A U.V. ASSAY FOR SERUM TRIGLYCERIDES USING ENZYMATIC HYDROLYSIS AND ENZYMATIC QUANTIFICATION OF GLYCEROL WITH A CENTRIFUGAL FAST ANALYZER

by

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INTRODUCTION

The analytical measurement of serum triglycerides poses more difficult technical problems than that of serum cholesterol. Before describing the historical development of serum triglyceride analysis, it is appropriate to discuss three techniques which measure the entire lipoprotein: ultracentrifugation, lipoprotein electrophoresis, and nephelometry.

ULTRACENTRIFUGATION

The ultracentrifugation technique offers a high degree of resolution for fractionating various lipoproteins (1). When sodium chloride with the density of 1.063 kg/liter is mixed with a serum specimen and is centrifuged in a preparative ultracentrifugation, the molecules less dense than 1.063 kg/liter undergo floatation. The lipids and lipoproteins that float on the top of the serum with sodium chloride with density of 1.063 kg/liter are referred to as the light density lipoproteins (LDL). After the top fraction is pipetted off and it is further subjected to an analytical ultracentrifugation technique as originally described by Svedberg, the low density lipoproteins (LDL) can be sub-classified according to their floatation rates into five Svedberg floatation classes: S_f 0 - 10, S_f 10 - 20, S_f 20 - 100, S_f 100 - 400, and S_f 400 - 1000 (chylomicrons). One Svedberg unit is defined as 1×10^{-13} cm/sec/dyne/g.

When the lipoprotein fractions of S_f 0 - 400 are subjected to electrophoresis, they migrate with the beta-globulins; thus, these

fractions are referred to as β-lipoproteins. The S_f 0 - 400 lipoproteins are conveniently grouped into two categories: (i) low density lipoprotein (LDL) or β-lipoprotein of cholesterol-bearing fraction (S_f 0 - 20); and (ii) very low density lipoprotein (VLDL) or pre-β-lipoprotein of glyceride-bearing fraction (Sf 20 - 400). These two classes respond differently to nutritional and pharmacological manipulation. The alpha-1 and alpha-2 lipoproteins which migrate with the alpha-globulins have densities between 1.063 and 1.210, and they are called the high density lipoproteins (HDL).

Gofman et al (1) used the ultracentrifugal floatation tehcnique to demonstrate the direct relationship between the amount of S_f 10 - 20 lipoprotein in serum and the incidence of atherosclerosis, but the S_f 5 - 8 class had no correlation with the development of atherosclerosis. The alimentary lipidemia showed no affect on the concentration of the S_f 10 - 20 class; however, those of S_f 40 and greater were markedly increased.

The ultracentrifugation technique is too time-consuming, cumbersome, and costly for the routine clinical workup of patients. However, the ultracentrifugal technique became a reference method to standardize and demonstrate the validity of the electrophoresis technique and the hyperlipoproteinemia classification system as described by Fredrickson, Levy and Lees (2).

LIPOPROTEIN ELECTROPHORESIS

Fredrickson and Lees (3) classified hyperlipoproteinemia into

five major classes. This phenotyping technique, based primarily upon lipoprotein electrophoresis and determinations of serum cholesterol and triglycerides, has gained a wide popularity and proven to be a sine qua non in establishing the relationship between abnormalities of lipid metabolism and the incidence of atherosclerotic heart disease as well as other pathological conditions. In 1970 the World Health Organization prepared a memorandum (4) outlining a system of classifying the different types of hyperlipoproteinemia. The purpose of this system was to aid in the diagnosis and therapy of hyperlipoproteinemia and to help in the understanding of the genetic, etiological and epidemiological factors that relate to hyperlipidemia. The classification of hyperlipoproteinemia is basically that of Fredrickson, Levy and Lees (2).

Type I (hyperchylomicronemia) in this classification system is characterized by an enormous amount of chylomicrons with normal to slightly increased amount of VLDL (pre-β-lipoproteins) accompanied with increased cholesterol and triglycerides (Chol/TG ratio of less than 0.2 or sometimes less than 0.1). Serum samples which have stood for 16 to 24 hours at 0 - 4°C show a creamy layer above a clear serum. Most chylomicrons are exogenous triglycerides. Type V (hyper pre-β-lipoproteinemia with hyperchlomicronemia) is similar to Type I, but in addition the VLDL (pre-β-lipoproteins) is increased. The Chol/TG ratio is greater than 0.15 with increased cholesterol and triglycerides. A lipoprotein electrophoresis reveals an increased pre-β band with the concominant presence of chylomicrons. Clinical signs may include

erruptive xanthomas, pancreatitis, or abdominal pain in both Types I and V .... all of which may indicate severe hyperglyceridemia.

Type II (hyper-β-lipoproteinemia) has abnormally increased LDL (β-lipoproteins). Type II has been sub-classified into IIa and IIb. Type IIb patients have increased VLDL (pre-β-lipoproteins) and LDL (β-lipoproteins), while in Type IIa, VLDL (pre-β) is normal. The cholesterol level is usually increased and the triglyceride level is usually normal. The Chol/TG ratio of greater than 1.5 is found in Type IIa; however, in Type IIb, both lipids are increased with a variable Chol/TG ratio. Tendon, tuberous and planar xanthomas are characteristic clinical features. Xanthelasma also occurs frequently in Type II.

Type III ("floating β ", "broad β " or "β-VLDL") shows a β-band and pre-β-band appearing in a broad band extending from the β area into the pre-β area. The serum cholesterol and triglyceride levels are usually elevated. The Chol/TG ratio of about 1.0 is frequently found, but it may vary from 0.3 to greater than 2.0. Tuberous xanthomas, "tubero-eruptive" lesions, planar xanthomas on the palms of the hands, and xanthelasma can be seen in Type III patients.

Type IV (hyperpre-β-lipoproteinemia) has a characteristic increase in VLDL (pre-β-lipoproteins) in which triglycerides are greatly increased with normal or increased cholesterol. The Chol/TG ratio is variable. Electrophoresis shows a distinct pre-β band with diminished β-band. Type IV hyperpre-β-lipoproteinemia is often found in patients

with diabetes.

The relationship between various lipid levels and predisposition to atherosclerotic heart disease has received intensive study (5,6,7,8, 9,10, and 11). Abnormally elevated serum triglycerides were also found in patients with fat-induced and carbohydrate-induced hyperlipoproteinemia, the nephrotic syndrome, diabetes mellitus, fatty infiltration of the liver, and certain of the glycogen storage disease (2,4 and 12). Among five classes of hyperlipoproteinemia, Types II, III, and IV patients have been shown to have a high incidence of atherosclerosis. Patients with Type II and IV are commonly found; however, those with Type I and V are rare (Table 1).

Katchman and Zipt (13) showed that serum glutamic-pyruvic transaminase (L-Alanine: 2-oxoglutarate aminotransferase, E.C.2.6.1.2) was increased along with serum triglycerides in certain individuals while alkaline phosphatase, SGOT and LDH remained normal. This elevation was hypothesized to be a response to metabolic stress induced by high-lipid diets.

A therapeutic armamentarium of dietary management, drug therapy and the last, but rather effective, alternative using surgical bypass of the small bowel has been aggressively used to lower serum lipids. A surgical ileal bypass is fully discussed by Buchwald (14) and this technique offers an excellent response by lowering the serum lipoproteins in Types II, III, and IV persons. A nutritional therapy is most effective in lowering Type I hyperlipoproteinemia; however, ineffective for Type II. Fredrickson (15) extensively reviewed the use and the

effect of four drugs (clofibrate, cholestyramine, D-thyroxine, and nicotinic acid) to treat patients who have hyperlipidemia. Clofibrate produces good responses in patients who have Types III, IV, and V. This drug interferes with cholesterol synthesis by impeding the conversion of acetate to mevalonate. Cholestyramine, an anion exchange resin, binds bile acids so that they are not reabsorbed through the intestine. Consequently this increase catabolism of cholesterol. Cholestyramine lowers serum cholesterol and β-lipoprotein levels of Type II hyperlipoproteinemia patients. Fallon and Woods (16) reported that fourteen patients with Type II hyperlipoproteinemia showed serum cholesterol levels lowered by 24 per cent; however, there was no significant decrease in serum cholesterol in other types of hyperlipoproteinemia and the drug had no effect on serum triglyceride values. Buchwald (14) reported that cholestyramine was one of the effective drugs used to lower serum cholesterol in Type II patients, but the ileal bypass achieved an average of 40 per cent reduction of serum cholesterol level. He recommended the use of clofibrate with or without cholestyramine to lower Type III hyperlipoproteinemia, while clofibrate alone for Type IV patients. D-thyroxine lowers cholesterol by catabolism of cholesterol, but it does not lower hypertriglyceridemia. Nicotinic acid impedes the release of free fatty acids from adipose tissue; thus it lowers the serum triglycerides levels. Table 1 summarizes the criteria used to phenotype and to classify five major hyperlipoproteinemias, their relationship with the hypercholesterolemia and hypertriglyceridemia, and the response to a therapeutic management using

Table 1. Classification of Hyperlipoproteinemia, Clinical Manifestation and Therapeutic Managements.

