Simplified fluorometric method for the determination of plasma glycerol

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SUMMARY A simplified method for determining plasma glycerol is described. This assay utilizes the fluorometric measurement of the reduced adenine dinucleotide, $NADH_2$ which is formed when glycerol is oxidized by glycerol dehydrogenase. Only three pipettings are necessary for each reaction tube, and a large number of samples can be included in each assay.

SUPPLEMENTARY KEY WORDS glycerol · glycerol dehydrogenase · $NADH_2$

THE current method for determining plasma glycerol concentrations involves the phosphorylation of glycerol and subsequent oxidation of α-glycerophosphate with formation of $NADH_2$ (1). This double-enzyme procedure is tedious and time-consuming since not only are many pipettings necessary but one or both reactions are also usually followed to completion entailing multiple instrument readings for each tube. In 1962 a spectrophotometric method was published utilizing the direct oxidation of glycerol by glycerol dehydrogenase with generation of $NADH_2$ (2). However, this procedure has never enjoyed wide popularity, possibly because of the exact timing required (absorbance continues to increase slightly) and the limited number of reaction tubes that can be included in an assay. The adaption of this single enzyme method to a fluorometer has produced a simplified assay for plasma glycerol which is capable of handling large numbers of samples.

Material. Glycerol dehydrogenase and NAD were purchased from Sigma Chemical Co., St. Louis, Mo. Glycerol was purchased from J. T. Baker Chemical Co., Phillipsburg, N.J.

Methods: Fluorometer. An Aminco-Bowman spectrophotofluorometer was used to measure the fluorescence of $NADH_2$ with excitation at 350 nm and emission at 465 nm.

Deproteinization. 1 ml of serum or plasma is added to 2 ml of 1 N perchloric acid in a centrifuge tube; the contents are mixed and centrifuged. The supernatant is decanted and neutralized (pH 7–8) with approximately 1 ml of 2 N potassium hydroxide. The tubes are cooled in ice for 10 min to ensure complete precipitation of potassium perchlorate, and then centrifuged. The resulting supernatant is decanted and brought up to a volume of 4.0 ml with water. 1-ml aliquots are taken for the reaction tube and sample blank as described below.

Blanks. The fluorescence of the enzyme and NAD remain constant when kept separately, but there is a

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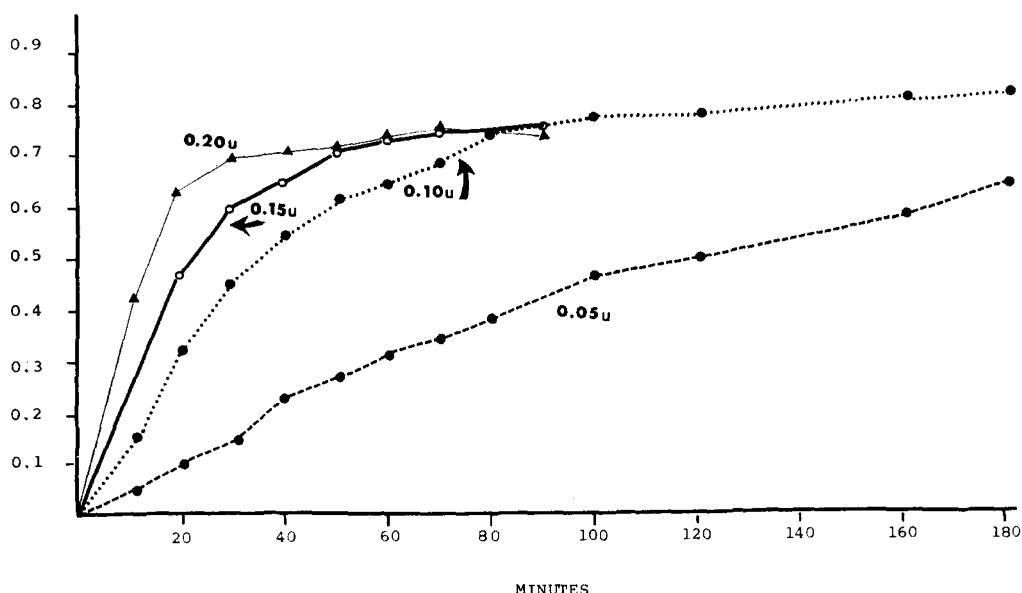

