Mouse plasma could be heated up to 62° without any loss in activity of the pH 7.4 enzyme. At 65° the pH 7.4 enzyme was completely inactivated. The pH 4.0 enzyme retained 45% of its activity when the plasma was maintained at 95° for 7 min.

Mouse plasma was chromatographed on Bio-Gel P-200 (Fig. 3) in the presence of 0.13 M NaCl. The material in the major peak at pH 7.4 (containing 54% of the activity) has an apparent molecular weight of 230 000. There is a small peak which elutes before the major peak (7% of the total activity). There is another slight peak (9% of the total activity) with a molecular weight corresponding to 190 000 and a fourth peak (14% of the total activity) with an apparent molecular weight of 52 000. The remaining 16% of the pH 7.4 activity was eluted between the third and fourth peaks. The 1.0 ml of plasma that was layered onto the column contained 91 units at pH 7.4. However, a quantity of activity amounting to 350 units was recovered from the column indicating that there was almost 4 times as much activity recovered as was applied to the column. It is possible that the high molecular weight of the ribonuclease may be due to interaction with other plasma proteins.

When the column fractions were assayed at pH 4.0 no well defined peak was observed. The activity was distributed among fractions eluting between 24 and 51 ml. It is unlikely that this was due to an inactivation of the enzyme since 74% of the activity layered onto the column was recovered in these fractions.

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Purification of rabbit muscle pyruvate kinase by CM-Sephadex and evidence for an endogenous inhibitor

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rabbit muscle is most often prepared by the method of TIETZ AND OCHOA[1] or by slight modifications of this procedure. REYNARD *et al.*[2] reported that this preparation has traces of adenylate kinase as well as ATPase activities. Further attempts at purification by these authors were unsuccessful. Recently COTTAM *et al.*[3] stated that their sample

isolated and crystallized according to TIETZ AND OCHOA[1] was homogeneous as judged by sedimentation in the ultracentrifuge and polyacrylamide gel electrophoresis. Comparison of the reports of REYNARD *et al.*[2] and COTTAM *et al.*[3] suggests that the method of TIETZ AND OCHOA[1] can yield an homogeneous enzyme preparation but only with a degree of uncertainty.

Enzyme prepared in this laboratory by the method of TIETZ AND OCHOA[1] gave a single symmetrical peak during ultracentrifugation and forms large (up to 5 mm long), soft and uniformly shaped crystals. This material, however, gave 4 bands on disc gel electrophoresis (Fig. 1A). The commercial preparation (see Table I) also exhibited the same heterogeniety. The contamination was removed by chromatography on CM-Sephadex. Disc electrophoretic patterns of the enzyme before chromatography and Fractions 5, 48 and 56 of the elution (Fig. 2) are shown in Fig. 1.

The applied sample gave rise to three minor and one major band (Fig. 1A). The protein from Fraction 5 (Fig. 1B), which accounts for 10% of the total protein, was completely devoid of any pyruvate kinase activity and contained the three protein peaks present as minor contaminants in the applied sample. The data from Fractions 48 (Fig. 1C) and 56 (Fig. 1D) show that both sides of the main protein peak contain only the material that is present in the major band of the applied sample. Protein recovery as judged by absorption at 280 nm using 0.54 $cm^{-1} \cdot M^{-1}$ as the absorptivity coefficient[8]

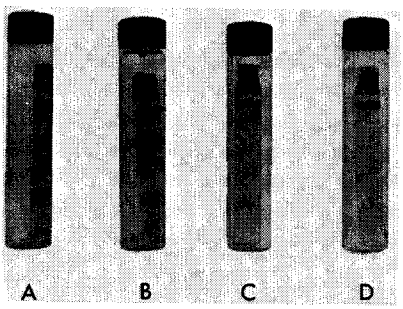

Fig. 1. Disc gel electrophoretic patterns of the protein applied and eluted from CM-Sephadex. The protein samples were dialyzed against 2 changes of the electrode buffer and applied to the top of the spacer gel in a solution to which a few crystals of sucrose were added. The electrophoresis was carried out in 7.0% polyacrylamide gel[6] according to the modification of WILLIAMS AND REISFELD[7] (anionic system). The pH of the running gel was 9.0. A current of 1.8 mA per gel was applied for 4 h at 1°. After electrophoresis the gels were stained with aniline blue black. Gel A is the pattern of crystalline pyruvate kinase applied to the CM-Sephadex column (about 100 μg of protein); Gel B is the pattern from Fraction 5 of the CM-Sephadex column (Fig. 1) (about 75 μg); Gels C and D are the patterns from Fractions 48 (about 60 μg) and 56 (about 75 μg), respectively.

was 95%, and the recovery of catalytic activity determined by the direct spectrophotometric assay[6] was 120%. The specific activities recorded are low because less than saturating amounts of substrates were used for the routine assays (*cf.* Table I for data under saturating conditions).