Type	Other name	Increased lipoprotein	Analytical centrifuge	Appearance of serum	Frequency	Affected age groups	Causes	Cholesterol	Triglyceride	Chol/TG ratio	PHLA*	Glucose tolerance	CHO sensitive	Atherosclerosis	Diet Therapy: Diet	Diet Therapy: Diet Response	Drug Therapy: Drug	Drug Therapy: Drug Response	Surgical response to ileal by-pass	Clinical features
I	hyperchylomicronemia	chylomicrons ↑	S_f 100-400 ↑	creamy top layer over clear serum	rare	early childhood	genetic, defective lipoprotein lipase activity	↑	↑↑↑ >1000mg/100ml	<0.2 or <0.1	↓	N	no	no	low fat	excellent	-	-	-	Eruptive xanthomas, pancreatitis, white retinal vessels, hepatosplenomegaly, abdominal pain
II IIa	hyper-β-lipoproteinemia	↑↑↑ β, N pre-β	S_f 0-12 ↑↑↑	clear or slightly opalescent	common 40%	early childhood (in severe case)	dominant gene, sporadic	↑↑↑	N	>1.5	N	N	no	yes	low chol., low sat. fat	poor	cholestyramine, choloxin, clofibrate	poor	excellent	Tendon, tuberous and palmar xanthomas, xanthelasma, arcus corneae (arcus senilis), ischaemic heart disease, vascular accidents
II IIb		↑↑↑ β, ↑↑ pre-β	S_f 0-12 ↑↑↑, S_f 12-400 ↑↑					↑↑↑	↑	variable										
III	floating β broad β β-VLDL	broad β-band	S_f 12-100 ↑↑, S_f 0-12 ↓	clear or turbid	relatively uncommon	>20 year old	recessive gene?, sporadic	↑↑ (variable)	↑ variable 175-1500mg/100ml	1.0	N	Abn	yes	yes	low CHO, low chol., low sat. fat	fair to good	clofibrate, cholestyramine, choloxin, oral hypoglycemics	fair to good	excellent	Tuberous xanthomas, "tuberoeruptive" lesions, palmar xanthomas, xanthelasma, hyperuricemia
IV	hyperpre-β-lipoproteinemia	↑↑↑ pre-β	S_f 12-400 ↑↑↑	turbid	frequent	adulthood	genetic, sporadic, increase endogenous glyceride synthesis, deficient in glyceride removal	↑	↑↑↑ (200-5000mg/100ml)	variable	N	Abn	yes	yes	low CHO, low chol., low sat. fat	fair to good	clofibrate, oral hypoglycemics	fair to good	excellent	Xanthomas, accelerated vascular accidents, hyperuricemia, exophytic subcutaneous xanthomas, diabetes.
V	hyperchylomicronemia and hyperpre-β-lipoproteinemia	chylomicron ↑↑ pre-β	S_f 20-400 ↑	creamy top layer with turbid serum	rare	early adulthood	genetic?	↑	↑↑	>0.15	N	Abn	yes	?	low fat, low chol., low CHO	fair to good	clofibrate, oral hypoglycemics, cholestyramine	fair to good	-	Same as Type I, hyperuricemia

* PHLA = plasma heparin lipase activity; ↑, increase; ↓, decrease; N, normal; and Abn, abnormal.

diet, drugs and partial ileal bypass (14 and 15).

NEPHELOMETRY

The nephelometric measurement of lipoproteins has recently gained popularity so that the analytical technique and principle are briefly discussed below. Nephelometric technique differs from turbidimetry. In nephelometry, light that is scattered at a right angle to the incident light is measured, while the turbidimetric measurement is made on transmitted light. The number and size of particles in the light beam are directly related to the amount of scatter.

Beta-lipoproteins may be quantified by complexing the lipoprotein with a polyanionic macromolecule such as mucopolysacchride and measuring the amount of precipitate by nephelometry. Bernfeld (17) used a finely-dispersed precipitate of β-lipoprotein with sulfated amylopectin at pH 8.6. The use of dextran sulfate is reported by others (18). A turbidimetric measurement of the precipitated β-lipoprotein is reported by several authors. Boyle and Moore (19) used K-agar which is a sulfated D-galactopyranose as a precipitating agent. The use of polyvinylpyrrolidone is also reported (18). Berenson et al (20) reported the use of heparin in the presence of calcium ion to yield insoluble complexes with low-density lipoproteins. They showed that measurement of pre-β plus β-lipoprotein concentrations might be more useful in screening for subtle abnormalities than the cholesterol or triglyceride determination alone. However, as in the fluorometric methods the

main source of error is the interference due to turbid substances in the sample and contaminants in the test tube. Fibrinogen has been known to produce a turbidity with sulfated amylopectin (17).

Nephelometric analysis of diluted serum before and after ultrafiltration for measuring pre-β-lipoproteins and chylomicrons was reported by Werner et al (21). Buckley et al (22) reported the use of MF12 filters (pore size of 0.05 μ) to remove chylomicrons from non-fasting specimens before measuring light scattering of the S_f 20 - 400 lipoprotein fraction. In this technique, there was a direct correlation between the total glycerides in the serum sample and the amount of light scattered at 650 nm. Helman et al (23) described a similar technique using two separate filtrations of the serum sample: the filtrate from a 0.45 μM filtration to determine chylomicrons and very low density lipoproteins (VLDL) and the filtrate from a 0.05 μM filtration to estimate triglyceride-containing VLDL. Nephelometric analysis is an indirect way of estimating serum triglycerides and suffers from the disadvantage that anything that scatters light will be measured as triglycerides.

ANALYSIS OF SERUM TRIGLYCERIDES

Many different analytical methods for the measurement of triglyceride have been developed. Determinations of plasma or serum triglycerides were devised to either measure the triglyceride molecule as a whole, or some part of it: the esterified fatty acids, the ester-

carbonyl bond, or the glycerol.

Triglycerides may be assayed by one of eight methods: 1) gravimetry, 2) infrared spectrophotometry, 3) gas-liquid chromatography, 4) TLC-charring, 5) nephelometry, 6) colorimetry, 7) fluorometry, or 8) enzymatic analyses.

The later four triglyceride methods are relatively simple, rapid, and reliable so that they are commonly used in clinical chemistry.

Indirect Methods for Measuring Total Fatty Acids

Indirect methods for estimation of triglyceride are based upon measuring total esterified fatty acids and then subtracting from that value the amount of fatty acids found in cholesterol esters and phospholipids.

In 1947 Bloor introduced a colorimetric oxidative dichromate method, which was later modified to a micro-procedure by Bragdon (24). In this method the extracted cholesterol and fatty acids reduce dichromate and the amount of dichromate reduced was measured photometrically. The oxidation of the lipids was 95 per cent complete (24). According to a titrimetric version of the dichromate method, the lipids were oxidized with a known amount of dichromate-sulfuric acid mixture and the unconsumed dichromate was titrated (25 and 26). Since cholesterol also reduces dichromate a correction must be made for this interference.

Titrimetric measurement of total esterified and non-esterified fatty acids utilizing alkali and thymol blue was reported by Dole and Meinertz (27).

The hydroxamic acid method is based upon the reaction of carboxylic acid esters of fatty acids with hydroxylamine to form hydroxamic acids, which then react with ferric ions to yield red colors (28 and 29). Various colored complexes were formed at different temperatures and pHs, with different solvents, and with different molar ratios of reactants. These factors all result in instability of the color (30). Cholesterol and large fatty acid molecules also impede the reaction with hydroxylamine (31).

Antonis (32) and Dirstine et al (33) described a method in which soluble copper soaps were formed from the fatty acids. The copper ion from the soap was subsequently complexed with diethyldithiocarbamate to yield a color.

Since the plasma triglycerides normally amounts to approximately ten per cent of the total lipids, methods using the indirect technique suffer from inaccuracy and poor precision because the errors of separate determinations are compounded in the final estimation of triglyceride. These indirect methods are also arduous and time-consuming to perform.

DIRECT METHOD OF MEASURING TRIGLYCERIDES

Amenta (34) proposed a direct measurement of each lipid component with acid dichromate solution. Various lipid components were first separated by thin-layer chromatography, then the reduction of dichromate by the specific lipid was quantified at 350 nm. Quantification by gravimetric analysis in conjunction with TLC is thoroughly reviewed by Privett et al (35).

Henry (18) recommended the gravimetric method of Sperry and Brand (26) as the method of choice in measuring the total serum lipids. The major difficulties for measuring lipid constituents by this technique are the requirement of a large amount of sample and the presence of organic impurities in the adsorbent on the TLC plate.

Colorimetry

In clinical chemistry, the early impetus in developing direct triglyceride assays was with colorimetric methods: phenylhydrazine-ferricyanide, chromotropic acid, and Hantzsch condensation. Methylbenzothiazolone hydrazone (MBTH) was introduced recently.

Analytical methods utilizing quantitative measurement of the liberated glycerol require extraction of triglyceride from biological samples, removal of phospholipids, and either acid or alkaline saponification of triglyceride. Randrup (36) and Galletti (37) modified and adapted the phenylhydrazine-ferricyanide method in which the liberated glycerol was oxidized by periodic acid to formaldehyde, which was then reacted with phenylhydrazine, potassium ferricyanide and concentrated hydrochloric acid at 0°C to give a red color (1,5-diphenylformazan). Galletti (37) used sulfuric-acetic acid instead of concentrated HCl to increase sensitivity and color stability. However, the red dye product has been reported to be unstable and the phenylhydrazine reagent to be questionable in purity (38).

A relatively simple, sensitive, reliable, and inexpensive colori-

metric technique is the chromotropic acid (1,8-dihydroxynaphthalene-3, 6-disulfphonic acid) method. Lambert and Neish (39) reported this method in which formaldehyde is generated using the same steps as the preceding method. MacFadyen's (40) procedure for quantifying formaldehyde with chromotropic acid was then used.

