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# Fluorometric and Colorimetric Enzymic Determination of Triglycerides (Triacylglycerols) in Serum

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We describe two fully enzymic methods, fluorometric and colorimetric, for determination of triglycerides (triacylglycerols) in serum. Samples are incubated with microbial lipase for 10 min, and the glycerol released from the triglycerides is oxidized by $NAD^+$ in the presence of glycerol dehydrogenase. In the fluorometric method, the resulting NADH is in turn oxidized by resazurin as catalyzed by diaphorase to form resorufin, a highly fluorescent compound. In the colorimetric method, the NADH is oxidized by coupling with a tetrazolium salt/diaphorase system to form formazan, a highly colored compound. Calibration curves, constructed by plotting change in fluorescence or absorbance vs concentration of triglycerides, were linear up to 6 and 5 g of triglycerides per liter of serum for the fluorometric and colorimetric methods, respectively. The assays require only 5 and 15 μL of serum for fluorometry and colorimetry, respectively. The CV was 0.59% for the fluorometric method, 0.91% for the colorimetric procedure. The time for analysis for either method is less than 15 min. The results correlate well with those obtained by the Dow Diagnostic Kit method, a colorimetric method in which glycerol kinase and glycerol-1-phosphate dehydrogenase form NADH from ATP and $NAD^+$ in the presence of glycerol and glycerol 1-phosphate.

Buccolo and David (*1*) have discussed evidence supporting the use of spectrophotometric procedures for enzymic analysis of triglycerides in serum. In these procedures, triglycerides are hydrolyzed by lipase to release glycerol, followed by a glycerol kinase–pyruvate kinase–lactate dehydrogenase coupled assay in which the oxidation of NADH is used to quantitate the amount of glycerol formed. This technique was successfully adapted for use in continuous-flow analysis (*2*). The same reaction sequence was utilized in a kinetic assay of serum or plasma triglycerides (*3*). The reaction is triggered by adding lipase at a time when free glycerol, pyruvate, or both are no longer present. A modified fluorometric method of Buccolo and David was described by Rietz and Guilbault (*4*).

Ziegenhorn (*5*) described an improved enzymic method for determining serum triglycerides by use of the same reaction sequence as Buccolo and David. The amount of glycerol released was determined by a kinetic fixed-time analysis, and the method was adapted for use with a centrifugal analyzer.

A similar approach was taken by Grossman et al. (*6*), who used glycerol dehydrogenase instead of the glycerol kinase–pyruvate kinase–lactate dehydrogenase system to catalyze the oxidation of glycerol by $NAD^+$ to produce NADH. In this method the initial velocity of the production of NADH is determined.

Most of the above-mentioned techniques involve many sequential reactions requiring expensive enzymes. The procedure described by Grossman et al. (*6*) considerably shortens the reaction sequence, but for good linearity requires excess $NAD^+$ and unusually high pH; NADH formation is measured in the ultraviolet. McGregor et al. (*7*) proposed that the nonlinearity associated with use of glycerol dehydrogenase probably resulted from the inhibition of the enzyme by the reaction products, NADH and dihydroxyacetone.

With these considerations in mind, we decided to improve the method of analysis by reacting the NADH released during oxidation of glycerol (equations 1 and 2) with a fluorogenic reagent, resazurin. The formation of resorufin, catalyzed by diaphorase (EC 1.6.99.–), is measured by monitoring the change of fluorescence (equation 3 below) ($\lambda_{ex}$ = 548 nm; $\lambda_{em}$ = 580 nm). Guilbault and Kramer (*8*) showed that the resazurin/diaphorase system also can be successfully applied to measure the activities of various dehydrogenases.

