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Measurement of serum triglycerides by thin-layer chromatography and infrared spectrophotometry*

KENNETH KRELL and SAMI A. HASHIM†

Department of Medicine, St. Luke's Hospital

and

Institute of Nutrition Sciences,
Columbia University
New York, N.Y.

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SUMMARY

A direct and nondestructive method for the quantitative determination of serum or plasma triglycerides has been developed. The lipid extract of 1 ml of serum is chromatographed on 1-mm wet-thickness silicic acid layers prepared from a water–acetone slurry. Following elution, triglycerides are quantified by infrared spectrophotometry. For both synthetic and naturally occurring triglycerides, a nonlinear relationship of absorptivity as a function of concentration has been characterized. Triglyceride fatty acid chain length and degree of unsaturation have no significant effect on analytical results under the conditions described. Precision of the method is 2% and accuracy 4%. Recoveries average 96%. The method is sensitive to serum concentrations as low as 20 mg/100 ml. Triglyceride levels of a group of 51 healthy males, age 40 to 59, were determined. The mean postabsorptive triglyceride value was 93 mg/100 ml, with a standard deviation of 43 mg/100 ml. The system permits preservation of the triglyceride molecule intact for subsequent procedures such as characterization of the fatty acid moiety and substitution pattern.

The plasma triglycerides are known to play an important role in lipid metabolism (1–4). However, progress in understanding their function in health and disease has been impeded by the elaborate analytical procedures needed to measure them in biological systems.

In recent years, adsorption chromatography, by means of thin layers of silicic acid applied to glass plates (5), has found wide application in lipid analysis. This procedure permits separation and excellent resolution of the major lipid classes. Usually, lipids separated by this method have been quantified by densitometry after charring (6), or by colorimetry following elution of the lipid spot (7). In addition, infrared methods have been used to measure lipid fractions following their separation by means of silicic acid column chromatography (8).

This paper describes the use of thin-layer silicic acid chromatography and infrared spectrophotometry in sequence to separate and quantify serum triglycerides. The procedure is direct, reproducible, and nondestructive.

MATERIALS AND METHODS

Reagents. All solvents used were either of spectrophotometric quality or redistilled analytical reagent grade. Before use, a portion of each batch of solvent, representing five times the volume required for one determination, was evaporated to dryness. The residue, if any, was dissolved in 0.5 ml carbon disulfide, and the infrared spectrum examined for interfering substances. The spectrum of each batch of carbon disulfide was obtained and compared to that supplied by the manufacturer.

The adsorbent used was Silica Gel G,[1] containing

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[1] E. Merck, A. G., Darmstadt, Germany.

13% $CaSO_4 \cdot {}^1/_2 H_2O$. The fluorescent dye incorporated into the adsorbent was rhodamine B, Color Index No. 45170.

Reference Compounds. Pure tripalmitin, triolein, and tristearin were obtained from the Hormel Institute, Austin, Minn. In addition, a naturally occurring mixed triglyceride (corn oil), and two synthetic triglyceride preparations were used, one containing 98% C8:0 fatty acids and the other 98% C12:0 fatty acids.[2] Triglyceride purity in every instance was demonstrated by thin-layer chromatography (TLC). Triglyceride fatty acid composition was determined by gas-liquid chromatography (GLC).

Instruments. Perkin-Elmer Model 137 and Model 21 double-beam infrared spectrophotometers were used to obtain the infrared spectra. The Model 137 was equipped with a sodium chloride prism and was operated with the slits at their narrowest setting. The Model 21, also equipped with a sodium chloride prism, was operated on a slit program of 927 and at a scanning speed of approximately 3 min/μ. In all cases, the scans were from 4,000 to 680 cm^{-1}. The samples in carbon disulfide solution were contained in 1-mm cells with sodium chloride windows. A matching cell containing only solvent was placed in the reference beam.

The relatively poorer resolution of the Model 137 spectrophotometer, as compared to the Model 21, did not preclude its use in quantifying triglycerides. Comparison of analytical results obtained from the two instruments for the same sample showed no significant differences. However, absorptivities were higher when measured on the Model 21, as might be expected.

