Journal of Chromatography, 383 (1986) 297—305
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3369

SEPARATION OF CITRIC ACID CYCLE INTERMEDIATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ION PAIRING

JAMES F. KEEFER and SHELDON M. SCHUSTER*

Department of Chemistry and School of Biological Sciences, University of Nebraska, Lincoln, NE 68588-0304 (U.S.A.)

(First received April 23rd, 1986; revised manuscript received August 7th, 1986)

SUMMARY

A method to separate underivatized tricarboxylic acid cycle intermediates within 20 min using the commonly available C_{18} high-performance liquid chromatography column has been developed. Ion pairing using tetrabutylammonium cations and isocratic conditions is used to separate the intermediates which are then detected at 210 nm. Separation was optimized by altering pH, the concentration of sodium sulfate and the pairing ion. This technique permits the detection of as little as 120 nmol of citrate to 0.5 nmol of fumarate. Physiological samples of rat liver mitochondria, human urine, and orange juice were analyzed.

INTRODUCTION

Detection and quantification of citric acid cycle intermediates is of fundamental biological interest. Important metabolites of carbohydrate, amino acid, and lipid metabolism are involved, so that separation and measurement is important in many metabolic studies and in the assessment of food quality. The structural similarities among the citric cycle intermediates and their lack of distinctive spectral properties have, however, conspired to make quantification and separation most difficult.

In order to quantify the acids of the citric acid cycle, many types of chromatographic methods have been developed. These include paper, thin-layer, gas, and conventional liquid and high-performance liquid chromatography (HPLC). The latter technique is of special interest owing to the recent rapid improvement of HPLC sorbent particles, detectors, and automation. Several HPLC methods have been developed, which require pre-column derivatization

0378-4347/86/$03.50

to form the dinitrophenyl hydrazones [1]. This is useful because these derivatives have a high molar absorptivity (20 000 l mol^{-1} cm^{-1}). However, techniques that require the preparation of the hydrazones are cumbersome, and the derivatives must be extracted several times in a tedious fashion. In addition to this, multiple peaks for single acids are sometimes found. For example, two peaks are formed both for the dinitrophenyl hydrazones of glyoxylate and pyruvate, and these are attributed to *syn–anti* isomers of the derivatives. While glyoxylate and pyruvate are not formally part of the citric acid cycle, they are intricately related to its function, so they, too, must be monitored. Further, due to the instability of oxaloacetate (OAA), it breaks down to two pyruvate peaks in addition to the OAA peak. This greatly complicates the interpretation of the chromatograms. Recently, a method for utilizing fluorescent quinoxalinol derivatives of keto acids has been developed [2] that requires little sample handling and provides high sensitivity. However, this technique requires sample derivatization that may be incomplete or result in sample breakdown. Most importantly, however, the technique fails to identify the non-keto acids of the citric acid cycle or the hydroxy acids related to the cycle.

The traditional method for detection of citric acid cycle intermediates uses anion-exchange chromatography [2, 3]. This technique requires a gradient of 3- 5 h per analysis including column re-equilibration. HPLC has been used to separate pyruvate and α-ketoglutarate using 10-μm silica-based anion-exchange beads [4]. This and other methods have enhanced sensitivity with fluorescent or highly UV-absorbing derivative formation via post-column reaction [5—7].

Turkelson and Richards [8] developed an HPLC method using strong cation-exchange resin. This technique was further optimized by Bio-Rad and other commercial companies [9] so that separation was achieved isocratically in 20 min using ion-moderated partition (IMP) to separate acids in order of decreasing pK_a values. The disadvantages of this system are considerable. Firstly, irreversible damage to the column can occur as a result of resin bed shrinkage due to pH or salt concentration changes; secondly, amines in biological fluids contaminate the column; thirdly, gradient elution cannot be effectively used; and fourthly, the column is relatively expensive and is useful only for specialized applications. Some of these problems are lessened by use of a guard column, but the overall difficulty and expense remain.

A more acceptable technique would use the common C_{18} reversed-phase columns. Some acids were separated using a C_{18} column and pH 2.5 10 m*M* $KHSO_4$ solvent [10, 11] but more resolving power is necessary to separate all the citric acid cycle intermediates. This communication reports that sufficient separation can be provided by an ion-pairing agent with the relatively inexpensive and more durable C_{18} column.

