STUDIES ON HUMAN PYRUVATE KINASE

by

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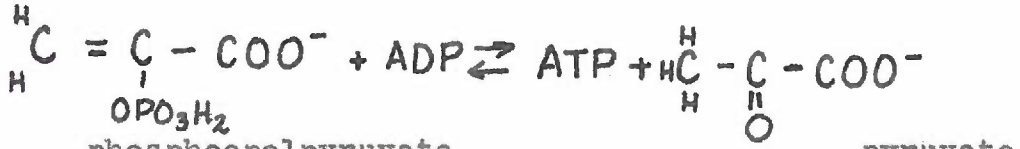
INTRODUCTION

Pyruvate kinase (PK) is an enzyme of the glycolytic pathway which catalyzes the conversion of phosphoenolpyruvate to pyruvate, coupled to the conversion of ADP to ATP:



phosphoenolpyruvate pyruvate

Recent observations have brought into focus questions regarding the nature of control mechanisms regulating the level of PK activity in various tissues. These observations have stemmed from investigations of a human metabolic disease associated with altered PK activity levels and from studies of the enzyme in various tissues of animals, especially as associated with different metabolic states.

In 1954, Selwyn and Dacie (1) were able to divide a group of familial diseases, nonspherocytic hemolytic anemia, into two groups: type I in which addition of glucose to incubating erythrocytes decreases the amount of hemolysis observed in 48 hours at 37° C.; and type II in which added glucose has no effect. It was subsequently shown (2,3,4) that abnormalities existed in the quantities of glycolytic intermediate metabolites in the erythrocytes of the type II disease. Valentine

and co-workers (5,6) demonstrated that the defect in this condition was specifically a decreased or absent PK activity in the erythrocytes of affected persons. Pedigree analysis and the finding of intermediate PK levels in parents and other relatives permitted classification of this disorder as a Mendelian autosomal recessive trait. An additional observation of importance with regard to the involved genetic mechanism was that the leukocyte PK levels were normal in these patients and their relatives.

Koler and collaborators (7) extended these observations to another series of patients and noted the same relationship between normal and affected persons, and between erythrocytes and leukocytes. No differences by ion-exchange chromatography could be demonstrated between the enzyme of either cell type obtained from normal subjects and that obtained from subjects with the anemia. In addition, they showed that human leukocyte and erythrocyte PK were different proteins by several chemical criteria. Comparison of the chromatographic patterns of tryptic peptides of highly purified enzymes from normal erythrocytes and normal leukocytes by these workers permitted the suggestion that the two forms of PK were similar, but not identical, in their primary structures. The structural studies were limited, however, by a paucity of pure material; thus, a

more precise definition of the nature of these structural similarities and differences was precluded. These findings led the authors to agree with the earlier suggestion of Parker and Bearn (8) that the PK deficiency type of nonspherocytic hemolytic anemia may be due to a control gene mutation.

The hypothesis of a control gene mutation in this disease might be challenged by evidence contained in two reports: a report by Busch (9) of a family in which PK activity is decreased in both erythrocytes and leukocytes; and a report by Waller and Löhr (10) that the enzyme from patients with the PK deficiency differs from that of normal individuals in its kinetic properties. The challenge from the former report could be met by proposing that Busch's family has a different disorder. The report of kinetic differences, if confirmed, would support the hypothesis that the lesion is due to a structural gene mutation, but this might also be explained on the basis of its representing a different disorder.

Epstein (11) has listed several points which he feels must be considered in applying the concept of control gene mutation to human disease. He emphasizes that the facts known about many hereditary diseases in man can be adequately explained by hypotheses based on structural gene mutations. He argues that, before a structural gene mutation can be discarded in favor of

a control gene mutation as an explanation, the involved protein must be shown to be unaltered in primary structure.

Weber and associates (12) showed that the level of PK activity in rat liver homogenates is reduced in the diabetic state and returns to nearly normal levels upon the administration of insulin. That this rise is blocked by actinomycin, puromycin, or ethionine was interpreted to indicate that the insulin effect is mediated by de novo enzyme synthesis (13).

Tanaka and co-workers (14) studied the effect of diabetes, insulin, and various diets on levels of PK activity in liver and muscle of the rat. They found various tissues contained two different forms of PK. The type which occurs as the only form in skeletal muscle, heart, and brain was designated as type M. Liver and kidney contained, in addition to type M, a second type which they called type L. It was demonstrated that only the type L enzyme was affected by the metabolic state of the animal, in that its level fell with starvation, high protein diet, or diabetes. This level rose toward normal with insulin administration. Muscle PK levels were unaffected.

Tanaka and associates (14) showed that the type L enzyme migrated in three bands on starch block electrophoresis. Von Fellenberg and associates (15) had pre-

viously demonstrated the existence of multiple electrophoretic forms of PK from rat tissues on agar gel electrophoresis. The relationship between these two studies is not clear at this time, due largely to the fact that different electrophoretic systems were employed.

The above-mentioned investigations have brought about several interesting questions regarding pyruvate kinase as it occurs in man: How many forms of the enzyme occur in different tissues? What is the distribution of the different types in these tissues? Are tissues other than the erythrocytes involved in the PK deficiency anemia? What is the relationship of these different forms in terms of structure and function? Is the PK of individuals with the anemia identical to normal PK in structure?

The pursuit of answers to these questions will, at the outset, require the establishment of methods for detection of different forms of the enzyme in human tissues, and of a scheme for isolating the various forms in quantity and purity suitable for the appropriate studies of structure and function. Of special importance is the need for a procedure which will supply PK from the erythrocytes of persons with the deficiency, in high yield and purity sufficient for investigation of its primary structure. Progress toward accomplishing

these requirements will be described in this thesis.

The salting-out characteristics of erythrocyte PK under various conditions and its heat stability have been studied. The occurrence of PK in several human tissues and the behaviour of the enzyme from some of these upon ion-exchange chromatography have been investigated. These experiments have resulted in the establishment of procedures for the purification of erythrocyte PK and the detection of multiple forms of the enzyme in different tissues.