In the colorimetric method we describe here, the NADH produced is coupled to the tetrazolium salt/diaphorase system. We measure the change in absorbance at 500 nm that results from the formation of a highly colored compound, called formazan, the reduced form of the tetrazolium salt (equation 4). This dye system has been previously applied in measuring the triglyceride concentration by the Dow Diagnostic Kit (Dow Chemical Co., Indianapolis, IN 46268) method, in which glycerol kinase coupled with glycerol-1-dehydrogenase produces NADH from ATP and $NAD^+$ in the presence of glycerol and glycerol 1-phosphate. In summary, the reactions in the present methods are:

$$\text{Triglycerides} \xrightarrow[\text{(EC 3.1.1.3.)}]{\text{lipase}} \text{glycerol + fatty acid} \quad (1)$$

$$\text{Glycerol + } NAD^+ \xrightarrow[\text{(EC 1.1.1.6)}]{\text{glycerol dehydrogenase}} \text{dihydroxyacetone + NADH + } H^+ \quad (2)$$

$$\text{NADH + resazurin} \xrightarrow[\text{(EC 1.6.99.–)}]{\text{diaphorase}} \text{resorufin + } NAD^+ \quad (3)$$

$$\text{NADH + 2-(}p\text{-iodophenyl)-3-(}p\text{-nitrophenyl)-5-phenyltetrazolium, oxidized (INT)} \xrightarrow{\text{diaphorase}}$$

$$\text{2-(}p\text{-iodophenyl)-3-(}p\text{-nitrophenyl)-5-phenyltetrazolium, reduced (formazan) + } NAD^+ \quad (4)$$

## Materials and Methods

### Apparatus

We used an Aminco Fluoromicrophotometer (American Instrument Co. Inc., Silver Spring, MD 20910) for all fluorometry; Corning and Wratten combination 1-60/58 and Wratten 22 filters serve as the primary and secondary monochromators to give $\lambda_{ex}$ 548 and $\lambda_{em}$ 580 nm. We used a linear recorder (Radiometer, Copenhagen, Denmark) to display the reaction rate.

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For the colorimetric method, we used a Model 124 double-beam grating spectrophotometer (Perkin-Elmer Corp., Coleman Instrument Division, Maywood, IL 60153) and a linear recorder, strip-chart Model SRB (Sargent-Welch Scientific Co., Dallas, TX 75235). All readings at 500 nm were taken in matched silica cuvets (Beckman Instruments, Inc., Fullerton, CA 92634).

We used a vortex-type mixer for sample mixing. Finnpipettes (Finnpipette KY, SF-00810 Helsinki 81, Finland), adjustable pipets with 5, 0–100, and 200–1000 μL capacities, were used to deliver serum and other reagents.

### Reagent and Control Sera

Glycerol dehydrogenase (268 kU/g), $NAD^+$, glycine, and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium, oxidized (INT) were obtained from Sigma Chemical Co., St. Louis, MO 63178.

Lipase from *Rhizopus arrhizus* (7900 kU/g) was a product of Fermco Biochemicals, Inc., Elk Grove Village, IL 60007.

Diaphorase (48 kU/g) was from Worthington Biochemical Co., Freehold, NJ 07728.

Resazurin was obtained from Aldrich Chemical Co. Inc., Milwaukee, WI 53233.

Control sera, Lipid-Trol and Monitrol I and II, were obtained from Dade Division, American Hospital Supply Co., Miami, FL 33152.

All the inorganic chemicals used were the purest grades available.

### Preparation of Reagents

*Hydrolysis reagent:* 10 850 U of lipase in 1.0 mL of phosphate buffer (50 mmol/L, pH 8.0).

*Glycerol dehydrogenase:* 10 700 U/L of phosphate buffer (50 mmol/L, pH 7.5).

*Diaphorase:* 17 200 U/L of phosphate buffer (50 mmol/L, pH 8.5).

*Glycine buffer,* 50 mmol/L, was adjusted to pH 9.75 with 50 mmol/L NaOH.

*$NAD^+$* was dissolved in doubly distilled water to give a 20 mg/L solution.

*INT* was dissolved in doubly distilled water to give a 2 g/L solution.