A widely used micro-colorimetric method, essentially as described by Lambert and Neish, was introduced by Van Handel and Zilversmit in 1959 (41). The method involves chloroform extraction of lipids, removal of phospholipids by Doucil (zeolite), alcoholic KOH saponification, oxidation of glycerol with periodate to formaldehyde, stopping oxidation by arsenite, and color development with chromotropic acid in sulfuric acid at 100°C for 30 minutes.

Carlson and Wadström (42) used column chromatography with activated silicic acid to remove phospholipids. The triglycerides were then eluted with chloroform-methanol. However, the Doucil-chloroform extraction and purification of Van Handel and Zilversmit are simpler and give a better yield than this column chromatographic technique of Carlson and Wadström.

Leffler (43) used 99 per cent isopropanol to precipitate serum proteins and to extract total cholesterol. Laurell (44) showed that 5 per cent ethanol in isopropyl-ether gave a rapid and complete extraction of triglyceride when activated silicic acid was used to adsorb phospholipids. A semiautomated chromotropic acid method was published by Lofland (45) in which isopropanol was used to extract triglycerides and zeolite to remove phospholipid as described by Leffler (43). Both

cholesterol and triglyceride may be determined using the same isopropanol extract. However, Lofland failed to remove glucose by copper-lime reagent as described by Kessler and Lederer (46) and Timms et al (47).

According to Ryan and Rasho (48), the use of ether-isopropanol extract with florisil to remove phospholipids gave consistently higher triglyceride results than the use of extraction-purification methods of chloroform-zeolite or isopropanol-zeolite mixtures.

Butler et al (49) adapted Van Handel and Zilversmit method for tissue triglyceride.

One major drawback for both chromotropic acid with sulfuric acid and phenylhydrazine-ferricyanide with HCl when carried out without extraction is the interference from serum constituents. Suehiro and Nakanishi (50) reported and it was subsequently confirmed by Levy and McGee (51) that increasing amounts of serum added to the chromotropic acid assay mixture inhibited the color development as much as 27 per cent. Levy and McGee further showed that a color inhibition up to 40 per cent was observed when increasing amounts of bovine serum albumin were added to formaldehyde and when deproteinization was performed just prior to color development. However, when deproteinization of the assay mixture was carried out just prior to the oxidation of glycerol to formaldehyde, color development was not affected. They concluded that formaldehyde was bound to protein which would consequently prevent formaldehyde from reacting with chromotropic acid.

The conventional non-extraction chromotropic acid method utilizing acid or alkaline saponification of serum triglyceride would denature protein so that protein would be unavailable for formaldehyde binding. Therefore, it is reasonable to assume that if the enzyme hydrolysis of triglyceride was carried out instead of alkaline saponification, the subsequent measurement of the liberated glycerol with chromotropic acid should require deproteinization prior to oxidation of glycerol to formaldehyde as recommended by Levy and McGee.

Nash (52) reported that formaldehyde from bacterial suspensions produced a yellow color when acetylacetone and ammonium salts were present. The yellow compound was found to be 3,5-diacetyl-1,4-dihydrolutidine which is produced from 1,4-pentanedione, ammonia, and an aldehyde *via* a Hantzsch condensation reaction. The Hantzsch condensation product is quantified either colorimetrically or fluorometrically, and all the methods using this reaction are discussed in the fluorometric section. Neeley *et al* (53) introduced a new automated colorimetric method in which formaldehyde was reacted with the primary amino group of 3-methyl-2-benzothiazolone hydrazone (MBTH) producing azide. MBTH was then oxidized by ferric chloride to yield the cation, which reacted with the azide giving a highly resonant compound. This compound has a molar absorptivity of 65,000 at 670 nm, while the molar absorptivity of 3,5-diacetyl-1,4-dihydrolutidine is 8,000 at 412 nm (52).

Fluorometry

Chelation of quinoline derivatives and Hantzsch condensation

methods are among the fluorometric techniques used to quantify triglyceride. Mendelsohn and Antonis (54) described the synthesis of quinoline derivatives in which liberated glycerol was heated with o-aminophenol, concentrated sulfuric acid, and arsenic acid to produce 8-hydroxyquinoline, which, in the presence of divalent metal ion in alkaline solution, yielded the fluorescent product.

The most commonly used fluorometric method in the clinical chemistry is the automated Hantzsch condensation method. Belman (55) utilized the fluorometric version of Hantzsch condensation reaction between an amine, a beta-diketone, and an aldehyde. Kessler and Lederer (46) popularized a semiautomated Autoanalyzer method in which isopropanol extracts were prepared manually, the phospholipids were adsorbed on zeolite and the glycerides in the phospholipid-free extract were saponified to free glycerol. The zeolite mixture also contained Lloyd reagent to remove bilirubin and other chromogenic substances, and copper-lime (copper sulfate and calcium hydroxide) to remove glucose from the serum samples. The liberated glycerol was then oxidized to formic acid and formaldehyde. The formaldehyde was condensed with 2,4-pentanedione and ammonium ion to give a fluorescent product, 3.5-diacetyl-1,4-dihydrolutidine.

Cramp and Robertson (56) modified the semiautomated method of Kessler and Lederer by reducing reagent concentrations and volumes to improve the Autoanalyzer flow pattern. A blank determination was also eliminated to simplify the assay system. This simplification did not

cause significant error unless the glucose was incompletely removed from the extracts or high concentration of free glycerol (200 μmol/liter or more) were present in the serum sample.

Royer and Ko (57) reported a simplified extraction procedure of triglyceride for use with the semiautomated fluorometric method of Kessler and Lederer. Nonane, isopropanol, and H_2SO_4 were mixed with plasma and vigorously shaken for 20 seconds by Vortex mixer and the nonane phase was used for automated anaylsis. In this system, no adsorption of phospholipids on silicic, Florisil and zeolite (Doucil) was necessary.

The reagent base line signal was stabilized to within 1 per cent of full scale or less and uniform bubble pattern was achieved by incorporating PC-1 pulse chambers, a jacketed H_3 fitting and standard pump tube suppressor (58). Noble and Campbell (59) also minimized the unsteady base line by mixing isopropanol and KOH in a single mixing coil to avoid deterioration of tubing which caused $\pm$ 10 per cent variation in delivery of the saponification reagent. These authors also used smaller pump tubes to reduce volume of reagents, to decrease flow rate, and to minimize axial streaming or turbulence. All of these modifications improved accuracy and precision of the determinations.

Fletcher (60) used a colorimetric, Hantzsch reaction, method in which isopropanol was not evaporated before saponification and a low concentration of periodate was used. Arsenite was not needed to oxidize excess periodate, since low levels of periodate were used. A

semiautomated adaptation of Fletcher's manual method was published by McLellan (38).

The acetylacetone reagent containing ammonium salt used for the Hantzsch reaction was known to be unstable and rapidly developed a yellow color on standing. Foster and Dunn (61) separated the mixing of acetylacetone with ammonium buffer as described by other authors. When the acetylacetone reagent was prepared in water-isopropanol containing no ammonia, neither yellow color nor deterioration were detected. Thus, the acetylacetone reagent was stable for about six months at room temperature.

Soloni (62) utilized alkoxide (ethoxide) transesterification technique after triglycerides were extracted by nonane-isopropanol-H_2SO_4 solvent. This eliminated the phospholipid adsorption on silicic acid as described by Royer and Ko (57). After transesterification chloroform was added and glycerol in the aqueous H_2SO_4 layer was measured colorimetrically or fluorometrically using the Hantzsch condensation reaction.

Infrared Spectrophotometry

The lipids were initially extracted with methanol and ethyl ether, column chromatographed with silicic acid - celite to elute three lipid fractions: fraction I being cholesteryl esters; fraction II being glycerides, unesterified fatty acids, and esterified fatty acids, and unesterified cholesterol; and fraction III being phospholipids. These three fractions were subsequently analyzed by a sodium chloride prism

infrared adsorption spectrometer (63 and 64). Freeman (65) reported a simultaneous measurement of cholesterol esters and triglycerides using an infrared spectrophotometer. The removal of phospholipids was accomplished by adsorption on silicic acid. The chloroform-methanol extracts of cholesterol esters and triglycerides were measured at 1730 cm^{-1} and 1745 cm^{-1}, respectively.

Krell and Hashim (66) described a method in which the extracted lipids were first chromatographed on thin-layer and then quantified by measuring the 1742 cm^{-1} ester carbonyl C=O stretching vibration.

Thin-layer-chromatography (TLC) - Charring Methods

The lipid constituents separated from a complex biological lipid mixtures by TLC can be charred with dichromate-sulfuric acid by heating and subsequently the charred spots can be quantified by densitometry (67). Privett et al (35) extensively reviewed recovery techniques after TLC separation and the principal lipid detection techniques utilizing dyes, charring and isotope methods. Louis-Ferdinand et al (68) compared the above dichromate-sulfuric acid charring method with established chemical lipid analyses. Marsh and Weinstein (69) and Marzo et al (70) used a chromatographic technique to separate lipid classes, which were then quantitatively analyzed by carbonizing with concentrated H_2SO_4 and measuring the eluted-carbonized color at 375 nm.

Silver nitrate-impregnated silica gel was used to separate triglycerides with varying degrees of unsaturation. The basis of this separation is the formation of a complex between silver ion and olefinic

bonds. Separations could also be obtained based on chain length and cis-trans isomerisms (71 and 72). Pelick et al (72) extensively reviewed some practical aspects of this technique including the effect of calcium sulfate as a binder, various systems of TLC, and some of the pitfalls and precautions.