MATERIALS AND METHODS

Materials

Carboxymethyl cellulose was obtained from Bio-Rad, carboxymethyl Sephadex C-50 medium from Pharmacia, and dextran type 100C from Sigma. Reagent dithiothreitol was purchased from Calbiochem and reagent grade beta-mercaptoethanol from Eastman or Matheson Coleman & Bell. All other chemicals were reagent grade. Distilled, deionized water was used in all experiments.

Enzyme Assay

PK assays were done at 37° C. according to the lactic dehydrogenase linked assay method of Tanaka et al. (6), using a Gilford model 2000 spectrophotometer. One unit of PK is defined as that amount of enzyme which converts one micromole of substrate per minute. Substrates and rabbit muscle lactic dehydrogenase essentially free of pyruvate kinase activity were obtained from Sigma.

General Methods

All pH determinations were done on undiluted solutions at room temperature with a Beckman model G or model 76 pH meter, using Beckman standard buffers for reference. Centrifugation was done in refrigerated centrifuges; unless specified otherwise, angle head rotors were used. Optical density determinations were made using quartz cuvettes with a 10 mm light path in a Zeiss

PMQ II spectrophotometer; the appropriate buffer was used as reference. Visking 8/32" tubing was used for dialyses.

Effect of Sulfhydryl Compounds on Enzyme Stability

Freshly drawn blood from a normal donor was treated as described in the following section on purification of the enzyme. 235 ml of packed erythrocytes were hemolyzed by addition of water to make a volume of one liter and filtered three times on a Buchner funnel, using S&S 202 filter paper. Ninety per cent and 10 per cent saturated ammonium sulfate solutions, 0.1 molal in dibasic potassium phosphate and adjusted to pH 7.5 with concentrated ammonium hydroxide or sulfuric acid were used for fractionation of the hemolysate.* Phase separations were effected by centrifugation at 4° C. The following fractions were taken successively: the 40 per cent saturation precipitate; the 10 per cent saturation supernatant; and the 35 per cent saturation precipitate. The resulting precipitate was washed twice with 35 per cent saturated ammonium sulfate by centrifugation and dissolved in 0.003 M potassium phosphate buffer at pH 7.3. Insoluble material was removed by centrifugation.

The resulting enzyme concentration was 0.53 units

*The method of calculating ammonium sulfate concentrations is described in the appendix.

per ml. Samples were weighed into dialysis tubing which had been soaked in the phosphate buffer. These were then dialyzed at 6° C. for 29 hours against 500 ml of the phosphate buffer containing beta-mercaptoethanol, dithiothreitol, or no sulfhydryl compound; the dialysis solution was changed at 18 hours. The dialyzed solutions were recovered quantitatively and assayed for enzyme activity. The composition of the different dialysis solutions and the calculated recovery of enzyme activity are shown in table 1.

Purification of PK from Erythrocytes

All procedures were carried out at 4 to 6° C., unless specified otherwise.

Step 1. 500 ml of blood were drawn from a normal donor and collected in a plastic bag containing 10 ml of a 15 per cent potassium EDTA solution. After storage in the cold for 36 hours, 100 ml of a 0.85 per cent sodium chloride solution containing 6 g of dextran were added to the blood. After settling in a one liter, glass beaker for 45 minutes, 270 ml of plasma and partially clumped leukocytes were drawn off by suction. The erythrocytes were mixed with 280 ml of an 0.85 per cent sodium chloride solution containing 6 g of dextran, allowed to settle, and decanted as before. The settling in dextran was repeated, and the erythrocytes were washed three times by mixing with an equal volume of 0.85

TABLE 1.

EFFECT OF SULFHYDRYL COMPOUNDS ON ENZYME STABILITY

Buffer composition	Number of determinations	Per cent recovery
0.003 M potassium phosphate pH 7.3	4	8 8 8 6
0.003 M potassium phosphate pH 7.3 0.01 M beta-mercaptoethanol	2	42 46
0.003 M potassium phosphate pH 7.3 0.001 M beta-mercaptoethanol	2	21 21
0.003 M potassium phosphate pH 7.3 0.00065 M dithiothreitol	1	31

per cent sodium chloride solution and centrifugation with a swinging bucket rotor at 500 x g for 15 minutes. The resulting packed erythrocytes contained 7.75×10^6 erythrocytes and 1350 leukocytes per mm^3. Hemolysis was effected by addition of an equal volume of cold water to 330 ml of erythrocytes, followed by inversion of the vessel every minute for 15 minutes.

Step 2. Various proportions of a 90 per cent saturated ammonium sulfate solution, 0.1 molal in dibasic potassium phosphate and adjusted to pH 7.1 with concentrated ammonium hydroxide, were added to 10 g aliquots of the hemolysate in small beakers. Addition of the salt solution was done on a top loading balance with a Pasteur pipette. After gentle mixing, the resulting suspensions stood for 30 minutes. Each was assayed for PK activity, and the samples were centrifuged for 30 minutes at 17,000 x g. The resulting supernatant solutions were assayed for enzyme activity. The proportions of salt solution and hemolysate, and the assay results are shown in table 2. The aliquots, the remaining hemolysate, and 58.2 g of the phosphate buffered, 90 per cent saturated ammonium sulfate solution were combined to make a suspension of such composition that the ratio of salt solution to hemolysate was 0.152. The suspension was held for 15 minutes and centrifuged for 45 minutes at 16,000 x g. 851.5 g of the phosphate buf-

TABLE 2.

DETERMINATION OF SOLUBILITY OF PK IN AMMONIUM SULFATE SOLUTIONS AT pH 7.1

Ratio of 90 per cent saturated ammonium sulfate solution to hemolysate	Suspension assay	Supernatant assay	Ratio of assays
0.181	1.027 units per ml	0.959 units per ml	0.933
.239	.918	.843	.918
.298	1.110	.874	.787
.340	1.071	.952	.889
.418	1.110	.949	.855
.492	1.090	.656	.602
.565	1.060	.306	.288
.658	.969	.136	.140
.759	1.071	.065	.061
.881	.942	.048	.051

fered, 90 per cent saturated ammonium sulfate solution were added to the resulting 740.4 g of supernatant solution and the resulting suspension stored in the cold for 7 days.

