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# TECHNIQUES FOR DETECTING ENZYMES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

TIMOTHY D. SCHLABACH and FRED. E. REGNIER**
*Department of Biochemistry, Purdue University, Lafayette, Ind. 74907 (U.S.A.)*

## SUMMARY

Techniques are described for the automated detection of a series of enzymes in a high-performance liquid chromatographic system. Detection was achieved by either a direct or a coupled enzyme assay using photometric detectors. In direct detection the immediate enzymatic product was monitored. Coupled enzyme assays required additional enzyme(s) to convert the product of the primary enzyme reaction into a more easily detectable form. The efficiency of both free and immobilized coupling enzyme(s) was evaluated. The detector sensitivity could be increased threefold by increasing the reaction temperature. This system is particularly suitable for isoenzyme profiling in biological materials.

## INTRODUCTION

The detection of chromatographically separated enzymes in the presence of large amounts of protein can be accomplished only by enzymatic assay. Classically, this has been accomplished by fraction collection and the subsequent enzymatic analysis of each fraction. Fraction collection, however, is not attractive for separations by high-performance liquid chromatography (HPLC). The separation of lactate dehydrogenase (LD) isoenzymes, for example, by HPLC can result in five peaks emerging in 10 min or less. Fraction collection by conventional instrumentation would probably entail a decrease in resolution, and enzymatic analysis of all of the fractions would require much more time than the actual separation itself. The high-speed HPLC analysis of isoenzymes in biological samples requires a continuous, on-line detection system that generates the entire isoenzyme profile shortly after the last isoenzyme peak emerges from the column.

On-line detection systems have been coupled to columns for the colorimetric or fluorescence detection of analytes in the column effluent. The first was introduced about 20 years ago for automated amino acid analysis[1]. More recently, post-separation chemical reaction detection systems have been incorporated in HPLC[2,3]. En-

* Journal Paper No. 7211 from the Purdue Agricultural Experimental Station.
** To whom correspondence should be addressed.

zymatic post-column reactions have been used for the continuous detection of LD isoenzymes[4,5] and creatine kinase (CK) isoenzymes[6] separated by HPLC.

The post-column detection of CK isoenzymes requires the introduction of coupling enzymes in the post-column system. The addition of soluble coupling enzymes to the post-column reagents creates extra problems and costs. Bulk amounts of valuable coupling enzymes are consumed that cannot be recycled because the assay solution is diluted with column buffers and contains sample enzyme activity that continuously changes the composition of the assay solution.

A simple approach to re-using coupling enzymes in the post-column detection system is to immobilize them in a packed column. Immobilized enzyme columns have been used in the detection of small substrates[7], but only recently have they been used in the measurement or determination of soluble enzyme activity[8]. Special attention must be given to the kinetic restraints of coupled enzyme systems. For example, the reaction product of a soluble enzyme must first accumulate to a level where it can become a substrate for the immobilized coupling enzyme. This occasionally leads to a time lag or initiation period in the detector. Sufficient coupling enzyme must also be present to convert the product of the soluble enzyme so that the limiting rate is due to the activity of the soluble enzyme.

An automated detection system must also be very flexible because it is necessary to perform a variety of different assays in order to detect various enzymes. With a detection system for a variety of enzymes, it should be possible to use the same HPLC column (anion exchange) and the same equipment to detect a series of different isoenzymes after resolution. If immobilized enzyme columns could be used in the automated detection system, the cost of detection would be reduced and the flexibility of the system increased.

## MATERIALS AND METHODS

### *Apparatus*

The Constametric I and IIG system with the Gradient Master and Dynamic Mixer (Laboratory Control Data, Riviera Beach, Fla., U.S.A.) was used for the gradient elution of isoenzymes. The ISCO Model 384 pumping system (Instrument Specialties, Lincoln, Nebr., U.S.A.) and the Milton Roy MiniPump (Riviera Beach, Fla., U.S.A.) were used as post-column reagent pumps. The Rheodyne Model 7120 valve with a 100-$\mu$l loop was purchased from Anspec (Ann Arbor, Mich., U.S.A.) and used for all sample injections. The Perkin-Elmer (Norwalk, Conn., U.S.A.) LC 55 variable-wavelength spectrophotometer was used for the detection of nitrophenol at 400 nm, and the Aminco (American Instrument, Silver Springs, Md., U.S.A.) FluoroMonitor was used for detecting NAD(P)H. Two attenuation scales were used, M10 and M100, and full-scale deflections occurred when 14 and 140 $\mu M$ NADH solutions, respectively, were passed through the flow cell in the fluorimeter.

### *Analytical columns*

Anion-exchange materials were prepared from controlled-porosity glass, particle size 5–10 $\mu$m, with 250-Å pores (Corning Glass Works, Corning, N.Y., U.S.A.), according to previously described procedures[9]. The anion-exchange material used for the bulk of this work had a hemoglobin ion-exchange capacity of about 80 mg per

cubic centimeter support, and was packed by the slurry method into a 25-cm column. The column used for the separation of CK isoenzymes was 30 cm in length and was packed with an anion-exchange material with a hemoglobin ion-exchange capacity of 47 mg per cubic centimeter of support.

*Post-column detection system*

A 40 × 0.5 cm column was packed with non-porous glass beads (Cataphote, Jackson, Miss., U.S.A., Catalog No. 2740) and used for the post-column detection of enzyme activity. The fluid entering the packed column was heated to 40° with a pre-column heater, and the column was heated with heating tape to maintain that temperature, unless otherwise indicated.