Thin-Layer Plate Preparation. Chromatographic plates were prepared with the Desaga[3] applicator. Fifty grams of Silica Gel G was slurried with 100 ml of distilled water–acetone 1:1 (v/v) containing 20 μg rhodamine B dye. Relatively thick layers of adsorbent were applied, and with the water–acetone system it was possible to avoid air inclusions, which otherwise caused crazing of the adsorbent layer upon activation. Addition of acetone also extended the setting time of the binder. The applicator was filled with the slurry and four 40x40-cm plates were coated with a 1-mm wet-thickness layer. The binder was allowed to set and the plates were activated at 115° overnight. The activated plates were stored in a desiccator until used.

Extraction. Serum lipids were extracted by a modification of the Folch procedure (9). Two milliliters of serum were slowly pipetted into a 50-ml glass-stoppered volumetric flask containing 20 ml of chloroform–methanol (C–M) 2:1 (v/v). The mixture was brought to a boil while being agitated, and was then cooled to room temperature and made up to volume with additional C–M. The flask was thoroughly shaken by hand and the mixture filtered through Whatman No. 43 filter paper previously extracted in a Soxhlet apparatus with C–M for 2 hr. The filtrate was collected in a 125-ml flask containing 15 ml distilled water that had been equilibrated with C–M. The filtrate and water were thoroughly shaken, then allowed to stand until the two layers reformed. A volumetric pipette was introduced through the water layer into the C–M layer, and a 25-ml aliquot was taken. The aliquot was evaporated to dryness under nitrogen in a 50° water bath. At this point, samples may be temporarily stored under nitrogen in desiccators at −4° until required for further analysis.

Chromatography. Each activated thin-layer plate was divided into four strips by scoring through the adsorbent layer, thus accommodating four samples. The dried lipid extracts were transferred to their respective strips on the plate in *n*-heptane solution by means of disposable Pasteur pipettes. Quantitative transfer of the residue was accomplished using small portions of solvent totaling 1 ml. Each sample was deposited in a spot 1 in. from the end of the scored strip. The *n*-heptane was allowed to evaporate, and the plates were developed by the ascending method in covered rectangular chromatography jars at 23° for 1 hr. The developing solvent system was diethyl ether–glacial acetic acid–*n*-hexane 25:2:73 (v/v/v).

Identification and Recovery of Triglycerides. After development of the plates, the solvent front was marked, and any residual solvent rapidly evaporated at room temperature. The dry plates were viewed under ultraviolet light of 375 mμ wavelength. Triglycerides appeared as a dark spot on the fluorescent background when unsaturated fatty acids were present. Triglycerides of saturated fatty acids appeared lighter than the background. Figure 1 shows a typical separation of both reference compounds and serum lipids. Triglycerides always migrated to the same position in relation to the other lipids present. In the rare instance when identification was in doubt, the spots in question were eluted and the infrared spectrum obtained. Examination of the spectra permitted unequivocal identification of the lipid class.

Upon visualization, the triglyceride spot was lightly circled. Each spot was then scraped off the plate into a microfilter funnel of 3-ml capacity, fitted with a "fine" pore size, fritted glass disc. The funnel was mounted on a side arm filter flask and emptied into a 15-ml

[2] Synthesized and kindly provided by V. K. Babayan, E. F. Drew Co., Boonton, N. J.

[3] Brinkmann Instruments, Inc., Great Neck, N. Y.