Step 3. The suspension from the previous step was centrifuged at 16,000 x g for one hour and the supernatant decanted by pouring. The precipitate was resuspended in one liter of pH 7.1, 0.1 molal potassium phosphate, 55 per cent saturated ammonium sulfate solution and centrifuged for one hour at 16,000 x g. The washing was repeated with 930 ml of a 50 per cent saturated, and 950 ml of a 40 per cent saturated ammonium sulfate solution, 0.1 molal in potassium phosphate and at pH 7.1. The resulting precipitate was resuspended in 250 ml of a pH 7.1, 10 per cent saturated ammonium sulfate solution, 0.1 molal in potassium phosphate; after 15 minutes, this suspension was centrifuged for 45 minutes at 16,000 x g. This was repeated with another 250 ml of the 10 per cent saturated ammonium sulfate solution, and the 10 per cent saturated ammonium sulfate supernatants combined.

Step 4. Aliquots of the supernatant from the previous step were heated rapidly to various temperatures, held at constant temperature for 5 minutes, and rapidly cooled. This was repeated with aliquots of the same solution, made 0.1 molal in beta-mercaptoethanol. The

results of assays for enzyme activity done on the cooled aliquots are shown in figure 1. The remaining 390 ml of solution were made 0.1 molal in beta-mercaptoethanol, heated to 60° C. in 6½ minutes, held at that temperature for 5 minutes, and rapidly cooled. The resulting suspension was centrifuged at 16,000 x g for 45 minutes and the 410 ml of supernatant saved for the next step.

Step 5. Aliquots of the supernatant from step 4 were brought to 33, 36, 39 and 42 per cent saturation with respect to ammonium sulfate by the addition of pH 7.1, 90 per cent saturated ammonium sulfate solution, 0.1 molal each in dibasic potassium phosphate and beta-mercaptoethanol. The resulting suspensions were held for 30 minutes and centrifuged at 17,000 x g for 30 minutes. The results of assays of the supernatant are shown in table 3. The remaining supernatant from step 4 was brought to 42 per cent saturation and centrifuged at 16,000 x g for 30 minutes after having stood for 30 minutes. The precipitate was washed once by suspension in 42 per cent saturated ammonium sulfate and centrifuged as before. The precipitate was suspended in pH 5.2, 10 per cent saturated ammonium sulfate solution, 0.1 molal each in potassium acetate and beta-mercaptoethanol; the suspension was centrifuged at 16,000 x g for 30 minutes, yielding 84.5 ml of supernatant upon decantation.

Figure 1. Effect of heat on PK activity. The first point of each curve represents the assay on material not subjected to high temperatures. Determinations were made in 0.1 molal beta-mercaptoethanol (X---X---X---X) and without mercaptoethanol (.___.___.___.).

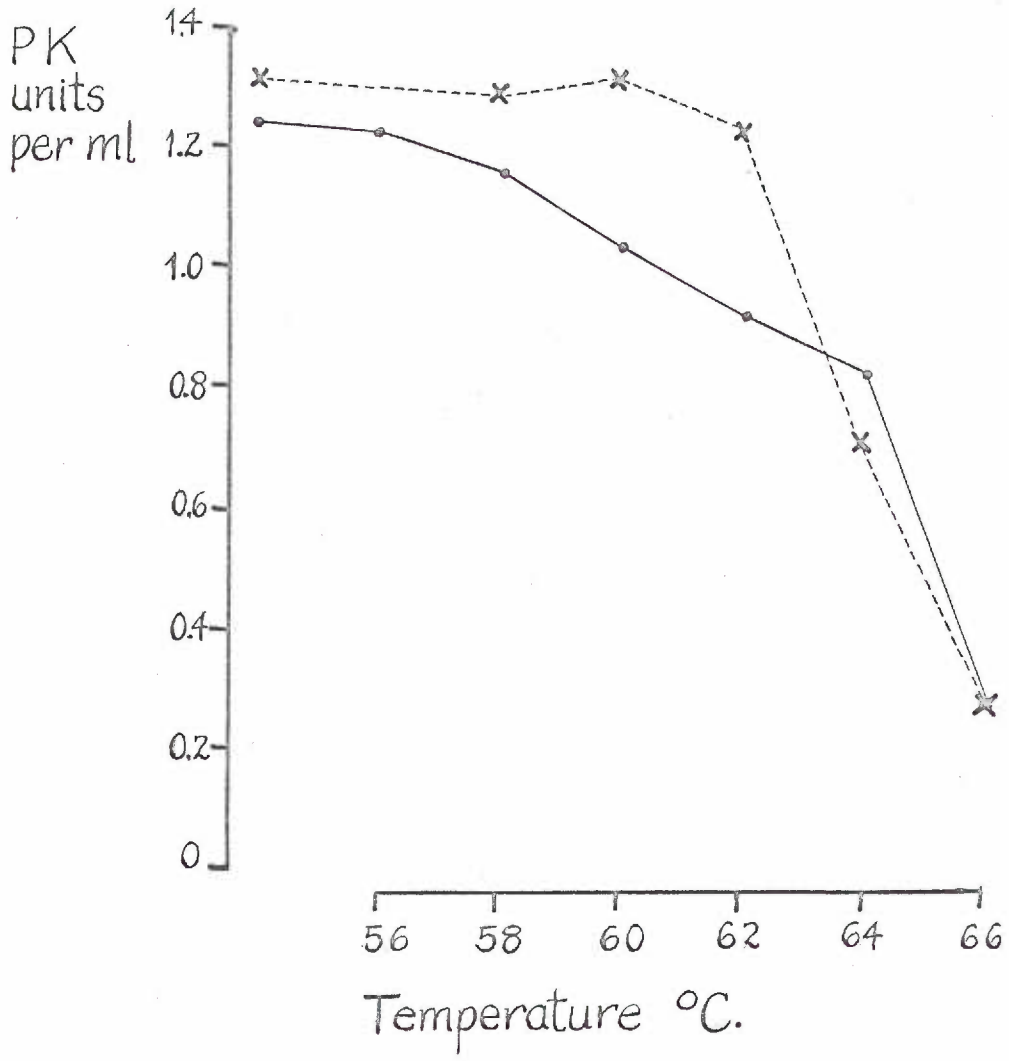

PK units per ml
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
56
58
60
62
64
66
Temperature °C.