EXPERIMENTAL

Chemicals and solvents

Tetrabutylammonium hydroxide (TBAH) was obtained from Aldrich (Milwaukee, WI, U.S.A.). All citric acid cycle intermediates were obtained from Sigma (St. Louis, MO, U.S.A.). A 300 m*M* stock solution of each organic acid

was prepared in 10 m*M* sodium phosphate buffer, pH 7, and was adjusted to pH 7.0 by the addition of hydrochloric acid or sodium hydroxide. Solutions were frozen or kept on ice at all times. Water was deionized and distilled in glass. All other chemicals were obtained from common suppliers.

Biological samples

Orange juice was squeezed directly from a whole orange. Rat liver mitochondria were prepared according to Pedersen et al. [12] and adjusted to pH 5 with concentrated sulfuric acid. Samples were frozen at −20° C. Before the analysis, the samples were thawed and then centrifuged for 10 min at 10 000 *g* to remove particles and denatured proteins.

Some samples were further purified by cation- and anion-exchange chromatography. A 2-ml volume of 50 X2-200 Dowex 50W was used for cation exchange chromatography. A 2-ml volume of 1 X2-200 Dowex 1 (Sigma) was used for anion-exchange chromatography. The resin was placed in empty Analytichem 4-ml tubes. Elution speed was enhanced by using the Vac-Elut vacuum apparatus (Analytichem International, Harbor City, CA, U.S.A.). For cation-exchange chromatography, the following procedure was followed: 3 × 2 ml of 0.5 *M* sulfuric acid, 4 × 2 ml of water, add sample and collect eluent, collect 5 ml of water column wash, clean column with 3 × 2 ml of 1 *M* ammonium hydroxide. Then the cycle can be repeated. For anion-exchange chromatography we used 4 × 2 ml of 1 *M* ammonium hydroxide, 4 × 2 ml of water, followed by the eluent from the cation-exchange column containing the sample, 2 × 2 ml of water, then collect 5 ml of 0.5 *M* sulfuric acid eluent. Then the cycle can be repeated. Each cycle takes 20 min and ten samples can be purified simultaneously. Using [1-^{14}C] citrate and [1-^{14}C] serine in 5 ml orange juice as standards for anions and cations, respectively, it was found that the cation-exchange chromatography step caused no citrate loss and removed 96% of the serine. The anion-exchange step recovered 91% of the citrate added. A 500-μl volume of anion-exchange eluent was neutralized before injection onto the HPLC system by adding 300 μl of 1 *M* ammonium hydroxide and 50 μl of 85% phosphoric acid adjusted to pH 8 using concentrated ammonium hydroxide.

Equipment

A Model 112 HPLC pump was used (Beckman, Berkeley, CA, U.S.A.). Injections were made using an Altex Model 210 sample injector (Beckman) fitted with a 100-μl loop. The chromatographic separations were performed on an Altex Ultrasphere ODS column (150 × 4.6 mm I.D. with 5-μm particles) (Beckman), preceded by a 50 × 4.6 mm guard column filled with 30–35 μm C_{18} particles (Whatman, Clifton, NJ, U.S.A.).

Chromatographic conditions and solvents

TBAH (20 m*M*) and sodium sulfate (20 m*M*, pH 7.0) solvent were prepared by adding 13.0 g of TBAH and 100 ml of a 200 m*M* sodium sulfate stock solution to 800 ml of water. The pH was adjusted using 85% phosphoric acid. This required 2.4 ml to reach pH 7.0. The solution volume was then adjusted to

1.0 l and filtered using a 0.45-μm pore size Type HA filter from Millipore (Bedford, MA, U.S.A.).

Separation was isocratic at a flow rate of 1.0 ml/min. In all cases, the HPLC column was washed with water before or after methanol was used. This prevented salt precipitation and consequent clogging of the frits. When the TBAH—Na_2SO_4 solvent was varied, the HPLC column was equilibrated for at least 20 min before a new sample was injected.