*Reagents*

Glass beads, Catalog No. 2740, were purchased from Cataphote. N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) was purchased from Aldrich (Milwaukee, Wisc., U.S.A.), and dimethyl adipimidate (DMA) and NIN-Sol (ninhydrin reagent) were purchased from Pierce (Rockford, Ill., U.S.A.). Alkaline phosphatase (AP) from beef liver, Type IX, AP from calf-intestine, Type VII, purified lactate dehydrogenase (LD), Type V, glucose-6-phosphate dehydrogenase (G-6-PDH), Type XXI, hexokinase (HK), Type C-300, HK/G-6-PDH combination (Catalog No. H8629), CK enzyme reference, glucose-6-phosphate, adenine triphosphate (ATP) and other reagents required for enzyme assays were purchased from Sigma (St. Louis, Mo., U.S.A.). LD and CK control materials, prepared from rat tissues, were purchased from Helena Labs. (Beaumont, Texas, U.S.A.). The LD heart control (lot No. 365919) and the CK control (lot No. 865131) were also purchased from Helena Labs.

*Alkylamine glass beads*

The glass beads from Cataphote were refluxed overnight in a 5% solution of aminopropyltriethoxysilane (Ohio Valley, Marietta, Ohio, U.S.A.) in toluene with paddle stirring. The product was washed with hot water and acetone.

*Assay of amino groups in alkylamine derivatives*

The determination of the primary amine groups in the aminopropyl derivative of glass beads was accomplished by the ninhydrin procedure as described by Greenstein and Winitz[10], except that 1 g of derivitized glass beads was used instead of 1 ml of sample. The amount of primary amine was determined from a standard graph prepared with various concentrations of $\beta$-alanine.

*Assay reagents used with the immobilized G-6-PDH column*

The assay reagents used in measuring the response of the G-6-PDH column to glucose-6-phosphate (G-6-P) were prepared in 0.05 *N* Tris–HCl (pH 8.0) containing 0.005 *M* magnesium chloride. The solution was made 1 m*M* in dithiothreitol (DTT) and 1 mg/ml of nicotinamide adenine dinucleotide (NAD) was added. The assay reagent used for the column assay of yeast hexokinase was prepared in 0.10 *M* Tris–HCl (pH 8.0) and contained 0.005 *M* magnesium chloride, 0.04 *M* D-glucose, 1 m*M* DTT, 1.5 mg/ml ATP and 0.75 mg/ml NAD.

*Assay reagents used with the immobilized HK/G-6-PDH column*

The assay reagent used for the column assay of ATP was prepared in 0.05 *M* Tris–HCl (pH 7.8) and contained 0.005 *M* magnesium chloride, 0.055 *M* D-glucose, 1 m*M* DTT and 1 mg/ml NAD. The assay reagent for the column assay of creatine kinase was prepared in 0.20 *M* imidazole (pH 6.9) and contained 0.025 *M* magnesium chloride, 0.04 *M* D-glucose, 1 m*M* DTT, 1 mg/ml ADP, 0.5 mg/ml NAD and 2 mg/ml creatine phosphate.

*Assay of HK activity*

The assay reagent for determining the stock HK activity was the same as that used for the detection of hexokinase by the G-6-PDH column but included about 0.5 unit/ml of G-6-PDH activity. A 100-$\mu$l aliquot of the stock yeast HK solution was combined with 2.0 ml of the substrate buffer, mixed and incubated for about 20 sec. The time required for an absorbance increase of 0.20 unit at 340 nm was subsequently recorded. The HK activity was calculated from the mean of three determinations.

*Assay of CK activity*

The stock CK activity was assayed by the Rosalki method[11] using the Rosalki reagents supplied by Smith Cline Diagnostics in tablet form. One tablet was combined with 2.5 ml of distilled water and thoroughly mixed. A 200-$\mu$l aliquot of the stock CK solution, prepared by diluting the CK material with a specified amount of distilled water, was combined with 2.5 ml of the assay solution, mixed and placed in the spectrophotometer. After an initial absorbance change of 0.10 units at 340 nm, the time required for an additional increase of 0.10 unit was measured. The stock CK activity was calculated from the mean of four determinations.

*Immobilization of G-6-PDH*

A 20.5 × 0.5 cm column was dry packed with glass beads (Cataphote Catalog No. 2740) that had been derivitized with 3-aminopropyltriethoxysilane (ATS) by refluxing overnight in a 5% solution of ATS in toluene with paddle stirring. The aminopropyl derivative was found to contain 1.26 $\mu$mole of primary amine per gram by the ninhydrin assay. The column was estimated to contain about 6 g of derivitized glass beads. The packing was activated by recirculating a solution of 2 mg/ml of DMA in 0.05 *M* sodium borate (pH 8.2) through the column at a flow-rate of about 1 ml/min. After 30 min, 20 ml of a solution containing 160 units of G-6-PDH and 10 mg of NAD was recirculated through the column for 3 h at the same flow-rate. This solution also contained 1 m*M* DTT and 0.005 *M* magnesium chloride in 0.05 *M* Tris–HCl, pH 8.0. After recirculation of this solution through the column, it was washed with 50 ml of buffer to remove unbound enzyme and then assayed with G-6-P for G-6-PDH activity.

*Immobilization of HK and G-6-PDH in one column*

A 30 × 0.5 cm column was dry packed with glass beads that were derivitized with aminopropyltriethoxysilane as previously described. This lot was found to have a primary amine content of 2.24 $\mu$mole per gram of beads. It was estimated that about 8 g of beads were packed into the column. About 100 units of both HK and

G-6-PDH were added to 20 ml of 0.05 *M* Tris–HCl (pH 8.0) that contained 0.005 *M* magnesium chloride and 1 m*M* DTT. A solution of 1 mg/ml of EEDQ in distilled water was prepared by heating and stirring to solubilize the compound. After this solution had cooled, 5 ml were added to the above 20 ml of solution, mixed and recirculated through the 30-cm packed column for 2 h at a flow-rate of about 1 ml/min. The column was then washed with 50 ml of buffer to remove unbound enzymes and subsequently assayed for activity in the conversion of ATP into G-6-P and subsequent formation of NADH.

