Enzymatic Assay of CHOLESTEROL OXIDASE
(EC 1.1.3.6)

PRINCIPLE:

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} H_2O_2 + \text{4-Cholesten-3-One}$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \xrightarrow{\text{Peroxidase}} 4\ H_2O + \text{Quinoneimine Dye}$$

Abbreviation:
4-AAP = 4-Aminoantipyrine

CONDITIONS: T = 37°C, pH 7.0, A_{500nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Potassium Phosphate Solution
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379.)

B. 100 mM Potassium Phosphate Solution
(Prepare 100 ml in deionized water using Potassium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. P-3786.)

C. 100 mM Potassium Phosphate Buffer, pH 7.0 at 37°C
(Prepare using Reagent B. Adjust to pH 7.0 at 37°C with Reagent A.)

D. 13.6 mM Cholesterol Solution
(Add 5 ml of Triton X-100, Sigma Stock No. X-100, to a 250 ml wide mouth Erlenmeyer flask and stir constantly with a stir bar while on a hot plate using the low heat settings. Add 500 mg of Cholesterol, Sigma Prod. No. C-8503, and stir constantly until all the Cholesterol is dissolved. Do not allow the Triton X-100 to yellow or boil. Once the Cholesterol is dissolved, continue stirring and gradually add 90 ml of deionized water by pipetting the water along the flask walls slowly. Cover the flask with aluminum foil and increase the heat to the high setting. Stir and allow to boil for 1 minute. Do not allow the

Cholesterol solution to boil over the top.

REAGENTS: (continued)

D. continued

The solution will be cloudy. Immediately cool under cold running water with gentle agitation to achieve a clear solution. Then add 2.6 g of Taurocholic Acid, Sodium Salt, Sigma Prod. No. T-4009. Continue to cool and mix well.

E. Color Reaction Mix (CRM)
(Prepare by adding 200 mg of Phenol, Sigma Prod. No. P-3653, to 100 ml of Reagent C. Mix thoroughly to dissolve by stirring. Then add 32 mg of 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382. Continue to mix by stirring. Transfer the solution into an amber bottle to protect from light.)

F. Cholesterol Oxidase Enzyme Solution (Cholesterol Oxidase)
(Prepare a solution containing 1 mg/ml of Cholesterol Oxidase in Reagent C. Immediately before use, dilute to 0.5 - 0.75 unit/ml with Reagent C.)

G. Peroxidase Enzyme Solution (POD)
(Immediately before use, prepare a solution containing 1900 units/ml in Reagent C using Peroxidase from Horseradish, Sigma Prod. No. P-8250.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes.

	Test	Blank
Reagent E (CRM)	2.80	2.80
Reagent D (Cholesterol Solution)	0.20	0.20
Reagent G (POD)	0.01	0.01

Mix by inversion and equilibrate to 37°C. Monitor the baseline at 500 nm. There should be no blank rate. Then add:

Reagent F (Cholesterol Oxidase)	0.02	------
Reagent C (Buffer)	------	0.02

Immediately mix by inversion and record the increase in A_{500nm} for approximately 5 minutes. Obtain the ΔA_{500nm}/minute using maximum linear rate for both the Test and Blank.

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CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\text{r } A_{500nm}/\text{min Test} - \text{r } A_{500nm}/\text{Blank})(3.03)(df)}{(0.5)(13.78)(0.02)}$$

3.03 = Total volume (in milliliters) of assay
df = Dilution factor
13.78 = Millimolar extinction coefficient of Quinoneimine Dye at 500 nm under the assay conditions
0.5 = Conversion factor based on one mole of H_2O_2 produces half a mole of Quinoneimine Dye
0.02 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will convert 1.0 µmole of cholesterol to 4-cholesten-3-one per minute at pH 7.0 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 3.03 ml reaction mix, the final concentrations are 94 mM potassium phosphate, 0.35% Triton X-100, 3.4 mM taurocholic acid, 0.9 mM cholesterol, 19.8 mM phenol, 1.5 mM aminoantipyrine, 19 units peroxidase and 0.01 - 0.015 unit cholesterol oxidase.

REFERENCE:

Allain, C.C., Poon L.S., Chan, C.S.G., Richmond, W. and Fu, P.C. (1974) Clinical Chemistry, 20, 470-475

NOTES:

1. This assay is based on the cited reference.

2. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

NOTES: (continued)

3. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin form pyrogallol in 20 sec at pH 6.0 at 20°C.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.