*Standard triglycerides solutions:* working standards of 0.950, 1.900, 3.800 and 5.700 g/L were made by appropriately diluting lipid-calibration serum with doubly distilled water.

### Procedure

Control serum (triglycerides concn, 1900 g/L) was used to study the effect of pH, lipase, glycerol dehydrogenase, $NAD^+$, resazurin, INT, and diaphorase on the reaction rate.

*Fluorometric method:* Into a 1.0 × 7.5 cm test tube place 5 μL of serum sample and 50 μL of lipase. Mix for 5 s on a vortex-type mixer and incubate for 10 min at room temperature (20 °C). Then add, in this order, 1.0 mL of phosphate buffer (pH 7.6), 25 μL of glycerol dehydrogenase, 25 μL of diaphorase, and 5 μL of resazurin. Shake the mixture for 5 s, then place the test tube in the cell holder inside the fluorometer and obtain a stable baseline. Remove the test tube, immediately inject 25 μL of $NAD^+$ solution into the tube, shake it, quickly replace it in the fluorometer, and record the fluorescence change at 580 nm ($\lambda_{ex}$ 548 nm) during 1 to 2 min.

To measure the free glycerol already in the serum before the hydrolysis with lipase, repeat the whole procedure, without adding lipase.

Plot the net change in fluorescence per minute (total triglycerides minus free glycerol) vs triglyceride concentration for a series of standard solutions. The calibration plot is linear from 0 to at least 6 g of triglycerides per liter of serum, without dilution.

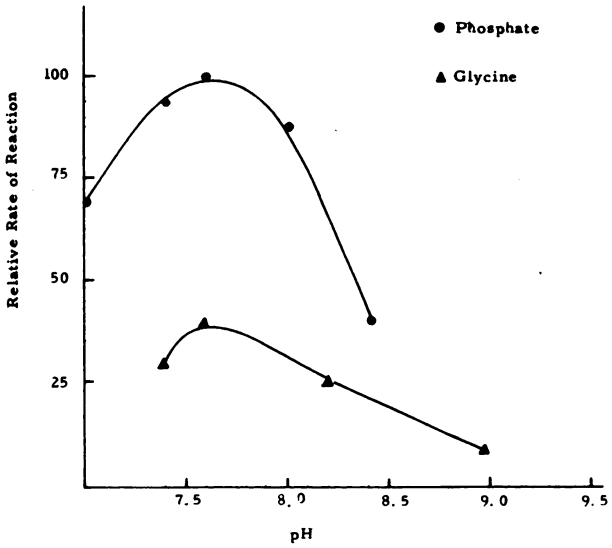


Fig. 1. Effect of pH on reaction rate in the fluorometric method

*Colorimetric method:* Mix 15 μL of serum sample and 50 μL of lipase, and incubate in a cuvet for 10 min at room temperature (20 °C). Then add 1.5 mL of the glycine buffer, 50 μL of glycerol dehydrogenase, 50 μL of diaphorase, and 50 μL of INT. Shake the mixture for 5 s and place the cuvet in the cell compartment of the spectrophotometer and monitor until a constant baseline is obtained. Then inject 50 μL of $NAD^+$, and read the absorbance change at 500 nm during 1 to 2 min.

Assay the free glycerol present before hydrolysis, by omitting the hydrolysis step.

Plot absorbance change per minute vs concentration of triglycerides from a series of standard solutions. The calibration plot is linear from 0 to at least 5 g of triglycerides per liter of serum. A twofold dilution is required for serum with triglyceride concentration exceeding 5 g/L.

## Results and Discussion

### Fluorometric Method

Figure 1 shows the relation between pH and reaction rate.

