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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE DETERMINATION OF GLYOXYLATE SYNTHESIS IN CHICK EMBRYO LIVER

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SUMMARY

The isolation and identification of three major α-keto end products (glyoxylate, pyruvate, α-ketoglutarate) of the isocitrate lyase reaction in 18-day chick embryo liver have been described. This was accomplished by the separation of these α-keto acids as their 2,4-dinitrophenylhydrazones (DNPHs) by high-performance liquid chromatography (HPLC). The DNPHs of α-keto acids were eluted with an isocratic solvent system of methanol–water–acetic acid (60:38.5:1.5) containing 5 m*M* tetrabutylammonium phosphate from a reversed-phase ultrasphere C_{18} (IP) and from a radial compression C_{18} column. The separation can be completed on the radial compression column within 15–20 min as compared to 30–40 min with a conventional reversed-phase column. Retention times and peak areas were integrated for both the assay samples and reference compounds. A relative measure of α-keto acid in the peak was calculated by comparison with the standard. The identification of each peak was done on the basis of retention time matching, co-chromatography with authentic compounds, and stopped flow UV–VIS scanning between 240 and 440 nm. Glyoxylate represented 5% of the total product of the isocitrate lyase reaction. Day 18 parallels the peak period of embryonic hepatic glycogenesis which occurs at a time when the original egg glucose reserve has been depleted.

INTRODUCTION

The conversion of acetyl-CoA into sugars via the glyoxylate cycle has been established in plants, bacteria, protozoa and nematodes[1,2]. But the operation of the cycle has never been convincingly demonstrated in avian and mammalian systems, even under conditions where net conversion of fat to carbohydrate might be expected to occur[3]. Recently, detection of enzymatic activity unique to the glyoxylate cycle has provided suggestive evidence for its presence in toad urinary bladder[4], rat epithelial

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tissue[5] and fetal guinea pig liver[6]. Isocitrate lyase activity has been detected in active low-energy state pigeon liver mitochondria[7]. The indispensible condition for glyoxylate production, according to Kondrashova[7], is the intensification of lipid catabolism coupled with the energy demands of growth or muscular activity.

The chick embryo develops in a self-contained environment rich in lipid and protein without a constant (maternal) glucose supply. Chemical analyses of the egg and of the 21-day embryo show little change in the carbohydrate and protein contents. The carbohydrate reserve of the egg is exhausted by day 10 of incubation[8] just prior to the appearance on day 11 of the hepatic enzymes obligatory for glyconeogenesis[8–12]. Late in the embryogenesis, and concurrently with the accumulation of hepatic glycogen, the RQ (respiratory quotient) of the embryo falls below 0.7 (ref. 8). These observations suggest there may be a net conversion of fat to carbohydrate.

Isocitrate lyase has never been detected in the extra-embryonic membranes and livers of developing chicks. Preliminary assays for isocitrate lyase in the microbody fraction of chick embryo liver yielded dinitrophenylhydrazone derivatives. Generally, progress in glyoxylate metabolism research in higher animal tissues has been impeded by lack of an efficient and sensitive method for conclusive identification of key metabolites.

We have succeeded in isolating and establishing the identification of three major α-keto acid end products (glyoxylate, pyruvate, α-ketoglutarate) of the isocitrate lyase reaction in 18-day chick embryo liver. This is achieved through the development of a very sensitive and reproducible method for the separation of 2,4-dinitrophenylhydrazone (DNPH) derivatives of these α-keto acids by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS*

Standard solutions

The DNPHs of α-keto glutarate, pyruvate and glyoxylate were prepared and recrystallized by the general method of El Hawary and Thompson[13]. The compounds were desiccated *in vacuo* at room temperature for several days and subsequently transferred to −20°C for permanent storage. To prepare a standard sample for HPLC analysis, known mg quantities of each compound were dissolved in 1.0–3.0 ml absolute methanol. Fresh preparations of α-keto acid mixtures were routinely used in HPLC for identification and quantitative purposes.

Instrumentation

The HPLC apparatus consisted of two Model 6000A solvent delivery systems and a Model 660 solvent programmer (Waters Assoc.). The eluted peaks were detected with a Model LC-75 (Perkin-Elmer) variable-wavelength autocontrol detector. Retention times and peak areas were electronically measured with a supergrator integrator (Columbia Scientific Industries). A reversed-phase Ultrasphere, C_{18} column (5 μm particle size; 25 cm × 4.6 mm I.D.) (Beckman) was used for these studies. The

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radial compression module (RC-100) and Radial-Pak C_{18} cartridge (10 μm particle size; 10 cm × 8 mm I.D.) were from Waters Assoc.