For preparative chromatography of pyruvate kinase, a CM-Sephadex column (2.5 cm × 40 cm) was prepared as described in Fig. 2 but equilibrated with 5 mM potassium phosphate buffer (pH 6.0). The packed column was flushed overnight with 5 mM potassium phosphate buffer (pH 6.0) and about 500 mg (in 10 ml) of dialyzed protein was applied to the top and washed with 250 ml of buffer. This represents about

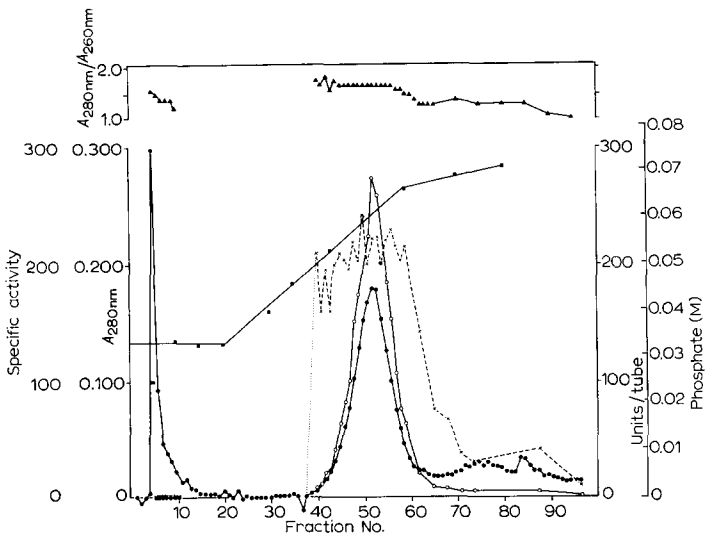

Fig. 2. Elution profile of crystalline pyruvate kinase from a CM-Sephadex (C-50) column (0.9 cm × 55 cm). The CM-Sephadex was converted to the K^+ form by the method suggested by Pharmacia[4] and equilibrated with 0.03 M potassium phosphate buffer (pH 6.0). After flushing the column with this buffer overnight, pyruvate kinase (20 mg) which had been dialyzed against 5 changes of 100-fold excess buffer at 0° was applied to the top. A linear gradient from 0.03 to 0.09 M potassium phosphate buffer (pH 6.0) was begun after five 1-ml fractions had been collected. The flow rate was 6–8 ml·h^{-1} by gravity flow. Fractions were assayed for protein (absorbance at 280 nm) (●—●), pyruvate kinase activity (○—○) and phosphate (determined by the method of Lowry and Lopez as described by LINDBERG AND ERNSTER[5]) (■—■). Also recorded are the $A_{280\ nm}/A_{260\ nm}$ ratios (▲—▲) and specific activities (×— — —×).

100 ml collected after the initial peak of inactive protein was eluted. The enzyme was eluted with 0.1 M potassium phosphate buffer (pH 6.0) at a flow rate of 44 ml·h^{-1} in 10 tubes of 10 ml each. The specific activity of the major peaks in the analytical, linear gradient column and the preparative two-step gradient column were identical. With 5 different columns, the increase in specific activity ranged from 1.5 to 1.9.

The specific activity of this preparation is compared to other preparations in Table I. NOLL *et al.*[10] described a preparation that was essentially free of adenylate kinase activity yet their activity appears low. This may not be a reflection of the purity, since the enzyme is less active at pH 7.6 than at pH 7.0 (ref. 14) and the effect of the buffer has not been examined. The only truly valid comparison among the different preparations is between Samples 4 and 5 (Table I), since in both cases the temperature, buffer, pH and protein determinations were the same and both samples were homogeneous on disc gel electrophoresis. Sample 4 has the highest specific activity of any preparation previously reported.