*Post-column reagents*

The assay solution for the detection of AP activity was prepared in 0.10 *M* Tris–HCl with 0.005 *M* magnesium acetate (pH 9.0) and contained 0.75 mg/ml of *p*-nitrophenyl phosphate (NPP), purchased from Aldrich. The assay solution for the post-column detection of LD activity was prepared in 0.20 *M* Tris–HCl (pH 8.8) and contained 0.75 *M* L-lactate and 1 m*M* NAD. The post-column reagent for the detection of CK activity was prepared in 0.10 *M* bis-Tris with 0.01 *M* magnesium acetate (pH 6.9) which contained 0.04 *M* D-glucose and 0.001 *M* DTT. To this buffer were added 2.0 mg/ml of creatine phosphate, 0.75 mg/ml of ADP, 0.33 mg/ml of NADP, 2 units/ml of hexokinase (HK) and 1 unit/ml of glucose-6-phosphate dehydrogenase (G-6-PDH). These coupling enzymes were specially prepared by Sigma for the enzymatic detection of ATP and were sold together. The reagent solution was placed in ice while it was kept in the reservoir for the Minipump, which introduced the reagent into the post-column detection system. The assay solution for the detection of HK activity was prepared in 0.10 *M* Tris–HCl, pH 8.0, containing 0.04 *M* of D-glucose, 0.01 *M* of magnesium acetate and 0.001 *M* of DTT. To this solution were added 0.75 mg/ml of NAD, 1.5 mg/ml of ATP and about 0.8 unit/ml of G-6-PDH (Type XXI). This solution was introduced into the post-column detection system with the ISCO pump.

*Rat liver and rat testis extracts*

Liver and testis extracts from 10-day-old and 10-week-old rats were prepared by J. Zysk by homogenization of the tissues in isotonic potassium chloride followed by dialysis overnight according to the method of DiPietro and Weinhouse[12].

*Serum samples*

A serum sample was obtained from an anonymous donor at St. Elizabeth Hospital (Lafayette, Ind., U.S.A.) with the cooperation of Dr. W. Jacobsen, Chief Pathologist, and J. Delkhoon. The serum sample was de-salted by gel filtration on a small G-25 column made from a Pasteur pipette. A 250-$\mu$l aliquot of serum was eluted with Tris buffer and about 0.5 ml of the band containing enzyme activity was collected.

*Column buffers*

The weak buffer used in column elution was 0.02 *M* Tris–HCl (pH 7.9), unless otherwise specified, and the strong buffers were prepared by adding sodium chloride to this buffer and re-adjusting the pH to 7.9 with hydrochloric acid.

## RESULTS AND DISCUSSION

### *System design*

The coupling of an HPLC column with an automated, post-column detection system is shown in Fig. 1. The column effluent was monitored for enzyme activity by the continuous flow analyzer (Fig. 1). The analyzer consisted of a reagent pump, a reaction vessel where the enzyme reaction occurs and a detector to monitor the enzymatic product. Different enzymes can be detected by simply changing the assay solution in the reagent pump. The assay solution is continuously added to the column effluent, and the combined solutions are passed through a packed column where the enzyme reacts with its substrate(s). A column packed with spherical, non-porous glass beads was used as the reaction vessel, because it was previously shown that a packed column produced less band spreading than a coil of capillary tubing[5].

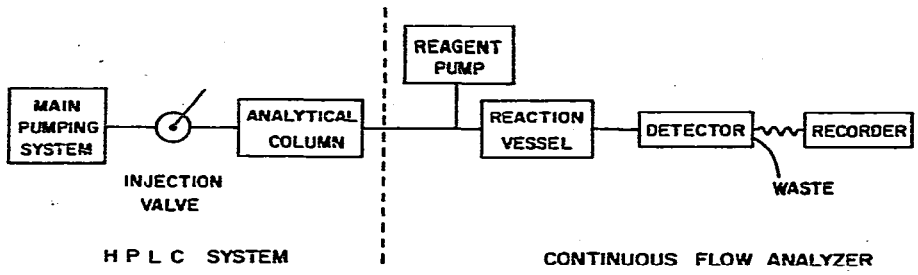


Fig. 1. Diagram of the system used for high-speed isoenzyme profiling.

### *Post-column heating*

A novel device was designed to heat the column effluent rapidly and assay solution just after they had been combined and just before they entered the packed column. This device was constructed by Micromeritics (Norcross, Ga., U.S.A.) and consists of a 16-cm length of 0.01 in. I.D. tubing brazed to a brass core containing a Nichrome heating element (Fig. 2). The cartridge heater was used in conjunction with heating tape to maintain elevated temperatures in the post-column detection

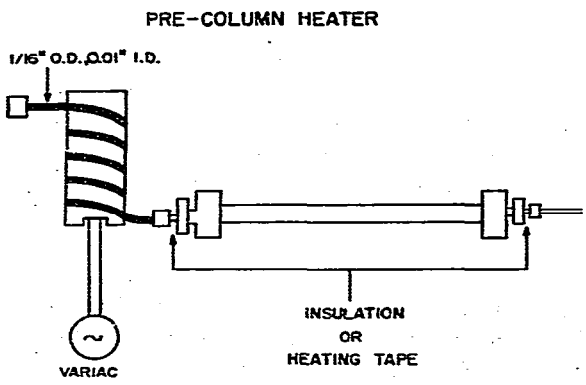


Fig. 2. Diagram of the heating system used in post-column detection of enzyme activity.

system. As a packed column is used as the reaction vessel, the cartridge heater can be referred to as a pre-column heater, even though it is used in the post-column detection system.