Chromatographic procedure

The α-keto acid dinitrophenylhydrazones were eluted with an isocratic solvent system of methanol–water–acetic acid (60:38.5:1.5) containing 5 m*M* tetrabutylammonium phosphate (Eastman Kodak). The column was washed at 1 ml/min successively with at least 30 ml of water and methanol, and was then equilibrated with eluting solvent. The injection volume was 10 μl with solvent flow-rate at 1.0 ml/min. Generated pressure and analysis time were dependent on the column used. Recorder speed was 0.5 cm/min but was increased to 2.0 or 5.0 cm/min during peak scanning.

Retention times and peak areas were determined for both the assay samples and reference compounds. A relative measure of the amount of α-keto acid in the unknown peak was calculated by comparison with the corresponding standard. Peak identifications were made on the basis of retention times, co-chromatography with standard compounds and stopped flow UV–VIS spectra, scanned between 240 and 440 nm.

Preparation of chick embryo liver homogenate

Fertile eggs of the Rock Cornish strain were incubated at 38°C under conditions of controlled humidity at the Poultry Science Research Lab., University of Wisconsin. At the indicated times, the embryos were removed from the eggs, decapitated and their livers quickly excised, chilled, and rinsed in 0.25 *M* sucrose. After weighing, the tissue was homogenized in the same medium (1:5, w/v) at 4°C. The homogenate was used immediately for the assay.

Enzyme assay

The homogenate was assayed for isocitrate lyase activity by a modified method of Daron and Gunsalus[14]. The reaction mixture was 62 m*M* Tris–HCl (pH 7.9), 3.1 m*M* $MgCl_2$, 1.2 m*M* cysteine·HCl, 3.75 m*M* DL-isocitrate and liver homogenate (1.0 ml) in a total volume of 16.0 ml. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 1 ml 50% trichloroacetic acid and the precipitated protein removed by centrifugation. After the addition of 3 ml of 0.2% 2,4-dinitrophenylhydrazine to the supernatant, the mixture was incubated for 20 min at room temperature. The α-keto acid DNPHs were selectively extracted in ethyl acetate and the free dinitrophenylhydrazine removed as described by El Hawary and Thompson[13]. The residue was immediately redissolved in 1–2 ml absolute methanol and stored at −20°C. After removal of microparticulate debris by passage through a Millipore 0.5-μm filter, the preparation was subjected to the HPLC analytical procedure.

RESULTS

The resolution and baseline separation of glyoxylic acid, α-ketoglutaric acid and pyruvic acid as their DNPH derivatives were achieved using a reversed-phase (Beckman) column and an isocratic solvent system containing tetrabutylammonium phosphate (TBAP) as an ion-paired reagent (Fig. 1A). The retention times and inte-

TABLE I

SEPARATION OF STANDARD DINITROPHENYLHYDRAZONE DERIVATIVES OF α-KETO ACIDS BY HPLC

HPLC on Beckman C_{18} IP column.

Peak No.	*Keto acid (as DNPH derivative)*	*Standard mixture (nmoles)*		*Retention time (min)*	*Fresh standard DNPH mixture*			*Isomerized standard DNPH mixture**		
					Counts (A_{365})	*nmoles*	*Recovery (%)*	*Counts (A_{365})*	*nmoles***	*Recovery (%)*
1	Glyoxylic acid			11.2	171,444	21.6		107,018	13.5	
1′	Glyoxylic acid	–	–	18.8	15,881	2.0		24,132	3.0	
1 + 1′	Glyoxylic acid		21.8		187,325	23.6	108	131,150	16.5	76
2	α-Ketoglutaric acid			15.1	157,736	19.8		136,424	17.2	
2′	α-Ketoglutaric acid	–	–	25.3	–	–		2387	0.3	
2 + 2′	α-Ketoglutaric acid		20.4		157,736	19.8	97	138,811	17.5	86
3	Pyruvic acid			16.9	32,920	4.1		28,944	3.6	
3′	Pyruvic acid	–	–	29.3	8853	1.1		7804	1.0	
3 + 3′	Pyruvic acid		5.1		41,773	5.2	102	36,748	4.6	90
	Total				386,834			306,709		

* Stored 1 week at 4°C.