FIG. 1. Effect of glycerol dehydrogenase concentration and length of incubation on $NADH_2$ generation from glycerol. A 1 ml aliquot of deproteinized standard (0.16 μmole/ml) was incubated at room temperature for different lengths of time with a final reaction mixture containing 6 mg of NAD and different amounts of enzyme (0.05, 0.10, 0.15, or 0.20 U) in 4 ml of 0.05 M glycine buffer, pH 9.5.

small gradual increase when the two are incubated together. The resulting fluorescence is slightly greater if 1 ml of water (carried through the deproteinization procedure) is included. This increase probably reflects the presence of a small amount of contaminating glycerol in the glycerol dehydrogenase preparation. In addition to this enzyme–coenzyme blank which must be subtracted from all standard and sample readings, a sample blank must also be subtracted from the corresponding sample reading. The components of these two blanks are described in the first two columns of Table 1.

Summary of Assay. 1 ml of plasma or serum and the four glycerol standards are deproteinized as described. 1-ml aliquots are incubated at room temperature for 90 min with a final reaction mixture containing 6 mg of NAD and 0.1 U glycerol dehydrogenase[1] in a total volume of 4 ml of 0.05 M glycine buffer, pH 9.5 (Table 1, last column). The fluorescence of both blanks is subtracted from the final reading of each sample. Only the enzyme–coenzyme blank is used for the standards. The long incubation period allows the inclusion of a large number of samples in each assay, and the completeness of the reaction and stability of $NADH_2$ (Fig. 1) eliminate the necessity of exact timing.

[1] 1 U oxidizes 1 μmole of glycerol per min to dihydroxyacetone at pH 10.0 at 25°C.

Temperature. Table 2 shows that the reaction rate is essentially the same at room temperature, 30°C, or 37°C. Since fluoresence is influenced by temperature (3), we chose to perform the assay at room temperature

TABLE 1 BLANK AND SAMPLE PROTOCOL

	Enzyme–Coenzyme Blank	Sample Blank	Sample or Standard
Supernatant	—	1 ml	1 ml
Deproteinized H_2O	1 ml	—	—
NAD*	2 ml	—	2 ml
Enzyme†	1 ml	—	1 ml
H_2O	—	3 ml	—

* 3.0 mg of NAD per ml of 0.1 M glycine buffer, pH 9.5.
† 0.1 U glycerol dehydrogenase per ml of H_2O.

TABLE 2 EFFECT OF TEMPERATURE ON REACTION RATE

Temperature	30 min	60 min	90 min
Room temperature	0.415*	0.580	0.665
30°C	0.435	0.600	0.710
37°C	0.430	0.600	0.720

The reaction mixture contained 6 mg of NAD, 0.1 U glycerol dehydrogenase, and 0.04 μM glycerol in 4 ml of 0.05 M glycine buffer, pH 9.5. Tubes incubated at 30°C and 37°C were kept at room temperature for 5 min before each reading.
* Fluorescence units.