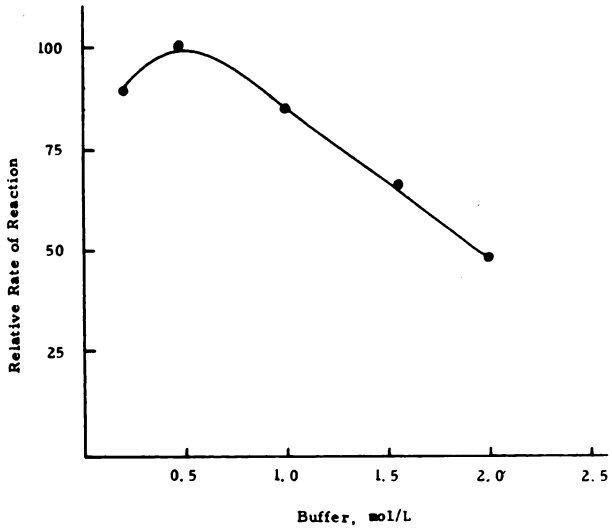


Fig. 2. Effect of ionic strength on the reaction rate in both methods

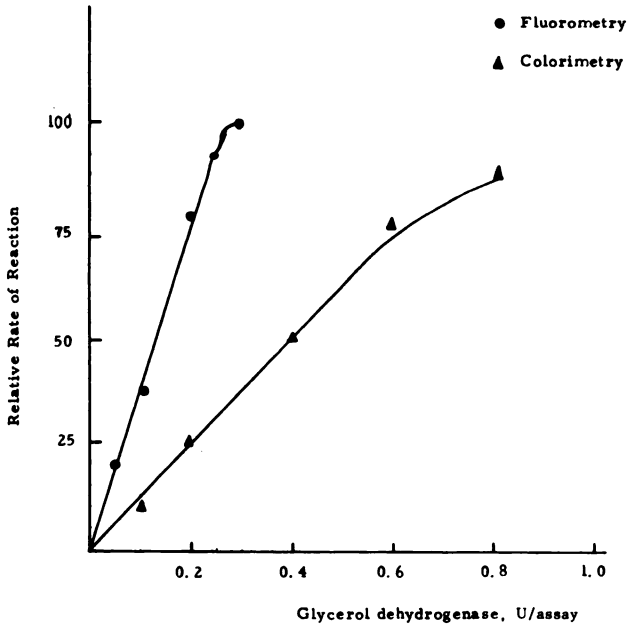


Fig. 3. Effect of glycerol dehydrogenase concentration on the reaction rate

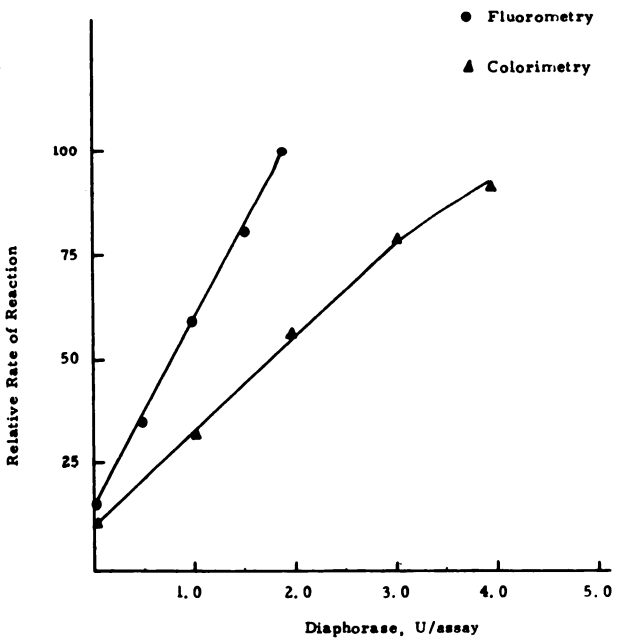


Fig. 5. Effect of diaphorase on the reaction rate

In the fluorometric method, phosphate buffer (pH 7.6) was optimum for the whole reaction sequence in the system.

Figure 2 indicates the effect of buffer concentration (ionic strength) on the reaction rate, showing that low ionic strength is favorable. This agrees with the study by Strickland and Miller (*9*) that glycerol dehydrogenase is inhibited by ionic strength.