** 7950 ± 920 counts/nmole DNPH derivative.

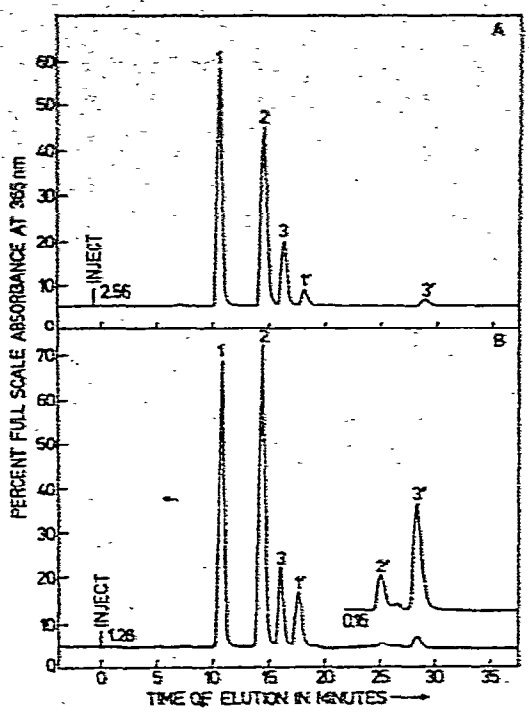

Fig. 1. Separation of standard mixture of DNPH derivatives of α-keto acids with Beckman C_{18} IP column. (A) Fresh; (B) one week storage at 4°C. Operating conditions: isocratic solvent system, methanol–water–acetic acid (60:38.5:1.5) containing 5 m*M* tetrabutylammonium phosphate; flow-rate, 0.5 ml/min; chart speed, 0.5 cm/min; pressure 1000–1200 p.s.i.; sample, 10, 6 and 10 mg each of glyoxylate DNPH, α-ketoglutarate DNPH and pyruvate DNPH, respectively, were dissolved separately in 3 ml methanol; 100 μl of each solution were mixed and 10 μl of the mixture were injected onto the column. Full scale absorbance was 2.56 or 1.28 at 365 nm. See Table I for peak identification.

grator counts per nmole were determined at 365 nm (Table I). The extent of isomerization of the three DNPH derivatives in the standard mixture after one week of refrigeration at 4°C is shown in Fig. 1B. After one week of storage, the area of the latter-eluting isomer (1′, 2′, 3′) of each compound was increased reciprocally with a decrease in that of the first isomer (1, 2, 3). Integrator counts after 1 week of storage fell 20% with the major decrease in the glyoxylic peak 1.

Identification of each compound and its isomer was achieved by running a scan between 240 and 440 nm of each peak using the stopped-flow technique during the HPLC procedure (Fig. 2). Each spectrum was characterized by a biphasic absorbance curve with the maximum absorption occurring within the 350–380 nm range. The spectra for each α-keto acid DNPH and its corresponding isomer (1,1′; 2,2′; 3,3′) showed superimposable fingerprints between 240 and 440 nm with absorption maxima of each at 354, 370 and 360 nm, respectively.

The application and validity of the above procedure for the detection and quantitation of these α-keto acids involved in the glyoxylate cycle have been tested in the well established biological system of the protozoan, *Tetrahymena pyriformis* E.[15,16]. The key enzymes of the glyoxylate cycle, isocitrate lyase and malate syn-