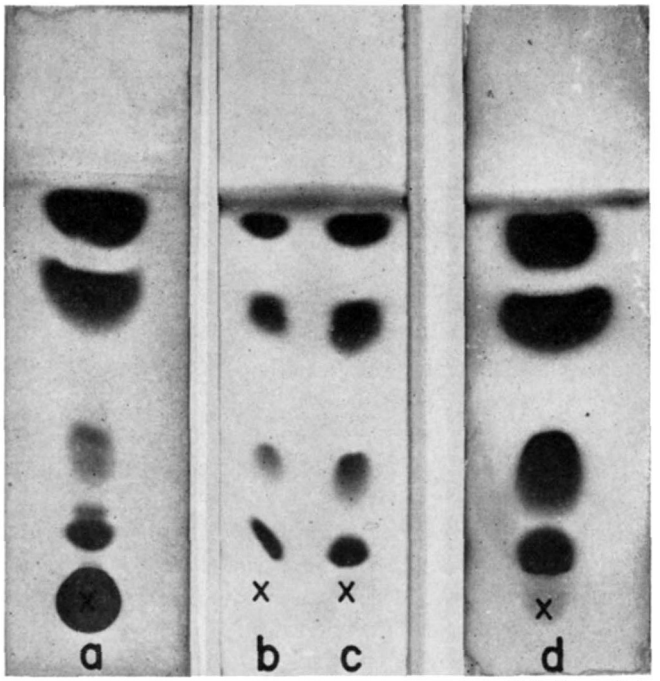

FIG. 1. Thin-layer chromatographic separation of lipids. Solvent system: diethyl ether–glacial acetic acid–*n*-hexane 25:2:73 (v/v/v). Development time: 1 hr. Indicator: sulfuric acid spray followed by charring. a: Serum lipid extract showing (from origin) phospholipid, cholesterol, monoglyceride, diglyceride, nonesterified fatty acid, triglyceride, and cholesterol ester; b, c, and d: reference compound mixture, 0.5, 1.0, and 5.0 mg total lipid, respectively. Spots are (from origin) cholesterol, oleic acid, triolein, and cholesterol oleate. The components of the reference compound mixture were present in equal amounts.

centrifuge tube positioned within the flask. The silicic acid was moistened with C–M and was broken down into a paste with the aid of a stirring rod to form a layer at the bottom of the funnel. With a mild negative pressure, 7–10 ml of C–M was allowed to pass through the funnel without disrupting the silicic acid layer. The eluate was then evaporated to dryness under nitrogen at 50°.

Infrared Spectrophotometry. The triglyceride residue was dissolved in 0.5 ml carbon disulfide. A 0.5-ml Ostwald-Folin pipette fitted to a 1-ml tuberculin syringe was used to fill the absorption cell. The infrared spectrum was scanned from 4,000 to 680 cm^{-1}, with a matched cell, containing carbon disulfide only, in the reference beam. Typical spectra of serum triglyceride, as well as reference compounds are shown in Fig. 2.

Absorbance[4] (A) values were measured at the 1,742 cm^{-1} band associated with the ester group carbonyl C=O stretching vibration (10). Computed spectral slit width at this frequency was 2.6 cm^{-1} for the Model 21. The base-line method of absorbance was used (11). Normally, the frequencies chosen for base-line construction lie close to the analytical frequency. Because of a strong solvent band adjacent to the 1,742 cm^{-1} band, and for reasons to be discussed later, the frequencies chosen were 1,900 and 925 cm^{-1}. Concentrations were calculated by means of an equation describing the nonlinear molar absorptivity (ϵ) found. Alternatively, concentrations may be determined by reference to a working calibration curve.

[4] A = log 1/T.

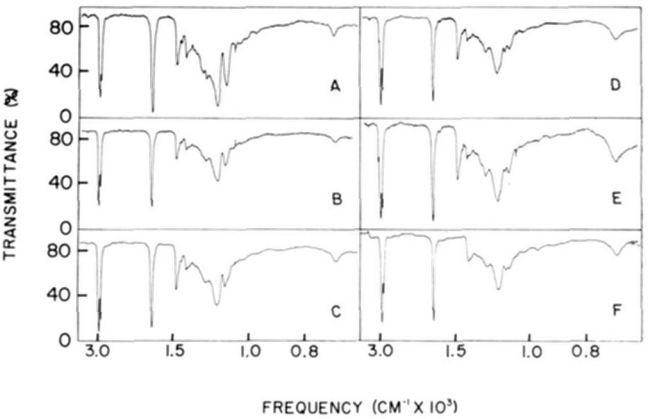

FIG. 2. Infrared spectra of triglycerides showing the carbonyl stretching band near 1,742 cm^{-1}. A: Tricaprylin; B: trilaurin; C: tripalmitin; D: triolein; E: corn oil. The above spectra were obtained from capillary films of the materials in the liquid state between sodium chloride plates. F: A serum triglyceride carbon disulfide solution in a 1-mm cell with matching cell containing carbon disulfide in the reference beam. There was no pen response in the regions 2,300–2,100 cm^{-1}, 1,700–1,400 cm^{-1}, and 880–845 cm^{-1}, due to solvent absorption.