TLC - Molybdate Method

Chedid et al (73) proposed TLC separation of lipids followed by staining lipid constituents with phosphomolybdic acid and perchloric acid, which were subsequently quantified by a densitometer.

Gas-liquid-chromatography (GLC)

Horrocks and Cornwell (74) described a simultaneous quantification of glycerol and fatty acids of triglycerides by gas-liquid chromatography. The triglycerides were initially converted by hydrogenolysis with lithium aluminum hydride to lithium aluminum alcoholates and free glycerol which were then acetylated with acetic anhydride to yield glyceryl triacetates and fatty alcohol acetates. The corresponding acetate esters were quantitatively analyzed by gas-liquid-chromatography (GLC) with known concentration of internal standard. Free glycerol could be directly acetylated with acetic anhydride to form glyceryl triacetate for GLC quantification (75 and 76).

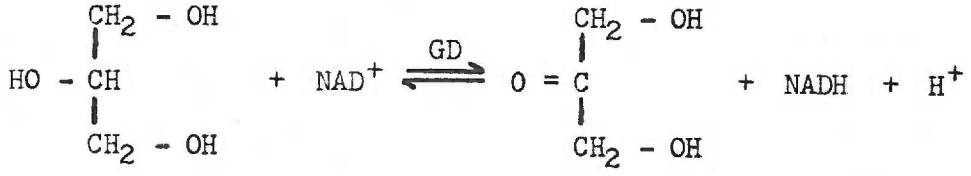
The transmethylation of triglycerides with methanol and sulfuric acid to form fatty acid esters for GLC technique was also reported

(77 and 78). Saifer and Goldman (79) used methylation of fatty acids on an anion exchange resin, 1RA-400. The chromatographic procedures, however, are poorly suited for the routine handling of large numbers of samples.

Enzyme Assays

After hydrolysis or saponification of triglyceride, the liberated glycerol can be enzymatically assayed by coupling with one of the following enzyme systems: (1) glycerol dehydrogenase as described by Burton and Kaplan in 1953 (80); (2) glycerokinase-pyruvic kinase-lactic dehydrogenase (GK-PK-LDH), Kreutz, 1961 (81 and 82); or (3) glycerokinase-glycerophosphate dehydrogenase (GK-GDH), Bublitz and Kennedy, 1954 (83 and 84).

Burton and Kaplan (85) partially purified glycerol dehydrogenase (glycerol:NAD^+ oxidoreductase, E.C.1.1.1.6) from Aerobacter aerogenes, which reduces NAD^+ in the presence of glycerol.



glycerol dihydroxyacetone

Lin et al (86) measured the production and the activity of glycerol dehydrogenase of Aerobacter aerogenes. The activity of this enzyme decreased during the aerobic metabolism of carbon compounds and

increased during anaerobic conditions.

Hangen and Hagen (87) used this enzyme to measure plasma glycerol which was produced by various hormonal stimuli. There was a slow reduction of NAD^+ by the enzyme in the absence of glycerol. This blank rate was subtracted from the sample readings.

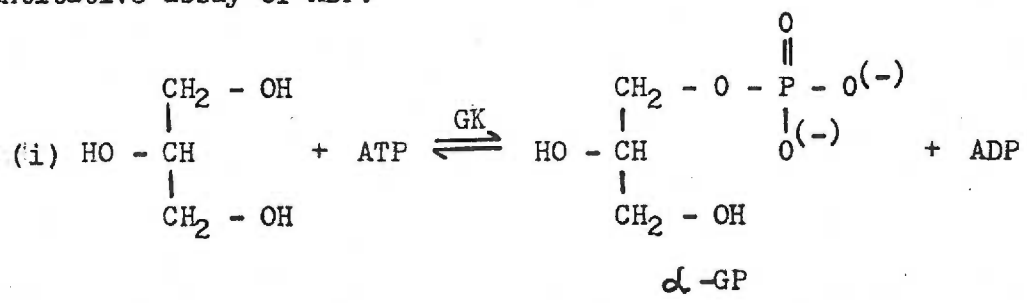
Strickland and Miller (88) reported that this enzyme was inhibited by high ionic strength and dihydroxyacetone concentrations above 0.4 mmol/liter, by lithium, sodium, and zinc, and strongly by 8-quinolinol and α,α-dipyridyl.

The enzyme does not have a high specificity for glycerol. According to Burton (85), 1,2-propanediol and 2,3-butanediol are oxidized at the same rate as glycerol by the glycerol dehydrogenase, and the following compounds are also oxidized: 1,3-propanediol at 37 per cent of the rate of glycerol, ethylene glycol at 20 per cent, 1,4-butanediol at 17 per cent, isopropanol at 17 per cent, i-inositol at 18 per cent and glycerol phosphate at 11 per cent. Strickland and Miller (88) reported that the affinity of this enzyme is greater for 1,2-propanediol than for glycerol.

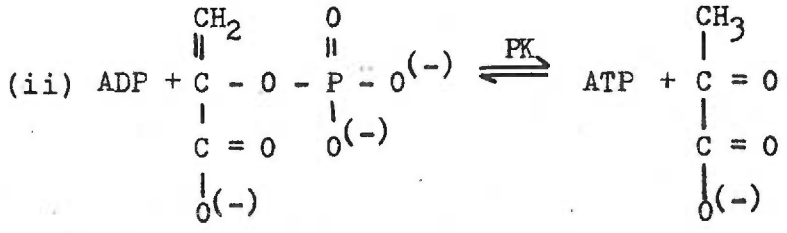
Hagen (89) reported that ethanol and glyceraldehyde-3-phosphate would also reduce NAD^+; however, glucose, lactate, malate, α-hydroxybutyrate, succinate, glutamate, α-glycerophosphate and α-oxoglutarate would not.

Therefore, the use of glycerol dehydrogenase for the assay of glycerol was abandoned since this enzyme will catalyze the oxidation of many polyhydroxy compounds as reported above.

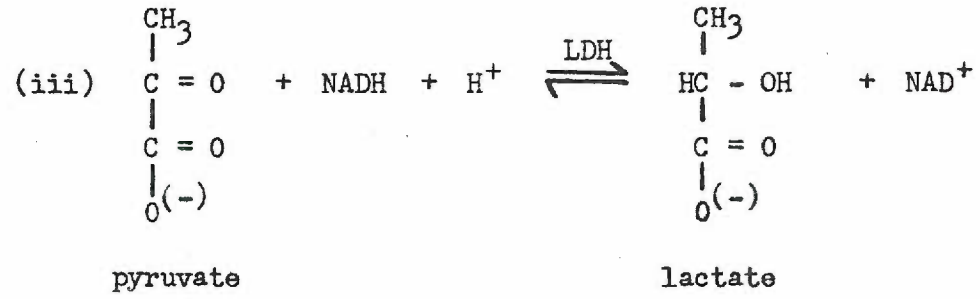
The enzyme assay utilizing GK-PK-LDH was first suggested by Kreutz (81 and 82) and later by Eggstein and Kreutz (90), Willams and Söling (91) and Berner and Guhr (92). Glycerol is phosphorylated with ATP in a reaction catalyzed by glycerokinase (ATP: glycerol phosphotransferase, E.C.2.7.1.30), yielding alpha-glycerolphosphate (α-GP) and ADP (i). The ADP produced is a substrate for the pyruvate kinase (ATP: pyruvate phosphotransferase, E.C.2.7.1.40) catalyzed reaction forming pyruvate and ATP(ii). The pyruvate is reduced by NADH under the catalytic influence of lactic dehydrogenase (L-lactate: NAD^+ oxidoreductase, E.C.1.1.1.27) to lactate and NAD^+(iii), giving a net reaction in which a mole of NADH is oxidized for each mole of glycerol that is initially present. The equilibrium is far in favor of lactate formation, giving a quantitative assay of ADP.

(i) 

α-GP

(ii) 

PEP pyruvate



Garland and Randle (93) measured glycerol after deproteinizing tissue or blood extracts with 10 per cent trichloroacetic acid. Dihydroxyacetone and glyceraldehyde were also reported to be measured by this technique although the initial velocity with glycerol was approximately fourty times faster than those of trioses. Pinter et al (94) modified the procedure of Garland and Randle (93) and reported that when these trioses were present in excess of 0.02 umol/liter, they would significantly interfere with the glycerol assay by being phosphorylated during the five minute assay period. Glyoxylate was also reported to interfere with the GK-PK-LDH technique.

Timms et al (47) modified Garland and Randle (93) by reducing reagent volume and by using potassium glycylglycine buffer (0.5 mol/liter, pH 7.4) instead of triethanolamine buffer (pH 7.6). Bell et al (95) automated the GK-PK-LDH technique for assaying glycerol. They corrected for non-glycerol oxidation of NADH by running the samples in a reagent containing no glycerokinase. The results obtained by this automated method were consistently lower than those obtained by the manual method and the normal range was also significantly wider with the manual method. Mallon and Dalton (96) adapted the manual method of Pinter et al (94)

to an automated fluorometric method. They found that the background fluorescence of the plasma was exceedingly high. However, the inclusion of acid reduced the background peak heights by 40 per cent and minimized protein binding as described by Falk (97). They postulated that protein binding of glycerol and fluorescence interference might have caused the low recoveries of glycerol added to human and dog sera. The background peaks were reported to be due to endogenous pyruvate, ADP, and fluorescent substances present in the serum.

