_4$ and 5 mM β-mercaptoethanol. This enzyme was applied to the three affinity columns (5 ml bed volume each). The columns were washed with the application buffer. N^6-(6-aminohexyl)-AMP alone retained the enzyme, but bleeding was observed. Changing the application solution to 1065 buffer arrested the bleeding.

Approximately 4 mg of protein per ml of gel were loaded on the N^6-(6-aminohexyl)-AMP column when the column was used in conjunction with the previously described purification steps. The purification was 3 fold and the yield was 18%. Overall purification was 180 fold.

The attempts to retain the enzyme on the before mentioned columns suggest the enzyme may have a preference in binding to an adenine nucleotide affinity column. But results presented here cannot be considered conclusive due to column differences in μmoles of ligand bound per ml gel (43). Before elucidation of the binding is possible additional studies must be performed.

Some kinetic properties of the enzyme which has been purified through all steps including N^6-(6-aminohexyl)-AMP were examined. Fructose 1,6-diphosphate activation assayed as described in Methods was 3 to 4 fold. The kinetic values for ATP inhibition and the reversal of this inhibition by fructose 1,6-diphosphate were in agreement with published values (23). The enzyme was preincubated in the assay mixture

(described in Methods) containing 3 mM ATP alone or together with 1 mM fructose 1,6-diphosphate. The reaction was started by the addition of phosphoenolpyruvate. The reaction with ATP alone showed 24% activity and the reaction with both ATP and fructose 1,6-diphosphate showed 50% activity. The enzyme reaction with fructose 1,6-diphosphate and no ATP was taken as 100% activity.

Figure 1. Ammonium sulfate gradient solubilization of type L pyruvate kinase from rat liver on a column of Celite. Enzyme activity is shown by the solid line, the decreasing salt concentration by the broken line. The salt saturation at the peak position is given above the curve.

TABLE I

PURIFICATION OF RAT LIVER TYPE L
PYRUVATE KINASE

	Total activity	Total protein	Specific activity	Recovery
	(Units)	(mg)	Units/mg	(%)
Homog	1021	5998	.17	100
Ammonium Sulfate	594	1036	.57	58
Celite	341	226	1.5	33
Seph-C_4-NH_2	182	18	10	18
N^6-AMP	182	6	30	18

Figure 2. Purification of type L pyruvate kinase on Seph-C_4-NH_2. After the unabsorbed protein (monitored by the absorbance at 280 nm shown as ●) was washed off, a linear phosphate gradient (---) was applied to elute the enzyme (Δ).