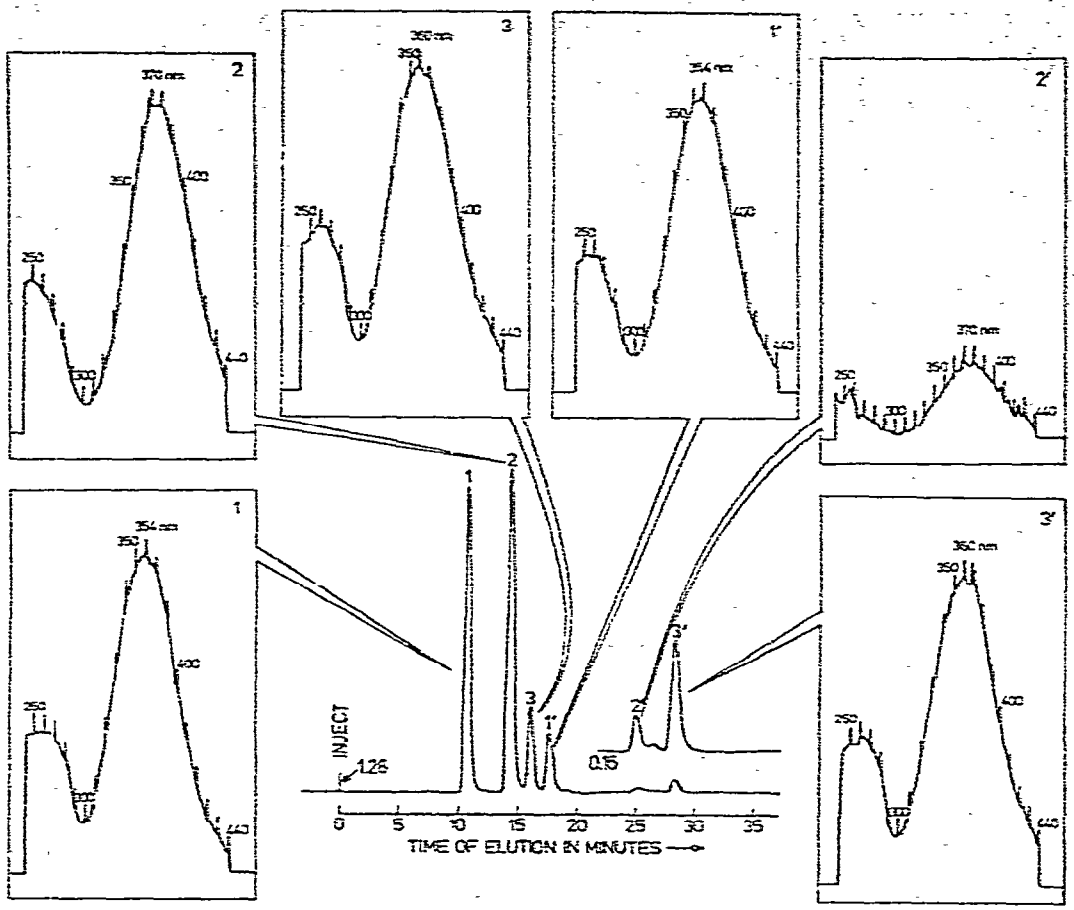

Fig. 2. Absorption spectra of major peaks in standard mixture (as shown in Fig. 1B) obtained between 240 and 440 nm by stop-flow technique during HPLC. Absorption maxima for 1,1'; 2,2'; 3,3' were 354, 370 and 360 nm corresponding to glyoxylate, α-ketoglutarate and pyruvate DNPH derivatives, respectively. Chromatographic conditions were the same as in Fig. 1A except the injected solution was diluted in 1:2 ratio with methanol.

thetase are induced upon transition from the logarithmic to the stationary phase. Glyoxylate, according to our HPLC analysis, accounted for 75–90% of the α-keto acid products of an isocitrate lyase assay of the homogenate of stationary phase *Tetrahymena pyriformis* (unreported).

A more challenging test of the methodology described above is the detection of glyoxylate formation by the embryonic chick liver. A homogenate of the 18-day embryonic liver was assayed for isocitrate lyase activity. The elution pattern of the DNPH derivatives of the α-keto acid end products of the assay is shown in Fig. 3. The tentative identification of each peak in the biological sample based on the elution times of samples and standards was confirmed with stopped-flow scanning of the spectrum (240–440 nm) which provided convincing evidence of each peak's identity, and due to the matched spectra of each isomeric pair the purity of the α-keto acids