EXPERIMENTAL PROCEDURE

Suitable concentrations of tripalmitin, tristearin, triolein, trilaurin, and tricaprylin were prepared by evaporating to dryness aliquots of standard solutions of these compounds and redissolving the residues in 0.5 ml carbon disulfide. These triglycerides were chosen because they differed so markedly in the chain length and the degree of unsaturation of their constituent fatty acids. The resulting concentrations in carbon disulfide ranged from 1.2 to 12.4 mM. The infrared spectra of the triglyceride solutions were obtained and base-line absorbance at 1,742 cm^{-1} was plotted as a function of concentration (Fig. 3). It was found that neither fatty acid chain length nor degree of unsaturation materially altered molar absorptivity of the carbonyl band. An additional band, near 1,159 cm^{-1}, tentatively assigned (12) to coupled vibrations of the C—C and C—O bonds in the ester group, also was examined. An attempt was made to correlate this band with triglyceride concentration, but it was found that fatty acid chain length significantly affected absorptivity because of superimposed skeletal C—C vibrations.

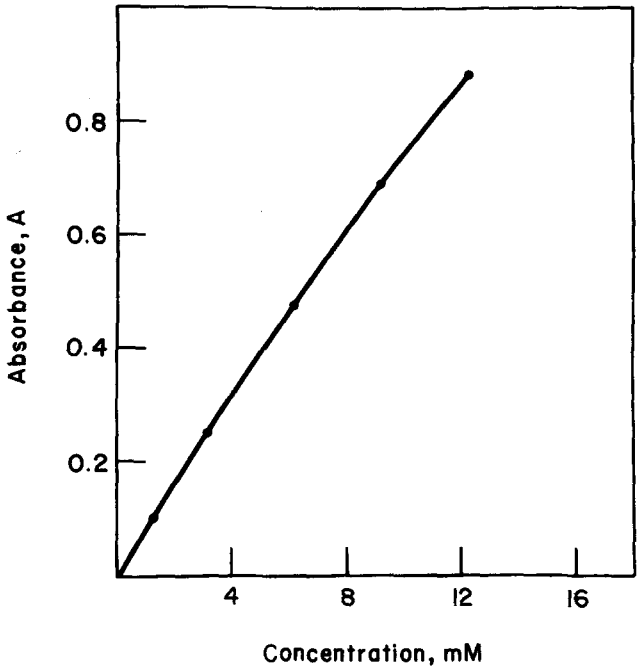

FIG. 3. Triglyceride carbonyl band absorbance as a function of concentration. The above plot is based on absorbance values obtained from spectra of tripalmitin carbon disulfide solutions in a 1-mm cell. Computed spectral slit width was 2.7 cm^{-1}.

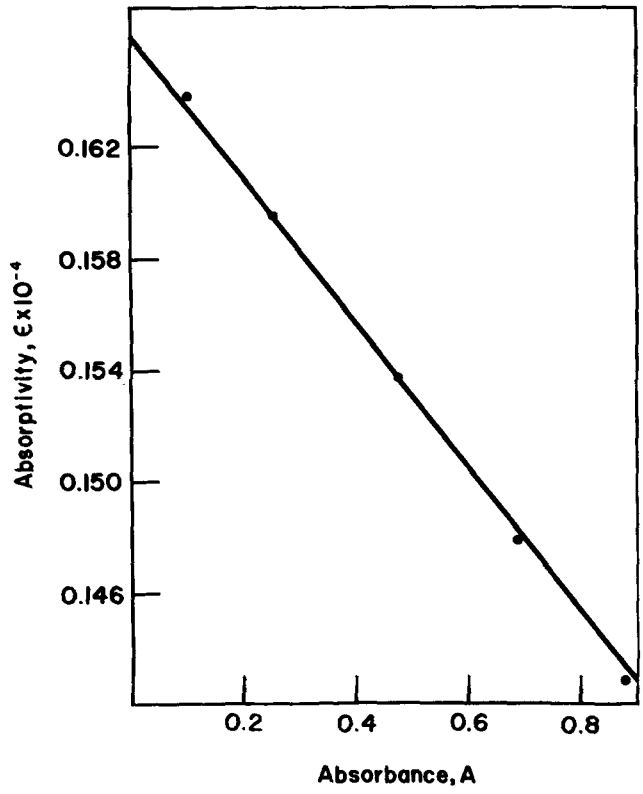

FIG. 4. Triglyceride carbonyl band absorptivity as a function of absorbance. Conditions are as described in Fig. 3.