RESULTS

Separation of the citric acid cycle intermediates was accomplished using C_{18} reversed-phase HPLC. A chromatogram of carboxylic acid standards analysed under standard conditions is shown in Fig. 1. *cis*-Aconitate was not used because it is an enzyme-bound intermediate between citrate and isocitrate. In cells the aconitase primarily converts citrate to isocitrate which is removed from the equilibrium by isocitrate dehydrogenase. *cis*-Aconitate does exist at equilibrium under physiological conditions in vitro [13]. First, attempts were made to enhance separation by decreasing the pH (Fig. 2). Three organic acids metabolically associated with the citric acid cycle, glyoxylate, lactate and pyruvate, are included. A drawback of decreasing pH is decreased resolution of α-ketoglutarate, isocitrate, and citrate. At pH 2 there is overlap of several

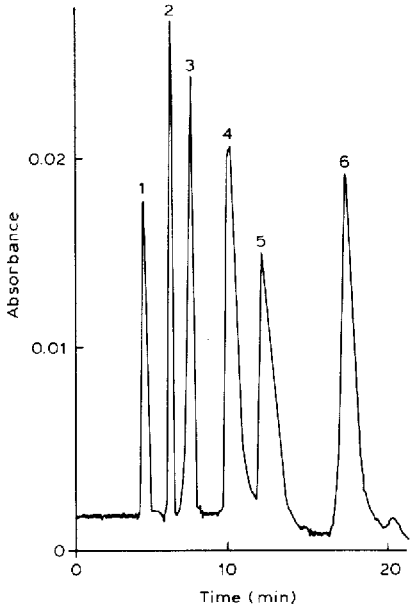

Fig. 1. Elution profile of citric acid cycle intermediates (absorbance versus time). The identities of the peaks and the quantities of solute are: (1) succinate, 150 nmol; (2) fumarate, 0.75 nmol; (3) α-ketoglutarate, 10 nmol; (4) citrate, 180 nmol; (5) isocitrate, 180 nmol; (6) oxaloacetate, 45 nmol. Concentrations of tetrabutylammonium hydroxide and sodium sulfate are each 20 m*M*, pH is 7.0. Flow-rate is 1.0 ml/min. Absorbance is measured at 210 nm. 100 μl of solution containing standards was injected. Oxaloacetate is a stable product of the OAA reaction with HPLC solvent before injection. See Experimental for further details.

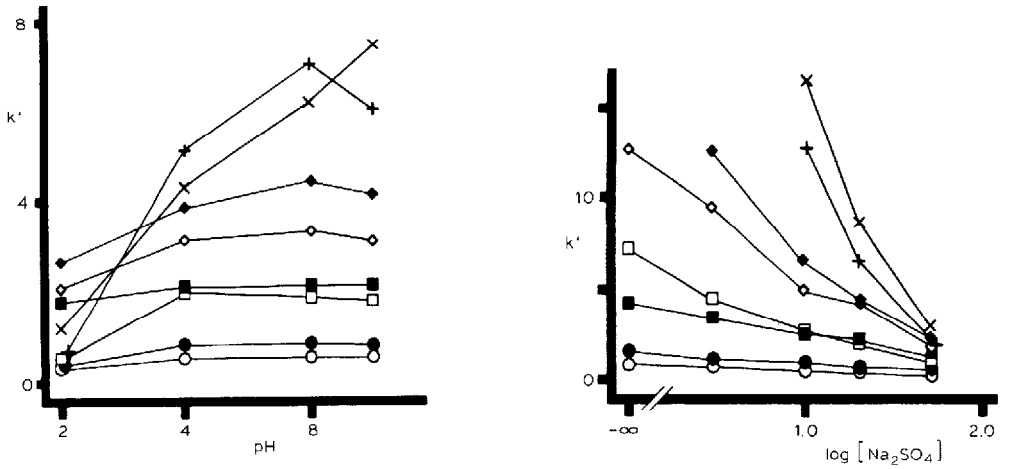

Fig. 2. Effect of pH upon the capacity factor, k', of citric acid cycle intermediates and metabolically related carboxylic acids. The pH was reduced by adding phosphoric acid. The organic acids used are identified as follows: (○) glyoxylate; (●) lactate; (□) succinate; (■) pyruvate; (◇) fumarate; (◆) α-ketoglutarate; (+) citrate; (×) isocitrate. Other chromatographic conditions were as in Fig. 1.