Mourad et al (98) used a Beckman "Discrete Sample Analyzer 560" to assay the saponified glycerol using the GK-PK-LDH method.

Bucolo and David (99 and 100) used Rhizopus delemar lipase (Glycerol-ester hydrolase, E.C.3.1.1.3) combined with proteolytic enzyme, α-chymotrypsin (Peptidyl-peptide hydrolases, E.C.3.4.4.5), to lipolyze triglyceride to glycerol and free fatty acids. The glycerol was then coupled with GK-PK-LDH and decrease in absorbance was measured at 340 nm. In some specimens a correction must be made for NADH oxidation which is not due to glycerol. This enzyme hydrolysis takes about 10 minutes to complete at 30°C and phospholipids are not hydrolyzed to glycerol.

Enzyme hydrolysis obviated the use of laborious, time-consuming, and often violent alkaline or acid saponification, extraction of triglyceride, and removal of phospholipids and other interferring substances from plasma sample.

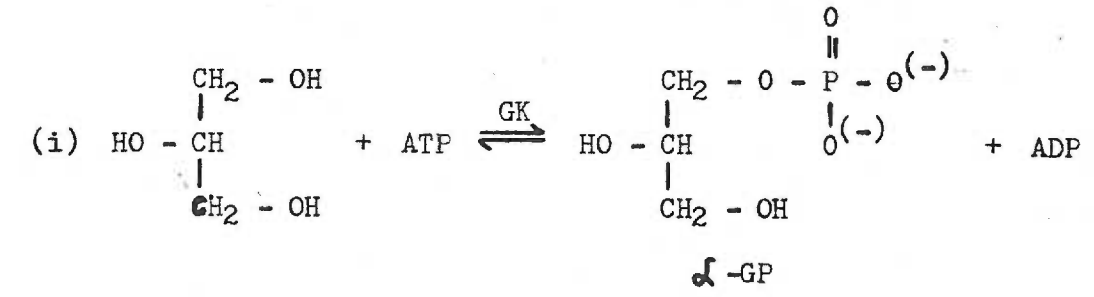
Phosphatases catalyze hydrolysis of phosphoenopyruvate and ATP to

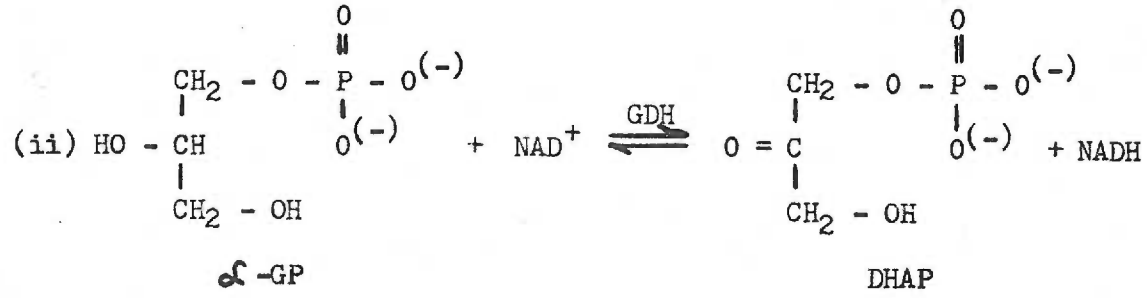
erroneously elevate triglyceride results (100). The presence of endogenous ADP and pyruvate would also give an erroneous triglyceride values (96). According to Antonis et al (101), normal pyruvate in fasting sera ranges from 50 to 150 μmol/liter. In a pathological condition, pyruvate, ADP, and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, E.C.3.1.3.1) levels may be higher. Hexokinase in the reaction mixture (ATP: D-hexose-6-phosphotransferase, E.C.1.7.1.1) will convert glucose to glucose-6-phosphate and ATP to ADP. The ADP produced by this enzyme will be measured as apparent glycerol (95).

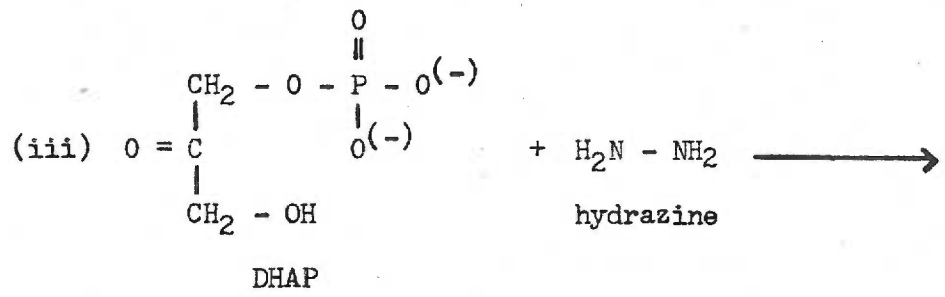
The main technical objection of the manual GK-PK-LDH system, e.g. Calbiochem Triglyceride Stat-Pack, is that a total of five calculations are required to arrive at a concentration of serum triglycerides after taking three consecutive absorbance readings of a serum sample. The initial absorbance (A_o) is taken after addition of a serum to a lipolysis reagent. The initial absorbance is multiplied by a factor to correct for a volume change (A_c). Then, glycerokinase is added to initiate the glycerol measuring reaction and the second absorbance reading (A_{10}) is recorded after 10 minutes at 30°C incubation. The second absorbance (A_{10}) reading is then subtracted from that of the first (A_c) to give a total absorbance change (ΔA_{total}). The correction for a blank rate caused by non-glycerol substances is made by taking the third absorbance reading (A_{20}) after incubating the same reaction mixture for additional 10 minutes at 30°C. The difference

between A_{20} and A_{10} is the blank absorbance, which is then subtracted from a total absorbance change (ΔA_{total}) to give a corrected absorbance reading (ΔA_{corr}). The corrected absorbance (ΔA_{corr}) is multiplied by an appropriate factor to give serum triglyceride concentration units.

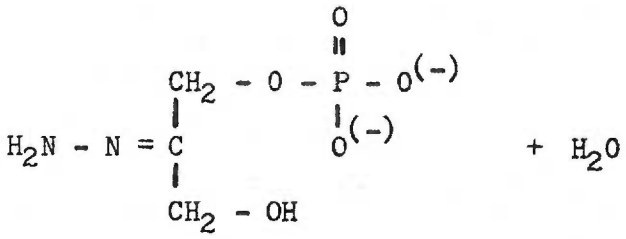
Bublitz and Kennedy (84) described the purification and properties of glycerokinase, which was isolated from rat liver and used the enzyme in a glycerol assay. The phosphorylated glycerol product, alpha-glycerophosphate (α-GP), was estimated by coupling with alpha-glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, E.C.1.1.1.8) and NAD^+. The product dihydroxyacetone phosphate was trapped by hydrazine at an alkaline pH.







DHAP



DHAP-hydrazone

Each mole of ATP formed one mole of alpha-glycerophosphate (i) as confirmed by Bublitz and Kennedy (84). Later, they demonstrated that glycerokinase phosphorylated either of the two primary hydroxyl groups of glycerol, producing a mixture of D-α-GP and L-α-GP instead of an optically pure product. Assay of glycerokinase, optimal pH, sulfhydryl requirement and inhibitors, optimal magnesium concentration, phosphorylation of dihydroxyacetone and glyceraldehyde, and phosphatide synthesis by glycerokinase are discussed (84). Wieland (102) utilized GK-GDH assay technique to measure glycerol in which glycerokinase of pigeon liver was used (103). Boltralik and Noll (104) prepared glycerokinase from Mycobacterium tuberculosis and critically reviewed the over-all glycerol assay reaction: a large quantity of glycerophosphate dehydrogenase, excess NAD concentration and albumin were found to ensure maximal velocity.

Bublitz and Wieland (105) thoroughly reviewed the purification, assay technique, properties and source of glycerokinase. According to them, this enzyme has been isolated not only from rat liver, pigeon liver and Mycobacterium tuberculosis as described above, but also from Oöspora lactis and Candida mycoderma.

Baranowski (106) isolated crystalline alpha-glycerophosphate dehydrogenase (GDH) from rabbit-muscle and found that this enzyme was stable in 50 per cent saturated ammonium sulfate. However, the diluted enzyme lost 90 per cent of the enzyme activity during 24 hours of storage at 0°C. Beisenherz et al (107) determined that the recrystallized preparation of alpha-glycerophosphate dehydrogenase maintained its activity for weeks when stored in 1.9 mol/liter ammonium sulfate at 0 - 4°C. Young and Pace (108) purified and characterized the crystalline alpha-glycerophosphate dehydrogenase from rabbit-muscle as described by Baranowski (106). They found that the enzyme was most stable at pH 5.7 and slightly less stable at pH 8.5. The optimal activity for reduction of dihydroxyacetone phosphate was at pH 7.5 and oxidation of L-alpha-glycerophosphate (α-GP) at pH 10.2. The enzyme was also reported to be relatively thermolabile in which no appreciable loss of activity was detected at 22°C for 30 minutes and 53 per cent loss at 55°C for one minute. The maximal rate of reduction of dihydroxyacetone phosphate was 100 fold faster than the rate of oxidation of alpha-glycerophosphate.

There are "soluble" and "particulate" alpha-glycerophosphate

dehydrogenases. The "soluble" alpha-glycerophosphate dehydrogenase requires NAD^+ as a cofactor (NAD^+-linked GDH), while the "particulate" or "mitochondrial" alpha-glycerophosphate dehydrogenase does not require NAD^+ ($non\text{-}NAD^+$-linked GDH).