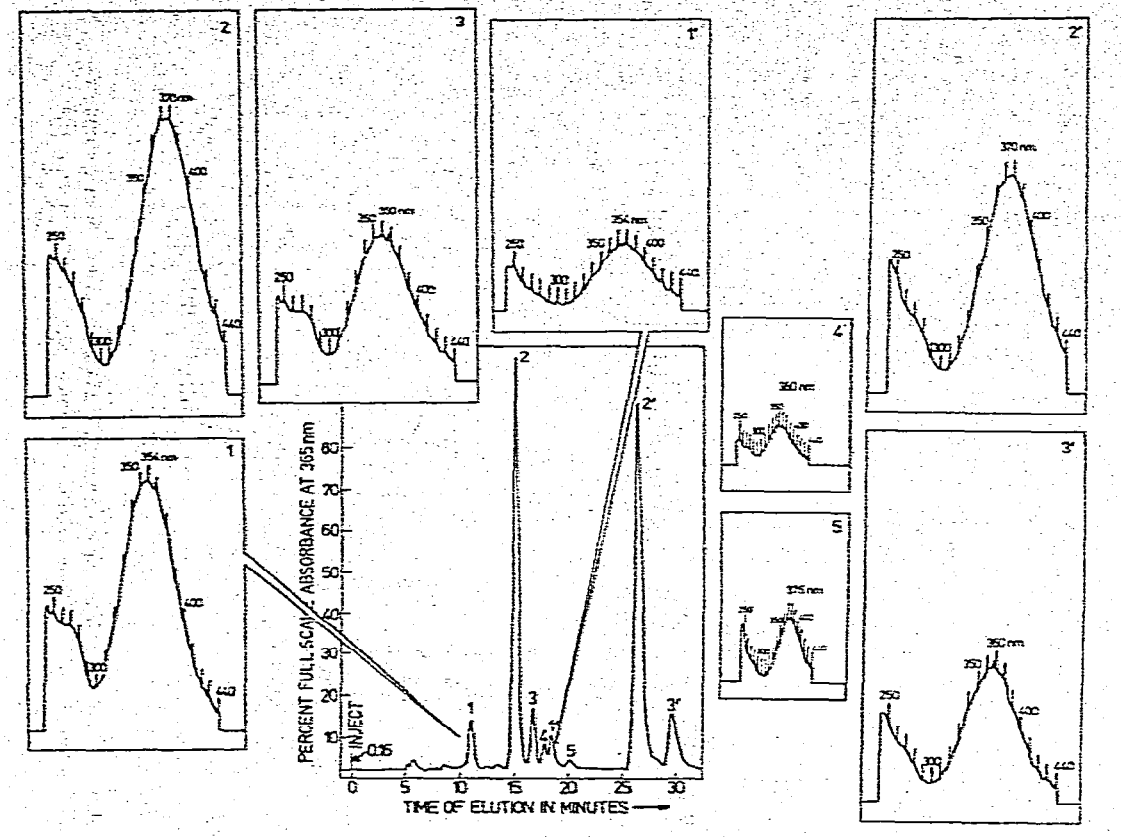

Fig. 3. Separation and spectral scanning of each peak of DNPH derived products of the isocitrate lyase assay in 18-day-old chick embryo liver homogenate (CEL-18). Peaks 4 and 5 are unidentified. Operating conditions were as given in Fig. 1A.

was demonstrated. The wavelength maxima of the biological isomers were in accord with those of the corresponding references (Fig. 2). The concentration of each α-keto acid and its isomer is presented in Table II. Apart from the identification of glyoxylic acid, pyruvic acid and α-ketoglutaric acid in the biological sample, there were two unidentified peaks labeled as 4 and 5 (Fig. 3).

Additional verification of the glyoxylate peaks was established by the addition of the authentic dinitrophenylhydrazone immediately prior to HPLC injection. The glyoxylate-DNPH spiked sample showed relative increases in the two peaks representing glyoxylic acid (Table II). The enlarged peaks were symmetrical, exhibiting no broadening or unusual shoulders in the elution pattern. Similarly, the sample spiked with the standard mixture showed excellent co-chromatography with enhancement of all identified peaks. The spectrum (240–440 nm) of each spiked peak duplicated those shown in Figs. 2 and 3, thus providing proof of peak homogeneity.

Although the absorption of the α-keto acid DNPHs is at maximum between 350 and 380 nm, the derivative can be efficiently detected at 254 nm (Fig. 4). The elution pattern monitored at 254 nm exhibits other large peaks (unknowns) one of