The heat-transfer capabilities of this device were tested by pumping dilute Tris buffer through the tubing, heating with a Variac voltage source and measuring the temperature of the emerging fluid with a thermometer. The plots in Fig. 3 show that the device can heat a fluid, flowing at rates commonly encountered in isoenzyme profiling, by up to 60° or more in less than 300 msec.

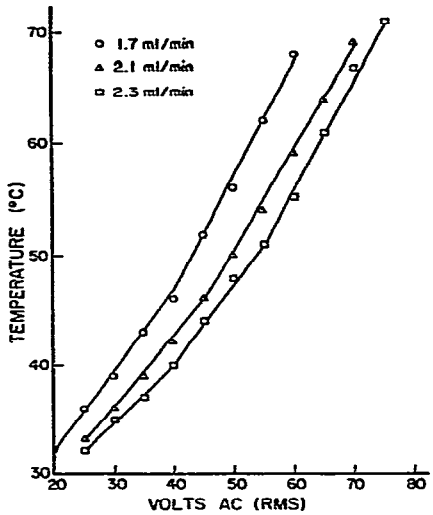


Fig. 3. Heating capacity of the pre-column (cartridge) heater.

The effect of post-column heating on the sensitivity of detection is often pronounced. It has been reported that LD activity increased 2.4-fold when the assay temperature is increased from 25 to 37°[13]. LD isoenzymes in a control material (Helena, lot No. 365919) prepared from rat heart tissue were separated on the analytical column and detected at post-column temperatures of 25° (ambient) and 45°. All of the LD isoenzyme peak heights, except $LD_5$, were increased about 3-fold at the higher temperature (Fig. 4). LD isoenzyme peaks which were difficult to identify at ambient temperature became much more pronounced at the higher temperature, because the fluctuations in the base-line fluorescence were not noticably affected by the change in temperature, whereas the enzyme activity was increased. Peaks that are increased by a moderate temperature elevation can be identified as activity peaks, while those which do not change are usually extraneous background peaks.

*Detection of AP isoenzymes*

The post-column detection of AP activity can be readily accomplished with the synthetic substrate *p*-nitrophenyl phosphate (NPP), according to the reaction

$$\underset{\text{NPP}}{H_2O + O_2N\text{–}C_6H_4OPO_3Na_2} \xrightarrow{AP} \underset{\text{NP}}{O_2N\text{–}C_6H_4\text{–}OH} + HOPO_3Na_2 \qquad (1)$$

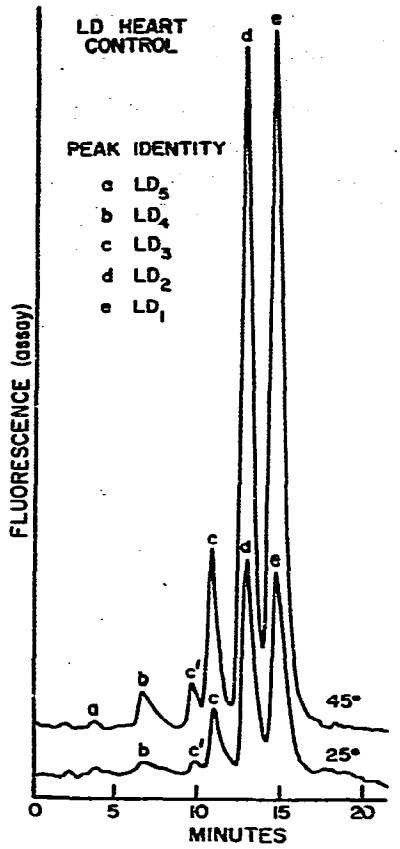


Fig. 4. Effect of post-column temperature on the LD isoenzyme profile. LD isoenzymes were eluted with a 15-min linear gradient reaching 0.15 *M* NaCl at a flow-rate of 1.5 ml/min. The LD assay solution was introduced into the column effluent at a rate of 0.8 ml/min. The post-column detection temperature was either 45 or 25° (ambient). The resulting NADH was detected by fluorescence.

The hydrolytic activity of AP liberates nitrophenol (NP), which can be readily detected in alkaline solution at 400 nm. The amount of NP produced by AP activity in this system was previously shown to have a broad linear dynamic range[5] that is limited only by substrate depletion and accumulation of product.

A solution of intestinal AP with an activity of 0.33 unit/ml and a solution of liver AP with an activity of 0.39 unit/ml were chromatographed separately under the above conditions. The intestinal and liver activities (Fig. 5) were detected at 6.3 and 8 min, respectively. The two solutions were combined in equal proportions and separated under the above conditions. The intestinal form eluted first (Fig. 6), as was confirmed by spiking the mixture with additional intestinal AP activity.

Urea was included in the elution buffers to reduce the affinity of the liver and intestinal forms for each other. In the absence of urea, the intestinal AP and liver AP eluted separately at the same positions observed in Fig. 5. The combined activities, however, tended to co-elute, and urea was found to be effective in preventing this effect.