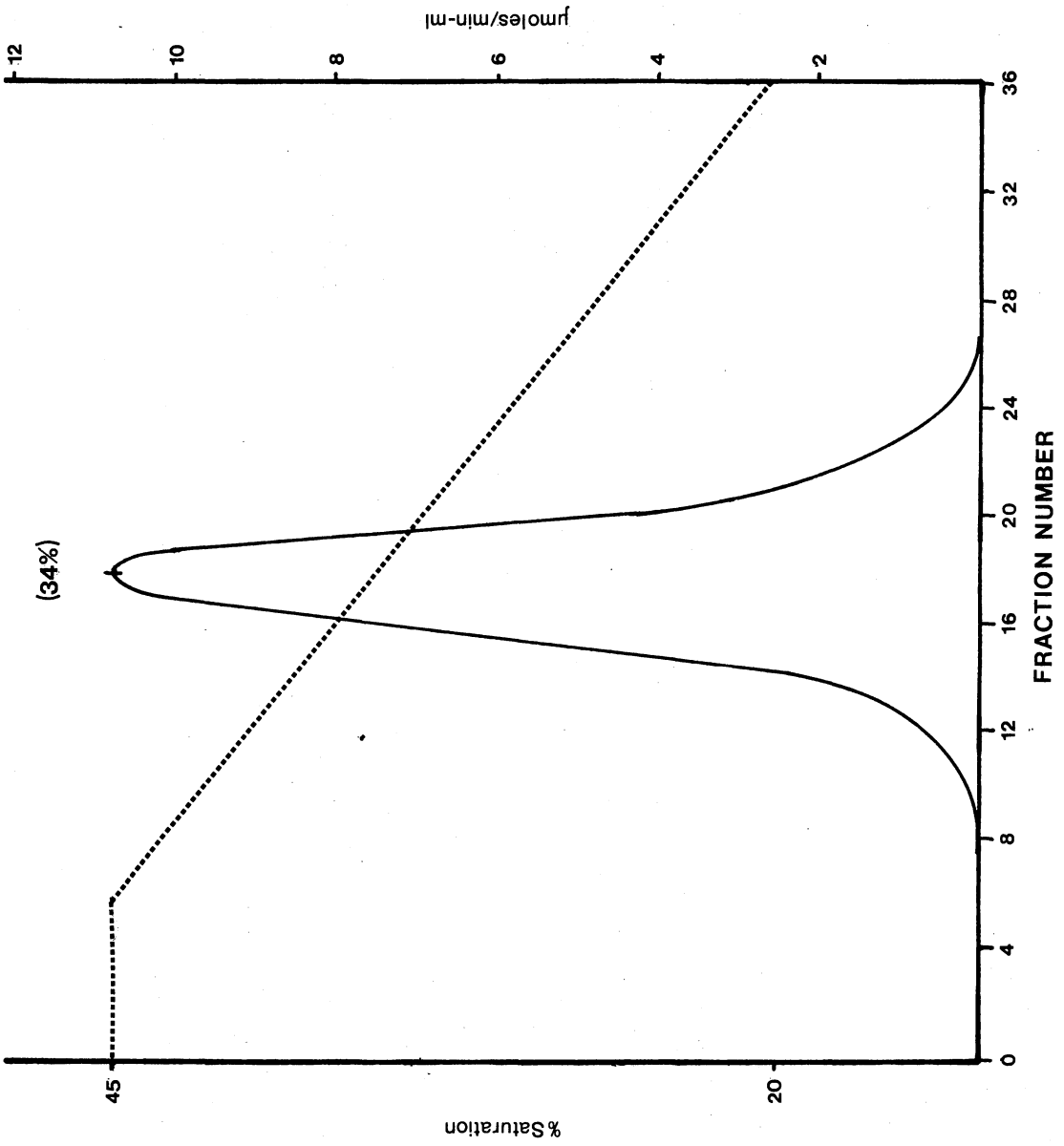
TABLE II

BINDING AND PURIFICATION OF RAT
LIVER TYPE L PYRUVATE KINASE
BY AMINOALKYL-AGAROSE

Abbreviation	Structure	Enzyme Retained	Specific Activity
Seph-C_3-NH_2	-NH-$(CH_2)_3$-NH_2	No	nd
Seph-C_4-NH_2	-NH-$(CH_2)_4$-NH_2	Yes	10
Seph-C_5-NH_2	-NH-$(CH_2)_5$-NH_2	Yes	5.6
Seph-C_6-NH_2	-NH-$(CH_2)_6$-NH_2	Yes	nd

Specific activity of enzyme applied to each column was 1.5.