TABLE 3

DETERMINATION OF PK SOLUBILITY AFTER HEAT DENATURATION

Per cent saturation of ammonium sulfate	Suspension assay	Supernatant assay
33	.690 units per ml	0.440 units per ml
36	.726	.165
39	.696	.080
42	.626	.068

Step 6. Aliquots of the supernatant from the preceding step were rapidly brought to 48, 50 and 52° C., held at constant temperature for 5 minutes and rapidly cooled. Results of assays on the cooled suspensions are shown in table 4. The remaining supernatant from step 5 was heated to 50° C. for 5 minutes and rapidly cooled.

Step 7. Aliquots of the suspension resulting from heat treatment in the previous step were taken to determine the solubility of the enzyme at several salt concentrations. This was achieved by the addition of pH 5.2, 90 per cent saturated ammonium sulfate solution, 0.1 molal each in beta-mercaptoethanol and potassium acetate, followed by standing for 30 minutes and centrifugation at 16,000 x g for 30 minutes. The suspensions and supernatants were assayed; the results are shown in table 5. The remaining 71 ml of suspension from step 6 were brought to 28 per cent saturation in ammonium sulfate by addition of the same 90 per cent saturated ammonium sulfate solution, held for 30 minutes, and centrifuged for 30 minutes at 16,000 x g. The supernatant was brought to 40 per cent saturation and centrifuged as before. The resulting precipitate was suspended in 20 per cent saturated ammonium sulfate, 0.1 molal each in beta-mercaptoethanol and potassium acetate, at pH 5.2. After 30 minutes, the suspension was centrifuged at 16,000 x g for 30 minutes and the supernatant taken.

A summary of the purification procedure appears in table 6.

TABLE 4. EFFECT OF HEAT ON PK AT pH 5.2

Temperature	Assay after Heating
not heated	3.36 units per ml
48° C	3.40
50°	3.38
52°	2.89

TABLE 5. SOLUBILITY OF PK IN AMMONIUM SULFATE SOLUTIONS AT pH 5.2

Per cent saturation ammonium sulfate	Suspension assay	Supernatant assay
28	2.41 units per ml	2.62 units per ml
32	2.72	1.53
34	2.55	0.61
37	2.30	0.29
41	2.30	0.03

TABLE 6. SUMMARY OF PURIFICATION PROCEDURE

	Volume	PK	Protein	Yield	Specific activity***
Step 1 hemolysate	660 ml	805 units	96* mg per ml		0.013 units per mg
Step 2 suspension after 7 days	1370	840		104%	
Step 3					
supernatant	1270	3			
55% wash	1000	0			
50% wash	930	11			
40% wash	950	35			
10% supernatant	495	522		65	
Step 4 supernatant after heat	410	482		60	
Step 5					
42% suspension	660	459		57	
42% supernatant	660	29			
42% wash	335	26			
10% supernatant	85	296	1.5**	37	2.4
Step 6 suspension after heat	83	235		29	
Step 7					
27% supernatant	90	224		28	
40% supernatant	108	3			
20% supernatant	110	202	0.26**	25	7.1

*hemoglobin, determined spectrophotometrically as cyanmethemoglobin.

**method of calculating protein concentration is discussed in the text.

***that purification was achieved in several steps where protein concentration could not be conveniently determined was apparent in that appreciable amounts of precipitate were discarded.

Occurrence of PK in Several Tissues

Tissues were obtained at autopsy within 12 hours of the death of a middle aged white male who had died either of exposure to cold or acute alcoholism. These were held at 6° C. until processed further, which was done within 36 hours in each case. After removal of adherent connective tissue, each was diced with a scalpel and homogenized in a Virtis homogenizer at low speed for 30 minutes at 0 - 2° C. with twice the tissue weight of .05 M potassium phosphate buffer at pH 7.4 and .01 M in beta-mercaptoethanol. The resulting homogenates were centrifuged at 40,000 x g at 0 - 2° C. for three hours. The supernatant fluid was decanted and passed through a glass wool plug to remove the lipid layer which had floated to the top of the fluid. Each supernatant was then brought to 80% saturation with respect to ammonium sulfate by the addition of the appropriate amount of solid salt over 20 - 30 minutes at 0 to 2° C. The resulting suspensions were then stored at 6° C. Tissue weights and yields of PK are shown in table 7.

In order that PK from erythrocytes could be compared to that obtained from these tissue preparations, a sample of blood from a normal donor was treated in the following manner: freshly drawn blood was centrifuged for 20 minutes at 1500 x g for 15 minutes at 4° C. and the plasma and white cell layer were removed by decantation. The erythrocytes were then washed with 0.9%

TABLE 7. OCCURRENCE OF PK IN SEVERAL TISSUES

Tissue	Wet weight	Total PK in homogenate supernatant	Total PK in ammonium sulfate suspension
Skeletal muscle	50 g	16,400 units	8,190 units
Heart	50 g	4,300	3,970
Liver	50 g	1,030	690
Kidney	50 g	1,240	1,600
Spleen	19 g	450	485
Brain	44 g	3,710	3,130

sodium chloride by suspension and centrifuged as before. The washing procedure was repeated twice and the cells lysed by addition of two volumes of cold water. After mixing by inverting the tube every minute for 15 minutes, the resulting hemolysate was centrifuged for 30 minutes at 15,000 x g at 4° C. Solid ammonium sulfate was added to 80 per cent saturation and the resulting suspension stored at 6° C.