In the present method, about 550 U of *R. arrhizus* lipase was used to hydrolyze the triglycerides in 5 μL of serum sample. A final 55-μL volume of reaction mixture was incubated for 10 min at room temperature (22 °C).

Grossman et al. (*6*) conducted the same hydrolysis study as we did, with the same lipase, and showed that 400 U of lipase is sufficient to hydrolyze completely as much as 5 g of triglycerides per liter of serum under conditions similar to ours. Figures 3–6 illustrate the effects of glycerol dehydrogenase, $NAD^+$, diaphorase, and resazurin concentration on the reaction rate. Per assay, 0.25 U of glycerol dehydrogenase, 0.4 μmol of $NAD^+$, and 1.5 U of diaphorase sufficed for 1.1 mL of reaction mixture. We used only 0.4 nmol of resazurin, even though the rate of reaction increases with increasing resazurin concentration, because the background fluorescence also increases owing to the presence of impurities in the resazurin.

## Colorimetric Method

Figure 7 shows the effect of pH on the reaction rate. We chose to use glycine buffer (50 mmol/L, pH 9.75). At pH values below 9.0, the reaction proceeded very slowly.

The optimum concentration of glycerol dehydrogenase, $NAD^+$, diaphorase, and INT are shown in Figures 3–5 and 8.

Per assay, 550 U of lipase, 0.8 U of glycerol dehydrogenase, 1.0 μmol of $NAD^+$, 4.0 U of diaphorase, and 0.5 μmol of INT per 1.715 mL of reaction mixture were sufficient.

## Analytical Variables

*Reproducibility and precision:* For six consecutive days we analyzed two sera containing different concentrations of triglycerides. Table 1 shows the excellent within-day and the