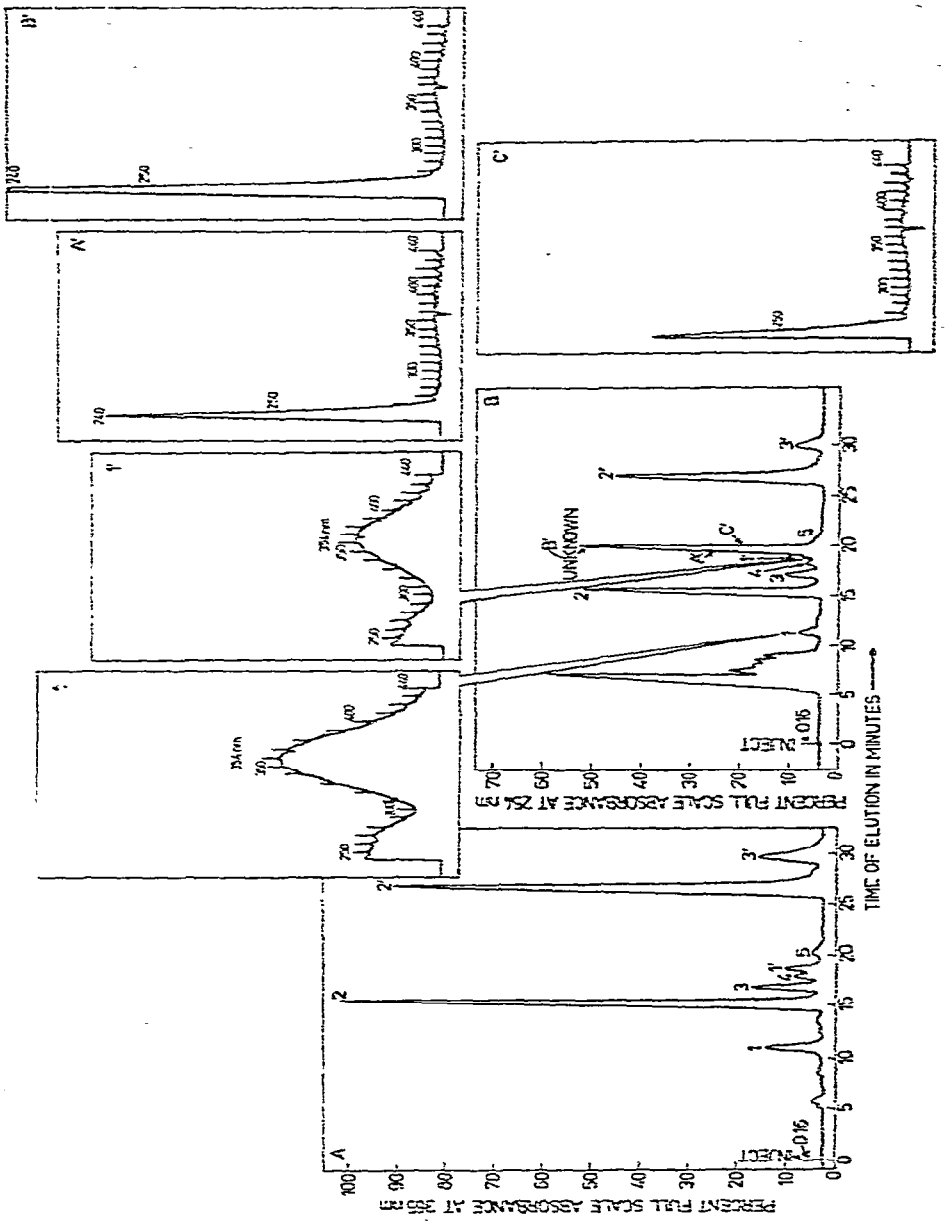

Fig. 4. The comparative elution profiles of the biological assay sample CEL-18 at (A) 365 nm and (B) 254 nm recorded under conditions identical to those given in Fig. 3. Absorption spectra (240–440 nm) of glyoxylate DNPH are shown. Spectra of the unknown peak were obtained at 3 different locations (A', B', C').

TABLE II

CONCENTRATION AND CONFIRMATION OF DIFFERENT α-KETO ACIDS IN THE CHICK EMBRYO LIVER HOMOGENATE BY HPLC

HPLC on Beckman C_{18} IP column.

Peak No.	Keto acid (as DNPH derivative)	Retention time (min)	CEL-18 Keto acid DNPH* (nmoles)	CEL-18 Keto acid DNPH + glyoxylic acid DNPH standard** (nmoles)	Recovery (%)
1	Glyoxylic acid	11.22–11.40	24.3	518	
1′	Glyoxylic acid	18.68–18.96	13.3	189	
1 + 1′	Glyoxylic acid		37.6	707	95
2	α-Ketoglutaric acid	15.14–15.41	333	320	
2′	α-Ketoglutaric acid	26.25–26.69	343	345	
2 + 2′	α-Ketoglutaric acid		676	665	98
3	Pyruvic acid	16.91–17.17	32.5	29.8	
3′	Pyruvic acid	29.51–29.83	41.0	38.8	
3 + 3′	Pyruvic acid		73.5	68.6	93

* 18-day chick embryo liver homogenate.

** 700 nmoles glyoxylate DNPH, isomerized standard, 80:20, 1:1′.

which is in the immediate vicinity of the glyoxylate DNPH (1′). The latter compound elutes as a shoulder of this unknown peak. Confirmation of each glyoxylate peak was achieved by scanning the spectrum between 240 and 440 nm. The spectrum of the unknown peak at three different locations (Fig. 4A′, B′ and C′) showed highest absorption at 240 nm. Several extractions of the DNPH derivative with ethyl acetate removed this compound.

The elution patterns of the standard α-keto acid DNPH mixtures (freshly prepared and stored 6 weeks at 4°C) from the radial compression C_{18} column (RCC) (10 μm particle size) are shown in Fig. 5A and B. Elutions with baseline separation were completed within 15–20 min, half the time required for the conventional column, but some loss in peak resolution was noted. The quantitative distributions of the isomer pairs present in the two standards eluted from the RCC are shown in Table III. These results demonstrate a progressive, quantitative conversion to the favored isomer during storage. The RCC elution pattern of the products of the isocitrate lyase assay of the 18-day-old chick embryo liver is shown in Fig. 5C.