The data obtained from the CM-Sephadex column, *i.e.* recovery of more than 100% of the catalytic activity and 1.9-fold increase in specific activity (Table I) with the recovery of 85% of the enzyme protein, are consistent with the removal of some inhibitor. Additional evidence for the presence of an endogenous inhibitor was obtained during the preparation of enzyme from fresh tissue. The water extract of muscle from two rabbits of four examined had no measurable pyruvate kinase activity. This lack

persisted through the $(NH_4)_2SO_4$ and ethanol fractionation steps; dissolution and dialysis were performed between these steps. Only after the heat step did activity appear. When 31 μg of active, purified enzyme were added to an assay mixture containing 173 μg of protein from the water extract, no activity was demonstrable. In the inverse experiment, an aliquot of water extract, added to an actively reacting assay, completely and instantaneously inhibited it. The other two fractions that showed no activity also possessed this inhibitor.

TABLE I

COMPARISON OF SPECIFIC ACTIVITIES OF DIFFERENT PREPARATIONS OF PYRUVATE KINASE

Samples 5–8 were assayed in this laboratory by the direct spectrophotometric method[9] with 7.2 mM $MgSO_4$, 72 mM KCl, 4.0 mM potassium phosphoenolpyruvate and 1.0 mM ADP (sodium salt). The other values were obtained from the literature, and the substrates are assumed to be saturating.

Sample No.	*Preparation*	*Buffer*	*pH*	*Temp.*	*Specific activity (μmoles · min^{-1} · mg^{-1})*
1	Ref. 10	Triethanolamine	7.6	25°	158*
2	Ref. 1	Imidazole–HCl	7.0	approx. 25°	268**
3	Ref. 11	Imidazole–HCl	7.0	approx. 25°	280**
4	Ref. 3	Imidazole–HCl	7.0	30°	300
5	From CM-Sephadex column	Imidazole–HCl	7.0	30°	464
6	From CM-Sephadex column	Tris–HCl	7.5	30°	418
7	Calbiochem***	Tris–HCl	7.5	30°	298
8	Crystalline enzyme before CM-Sephadex chromatography	Tris–HCl	7.5	30°	225

* The value reported was 9000 units · mg^{-1} as defined by BÜCHER AND PFLEIDERER[8]. One such unit is equivalent to 17.6 nmoles · min^{-1} (ref. 12).

** The specific activities reported by TIETZ AND OCHOA[1], and KUPIECKI AND COON[11] based on protein determined by the method of WARBURG AND CHRISTIAN[13] have been corrected by REYNARD *et al.*[2].

*** This preparation was purchased from Calbiochem and was labeled 308 Enzyme Commission units · mg^{-1} at 30°.

The relationship between the inhibitors evidenced by the two procedures is not known. If they are the same, then the binding of the inhibitor to pyruvate kinase, suggested by the CM-Sephadex column, would render the inhibitor heat stable. Thus its presence after the heat step is explained. The variable specific activity of commercially available pyruvate kinase could be attributed to variable amounts of inhibitor.

The column has the advantage of binding pyruvate kinase more specifically thus allowing it to be washed free of contaminating material. In addition it removes what appears to be an heretofore unknown inhibitor.

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Hydroxylation of proline and lysine in protocollagen involves two separate enzymatic sites

The hydroxyproline and hydroxylysine in collagen are synthesized by the hydroxylation of proline[1] and lysine[1,2] after these amino acids have been incorporated into a polypeptide precursor of collagen called protocollagen[1]. Most of the enzymatic activity for the hydroxylation of both the proline and lysine in protocollagen is found in the 150 000 × *g* supernatant fraction of chick embryo homogenates[3], and the cofactor or cosubstrate requirements for both hydroxylations are O_2, Fe^{2+}, α-ketoglutarate, and probably L-ascorbate[1–6]. It therefore seemed possible that the same enzyme is involved in both hydroxylations[3,5]. Results to be reported in this paper indicate that the two hydroxylations require separate enzymatic sites and probably separate proteins.

[^{14}C]Proline-labeled and [^{14}C]lysine-labeled protocollagen were prepared from chick embryo cartilage incubated with α,α′-dipyridyl and L-[$^{14}C_5$]proline (New England Nuclear Corp.), 209 μC/μmole, or L-[$^{14}C_6$]lysine (New England Nuclear Corp.), 248 μC/μmole[7]. The enzymatic reactions for the synthesis of [^{14}C]hydroxyproline and [^{14}C]hydroxylysine were carried out for 1 h at 37° in a final volume of 4 ml which