When absorptivity was plotted as a function of absorbance, a straight line with negative slope, m, was obtained as shown in Fig. 4. The equation of this line is

$$\epsilon = mA + b, \quad (1)$$

where b is the y-axis intercept, or absorptivity at infinite dilution. Substitution of eq. (1) into the Beer's law equation,

$$c = \frac{A}{l\epsilon} \quad (2)$$

where l is the path length, yielded the equation,

$$c = \frac{A}{l(mA + b).} \quad (3)$$

Equation (3) thus characterized the relationship of absorbance to triglyceride concentration when absorptivity was a variable. In practice, absorptivity was calculated from eq. (1) and was substituted into eq. (2) to obtain triglyceride concentration.

The relative error in assuming a straight-line relationship of absorbance as a function of concentration was compared to that associated with the nonlinear absorbance function. Four concentrations of tripalmitin were analyzed and quantified by the two approaches. In the case of the straight line assumption, the absorptivity value used was calculated on the basis of least squares. Results are shown in Table 1. The average relative error for constant absorptivity was 4% while that for variable absorptivity was 2%, a two-fold increase in accuracy.

In order to determine the precision of the method, a sample of corn oil was analyzed at two different concentrations, each in quadruplicate. In addition, four samples of serum obtained at 15-min intervals from a dog that had been fasted for 12 hr also were analyzed for triglyceride concentration, and the results are shown in Table 2. Molar concentrations were converted to mg/100 ml on the basis of triglyceride fatty acid composition as determined by GLC. The mean relative standard deviation was 2%. Each series was performed by a different operator and was carried out on different days.

In order to evaluate triglyceride recovery, a hyperlipemic serum sample was diluted with isotonic saline to yield $^2/_3$, $^1/_3$, $^1/_5$, and $^1/_{10}$ of the original concentration. Aliquots of the diluted samples as well as the undiluted serum were analyzed by the TLC–infrared method described above. The analytically determined triglyceride concentrations were 551 mg/100 ml for the undiluted serum, and 360, 173, 105, and 52 mg/100 ml for the respective serum dilutions. Molar concentrations were converted to mg/100 ml using a triglyceride

TABLE 1. COMPARISON OF TRIGLYCERIDE ANALYSIS OF TRIPALMITIN SOLUTIONS USING CONSTANT AND VARIABLE ABSORPTIVITY VALUES

Absorbance	mg/100 ml* Taken	mg/100 ml* Found†	mg/100 ml* Found‡
0.101	50	53	50
0.254	125	133	128
0.474	250	248	249
0.688	375	360	376

* M.W. = 256.
† ϵ = 1527.
‡ ϵ = 1665–270A.

average molecular weight of 840 as determined by GLC. The values obtained by analysis were compared with those calculated on the basis of the triglyceride concentration of the undiluted serum as measured by the TLC–infrared method. The mean triglyceride recovery for this series was 96%.

The effect of large amounts of triglyceride present in the sample on discreteness of the triglyceride spot on thin-layer plates was investigated. A hyperlipemic serum, later found to contain 47 mg/ml triglyceride, was analyzed as described above. Under ultraviolet light, the triglyceride spot, although larger than usual, did not overlap other lipids. The infrared spectrum of the recovered triglyceride showed no trace of contamination.

The TLC–infrared procedure was used to measure serum triglyceride levels in 51 healthy men, age 40 to 59. The mean value obtained was 93 mg/100 ml with a standard deviation of 43 mg/100 ml. The range was 20 to 175 mg/100 ml. The frequency distribution for this group is shown in Fig. 5.

DISCUSSION

The three most widely employed nongravimetric approaches to quantitative determination of serum or plasma triglycerides have been (*a*) estimation by difference, either of cholesterol ester and phospholipid fatty acids from total titrable fatty acids (1, 13), or of cholesterol and phospholipid from total lipid as measured colorimetrically following dichromate–sulfuric acid oxidation (14); (*b*) determination of glyceride-glycerol, after alkaline hydrolysis and periodate oxidation, by measurement at 570 mμ of the chromotropic acid-formaldehyde complex formed (15–17); (*c*) determination of glyceride fatty acids by formation of their hydroxamic acids (18–20).