Fig. 3. Effect of sodium sulfate concentration upon the k' of citric acid cycle intermediates and related carboxylic acids. Concentrations of 0, 3, 10, 20, and 50 m*M* sodium sulfate were used. Other conditions are the same as in Fig. 1.

solutes but separation of pyruvate and succinate is enhanced. Citrate and isocitrate elute earlier and ionization that results in tailing peaks at pH 7 is suppressed.

Sodium sulfate was added to decrease the retention time of the solutes (Fig. 3). This removal of solutes by high salt concentration is analogous to removal of compounds from an ion-exchange column by addition of high salt concentrations. At 50 m*M* sodium sulfate many peaks overlap, but a concentration of 20 m*M* provides good resolution. Concentrations of less than 10 m*M* give an unacceptably large capacity factor (k') for citrate and isocitrate but increase the resolution of early-eluting compounds. If these early-eluting compounds are of primary interest, later-eluting compounds can be eluted quickly by increasing the concentration of sodium sulfate to 100 m*M* with a gradient. A methanol gradient could also be used but this results in high back-pressure and some salt precipitation on the column. Acetonitrile is an alternative solvent but it may also cause salt precipitation at high concentrations [14].

Not shown is the effect of lowering or increasing the concentration of TBAH. Theoretically, k' increases up to a certain value, with increasing concentration of pairing agent and then reaches a plateau. However, we found that decreasing the concentration of TBAH alone (no sodium sulfate in solution) increases the k' (data not shown). For example, with no sodium sulfate the k' value for pyruvate using 20 and 1 m*M* TBAH are 4.3 and 18, respectively. This increase in the k' may be due to the decrease in phosphate as the concentration of TBAH decreases. At low TBAH concentrations, solute k' values and peak shapes become very sensitive to salt concentration in the injection volume. For example, a TBAH concentration of less than 0.1 m*M* gave a broad asymmetric peak for glyoxylate.

TABLE I

DETECTION LIMITS OF CITRIC ACID CYCLE AND RELATED INTERMEDIATES

Signal-to-noise ratio is 2.

Compound	Detection limit (nmol)	Compound	Detection limit (nmol)
Glyoxylate	60	α-Ketoglutarate	1
Lactate	1500	Malate	100
Succinate	100	Citrate	120
Pyruvate	1	Isocitrate	120
Fumarate	0.50	Oxaloacetate	60

Maximum sensitivity ranges from 1500 nmol of lactate to 0.50 nmol of fumarate at a signal-to-noise ratio of 2:1 (Table I). Absorbance versus concentration was linear from 1 to 320 nmol for pyruvate and α-ketoglutarate. Sensitivity was approximately five times higher than that reported by Turkelson and Richards [8] owing to the larger loop used. A 100-μl injection loop was used and the original solutions of mixed citric acid cycle intermediates were diluted in the elution buffer. Dilution in other solutions may cause changes in absorbance that render unreadable peaks eluting before 6 min, depending on the absorbance scale used. It was noticed that with standard HPLC elution conditions, monocarboxylic acids elute first and tricarboxylic acids elute last. The structure of the OAA breakdown product is unknown and thus its k' of 9.2 cannot be explained. Fresh OAA elutes at a k' of 2.9, approximately where expected due to its −2 charge. However, acids do not always elute according to electrostatic attraction. In some cases, solute with a −2 charge will be retained less than a solute with a −1 charge in the presence of oppositely charged ion-pairing reagents [11]. Fresh malate has k' values of 2.2 and 3.6, with areas of 47 and 53% of the total absorbance. Neither of these peaks were

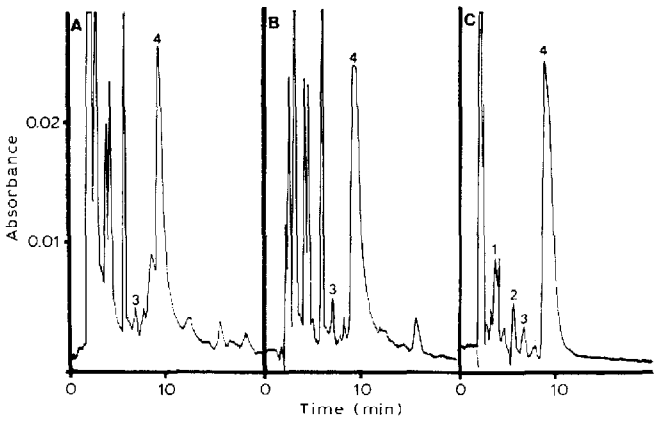

Fig. 4. Chromatogram of juice from a whole orange. (A) Juice diluted 1:20 in TBAH—Na_2SO_4 HPLC elution buffer. (B) Juice after strong cation-exchange purification, diluted 1:10 in HPLC elution buffer. (C) Juice neutralized after strong anion-exchange purification, diluted 1:5 in HPLC elution buffer. All other chromatographic conditions were the same as in Fig. 1. Peaks: 1 = succinate; 2 = fumarate; 3 = 2-ketoglutarate; 4 = citrate.