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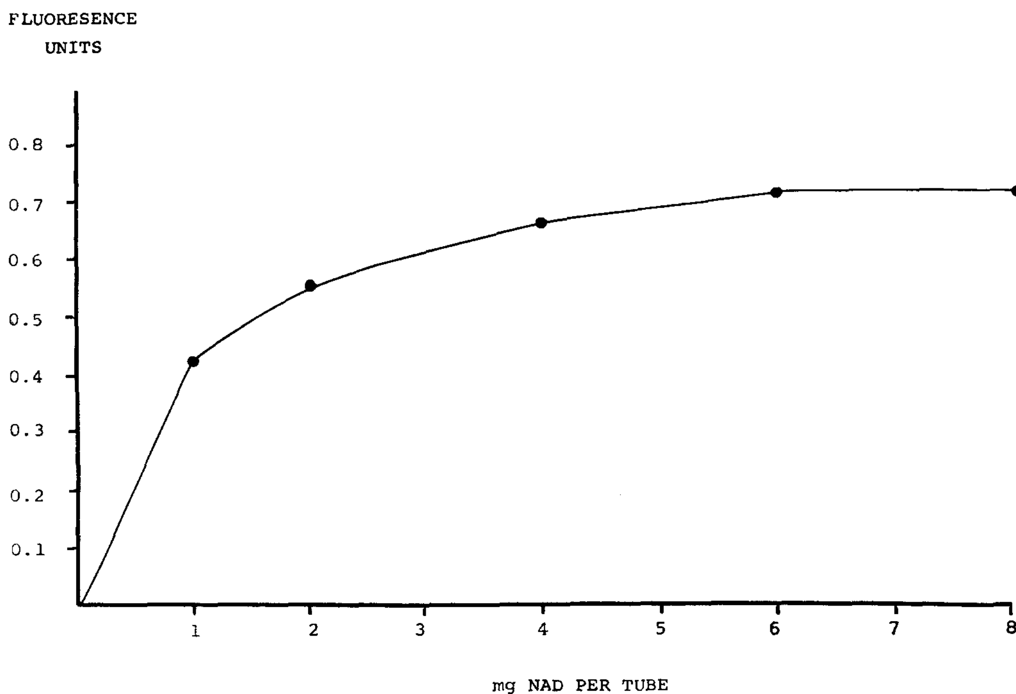

FIG. 2. Effect of NAD concentration on $NADH_2$ generation from glycerol. A 1 ml aliquot of deproteinized standard (0.16 μmole/ml) was incubated at room temperature for 90 min with a final reaction mixture containing 0.1 U of glycerol dehydrogenase and different amounts of NAD in 4 ml of 0.05 M glycine buffer, pH 9.5.