DISCUSSION

Assays of glyoxylate synthesis by biological systems generally involve the derivatization of the α-keto acids with DNPH, separation of the derivatives and then quantification, often by different presently known methodologies lacking in sensitivity. Recently, Hemming and Gubler[17] reported the separation via linear gradient elution of an homologous series of monocarboxylic α-keto acid DNPHs by ion-paired reversed-phase HPLC. However, they do not report the separation of a mixture of both mono- and dicarboxylic α-keto acids such as the glyoxylate, α-ketoglutarate and pyruvate triad. These investigators experienced many of the problems inherent to the use of the gradient elution including significant baseline deviation,

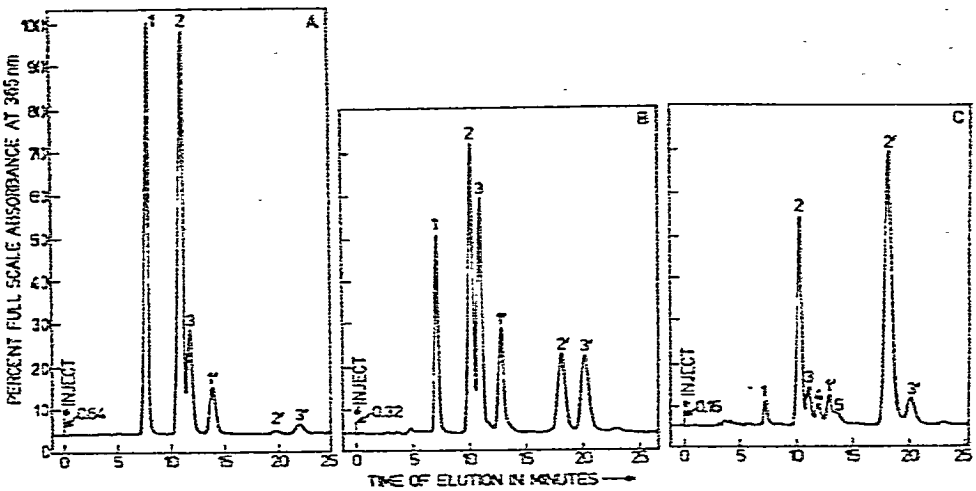

Fig. 5. Separation of α-keto acid DNPHs of (A) standard mixture, (B) standard mixture, stored 6 weeks, and (C) fresh biological assay sample CEL-18 using radial compression column chromatography. Flow-rate, 1.0 ml/min; pressure, 150–200 p.s.i.; remaining conditions were identical to those given in Figs. 1 and 4. Full scale absorbance values are indicated in the figure.

retention time variance in excess of half a peak width and inaccurate quantitative analysis by measurement of peak area.

The method described herein employs a reversed-phase column and isocratic eluting solvent system which not only provided baseline separation of closely related mono- and dicarboxylic α-keto acids but also resolved each isomer of the DNPH derivatives.

When subjected to HPLC, each α-keto acid DNPH appeared in doublet form. Terada *et al.*[18] observed earlier that the HPLC-chromatogram of pyruvate DNPH showed two peaks which, after careful study of conditions influencing changes in the peak area ratios, they attributed to *syn–anti* isomerism. They concluded that formation of the *syn* isomer becomes more favorable over time than that of the *anti* isomer due to steric interaction between the R group of the acid and the dinitrophenyl moiety. The isomerization is enhanced by an aqueous medium and is significantly depressed by cooling[17]. In the reported HPLC methods[17,18], glyoxylate and pyruvate, but apparently not α-ketoglutarate, derivatized into isomeric pairs. This report is contrary to our observation of a smaller, later eluting peak coupled with the prominent α-ketoglutarate DNPH peak. The peaks share identical UV–VIS spectra (373 nm). The relative reversal of the paired peak area ratios during storage is believed to be due to the presence of geometric isomers. Further isomerization was minimized by maintaining the samples dissolved in absolute methanol at −20°C.

DNPH derivatization has been criticized for imperfect qualitative and quantitative chromatographic behavior because DNPH is subject to isomerization and decomposition by hydrogen ions of absorbents and solvents[19,20]. We took advantage of the properties of geometric isomers as a valuable aid in the characterization of the α-keto acids. Quantitation was complicated by the isomerization and absorbance counts/nmoles for each compound was necessarily determined from the sum of both isomers. To measure DNPH stability, standard DNPHs of glyoxylate, α-ketoglu-

tarate and pyruvate were carried through the complete extraction procedure used for the chick embryo liver analysis. No alterations in retention time or appearance of new peaks suggesting breakdown products were observed (results not shown). Percentage recovery for the polar α-keto acids (*e.g.*, glyoxylate, α-ketoglutarate, pyruvate) by the "salting-out" extraction used in the present study reportedly varies between 84 and 100% when assessed by the colorimetric method of Friedemann and Haugen[21].