Ohkawa et al (109) assayed soluble alpha-glycerophosphate dehydrogenase (NAD^+-linked GDH) using dihydroxyacetone phosphate (pH 7.5) with β-NADH and also assayed the mitochondrial enzyme ($non\text{-}NAD^+$-linked GDH) in brown adipose tissue of rat using alpha-glycerophosphate (pH 7.6) without coenzyme. The soluble NAD^+-linked GDH had been reported to be higher in the brown tissue than in the other organs, but the activity of mitochondrial GDH in the brown fat was 10 times that in the liver, greater than 20 times that in the white adipose tissue, and 9 times that in the kidney. They postulated that high mitochondrial GDH in the brown adipose tissue is to regulate the synthesis of esterified phospholipids and triglycerides, to control thermogenesis, and to participate in the rapid mitochondrial oxidation of NADH as a cytoplasmic electron shuttle.

Several authors have reported kinetic constants for "soluble" α-glycerophosphate from rabbit muscle. The Km for α-glycerophosphate was reported as 1.4×10^{-4} mol/liter at pH 7.0 at 22°C (106); 1.25×10^{-3} mol/liter, Green (106); 1.2×10^{-3} mol/liter at pH 7.7, Sellinger and Miller (110); and 1.1×10^{-4} mol/liter, Young and Pace (108).

In 1965, Borreback et al (111) reported that the pH optimum for the reduction of dihydroxyacetone phosphate by α-glycerophosphate dehydroge-

nase was higher with NADH than with NADPH. They also found that NAD^+ inhibited the rabbit-muscle enzyme when NADPH served as the coenzyme, but was not inhibited when NADH was used. Pfleiderer and Auricchio (112) showed that two moles of NADH are bound per mole of rabbit-muscle L-alpha-glycerophosphate dehydrogenase (mw 78,000), which was later confirmed by Kim and Anderson (113 and 114). Marquardt and Brosemer (115) described the purification, the physical and the chemical properties of the honeybee (Apis mellifera) glycerophosphate dehydrogenase, which differed in ultracentrifugal, immunological and electrophoretic properties from that of the rabbit-muscle enzyme. Later, the enzyme properties revealed that the honeybee enzyme has a broad pH optimum between pH 6 and 7, while the rabbit-muscle enzyme has a sharp optimum at pH 7.7. Glutathione was reported not to stimulate the activity of either enzyme after the enzymes were inhibited by p-mercuribenzoate and N-ethylmaleimide. The amino acid composition has shown that the honeybee enzyme is about 120 amino acid residues smaller than the rabbit-muscle enzyme. Brosemer and Marquardt (116) reported that the Michaelis constant (Km) for dihydroxyacetone phosphate was 3.3×10^{-4} mol/liter for the honeybee alpha-glycerophosphate dehydrogenase. Concentrations of α-GP above 5.0×10^{-4} mol/liter showed no increase in activity, and substrate inhibition was observed above 10^{-3} mol/liter.

Ultrastructural studies of enzymes precipitating with osmium tetroxide result in characteristic forms and shapes (117). Lactic dehydrogenase forms cuboidal units while L-glutamic dehydrogenase

showed tubules. However, alpha-glycerophosphate dehydrogenase formed elongated plates.

Ciaccio et al (118) modified the GK-GDH method of Bublitz and Kennedy (84) to measure the level of alpha-glycerophosphate during anaerobic glycolysis in malignant tissues. Later in 1962, Ciaccio (119) described a kinetic assay for quantifying alpha-glycerophosphate rather than measuring the total NADH produced at equilibrium. The rate of lipolysis of triglycerides and the rate of esterification of free fatty acid (FFA) in vivo were determined by using the GK-GDH technique of Bublitz and Kennedy (84) to assess the net change in glycerol content (120). Carlson and Oro (121) in 1963 and Shafrir and Gorin (122) in 1963 used Wieland's method to determine the effect on plasma glycerol concentrations when norepinephrine, glucose and insulin were administered.

Table 31 lists a summary of the concentrations and conditions used by various authors. Spinella and Mager (123) slightly modified the concentrations and conditions used by Wieland. Their assay was later adapted by Parijs et al (124). Laurell and Tibbling (125) described a fluorometric micromethod, which was later automated by Ko and Royer (126) and Harding and Heinzel (127).

Fried and Antopol (128) measured alpha-glycerophosphate dehydrogenase activity of the obese-hyperglycemic mice utilizing the tetrazolium technique in which 2-p-iodo,3-p-nitro,5-phenyltetrazolium (INT) was reduced to the insoluble formazan, which was then measured photometrically at 490 nm.

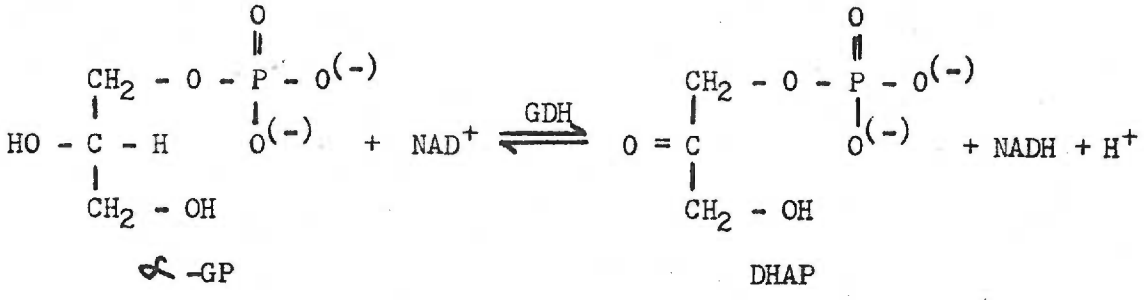
Wassef et al (129) used the spectrophotometric methods of Bublitz and Kennedy (84) and Kennedy (130) to assay the presence of alpha-glycerophosphate produced by Halobacterium curirubrum in which the activities of glycerokinase and alpha-glycerophosphate dehydrogenase were shown.

Sellinger and Miller (131) reported that an appreciable loss of activity was found when an anion such as sulfate or phosphate, was added to a dialyzed, anion-free, alpha-glycerophosphate dehydrogenase, but glycine and ethylene diamine tetracetate (EDTA) produced no loss of enzymatic activity. However, in the presence of sulfate the enzyme was stable prior to dialysis, which indicates that the dialysis exposes the active sites on the enzyme molecule causing sensitivity to anions.

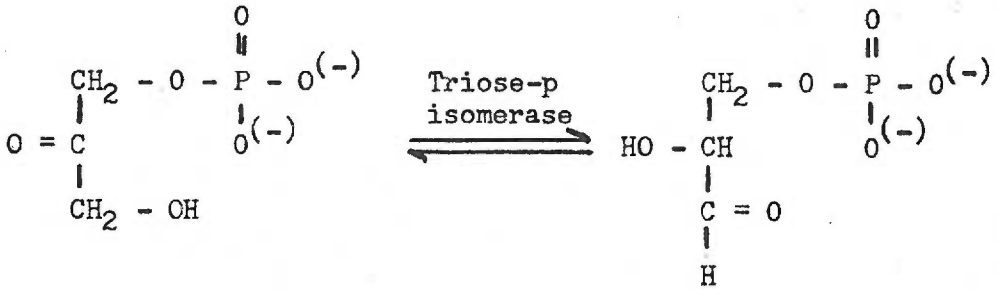
In 1968, Kim and Anderson (132) demonstrated that adenosine diphosphoribose, adenosine diphosphate and the positively charged nicotinamide ring were NAD competitive inhibitors in the alpha-glycerophosphate dehydrogenase-catalyzed oxidation of alpha-glycerophosphate. The same compounds were also found to be the NADH-competitive inhibitors in the alpha-glycerophosphate dehydrogenase-catalyzed reduction of dihydroxyacetone phosphate (133). Alpha-glycerophosphate dehydrogenase was completely inhibited by N^1-alkylnicotinamide chlorides and the inhibition was found to be competive with respect to NAD, which suggested that the binding sites of the enzyme were at a "pyridium ring" for the inhibitors and NAD. Aliphatic carboxylic acids and n-alkylammonium chlorides were also shown to competitively inhibit the enzyme in oxidation of L-α-glycerophosphate with NAD. Increasing the chain length of

the inhibitors increased binding of n-alkylammonium chlorides to the enzyme indicated a non-polar interactions with a hydrophobic site of the enzyme.

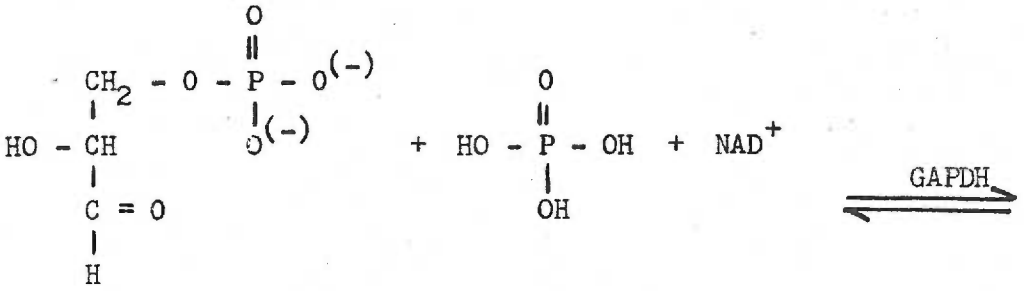
Using in vivo rat heart and adipose tissue it was observed that the free fatty acid, octanoate, competitively inhibited alpha-glycerophosphate dehydrogenase activity (134). Schwark et al (135) later showed that the same fatty acid competitively inhibited the enzyme in cerebral cortex and cerebellum.