Figure 3. Purification of type L pyruvate kinase on N^6-(6-aminohexyl)-AMP. Unabsorbed protein washed off before the gradient is not shown. A linear phosphate gradient was used to elute the enzyme (Δ). The elution of protein was monitored by the absorbance at 280 (●). The inset shows the structure of the immobilized nucleotide absorbent.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8
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(34%)
45
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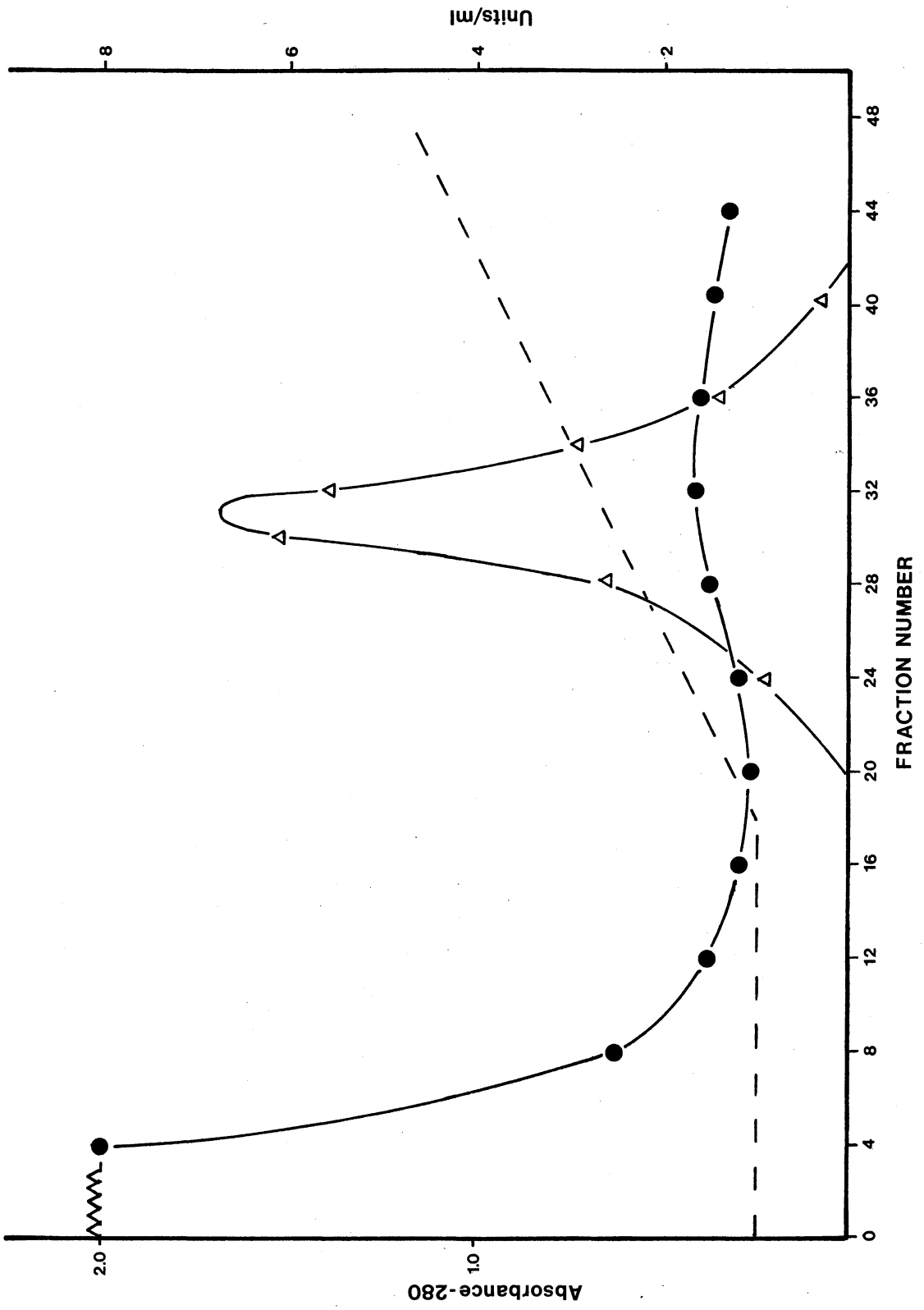
% Saturation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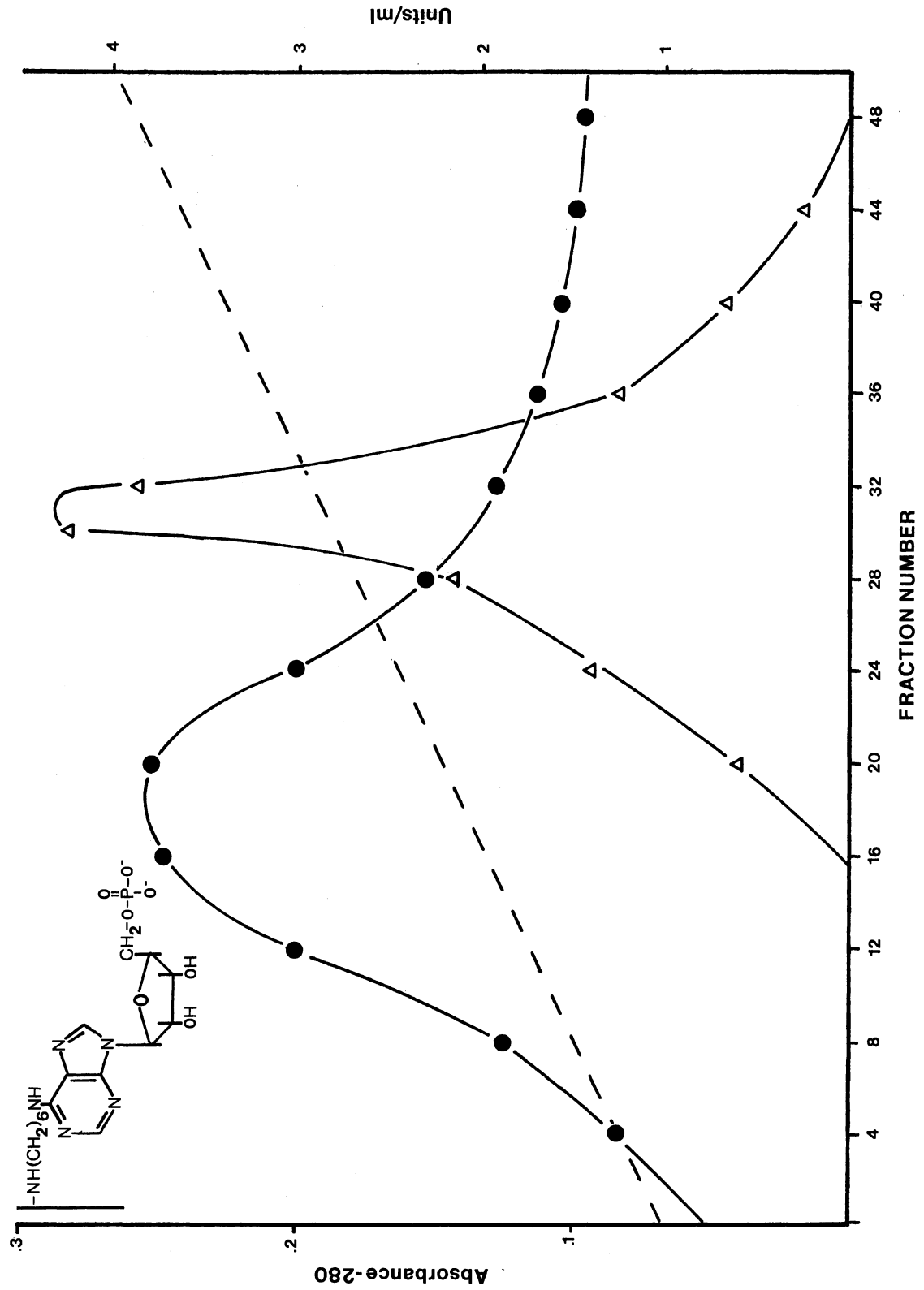
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.1
4
3
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4
8
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28
32
36
40
44
48
–NH(CH2)6NH
CH2O–P–O-
O=
O-
OH
OH
O
N