Carboxymethyl Cellulose Chromatography of Liver PK

5 g of carboxymethyl cellulose were stirred with 500 ml of water at room temperature for 30 minutes. 50 ml of a solution 1 M in sodium chloride and 1 M in sodium hydroxide were added and stirring continued for 30 minutes. After 2 minutes, the suspension was taken from the fast-settling material by pouring. Fines were decanted by pouring after 20 minutes. The exchanger was stirred for 15 minutes with 4 liters of water and the fraction settling between 3 and 30 minutes taken. This step was repeated, and the material was washed on a Buchner funnel using Whatman number 1 filter paper with 1 liter of water, four times. It was then washed with 500 ml of a pH 5.28 buffer made by dissolving 23.55 g potassium acetate, 3.60 g acetic acid, and 4.69 g beta-mercaptoethanol in 6 liters of water. The exchanger was suspended in 300 ml of the acetate buffer and allowed to settle for 30 minutes. Excess buffer

was poured off and the material resuspended in a volume of buffer equal to that of the settled material. A 0.9 x 15 cm glass chromatographic column, fitted with a medium porosity sintered glass filter disc, and cooled at 6° C. by means of a water jacket and circulating water bath was used. The column was prepared by pouring the suspended exchanger into the column and extension tube, allowing it to settle to 1 cm by gravity, and packing with air at a pressure of 10 pounds per in^2 to a height of 10 cm. Buffer was pumped through the column at 20 ml per hour for 20 hours prior to loading; a Milton Roy Minipump was employed. 2 ml of the ammonium sulfate suspension of liver homogenate was dialyzed against 500 ml of the acetate buffer at 6° C. for 18 hours; the dialysis solution was changed at 12 hours. A brown-colored precipitate was removed by centrifugation at 20,000 x g for 30 minutes at 4° C. The resulting 7.1 ml of solution contained 2.45 units of PK per ml; 3 ml were loaded on the column.

Linear gradient elution was done using two vessels of a Varigrad system (Buchler Instruments). The mixing vessel contained 100 ml of the acetate buffer described above and the reservoir 100 ml of the same buffer made 0.3 M in potassium chloride. 5 ml fractions were collected in a fraction collector at 4-6° C.; the flow rate was 20 ml per hour.

Carboxymethyl Sephadex Chromatography of PK from Liver, Kidney, Skeletal Muscle, and Erythrocytes

Citrate-phosphate buffer was prepared by dissolving 15.13 g citric acid, 29.26 g dibasic potassium phosphate, and 4.69 g beta-mercaptoethanol in 6 liters of water and adjusting the pH to 6.0 with 10 M potassium hydroxide. This buffer was used for preparing the ion-exchange material, dialysis, and as the beginning buffer in elution of the columns.

12 g of carboxymethyl Sephadex were added slowly with stirring to 4 liters of water and allowed to stand for 24 hours. The material was then washed by stirring for 30 minutes, settling for 30 to 60 minutes, and decanted by pouring, using each of the following in succession: 4 liters of water; 2.5 liters of 0.5 N hydrochloric acid; 4 liters of water, three times; 2.5 liters of 0.5 N potassium hydroxide; 4 liters of water, three times; and 1 liter of beginning buffer, three times. The exchanger settled to a volume of 250 ml; an equal volume of beginning buffer was added, and the material was stored at 4° C. until pouring of the columns.

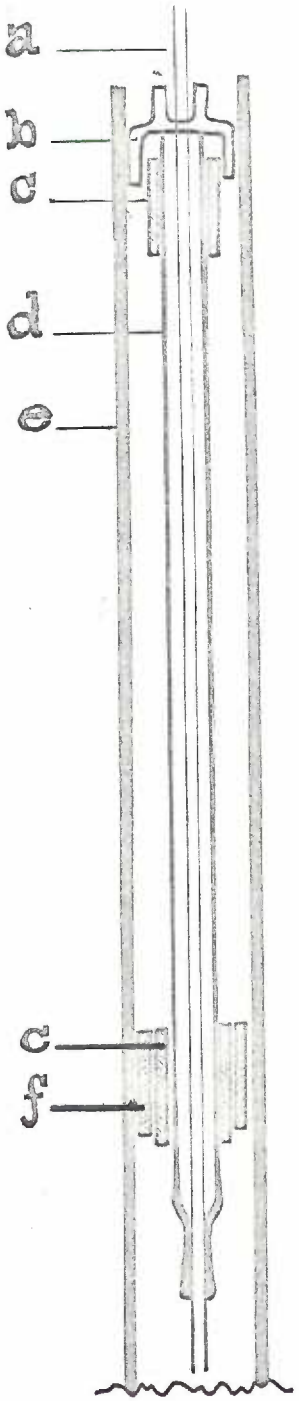
Columns of the type described in the previous section were used at 6° C. The columns were filled with buffer, and suspended exchanger was added to an extension tube. After 15 minutes, flow was begun by opening the bottom of the column. The material settled to a

height of 11 to 12 cm. 150 to 250 ml of beginning buffer were passed through the column at about 10 ml per hour prior to loading.

One ml fractions of the ammonium sulfate suspensions of liver, kidney, and skeletal muscle homogenates were dialyzed against 500 ml of the beginning buffer for 20 hours at 6° C.; the dialysis solution was changed at 12 hours. A 5 ml fraction of the ammonium sulfate suspension of the erythrocyte preparation was similarly dialyzed. The dialyzed suspensions were centrifuged at 20,000 x g for 30 minutes at 4° C. before loading. The column loads were as follows: liver, 1 ml containing 5.9 PK units; kidney, 1 ml containing 5.0 PK units; skeletal muscle, 0.17 ml containing 5.6 PK units; and erythrocytes, 3 ml containing 0.75 PK units.