identifiied but they do co-elute with succinate and fumarate, respectively. Perhaps use of other pairing agents would improve the resolution of "malate" and other citric acid cycle intermediates. Tetramethyl, tetraethyl, tetrapropyl, tetrahexyl, and other ammonium compounds (in addition to the tetrabutylammonium hydroxide used for this article) are commercially available.

Figs. 4, 5, and 6 show separation of the components of physiological samples. Tentative identification of peaks was made by comparison of the k' values with the k' values of standards. All samples were diluted in the standard HPLC buffer. Citrate can easily be visualized without sample pretreatment (Fig. 4A). However, cation-exchange pretreatment is easily performed, dilutes the sample little, and removes many interfering compounds, especially early eluting compounds (Fig. 4B). Other acids, such as succinate and fumarate, are only decreased after anion-exchange pretreatment (Fig. 4C). Use of ion-pairing chromatography for an elution profile of urinary organic acids is shown in Fig. 5. Mitochondrial organic acids are shown in Fig. 6. This chromatogram is especially relevant because the citric acid cycle is located in mitochondria.

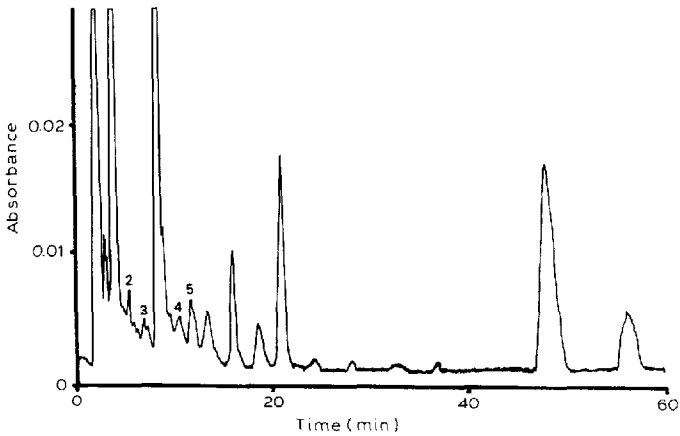

Fig. 5. Chromatogram of human urine. Preparation, dilution, and chromatography as in Fig. 4B. Peaks: 2 = fumarate; 3 = 2-ketoglutarate; 4 = citrate; 5 = isocitrate.

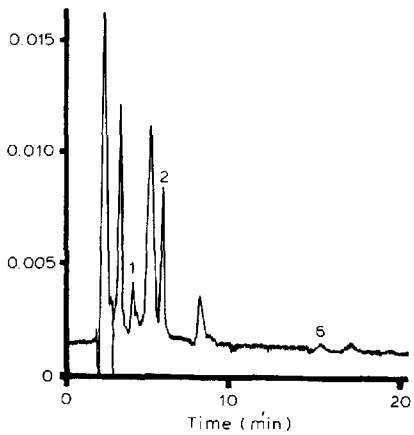

Fig. 6. Chromatogram of rat liver mitochondria. Diluted 1:3 with HPLC elution buffer. Preparation and chromatography as in Fig. 4B. Peaks: 1 = succinate; 2 = fumarate, 6 = oxaloacetate.

DISCUSSION

We have presented a reversed-phase ion-pairing method that will separate the citric acid cycle intermediates in 20 min using a typical C_{18} reversed-phase column. No other reversed-phase methods that separated these acids have been reported. This method also requires practically no sample handling before injection.