CHAPTER V

SUMMARY

Tris buffer (5 mM) was used for homogenization, ammonium sulfate, and Celite steps during the purification of Type L pyruvate kinase from rat liver.

The Celite technique when perfected gave reproducible results and significant purification. The Celite to protein ratio was 30 to 90 grams (dry weight) Celite per gram protein. The gradient had a decrease of 0.2% saturation in ammonium sulfate per ml and the pH was controlled at 7.4. A specific activity as high as 2.1 was obtained with this method.

A Seph-C_4-NH_2 gel was selected for the hydrophobic column. The protein load was 20 to 25 mg per ml gel. A linear gradient of 10 mM phosphate to 50 mM phosphate was effective in eluting the enzyme. Cumulative purification was 59; step purification was 6; specific activity was 10.

Three adenine nucleotide affinity columns were used for affinity chromatography studies of these only N^6-(6-aminohexyl)-AMP would retain the enzyme. Approximately 4 mg of protein per ml gel was bound to this column when it was used in conjunction with all other steps. The cumulative enzyme purification achieved was 180 fold (step purification was 3) and the yield from the previous step was 98%.

A SELECTED BIBLIOGRAPHY

(1) Lohmann, K. and Meyerhof, O. (1934) Biochem. Z. 273, 60-74.

(2) Parnas, J., Ostern, P. and Mann, T. (1934) Biochem. Z. 272, 64-70.

(3) Parnas, J., Ostern, P. and Mann (1934) Biochem. Z. 275, 74-86.

(4) Lehmann, H. (1935) Biochem. Z. 281, 271-291.

(5) Lutwak-Mann, C. and Mann, T. (1935) Biochem. Z. 281, 140-156.

(6) Needham, D. M. and Van Heyningen, W. E. (1935) Biochem. J. 29, 2040-2050.

(7) Boyer, P. D., Lardy, H. S., and Phillips, P. H., (1942) J. Biol. Chem. 146, 673-682.

(8) Meyerhof, O. and Junowiez-Kocholaty, R., (1942) J. Biol. Chem. 145, 443-456.

(9) Boyer, P. P., Lardy, H. and Myrbäck, K., (1962) "The Enzymes" 6, 95-113.