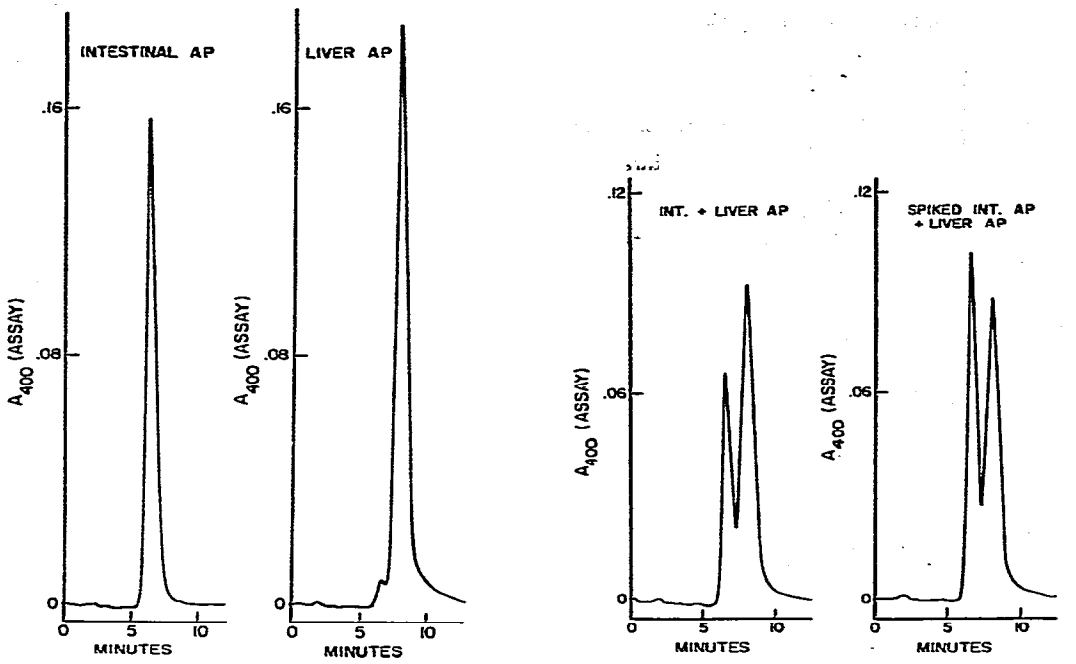


Fig. 5. Peak positions for intestinal and liver AP eluted individually. Liver and intestinal AP were chromatographed individually and eluted with a 15-min linear gradient reaching 0.15 *M* NaCl at a flow-rate of 1.5 ml/min. Both the weak and strong buffer contained 1 *M* urea. The AP assay solution was introduced into the column effluent at a rate of 2.0 ml/min. The post-column temperature was 40° and the resulting nitrophenol was detected by absorbance at 400 nm.

Fig. 6. Separation of a combined solution of liver and intestinal AP, and the separation of the same solution spiked with intestinal AP. Conditions as in Fig. 5.

*Detection of LD isoenzymes*

The post-column detection of LD activity was accomplished with a conventional assay, according to the reaction

$$\text{L-Lactate} + \text{NAD}^+ \xrightarrow{\text{LD}} \text{pyruvate} + \text{NADH} + \text{H}^+ \qquad (2)$$

The reduced cofactor, NADH, can be readily detected by either absorbance at 340 nm or by fluorescence. In this work, fluorescence was used because of its greater sensitivity.

A control material containing LD isoenzyme prepared from rat tissue (Helena, lot No. 875131) was spiked with purified $LD_5$ and separated on the analytical column. Base-line resolution of nearly all the LD isoenzyme (Fig. 7) was achieved in less than 15 min. The $LD_5$ peak was of sufficient magnitude to obscure partially the smaller $LD_4$ peak in this sample.

A serum sample from a patient with a mildly elevated serum LD activity was chromatographed under the same conditions used above except that the sensitivity of the fluorimeter was doubled. The serum was first desalted on a small G-25 column before it was injected into the column. All five isoenzymes were resolved with ex-

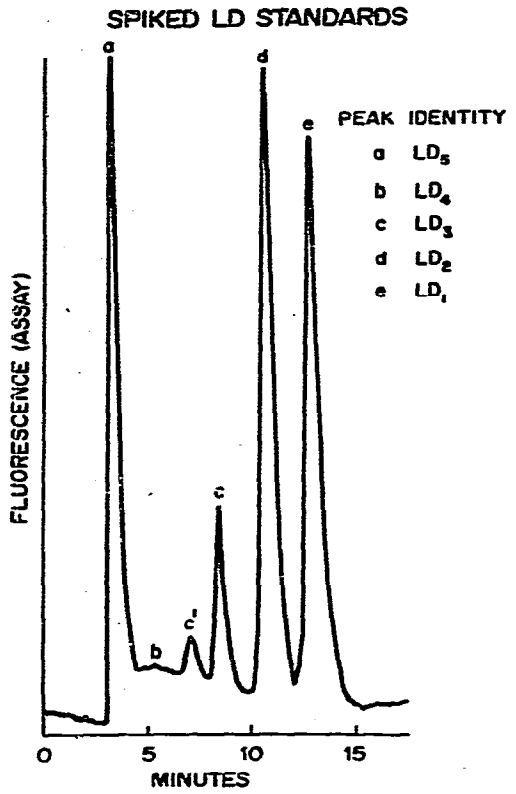


Fig. 7. Separation of LD isoenzymes in a control material that was spiked with purified $LD_5$. LD isoenzymes were separated with a 15-min linear gradient reaching 0.15 *M* NaCl at a flow-rate of 1.5 ml/min. Immediately after injection, the eluent was advanced to 20% strong buffer and the gradient was started from this point. The LD assay solution was introduced into the column effluent at a rate of 0.8 ml/min. The post-column temperature was 40° and the resulting NADH was detected by fluorescence.

cellent resolution of the clinically important $LD_2$ and $LD_1$ isoenzymes (Fig. 8). When the serum $LD_1$ activity exceeds the $LD_2$ activity, which is normally predominant, it is indicative of myocardial damage[14]. In this serum sample, the $LD_5$ activity appears elevated, which suggests possible liver or muscle damage.