Analysis of phsyiological samples by HPLC provides only one criterion for sample identification. This makes interpretation of results sometimes difficult since an absorbance peak may not necessarily represent a citric acid cycle intermediate. This is a difficulty for most HPLC methods using underivatized samples, especially those methods using absorbance at 210 nm. Turkelson and Richards [8] used a cation-exchange column that retained amines indefinitely, thus partially purifying the sample and simplifying interpretation of the chromatogram. Samples separated by the method presented here could be eluted from a cation-exchange resin before adding them to the reversed-phase ion-pairing method (see Figs. 5B, 6, and 7). This achieves results similar to the IMP method. The extent of sample purification necessary depends on the information desired. Both the IMP and ion-pairing chromatographic methods may show interfering carbohydrates. Removal of these requires an anion-exchange step and greater sample manipulation before injection. However, if large amounts of raw samples are available, this would be an excellent purification and concentration technique (see Fig. 5C).

Another advantage of ion pairing over IMP is in separation of acid-unstable compounds. α-Ketosuccinamate (α-KSA), the α-keto form of the amino acid asparagine, is unstable in acid [15]. There is less breakdown on the column and in the elution solvent at pH 7 than with the IMP method using 10 m*M* sulfuric acid.

Ion pairing is a straightforward technique for measuring radioactive label flow of citric acid cycle intermediates as well as immediate precursors and products. Rapid separation is required for the numerous samples, but since few radioactive peaks appear, sample clean-up is less important. A variation of this was used to examine rat liver asparagine and glycine metabolism [16]. This application is especially appealing to label flow studies due to the lack of sample handling and subsequent sample loss from very small sample volumes. Radioactivity detectors for HPLC are becoming more prevalent and should make this type of procedure more precise, with higher resolution of radioactive peaks than obtainable by collecting individual fractions. The ion-pairing method for organic acid separation may also be used for enzyme assays, especially when working with a purified enzyme and a highly absorbent organic acid product. This method may also be useful for measuring chromatographic profiles of, for example, blood, urine or food products as an initial screening for disease or as a quality-control step.

In conclusion, a reversed-phase ion-pairing HPLC technique has been developed that separates the citric acid cycle intermediates. The advantages over currrent techniques include: no requirement for a specialized column; column matrix unaffected by changes in salt concentration; high adaptability of the system to specialized separation application by altering ion-pair concentration,

salt concentration of pH; and the ability to use fast gradient methods. The system has versatility so that it can be easily modified to perform specific separations necessary for metabolic and other studies.

ACKNOWLEDGEMENT

This work was supported by Grant CA 28725 from the National Cancer Institute, DHHS.

REFERENCES

1 B.C. Hemming and C.J. Gubler, Anal. Biochem., 92 (1979) 31—40.
2 D.J. Kieber and K. Mopper, J. Chromatogr., 281 (1983) 135—149.
3 K. LaNoue, W.J. Niekks and J.R. Williamson, J. Biol. Chem., 245 (1970) 102—106.
4 Y. Kasai, T. Tanimura, Z. Tamura and Y. Ozawa, J. Biol. Chem., 49 (1977) 655—658.
5 H. Nakamura and Z. Tamura, Anal. Chem., 51 (1979) 1679—1683.
6 W. Lindner and W. Santi, J. Chromatogr., 176 (1979) 55—64.
7 S. Katz, W.W. Pitt, Jr. and G. Jones, Clin. Chem., 19 (1973) 817—820.
8 B.T. Turkelson and M. Richards, Anal. Chem., 50 (1978) 1420—1423.
9 Bio-Rad Laboratories, The Liquid Chromatographer, 4 (1980) 2—6.
10 R. Schwarzenbach, J. Chromatogr., 251 (1982) 339—358.
11 B.A. Bidlingmeyer, LC, Liq. Chromatogr. HPLC Mag., 1 (1983) 355—359.
12 P.L. Pedersen, J.W. Grenawalt, B. Reynafarje, J. Hullichen, G. Decker, J.W. Soper and E. Bustamente, Methods Cell Biol., 20 (1978) 411—481.
13 C. Walsh, Enzymatic Reaction Mechanisms, W.H. Freeman, San Francisco, CA, 1979, p. 526.
14 R. Gloor and E.L. Johnson, J. Chromatogr. Sci., 15 (1977) 413—423.
15 R.A. Stephani and A. Meister, J. Biol. Chem., 246 (1971) 7115—7118.
16 K. MacPhee-Quigley, J.F. Keefer and S.M. Schuster, submitted for publication.