(10) Bücher, P. and Pfleiderer, G., (1955) "Methods in Enzymology" Vol. I, 435.

(11) Beisenherz, V. G., Boltze, H. T., Bücher, T., Czok, R., Garbade, K. H., Meyer-Arendt, E. and Pfleiderer, G., (1953) Z. Naturforssh. 86, 555-577.

(12) Tiertz, A. and Ochoa, S., (1958) Archives Biochem. Biophys. 78, 477-493.

(13) Kubowitz, F. and Ott, P., (1944) Biochem Z. 317, 193-196.

(14) Dvornikova, P. D., Gulyl, M. F., Fedorchenko, E. Y., and Martinenko, F. P., (1960) Ukrain. Biochem. J. 32, 783-790.

(15) Krebs, K. A., Eggleston, L. A., (1965) Biochem. J. 94, 3C.

(16) Weber, G., Singhal, R. L., Stamm, N. B., and Strivastava, S. K., (1965) Federation Proc. 24, 745-754.

(17) Tanaka, T., Harano, Y., Morimura, H., and Sue, F., (1965) 'Proceedings of Symposium on Enzyme Chemistry' Vol. 17, 341.

(18) Tanaka, T., Harano, Y., Morimura, H. and Mori, R., (1965) Biochem. Biophys. Res. Comm. 12, 55-60.

(19) Strandholm, S., Cardenas, J. M. and Dyson, R. D., (1975) Biochem. 14, 2242-2246.

(20) Tanaka, T., Harano, Y., Sue, F., and Morimura, H., (1967) J. Biochem. 62, 71-91.

(21) Hess, B., Haeckel, R., and Brand, K., (1966) Biochem. Biophys. Res. Comm. 24, 824-831.

(22) Taylor, C. B., and Bialey, E., (1967) Biochem. J. 102, 32C-33C.

(23) Tanaka, T., Sue, F., and Morimura, H. (1967) Biochem. Biophys. Res. Comm. 29, 444-449.

(24) Carminatti, H., Rozengurt, E., and Jimenez de Asua (1969) FEBS Letters 4, 307-310.

(25) Staal, G. E. J., Koster, J. F., Kamp, H., Van Milligen-Boersma, L., and Veeger, C., (1971) Biochem. Biophys. Acta. 227, 86-96.

(26) Blume, K. G., Hoffbauer, R. W., Busch, D., Arnold, H., and Löhr, G. W., (1971) Biochem. Biophys. Acta 225, 364-372.

(27) Haeckel, R., Hess, B., Lauterborn, W., and Wuster, K. H. (1968) Hoppe-Seyler's Z. Phys. Chem. 349, 699-714.

(28) Chern, C. J., Rittenberg, M. B., and Black, J. A. (1972) J. Biol. Chem. 247, 7173-7180.

(29) Cardenas, J. M., Dyson, R. D., and Strandholm, J. J., (1973) J. Biol. Chem. 248, 6931-6937.

(30) Cardenas, J. M., and Dyson, R. D. (1973) J. Biol. Chem. 248, 6938-6944.

(31) Dyson, R. D., and Cardenas, J. M. (1973) J. Biol. Chem. 248, 8482-8488.

(32) Ljungström, O., Hjelmquist, G., and Engström, L., (1974) Biochem. Biophys. Acta 358, 289-298.

(33) Llorente, P., Marco, R. and Sols, A., (1970) Eur. J. Biochem. 13, 45-54.

(34) Walker, P. R. and POtter, V. R., (1973) J. Biol. Chem. 248, 4610-4616.

(35) Pogson, C. I., (1968) Biochem. J. 110, 67-77.

(36) Flory, W. and Koeppe, R. E., (1973) Biochem. J. 133, 391-394.

(37) Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., (1951) J. Biol. Chem. 193, 265-275.

(38) Shaltiel, S. and Er-el, Z., (1973) Proc. Nat. Acad. Sci. USA 70, 778-781.

(39) Axen, R., Porath, J., Ernback, S., (1967) Nature 214, 1302-1304.

(40) King, T. P., (1972) Biochem. 11, 367-371.

(41) Hofstee, B. H. J., (1973) Biochem. Biophys. Res. Comm. 50, 751-757.

(42) Jennissen, H. P. and Heilmeyer, L. M. G., (1975) Biochem. 14, 754-760.

(43) Harvey, M. J., Lowe, C. R., Craven, D. B. and Dean, P. D. G., (1974) Eur. J. Biochem. 41, 335-340.

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