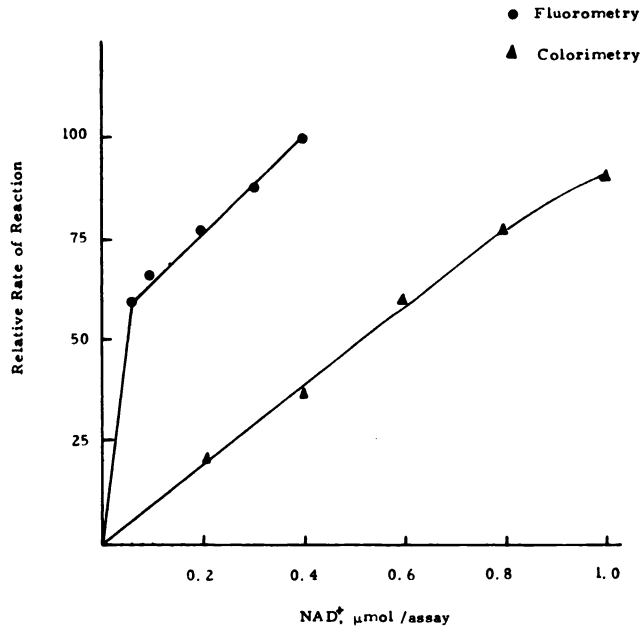


Fig. 4. Influence of $NAD^+$ concentration on reaction rate

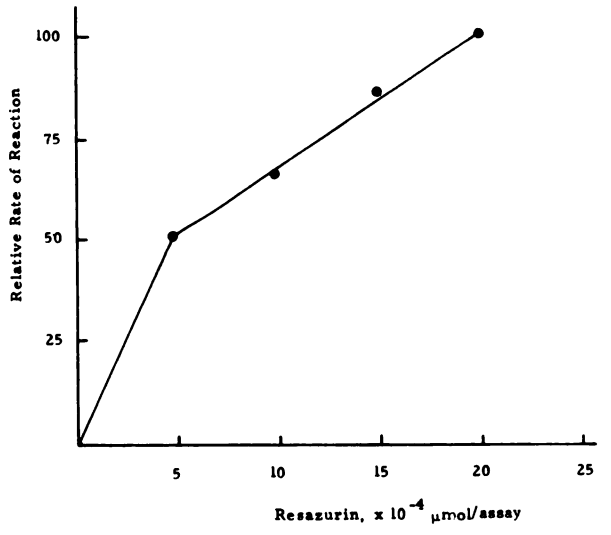


Fig. 6. Effect of resazurin concentration on the reaction rate

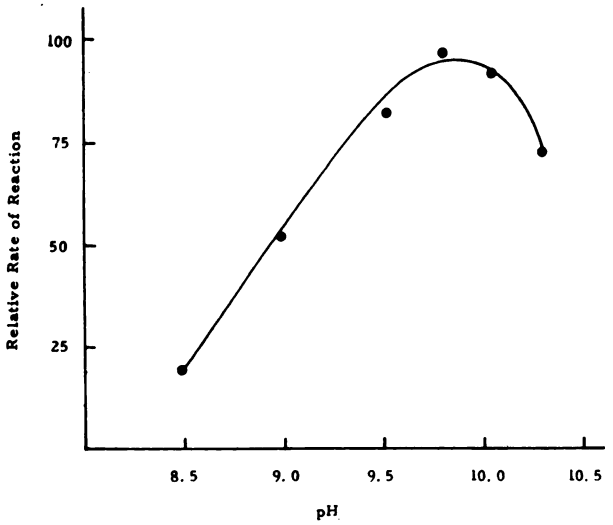


Fig. 7. Effect of pH on reaction rate in the colorimetric method

day-to-day precision for both the fluorometric and the colorimetric method.

*Recovery.* On addition of control sera to a serum that had been previously assayed, the analytical recovery ranged from 99 to 103% for fluorometry and 98 to 101% for colorimetry (Table 2).

*Effects of some other substances.* We found that surfactants such as Triton X-100 were inhibitory in both methods. Also, addition of monovalent cations such as $K^+$ or $NH_4^+$, which reportedly are activators for glycerol dehydrogenase (*10*), did not substantially alter the reaction rate in either method, so we did not include these reagents in our assay mixtures.

The following common interfering substances, added to a control serum (950 mg/L), had no notable effect (gave values within 3% of the mean for both methods) up to the concentration indicated:

| | *Concn, g/L* |
|---|---|
| Ascorbic acid | 0.2 |
| Uric acid | 0.2 |
| Bilirubin | 0.3 |
| Glucose | 2.0 |
| Ethanol | 2.0 |

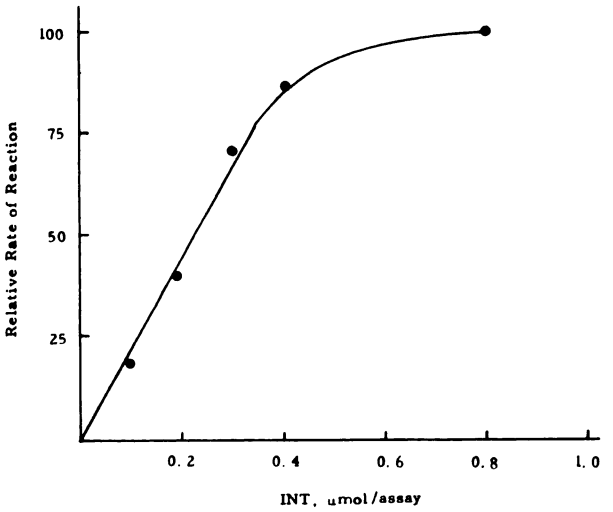


Fig. 8. Effect of INT concentration on the reaction rate

**Table 1. Precision of the Two Assays for Serum Triglycerides**

| | Fluorometry | | Colorimetry | |
|---|---|---|---|---|
| | *Within-day* ($n$ = 10) | | | |
| Mean, g/L | 0.952 | 3.807 | 0.768 | 2.613 |
| SD, g/L | 0.006 | 0.021 | 0.005 | 0.031 |
| CV, % | 0.63 | 0.55 | 0.65 | 1.18 |
| | *Day-to-day* ($n$ = 6) | | | |
| Mean, g/L | 1.208 | 3.063 | 0.879 | 2.314 |
| SD, g/L | 0.027 | 0.044 | 0.013 | 0.064 |
| CV, % | 2.24 | 1.43 | 1.47 | 2.76 |