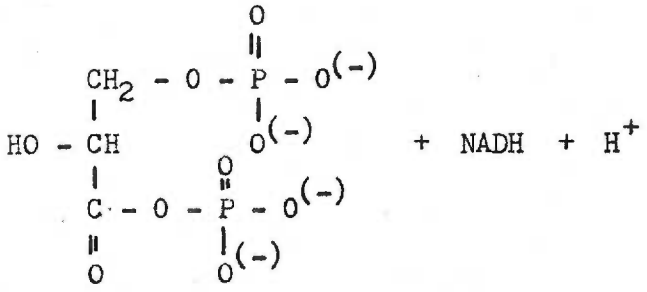


α-GP DHAP



DHAP glyceraldehyde-phosphate





1,3-diphospho-glycerate

According to Lowry and Passonneau (136), Matschinsky (1964) proposed the above assay technique in which two additional enzymes, triose-phosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, E.C.5.3.1.1) and glyceraldehyde-3-phosphate-dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase, E.C.1.2.1.12) are used to shift the equilibrium of glycerophosphate dehydrogenase reaction in the favor of dihydroxyacetone phosphate production; thus, it avoids the use of hydrazine. However, in contrast to GK-GDH assay technique, two additional enzymes are needed to measure α-glycerophosphate. The one advantage is that two moles of NADH are formed per mole of α-glycerophosphate.

CENTRIFUGAL FAST ANALYZER (C.F.A.):

Centrifugal Fast Analyzers are a combination of centrifuge, double-

beam spectrophotometer and computer in which a temperature controlled multiple-cuvette assembly is mounted at the periphery of a rotor. The initial rotary motion of the cuvette assembly produces centrifugal force which results in mixing and quantitative transfer of a number of discrete aliquots of samples and reagents into their appropriate cuvettes. This initiates simultaneous chemical or enzymatic reactions in each cuvette, minimizing analytical problems owing to physical, chemical, procedural and electronic variations. The simultaneous mixing of solutions in several cuvettes allows precise, simultaneous measurement of absorbances at accurately measured time intervals. The rotor speeds range from 500 - 1500 rpm (CentrifiChem Fast Analyzer uses 1000 rpm). The multiple-cuvette assembly moves through a single, fixed optical system consisting of a light source, interference filter, and photomultiplier. The absorbance values generated from the reaction media of each cuvette are first referenced to the reference cuvette, which normally contains water, and the average absorbance of eight successive revolutions of the rotor is calculated and processed to improve the signal-to-noise performance of the instrument. The net effect of combining continuous referencing with signal averaging minimizes interference from electronic, light and mechanical noises. The absorbance values are then transferred to an oscilloscope (CRT) and a focal program computer which calculates changes in concentrations or activity units with appropriate conversion factors. Detailed descriptions, principles and summaries of the three currently available Centrifugal Fast Analyzers are described in the ASCP Workshop Manual (137).

Figures 1 to 3 show the CentrifiChem Fast Analyzer, Autopipettor, Sample Holder, Transfer Disc, and 30°C Water Bath.

Fig. 1 CentrifiChem Fast Analyzar

Fig.2 Autopipettor with sample holder and transfer disc (left), and 30°C water bath (right).

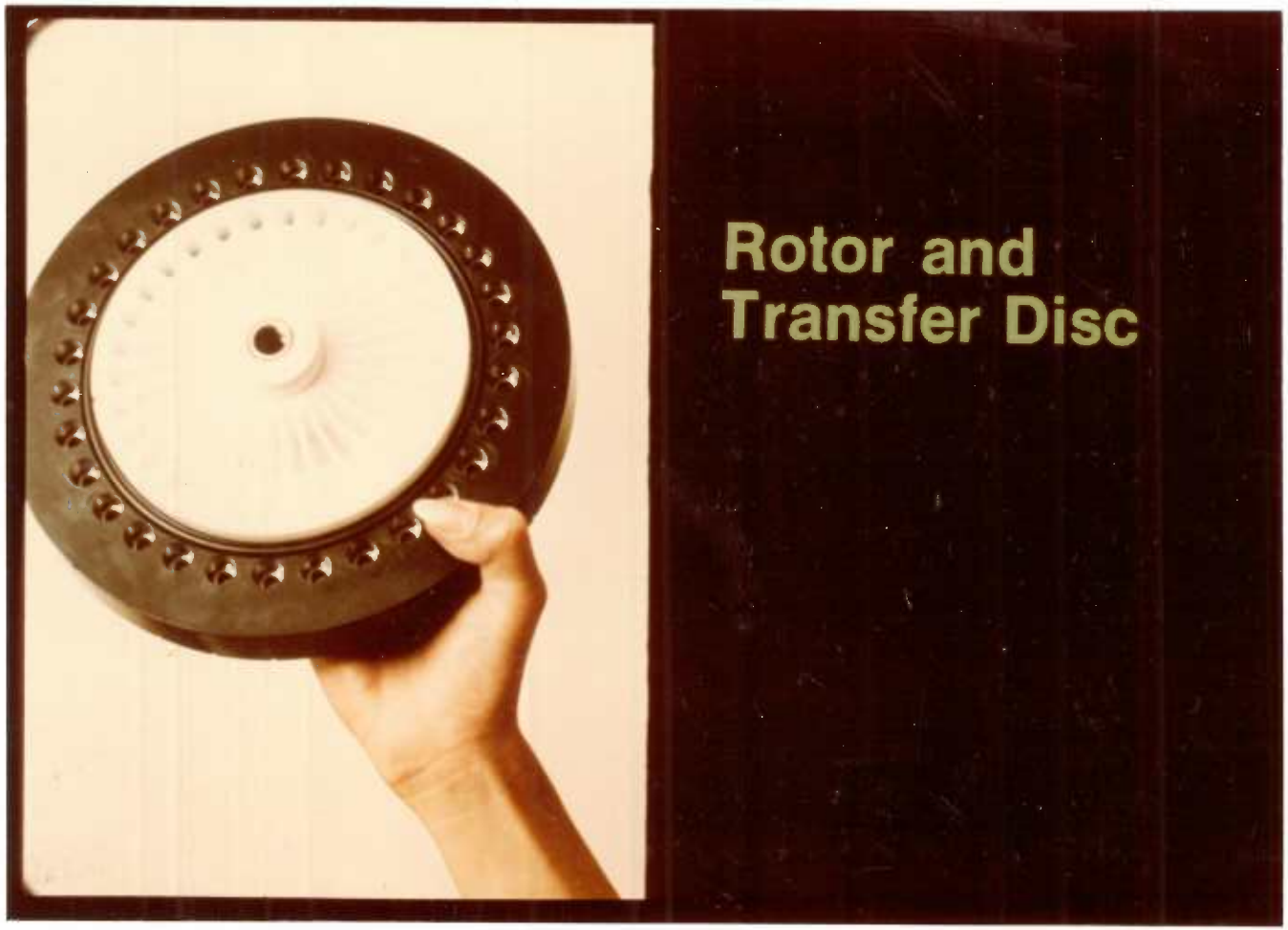


Fig. 3 Transfer disc (center) and rotor cuvettes (outside).

STATEMENT OF THE PROBLEM

In the Fall of 1971, we had learned that serum triglycerides could be analyzed without extracting the glycerides; without removing phospholipids, glucose, bilirubin, and other chromogenic substances from a serum sample; and without using the violent alkaline saponification of triglycerides to free glycerol. Such a triglyceride assay reagent is commercially available from Calbiochem, La Jolla, California 92037, which utilizes lipase and α-chymotrypsin to hydrolyze triglyceride ester bonds to yield quantitative amounts of free glycerol. The liberated glycerol is measured by the GK-PK-LDH method as described above. This commercial assay has some drawbacks which have already been mentioned. We wanted to examine a different glycerol measuring system and try to come up with an improved assay for triglycerides using an enzymatic hydrolysis and analysis of glycerol using the Centrifugal Fast Analyzer. The initial aim was to seek a complete enzymatic quantification of serum triglycerides: enzymatic hydrolysis of triglycerides and enzymatic measurement of the liberated glycerol. The second aim was to find an enzymatic glycerol measuring technique which can be easily adapted for use in Centrifugal Fast Analyzer. The problem was broken down into the following areas: finding optimal conditions for lipolysis, optimal conditions for glycerol measurement, and the best condition for carrying out glycerol measurement on lipolyzed serum. During the course of developing this method, it was necessary to calibrate the 340 nm filter of CentrifiChem Fast Analyzer, to determine the accuracy and precision of automatic pipettor, to utilize the GK-PK-LDH method of

Calbiochem Triglyceride Fast-Pack Reagent and the Automated Fluorometric Hantzsch Condensation Method for correlation study, to confirm the completeness of enzyme hydrolysis by using Thin-Layer Chromatography, and to determine the stability and activity of α-chymotrypsin solutions. The findings of the research project are described below.