*Detection of HK isoenzymes*

The post-column detection of HK activity was accomplished with a coupled enzyme reaction according to the reactions

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \quad (3)$$

and

$$\text{Glucose-6-phosphate} + \text{NAD}^+ \xrightarrow{\text{G-6-PDH}} \text{NADH} + (\text{gluconolactone-6-phosphate}) + \text{H}^+ \quad (4)$$

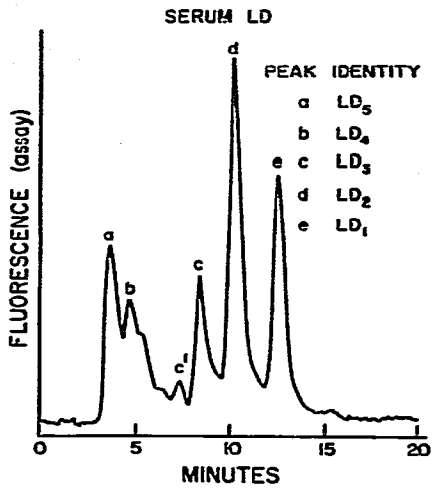


Fig. 8. Separation of serum LD isoenzymes from a patient with a mildly elevated total serum LD activity. Conditions as in Fig. 7.

The enzymatic production of the HK activity is coupled to the reduction of NAD by the coupling enzyme, glucose-6-phosphate dehydrogenase (G-6-PDH). Again, NADH was detected by fluorescence.

HK isoenzymes in crude rat tissue extracts were separated on the analytical column and detected by the reactions outlined above. All of the reactants and the coupling enzyme (G-6-PDH) were introduced with the reagent pump. The HK isoenzyme profiles observed in rat liver and testicular tissue are shown in Fig. 9. Both tissues have been reported to contain multiple HK activity[15]. The rat-liver HK isoenzyme profile resembled the profile observed in DEAE-Sephadex chromatography, where the two major peaks were reported to be HK Type I and Type IV[16].

*Detection of HK activity with an immobilized coupling enzyme*

The G-6-PDH coupling enzyme was immobilized in a 20.5-cm packed column with a bifunctional imidoester (as described under Materials and Methods), and the response of the immobilized enzyme column to its substrate (G-6-P) was tested in order to determine the linear response range at chromatographic flow-rates. At a flow-rate of 1.33 ml/min, which produced a residence time of 1.31 min, the peak-height response was found to be linear from a concentration of 0.02 to 0.50 m*M* G-6-P (Fig. 10).

The response to G-6-P was tested under the same conditions 7 months later and found to be linear over the same concentration range with no loss in enzyme activity. The column was used occasionally during this period, and between use it was stored in the refrigerator with the ends sealed to prevent moisture loss. No decrease in enzymatic activity has been observed over a 10-month period.

The response of the immobilized G-6-PDH column to varying levels of HK activity was evaluated by eluting HK (purified yeast preparation) from the analytical column with 0.40 *M* sodium chloride in Tris buffer at a flow-rate of 1.0 ml/min. The

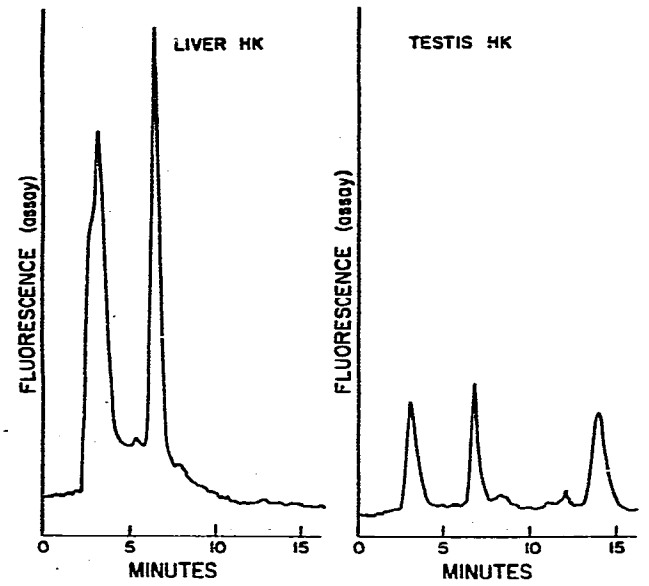


Fig. 9. Separation of HK isoenzymes in rat liver and testicular tissue extracts. The 105,000 *g* supernatant from each tissue extract was directly chromatographed with a 15-min linear gradient reaching 0.4 *M* NaCl at a flow-rate of 1.5 ml/min. The HK assay reagent was introduced at a rate of 0.8 ml/min into the column effluent. The post-column temperature was 40° and the resulting NADH was detected by fluorescence.

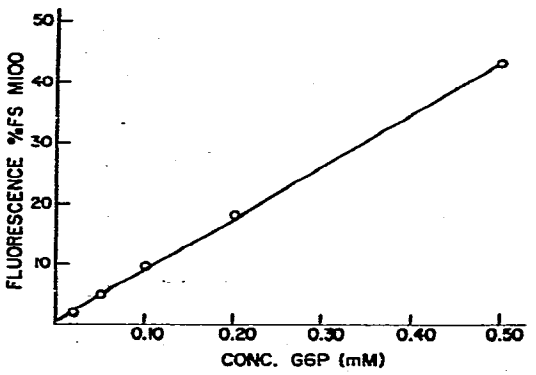


Fig. 10. Peak-height response of the immobilized G-6-PDH column to varying solutions of G-6-P. Solutions of G-6-P were injected into the system in the absence of an analytical column and passed through the immobilized enzyme column in 1.31 min at a flow-rate of 1.33 ml/min. The NADH peaks resulting from the conversion of G-6-P at ambient temperature (25°) were detected by fluorescence.

residence time of the HK activity in the immobilized enzyme column was approximately 1 min, which was found to be sufficient time to produce a linear response (Fig. 11) to HK activity over the range from 0.05 to 0.50 unit/ml.