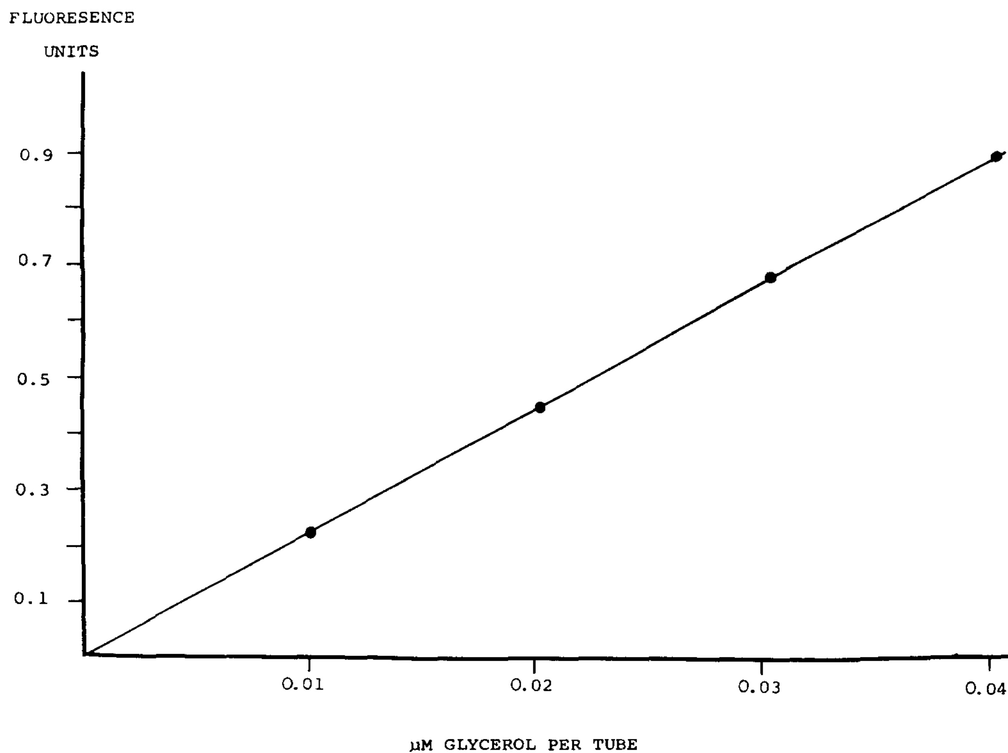

FIG. 3. Typical standard curve. 1-ml aliquots of deproteinized standards were incubated at room temperature for 90 min with a final reaction mixture containing 6 mg of NAD and 0.1 U of glycerol dehydrogenase in 4 ml of 0.05 M glycine buffer, pH 9.5.

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TABLE 3 Plasma Glycerol Response to 1 g of Intravenous Sodium Tolbutamide in Seven Young Women*

	Time in Minutes									
	0	2	5	10	20	30	45	60	90	120
Mean	0.075†	0.072	0.065	0.057	0.035	0.035	0.052	0.069	0.068	0.062
SE	0.009	0.008	0.008	0.008	0.008	0.008	0.013	0.009	0.011	0.010

* A 1 ml aliquot of deproteinized plasma was incubated at room temperature for 90 min with a final reaction mixture containing 6 mg of NAD and 0.1 U glycerol dehydrogenase in 4 ml of 0.05 M glycine buffer, pH 9.5.

† µmoles/ml.

to avoid the variable cooling of the reaction mixture between incubation and reading.

Enzyme Concentration and Time of Incubation. Fig. 1 shows that although the reaction goes to completion faster with higher concentrations of glycerol dehydrogenase, 0.1 U of the enzyme is sufficient for the reaction to be completed by 90 min. Even after 3 hr the reaction is incomplete with 0.05 U of glycerol dehydrogenase. Doubling the amount of NAD does not increase the rate with 0.05 U of enzyme (not shown).

NAD Concentration. Fig. 2 depicts the effect of NAD concentration on the reaction. Based upon this observation 6 mg of the coenzyme per tube was chosen for the assay.

Standards. To compensate for any loss of substrate during the deproteinization procedure, 1 ml of the glycerol standards (0.04, 0.08, 0.12, and 0.16 µmole/ml) is treated exactly as the unknown sample. A 1 ml aliquot of the final supernatant which is added to the reaction tubes represents one-fourth of the original amount. A typical standard curve is shown in Fig. 3.

Results: Duplication and Recovery. The mean ± SEM of 10 aliquots of a plasma sample deproteinized separately and assayed on the day of collection, was 0.076 µmoles/ml ± 0.001. When nine of these filtrates were frozen and reassayed 10 days later, the mean ± SEM was 0.079 µmoles/ml ± 0.002. The average absolute difference between a fresh filtrate and its frozen counterpart was 0.0037 µmoles/ml (range 0.005–0.0085). We routinely freeze the supernatants obtained after deproteinization and perform the assay within 2 wk. Recovery of glycerol added to plasma in six separate experiments ranged from 85 to 113% with a mean ± SEM of 99.7% ± 3.9.

Changing Glycerol Levels. The usefulness of the assay was demonstrated by its ability to discern physiological changes in glycerol concentrations. Table 3 records the expected decrease of glycerol levels during a tolbutamide[2] tolerance test in seven normal young women. A female patient with a fasting glycerol concentration of 0.075 µmoles/ml had increases of 74%, 158%, and 107% 4, 7, and 9 hr, respectively, after the intramuscular injection of 40 U of adrenocorticotropin.

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[2] Generously supplied by the Upjohn Co., Kalamazoo, Mich.

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