*Effect of pH.* We investigated the rather large difference in pH optima between the fluorometric and colorimetric method. For the fluorometric method, we monitored the rate of NADH oxidation by resazurin in the presence of diaphorase only, and found that resorufin is produced much faster at a pH near 7.6 than at a higher pH. Therefore, even though the production of NADH itself, which is catalyzed by glycerol dehydrogenase, is actually favorable at higher pH, the net rate is much faster at pH 7.6 because the oxidation of NADH to $NAD^+$ in assay system by resazurin pushes to the right the reaction shown in equation 3.

In the colorimetric method, however, the rate of oxidation of NADH by INT tends to increase with increasing pH, so that the optimum overall pH is 9.75.

*Reagent stability.* The lyophilized enzymes are stable for longer than six months when stored at <0 °C (lipase and glycerol dehydrogenase) or 2 to 8 °C (diaphorase). Aqueous solutions of these enzymes maintained their activity for at least four to five days if kept frozen when not in actual use.

## Comparison Studies

We determined the triglyceride concentration of two sets of 25 fresh sera (range, 0.52 to 6.06 g/L) obtained from two local hospitals (Oschner and Charity, New Orleans, LA) by both methods, and compared the results with those obtained by a kit method that is routinely used by these hospitals (Dow Diagnostic kit method mentioned above). The linear regression analysis of triglyceride values gave coefficients of correlation of 0.999 and 0.997 for the fluorometric and colorimetric procedures. The equations for the comparison were fluorometric ($y$) = 1.007$x$ + 0.43 mg/L and colorimetric ($y$) = 0.97$x$ + 0.51 mg/L.

**Table 2. Analytical Recovery for the Two Methods**

| Triglycerides, g/L | | |
|---|---|---|
| Added | Found | Recovery, % |
| | *Fluorometry* | |
| 0 | 1.460[a] | |
| 0.450 | 1.948 | 103 |
| 0.900 | 2.430 | 102 |
| 1.800 | 3.293 | 101 |
| 3.600 | 5.009 | 99 |
| | *Colorimetry* | |
| 0 | 0.980[a] | |
| 0.375 | 1.369 | 101 |
| 0.750 | 1.747 | 101 |
| 1.500 | 2.455 | 99 |
| 3.000 | 3.900 | 98 |

[a] Independently assayed by the Dow Diagnostic method.

## Correction for Free Glycerol

Stinshoff et al. (*11*) evaluated free glycerol and triglycerides in human sera and concluded that subtracting 0.11 mmol of triglycerides per liter from the measured value for total glycerol concentration (after hydrolysis) could be justified in clinical routine. This would eliminate the necessity of measuring the free glycerol before hydrolysis (blank) and would obviate the subtraction of individual values for sample blanks. Our studies show that this suggestion is quite reasonable, but for optimum precision, all the results reported here have had individual sample-blank values for free glycerol subtracted.

We conclude that both methods proposed here are reasonably simple, easy to use, and have excellent precision, accuracy, and sensitivity. Coupling the NADH produced in reactions 1 and 2 to resazurin or INT (reactions 3 and 4) gives linear reaction rates that persist fairly long after the period of initial mixing; this allows easy calculation of triglyceride concentration and also permits adaptation to mechanized measurements. Because of the inherent simplicity of the three-step fluorometric or colorimetric sequence, compared with the previously developed four-step glycerol kinase procedure, we believe that these new procedures are a distinct improvement in current triglyceride methodology.

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