The rapid and sensitive response of the immobilized enzyme column is largely attributable to the low Michaelis constant of the enzyme for G-6-P, which was re-

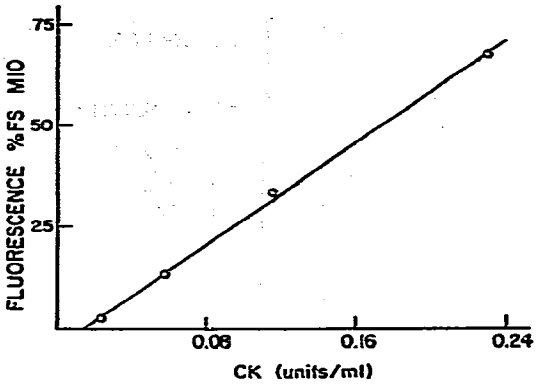


Fig. 11. Peak-height response of the immobilized G-6-PDH column to solutions of varying HK activity. HK was eluted isocratically from the analytical column with 0.4 *M* NaCl in the usual Tris buffer at a flow-rate of 1.0 ml/min. The HK assay reagents were introduced into the column effluent at a rate of 0.8 ml/min. The post-column temperature was 40° and the resulting NADH was detected by fluorescence.

ported to be 64 $\mu M$ for G-6-PDH from *L. mesenteroides*[17]. The strong affinity of the enzyme for its substrate enabled the immobilized enzyme to begin conversion of the hexokinase product promptly without a long lag time for the accumulation of G-6-P.

HK isoenzymes in a rat liver extract were detected using the immobilized G-6-PDH column (Fig. 12). The column was developed with a stronger salt gradient, but the resulting profile resembles the HK profile shown in Fig. 9. As this sample is the same as that used in Fig. 9, the smaller initial peak is probably the result of loss of enzyme activity during the subsequent 48 h of cold storage. The total HK activity in the rat liver extract was only about 0.15 unit/ml, but there was sufficient sensitivity to detect even lower activities under these conditions.

*Detection of CK isoenzymes*

The post-column detection of CK activity was accomplished with a coupled enzyme assay that requires two coupling enzymes according to the reactions

$$\text{Creatine phosphate} + \text{ADP} \xrightarrow{\text{CK}} \text{creatine} + \text{ATP} \quad (5)$$

$$\text{ATP} + \text{D-glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP} \quad (6)$$

and

$$\text{Glucose-6-phosphate} + \text{NAD(P)}^{+} \xrightarrow{\text{G-6-PDH}} (\text{gluconolactone-6-phosphate}) + \text{NAD(P)H} + \text{H}^{+} \quad (7)$$

The use of either NAD or NADP in reaction 7 depends on whether the G-6-PDH coupling enzyme is from yeast or from *L. mesenteroides*. The latter utilizes NAD better while the yeast enzyme is specific for NADP.

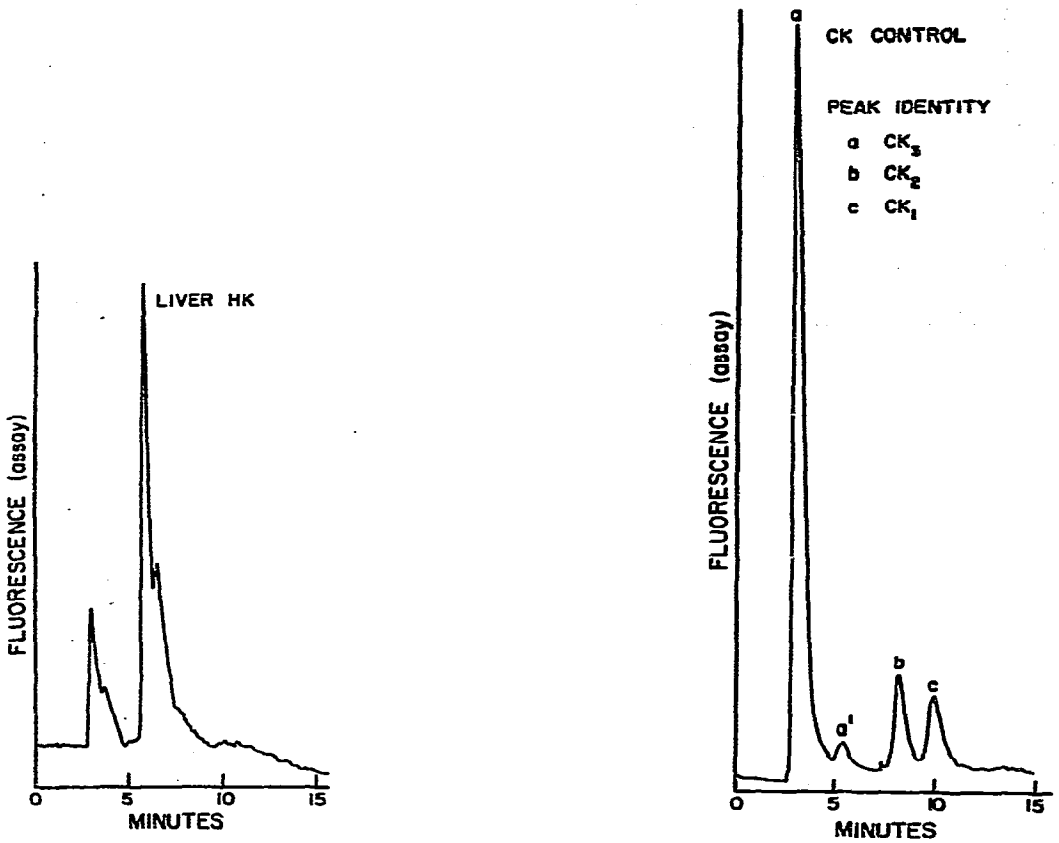


Fig. 12. Detection of chromatographically separated HK isoenzyme with the immobilized G-6-PDH column. The 105,000 *g* supernatant of a rat liver extract was chromatographed with a 10-min linear gradient reaching 0.5 *M* NaCl at a flow-rate 1.2 ml/min. The HK assay reagents were introduced into the column effluent at a rate of 0.8 ml/min. The post-column temperature was 40° and the resulting NADH was detected by fluorescence.