In preparation of the final concentrate for chromatography, slight losses of DNPH would be expected during the lengthy extraction and drying processes. HPLC of an authentic oxaloacetic acid DNPH yielded two peaks corresponding to the pyruvate isomers. Oxaloacetate behaves as a β-keto acid making it very susceptible to decarboxylation, in activities which has been demonstrated in numerous chromatographic procedures including HPLC[22,23]. Thus, in the biological assay, quantitation of pyruvic acid DNPH may be inaccurate reflecting the sum of both pyruvate and oxaloacetate DNPHs. The order of elution of the α-keto acid DNPHs was not strictly based on carbon number since the first isomer of α-ketoglutarate elutes prior to the pyruvate derivatives. A linear log plot of retention time *vs.* carbon number was reported for the separation of a straight-chain homologous series of α-keto acid DNPHs[17].

Incorporation of the large organic counter-ion, TBAP, was essential for adequate separation. Many of the problems of the more traditional ion-exchange method, such as precise pH control, reproducibility, and short column life were avoided. TBAP added to the mobile phase forms a reversible lipophilic complex with the ionized sample. Separation is achieved because of varying degrees of attraction of this non-polar ion-pair complex for a non-polar stationary phase and polar mobile phase. The degree of retention depends on the charge of the α-keto acid and the hydrophobic area of the tetrabutylammonium ion[24].

As in many HPLC applications, the tentative identification of a compound by its retention time must be confirmed by other methods. In the case of multiple derivatization, extra peaks must be identified either as breakdown products, contaminants, or isomers. Hydrogen peroxide treatment has been used in HPLC methods to selectively oxidize α-keto acids to their corresponding aldehydes which then react with free DNPH. A shift in retention time signals a peak arising from an α-keto acid DNPH[17]. But such analysis is cumbersome and difficult to interpret compared to the ease and certainty of UV–VIS scanning. Other traditional means of peak identification include fluorescence, absorbance ratioing and enzymatic peak shift techniques. However, the present method describes the identification of each peak on the basis of retention time matching and co-chromatography with authentic compounds and stopped flow UV–VIS scanning between 240 and 440 nm. The UV–VIS spectrum provided a powerful tool for identification of the DNPH derived α-keto acid.

This sensitive technique allowed us to detect measurable glyoxylic acid in the products of the isocitrate lyase assay of the 18-day chick embryo liver homogenate. Glyoxylic acid represented approximately 5% of the total product formed. Higher activities have been noted in preliminary studies of the microbody fraction of the liver homogenates of 14–20-day chick embryos[25].

The unknown peak 4 on Figs. 3, 4 and 5 appears to be the DNPH derivative of hydroxypyruvate. Identifications of this α-keto acid and of glyoxylate are supportive of the metabolic pathway proposed by Snell *et al.*[26] wherein glyoxylate is the sub-

TABLE III

HPLC OF STANDARD DINITROPHENYLHYDRAZONE DERIVATIVES OF α-KETO ACIDS BY RCC

Peak No.	*Keto acid (as DNPH derivative)*	*Standard mixture (nmoles)*	*Retention time (min)*	*Fresh standard DNPH mixture*			*Isomerized standard DNPH mixture**		
				Counts (A_{365})	*nmoles*	*Recovery (%)*	*Counts (A_{365})*	*nmoles***	*Recovery (%)*
1	Glyoxylic acid		7.5	350,472	22.4		249,128	15.9	
1′	Glyoxylic acid		13.57	33,762	2.1		40,564	2.6	
1 + 1′	Glyoxylic acid	21.8		384,234	24.5	112	289,692	18.5	85
2	α-Ketoglutaric acid		10.6	364,192	23.2		355,688	22.7	
2′	α-Ketoglutaric acid		18.7	–			4175	0.3	
2 + 2′	α-Ketoglutaric acid	20.4		364,192	23.2	114	359,863	23.0	113
3	Pyruvic acid		11.5	52,589	3.4		43,451	2.8	
3′	Pyruvic acid		21.3	19,027	1.2		25,912	1.7	
3 + 3′	Pyruvic acid	5.1		71,616	4.6	90	69,363	4.4	86

* Isomerization was carried out at 4°C for six weeks.

** 15,675 ± 2240 counts/nmole DNPH derivative.

strate for hydroxypyruvate formation in the pathway bypassing the phosphoenolpyruvate carboxykinase reaction leading to the net synthesis of glucose. Determination of the relative contributions of isocitrate, hydroxyproline and glycine to the glyoxylate pool will show whether or not the conversion of lipids to carbohydrates plays a central role in embryonic development.

The capacity of the 14-day-old chick embryo to synthesize 11-deoxycorticosterone, corticosterone, and aldosterone from acetate has been demonstrated by Pednera and Lantos[27]. Toad hemibladders pretreated with aldosterone and incubated in the presence of fatty acid had higher tissue glycogen content than did non-hormone-treated control tissue. The recently discovered glyoxylate cycle enzyme activity in toad bladder provides the necessary route for the conversion of lipid carbon to carbohydrate[4]. A similar hormonal regulation might be operative in chick embryo tissue.

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