After elution with at least 15 ml of beginning buffer, gradient elution was begun, using 2 chambers of a Varigrad gradient apparatus. The reservoir contained beginning buffer which had been adjusted to pH 7.8 with 10 M potassium hydroxide. The kidney, liver and skeletal muscle columns were run simultaneously; the apparatus described in figure 2 was used so that the developer for each column flowed from a common gradient device, containing 225 ml in each chamber. A pump was used for elution of the erythrocyte column; 100 ml of buffer was in each chamber of the gradient

Figure 2. Apparatus for varying the hydrostatic head on a chromatographic column. The assembled apparatus is shown in section; the parts are: a. 1 mm inside diameter Teflon tubing; b. vaccine cap; c., f., g., i. Tygon tubing of various dimensions; d. Pasteur pipette; e. 14 mm inside diameter glass tubing; h. cut end of plastic syringe barrel. Parts c. and f. are of such dimensions that a small clearance is left between parts e. and f. The hydrostatic head across the column is varied by raising or lowering the pasteur pipette assembly in the glass tubing which has been carefully mounted so that its long axis is exactly vertical. The effluent tubing from the apparatus is adjusted over the fraction collector so that the syringe barrel end contains the smallest volume without emptying completely. One such assembly was used for each of the columns eluted from the same reservoir.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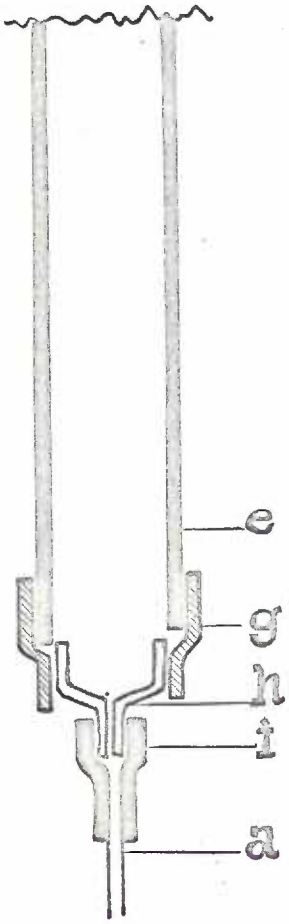
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device. Fractions of 2 to 4 ml were collected in a refrigerated fraction collector at flow rates of 8 to 12 ml per hour.

RESULTS AND DISCUSSION

Effect of Sulfhydryl Compounds on Enzyme Stability

Erythrocyte PK is unstable upon dialysis against low ionic strength phosphate buffer at neutral pH. The enzyme is partially protected against loss of activity under these conditions by dithiothreitol or beta-mercaptoethanol; this protection is dependent to some extent upon the concentration of the sulfhydryl compound. These findings suggest that some inactivation of the enzyme is due to oxidation of cysteine residues. The use of sulfhydryl compounds during procedures for purification of this enzyme is indicated when low ionic strengths are to be used.

Purification of Erythrocyte PK

Protein concentrations were calculated from optical density readings at 280 millimicrons and from the assumption that the PK at a concentration of one mg per ml gives an optical density of 0.54 at this wavelength. This assumption is based on published extinction coefficients of 0.54, 0.54 and 0.53 for PK from skeletal muscle of rabbit, man and rat, respectively (16).

Purification of human erythrocyte PK has been reported by Koler *et al.* (7). Comparison of the final specific activity of 7.1 units per mg, in this paper, with the formerly reported value, is not possible because, in the latter instance, protein concentration was deter-

mined by alkaline hydrolysis and reaction with ninhydrin. The previously reported overall yield was 0.84 per cent; in that procedure, losses of about 90 per cent were encountered in each of two steps consisting of procedures at low ionic strengths. It is suggested that the higher yield (25 per cent) in the present procedure can be attributed, to some extent, to the avoidance of steps at low ionic strength.

Attempts to establish a procedure in which predetermined ammonium sulfate concentrations were used were unsuccessful. This was probably due, at least in part, to the influence of the great relative amounts of hemoglobin and the variability of the PK to hemoglobin ratios in different hemolysate preparations. The heat stability of the enzyme seemed to be affected by the concentration of protein, also. Because of these difficulties, it was decided that the greatest efficiency of salting out and heat denaturation techniques could be achieved by determinations of these characteristics on aliquots of the solution during the purification procedure. This would not have been possible without a simply performed, rapid enzyme assay. With these considerations, it is anticipated that the method described can be applied to the purification of PK from other human tissues.

At the stages in the second and fifth steps where

the enzyme is present as a suspension at salt concentrations rendering it insoluble, it is stable to loss of activity during storage for several days. This stability should allow the accumulation of greater amounts of PK at these stages of purification. This would be an advantage in terms of efficient utilization of time as greater amounts of enzyme are prepared. In addition, attempts to crystallize the enzyme should be enhanced because greater protein concentrations and volumes large enough to be effectively processed could be achieved.

Ion Exchange Chromatography of PK from Different Tissues

The resolution of the apparent components of liver PK on carboxymethyl cellulose shown in figure 3 could be improved neither by use of a more gentle ionic strength gradient nor by the use of phosphate rather than acetate buffers. Comparison of the behaviour of liver PK on carboxymethyl cellulose, using an ionic strength gradient, and on carboxymethyl Sephadex (figure 4), using a pH gradient, shows the superior resolving power of the latter, with the buffer systems employed.

That at least two chromatographic forms of the enzyme are present in liver and kidney can be seen in figure 4. The relationship between the different chromatographic zones from the several tissues can be more precisely established by rechromatography experiments. This chromatographic system may have some application in proce-

Figure 3. Chromatography of liver PK on carboxymethyl cellulose.