Fig. 13. Separation of CK isoenzymes in a control material using soluble coupling enzymes. CK isoenzymes in the control material were separated with a 15-min linear gradient reaching 0.3 *M* NaCl at a flow-rate of 1.5 ml/min. Immediately after injection, the eluent was advanced to 15% strong buffer and the gradient was initiated from this point. The CK assay solution was introduced into the column effluent at a rate of 1 ml/min. The post-column temperature was 40° and the resulting NADPH was detected by fluorescence.

CK isoenzymes in a control material (Helena, lot No. 865131) were separated on the analytical column and detected with soluble coupling enzymes in the assay solution. All three CK isoenzymes in this material were separated (Fig. 13) with baseline resolution. An extra peak was resolved, which was tentatively designated $CK_2'$. An extra CK peak has also been found in human heart tissue, and was eluted in about the same region from an anion-exchange column[18].

*Detection of CK activity with immobilized coupling enzymes*

The peak-height response of the 30-cm packed column containing both immobilized HK and G-6-PDH to varying concentrations of ATP was examined. At

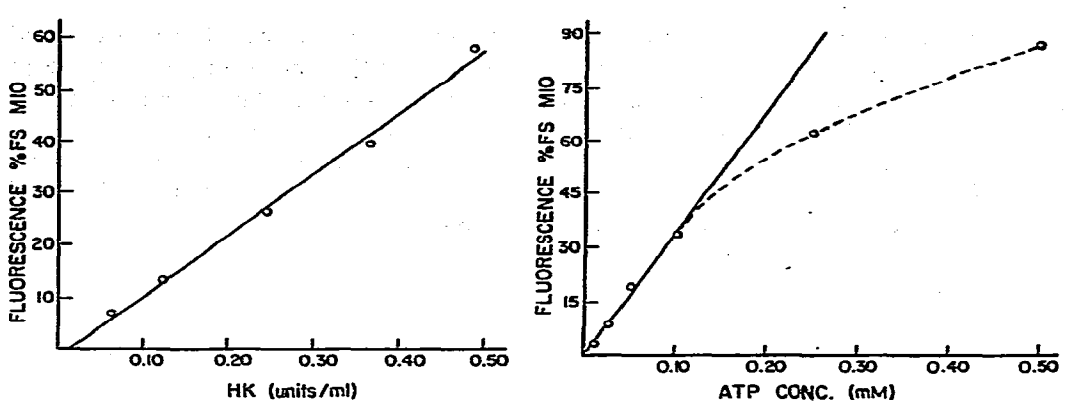


Fig. 14. Peak-height response of the immobilized HK/G-6-PDH column to varying ATP solutions. Solutions of ATP were injected into the system in the absence of an analytical column. The flow-rate through the column was 1 ml/min and the residence time of ATP solutions in the column was 2.7 min. The reaction proceeded at ambient temperature and the resulting NADH was detected by fluorescence.

Fig. 15. Peak-height response of the immobilized HK/G-6-PDH column to solutions of varying CK activity. Solutions of CK were injected into the system in the absence of an analytical column. The flow-rate through the column was 0.72 ml/min and the residence time of CK solutions in the column was 3.6 min. The reaction proceeded at ambient temperature and the resulting NADH was detected by fluorescence.

a flow-rate of only 1 ml/min, the residence time for the ATP solutions in the immobilized enzymes column was about 2.7 min. The response was found to be linear (Fig. 14) over a limited range of ATP concentrations (from 0.02 to 0.10 m*M*). The deviation form linearity at higher concentrations is due to the inability of the immobilized enzymes to convert the ATP solution completely into NADH.

The peak-height response of this column to CK activity was examined by injecting CK solutions into the system in the absence of an analytical column. The flow-rate through the column was decreased to 0.72 ml/min to increase the reaction time to 3.6 min. The response to CK activity was found to be linear (Fig. 15) but not very rapid or sensitive. The stock CK solution used in this study was about two to three times above the upper limit of normal CK activity in serum.

If a pre-column was used to allow the CK activity to increase ATP levels, it was found that the response could be increased by 30%. However, the pre-column was found to increase the dilution factor and the peak widths.

The lag time in the CK assay is well known. It usually takes 1 min or more before the production of NAD(P)H accurately reflects the CK activity, owing to the need to build up intermediate substrates, ATP and glucose-6-phosphate for the coupling enzymes. It appears that substantial HK activity is required to reduce this lag time. To immobilize more of the HK activity in the packed column, it is probably necessary to use a porous support.

## CONCLUSIONS

The automated enzyme detection system for HPLC was shown to function adequately in the detection of three different types of enzyme activities, a hydrolase,

two transferases and an oxidoreductase. This system performed both direct and coupled enzyme assays, and it was possible to immobilize one coupling enzyme in the system without losing efficiency. Using detection times of 2 min or less, it was possible to detect isoenzymes in serum and crude biological extracts.

Preliminary work with the immobilized G-6-PDH column indicates that it may be useful in detecting phosphoglucomutase and phosphoglucoisomerase, both of which have been found to have distinct isoenzymes[19].

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