TABLE 2. PRECISION OF QUADRUPLICATE DETERMINATIONS OF TRIGLYCERIDE CONCENTRATION BY THIN-LAYER CHROMATOGRAPHY AND INFRARED SPECTROMETRY

Sample	Triglyceride Conc. (mg/100 ml)				Relative Standard Deviation
Corn oil	171*	168	160	159	3.5
Corn oil	96*	94	95	95	1.1
Dog serum	124†	120	119	124	2.1

* M.W. = 869.
† M.W. = 854.

A fundamental feature of the TLC–infrared method is its direct and nondestructive nature. Due to their unique infrared spectra, triglycerides, after separation and recovery, can be positively identified before quantification. The nondestructive aspect of the procedure allows, after quantification, the characterization of triglyceride fatty acids by GLC. Alternatively, fatty acid substitution patterns may be determined on the still intact triglyceride molecule.

The initial separation of the triglycerides on 1-mm wet-thickness layers of silicic acid was consistent and reproducible under the conditions described. The introduction of acetone into the silicic acid slurry facilitated the preparation of these relatively thick adsorbent layers and contributed significantly to their uniformity. The use of thick rather than thin layers of silicic acid allowed excellent separation of large quantities of triglycerides from other lipids.

Since neither chain length nor degree of unsaturation of triglyceride fatty acids had any significant effect on carbonyl absorptivity at 1,742 cm^{-1}, the method was not dependent for standardization upon any particular triglyceride or mixture of triglycerides. Results were re-

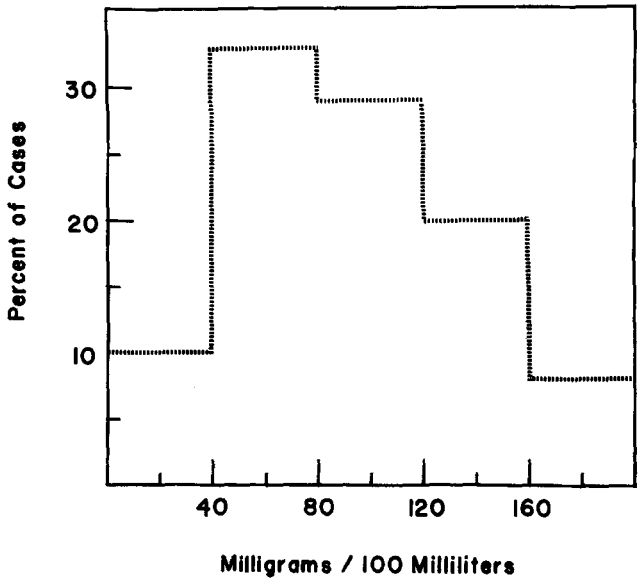

FIG. 5. Frequency distribution of serum triglyceride concentrations in a group of 51 clinically healthy men, age 40 to 59.

ported as molar concentrations and converted, when necessary, to mg/100 ml on the basis of triglyceride fatty acid composition as determined by GLC. Also, the conversion can be made on the basis of mean values of serum triglyceride fatty acid composition.

Triglyceride carbonyl absorptivity at 1,742 cm^{-1} was found to be a curvilinear function of concentration, and thus, Beer's law was not strictly applicable. Such a departure from linearity can be explained in two ways. The absolute concentrations (those contained in the absorption cell) used for measurement ranged as high as 12.4 mM, near the upper limit normally assigned to conformity with the Beer law (21). Moreover, since a constant volume of carbon disulfide was added to each sample, the final concentration of the solution varied with sample contribution to final volume. Thus, to ascribe a straight-line nature to what is an essentially curvilinear relationship of absorbance to concentration, could not fail to decrease the accuracy of quantification.

Meaningful studies involving recovery of lipid material are always difficult to design with biological systems. The validity of adding free triglycerides to serum or serum extracts, assuming equivalence of free triglyceride and protein-bound lipid, is questionable. Therefore, an additional attempt was made to test the procedure by analysis of serial dilutions with isotonic saline of a hyperlipemic serum. Results revealed good agreement between analytical and predicted values.

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