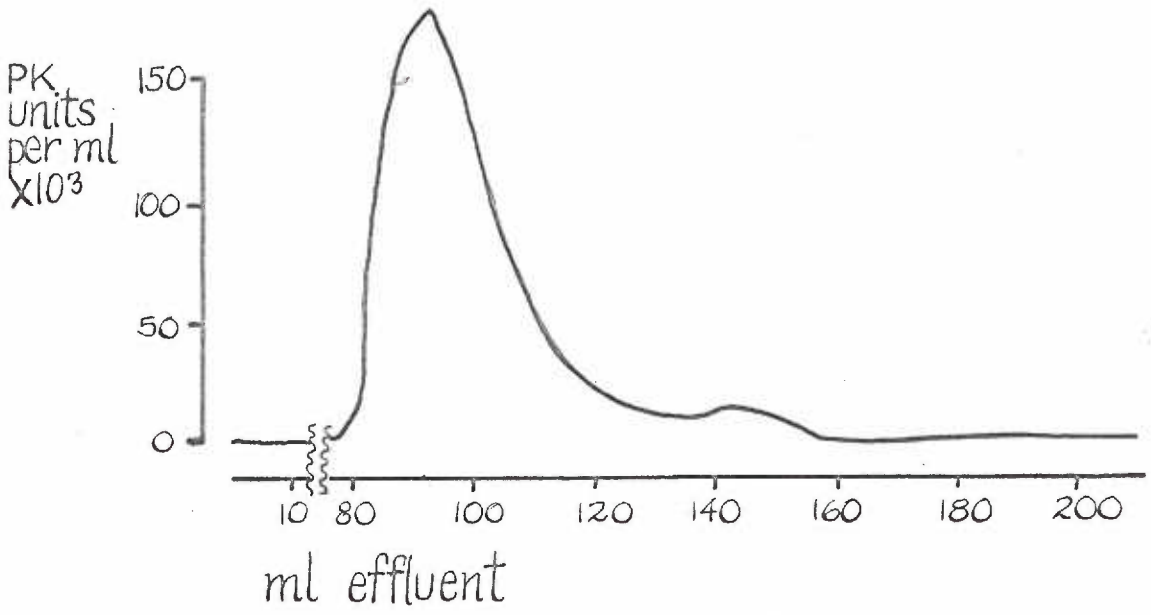
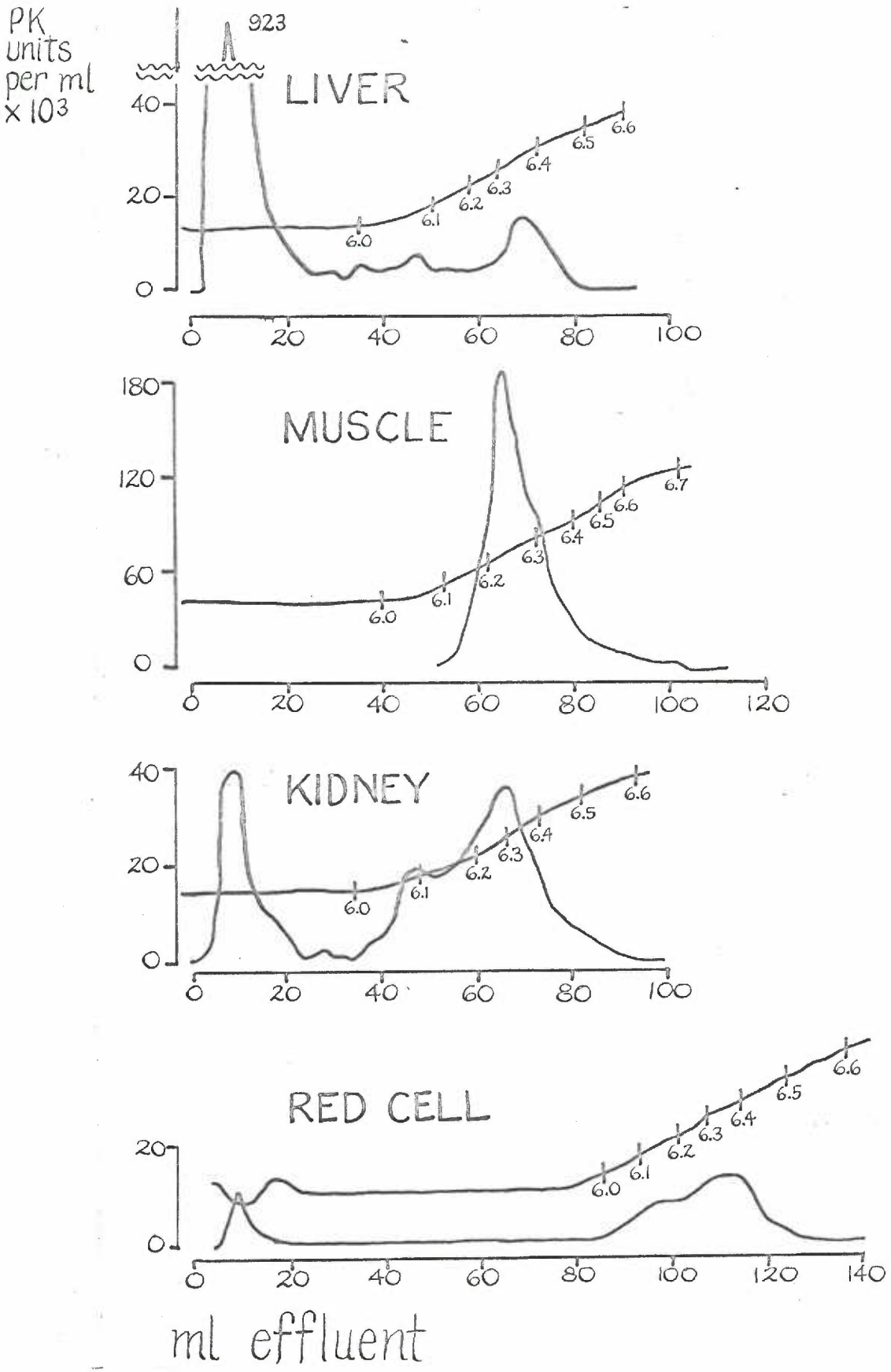

PK units per ml X10³
150
100
50
0
10
80
100
120
140
160
180
200
ml effluent

Figure 4. Chromatography of PK from several tissues on carboxymethyl Sephadex. The line marked with vertical bars indicates the effluent pH.


PK units per ml x 10³
923
LIVER
40
20
0
6.0
6.1
6.2
6.3
6.4
6.5
6.6
0
20
40
60
80
100
MUSCLE
180
120
60
0
6.0
6.1
6.2
6.3
6.4
6.5
6.6
6.7
0
20
40
60
80
100
120
KIDNEY
40
20
0
6.0
6.1
6.2
6.3
6.4
6.5
6.6
0
20
40
60
80
100
RED CELL
20
0
6.0
6.1
6.2
6.3
6.4
6.5
6.6
0
20
40
60
80
100
120
140
ml effluent

dures to be employed for the purification of PK from different tissues.

SUMMARY AND CONCLUSIONS

Sulfhydryl compounds protect human erythrocyte pyruvate kinase against loss of activity during dialysis against phosphate buffers at low ionic strength. It is inferred that the enzyme contains cysteine residues which must be kept in the reduced state for maintenance of enzyme activity.

A method of purifying erythrocyte pyruvate kinase is described which results in a preparation with a specific activity approximately equal to that reported previously and a thirty-fold increase in yield. The nature of the procedure is such that it is anticipated that it can be applied to the purification of pyruvate kinase from other tissues.

A chromatographic system has been established which resolves at least two zones of pyruvate kinase activity from liver and kidney homogenates. The chromatographic patterns of pyruvate kinase from liver, kidney, skeletal muscle, and erythrocytes are compared.

APPENDIX

CALCULATIONS FOR MAKING AMMONIUM SULFATE SOLUTIONS

Traditionally, the concentrations of ammonium sulfate solutions used during salting out procedures are expressed in "per cent saturation;" for example, equal volumes of protein solution and a solution saturated with ammonium sulfate are mixed and the resulting solution taken to be "50 per cent saturated."

Green and Hughes (17) have discussed the difficulties encountered in this method. The concentration of a saturated solution varies with the temperature; at 0°, a saturated ammonium sulfate solution is 3.9 M; at 25°, it is 4.1 M. Furthermore, such a system does not result in a linear relationship between "per cent saturation" and salt concentration as expressed in molarity. Second order volume changes invalidate the assumption inherent in that system, that the addition of one gram of salt increases the volume by the same amount, regardless of the concentration of the solution.

Green and Hughes (17) have given a table which presents grams of ammonium sulfate necessary to take one liter of solution from one "per cent saturation" to another, meaning by "per cent saturation," per cent of 4.1 M. From these values the fraction of the solutions by weight which is water has been calculated and is given in the

third column of table 8. Similar calculations have been made for solutions made with ammonium sulfate and dilute aqueous solutions, substituting the weight of solution containing 1000 g of water for 1000 g. For example, the value for 40 per cent saturation and 0.1 molal dibasic potassium phosphate (FW = 174) was obtained by:

$$B_{40\%} = \frac{1000 + 17.4}{1000 + 17.4 + 243}$$

The calculated values, termed here "B values," are also given in table 8.

If w_1 and w_2 grams are taken of solutions having B values of B_1 and B_2 respectively, upon combining them $(w_1 + w_2)$ grams of a solution having a B value of B_3 is obtained, in accordance with the equation:

$$B_3 = \frac{B_1 w_1 + B_2 w_2}{w_1 + w_2}$$

which, upon rearranging,

$$w_1 + w_2 = \frac{B_1 w_1}{B_3} + \frac{B_2 w_2}{B_3}$$

dividing by w_1,

$$1 + \frac{w_2}{w_1} = \frac{B_1}{B_3} + \frac{B_2}{B_3} \frac{w_2}{w_1}$$

rearranging and collecting terms,

$$\left(1 - \frac{B_2}{B_3}\right) \frac{w_2}{w_1} = \frac{B_1}{B_3} - 1$$

multiplying by B_3,

$$\left(B_3 - B_2\right)\frac{w_2}{w_1} = B_1 - B_3$$

and rearranging gives

$$\frac{w_2}{w_1} = \frac{B_1 - B_3}{B_3 - B_2}$$

The use of the derived equation is illustrated in the following example:

> To make a 40 per cent saturated solution from a 10 per cent saturated solution and a 90 per cent saturated solution, the proportions are calculated as follows:

$$\frac{w_{90\%}}{w_{10\%}} = \frac{B_{10\%} - B_{40\%}}{B_{40\%} - B_{90\%}} = \frac{.947 - .805}{.805 - .602}$$

$$= \frac{.142}{.203} = .700$$

B values for a solution with no ammonium sulfate and for solid ammonium sulfate are 1 and 0, respectively. B values for a "per cent saturation" not given in the table can be obtained by interpolation.

TABLE 8. B VALUES FOR DIFFERENT SOLUTIONS

Per cent saturation	Ammonium sulfate to be added to one kilogram	B values for different solutions*			
		1	2	3	4
10	56	.947	.948	.948	.947
20	114	.898	.899	.900	.899
25	144	.874	.876	.877	.875
30	176	.850	.853	.853	.852
35	209	.827	.830	.831	.829
40	243	.805	.807	.808	.806
45	277	.783	.786	.787	.785
50	313	.762	.765	.766	.763
55	351	.740	.744	.745	.742
60	390	.719	.723	.724	.721
65	430	.699	.703	.705	.701
70	472	.679	.683	.685	.681
75	516	.660	.664	.665	.662
80	561	.641	.645	.646	.643
90	662	.602	.606	.608	.604
100	767	.566	.570	.572	.568

*Ammonium sulfate is added to these solutions, corresponding to the last 4 columns: column 1, water; column 2, 0.1 molal dibasic potassium phosphate or 0.1 molal each in potassium acetate and mercaptoethanol; column 3, 0.1 molal each in dibasic potassium phosphate and mercaptoethanol; column 4, 0.1 molal potassium acetate.

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