MATERIALS AND EXPERIMENTAL METHODS

EQUIPMENT

1. CentrifiChem Fast Analyzer with Autopipettor .... Union Carbide Corp., Tarrytown, N.Y. 10591
2. Beckman DB Ultraviolet Spectrophotometer .... Beckman Instruments, Inc., Fullerton, Ca. 92634
3. Beckman DU Spectrophotometer with Gilford Attachments: Model 240, Model 208 Auxiliary Offset Control, Model 210 Automatic Cuvette Positioner, and Model 242 Recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Temperature adjustable circulating water bath (Labline, Inc., Chicago, Ill.)
4. Beckman Acta CIII UV-Visible Spectrophotometer
5. Technicon Autoanalyzer Sampler II with Fluorometer (400 nm primary and 485 nm secondary filters). Technicon Corp., Tarrytown, N.Y. 10591
6. pH Meter, Model 245 Delta-Matic with combination electrode #14043, Instrumentation Laboratory Inc., Watertown, Mass. 02172
7. Thin-Layer Chromatography .... Glass plates, 20 X 20 cm, precoated Silica Gel 60, 0.25 mm thick, E. Merk Laboratories, Inc., Darmstadt, Germany and Emsford, N.Y. 10523
8. Developing Tank, Multi-Plate, 3 5/8" X 11 5/8" X 10"
9. Hamilton 10 µl Syringe with PB600 Repeating Dispenser, Hamilton Co., Whittier, Ca. 90608
10. Eppendorf Push-Button Microliter Pipettes with Disposable Tips, Brinkmann Instruments, Inc., Westbury, N.Y. 11590
11. 5, 25, and 100 µl Disposable Micropipettes (TC) .... Clay Adams,

Parsippany, N.J. 07054

12. 10, 20, and 50 µl Micropipettes (TC) .... Dade, Miami, Fla. 33152

13. 20 X 125 mm srew-capped test tubes with Teflon lined caps

14. Vortex Mixer .... Model K-500-J, Scientific Industries, Inc., Queens Village, N.Y.

15. Reciprocating Shaker, Model 73441, AAPCO, National Appliance Co., Portland, Ore.

16. Chromatographic Sprayer, atomizer glass unit

REAGENTS

1. Acetone, glacial acetic acid, ammonium acetate, calcium hydroxide, cupric sulfate, hexane, hydrochloric acid, isopropanol, lactic acid, methanol, potassium hydroxide, and sodium hydroxide were purchased from Mallinckrodt Chemical Works, St. Louis, Mo. 63160.
2. Adenosine-5'-Triphosphate (ATP), disodium (99-100%); β-Nicotinamide Adenine Dinucleotide (β-NAD), 99%, grade III (yeast), Lot No. 121C-7290; Calcium chloride ($CaCl_2 \cdot 2H_2O$), grade I, 99%, Lot No. 122C-1850; Imidazole, grade III, low fluorescence, Lot No. 32C-5330; Magnesium cloride ($MgCl_2 \cdot 6H_2O$), Lot No. 42C-2670; and Tris (hydroxymethyl) amino-methane, 99-99.5%, Lot No. 52C-24635, were obtained from Sigma Chemical Co., St. Louis, Mo. 63118.
3. Benzene; glycerol, spectroquality with 0.05% water, GX175; and glycine, 99.5%, ammonium-free, were products of Matheson Coleman and Bell, Norwood, Ohio.
4. Adenosine-5'-Triphosphate (ATP), dipotassium, A grade; N-Benzoyl Tyrosine Ethyl Ester Hydrochloride (BTEE), A grade, 4.42% nitrogen, MP 110-115°C; Calbiochem Glucose Stat-Pack; Calbiochem Triglyceride Stat-Pack; and Deoxycholic acid, A grade, 73.32% carbon, 10.17% hydrogen, MP 172-174°C, were purchased from Calbiochem, La Jolla, Ca. 92037.
5. Chloroform, GC-Spectrophotometric Quality; Diethylether (99.9%); potassium dichromates (primary standard); and sodium meta-periodate, were products of J.T. Baker Chemical Co., Phillipsburg, N.J.
6. pH Buffer Solution .... Type S-1510, pH 7.410 at 25°C, Radiometer-Copenhagen, Denmark

7. pH 10 Buffer Solution .... Beckman Instruments, Inc., NBS Certified
8. Gum Arabic (acacia) .... Fisher Scientific Co., Fairlawn, N.J. Lot No. 787413
9. Ethanol, 100% .... Commercial Solvents Corp., Terre Haute, Indiana
10. Acetylacetone (2,4 Pentanedione) .... Eastman #1088
11. Lloyd Reagent .... Hartman-Leddon Co., Philadelphia, Pa.
12. Sodium chloride .... Merck and Co., Rahway, N.J.
13. Zeolite .... W.A. Taylor and Sons, Baltimore, Md.
14. Ketone Trapping Agents:
    a. (Aminooxy) acetic acid Hemihydrochloride .... Kodak Eastman, Rochester, N.Y. 14650 No. 5336
    b. Hydrazine Dihydrochloride .... Matheson Coleman and Bell
    c. Hydrazine Hydrate .... 99%, Mallinckrodt Chemical Works
    d. Hydrazine Sulfate .... Sigma Chemical Co., Lot No. 52C-3290
    e. Hydroxylamine Hydrochloride .... 96%, Analytical Reagent, Mallinckrodt Chemical Works
    f. Phenylhydrazine .... Mallinckrodt Chemical Works
    g. Semicarbazide Hydrochloride .... Mallinckrodt Chemical Works
15. Serum Albumin:
    a. 35% Bovine Serum Albumin .... Sterile and no preservative added, Sigma Chemical Co., A5128
    b. Fatty Acid Free Bovine Serum Albumin .... Less than 0.005% FFA, Sigma Chemical Co., A6003
    c. Bovine Serum Albumin Fraction V .... 96-99% albumin, Sigma Chemical Co., A4503

d. Human Fraction V Albumin .... Pentex, Inc., Kankakee, Ill. 60901

e. Bacto Bovine Albumin .... 30%, Difco Laboratories, Detroit, Mich.

f. Bovine Serum Albumin V .... Metrix, Armour Pharmaceutical Co., Chicago, Ill. 60690, Lot No. J41010

16. Lipid Standards:

a. Cholesterol linoleate (Δ 5-cholesten-3-ol-linoleate)

b. Cholesterol oleate (Δ 5-cholesten-3-oleate)

c. Dipalmitin

d. Triolein

(Purchased from Sigma Chemical Co., St. Louis, Mo. 63118)

e. Lecithin (1-octadec-9-enyl,2-hexadecyl)

f. Linolenic acid

g. Oleic acid

h. Sphingomyelin

(Purchased from Calbiochem, La Jolla, Ca. 92037

17. Lipase:

a. Candida cylindracea Lipase .... 1033 U/mg, Worthington Biochemical Corp., Freehold, N.J. 07728

b. Hog Pancreas Lipase .... Lyophilized powder, Type V, 11450 U/mg at pH 7.4 and 37°C, Sigma Chemical Co., Lot No. 52C-1520

c. Rhizopus delemar Lipase .... 7040 U/mg, Seikagaku Kogyo Co., LTD., Tokyo, Japan and Miles Laboratories, Inc., Kankakee, Ill. 60901

18. Alpha-Chymotrypsin:

a. Calbiochem .... 37 I.U./mg at 30°C (BTEE as a substrate), 0.68% Trypsin, B grade, Pancreas, Lot No. 801634

b. Miles-Seravac .... 1130 N.F. Units/mg, #1507A

19. -SH Reagents:

a. L-Cysteine hydrochloride monohydrate .... A grade, Calbiochem, Lot No. 901454

b. Dithiothreitol (DTT) .... Sigma Chemical Co., Lot No. 92C-0140

c. Glutathione (reduced) .... 13.50% nitrogen, 10.40% sulfur, MP 192°C, SH 97%, A grade, Calbiochem, Lot No. 100563

d. 2-Mercaptoethanol (Monothioethylene glycol) .... Matheson Coleman and Bell, Lot No. 6377

20. Glycerokinase (GK):

a. Calbiochem .... E. coli, glycerol-free, 197 I.U./ml at 30°C, 2 mg protein/ml, 0.0015% GDH, in 2.6 mol/liter ammonium sulfate, Lot No. 244019

b. Sigma .... Candica mycoderma, 180 U/ml at 37°C and pH 9.8, 2 mg protein/ml, in 3.2 mol/liter $(NH_4)_2SO_4$ at pH 6.0 and 1% ethylene glycol (v/v), Lot No. 122C-059185

c. Boehringer-Mannheim (BMC) .... Candida mycoderma, 85 U/ml at 25°C and in 2.4 mol/liter $(NH_4)_2SO_4$ of pH 6.0 and 1% (v/v) ethylene glycol .... Less than 0.01% each of GDH and NADH-oxidase, less than 0.02% each of hexokinase, myokinase and TIM

21. α-Glycerophosphate Dehydrogenase (GDH):

a. Calbiochem .... rabbit muscle, 779 U/ml at 30°C, 4.75 mg protein/ml, 0.02% LDH, 0.001% GAPDH, 0.02% PK, less than 0.001%

ALD, in 2.0 mol/liter ammonium sulfate, Lot No. 130043

b. Sigma .... rabbit muscle, 160 U/mg protein at 25°C and pH 7.4, 0.003% LDH, less than 0.003% PK, less than 0.003% Aldolase, less than 0.003% GAPDH, 0.009% Triosephosphate isomerase, in 2.0 mol/liter $(NH_4)_2SO_4$ of pH 6.0 containing 100 ug EDTA/ml

c. Sigma .... rabbit muscle, lyophilized and sulfate-free powder with 30% buffer salts as EDTA and citrate, 92 U/mg protein at 25°C and pH 7.4, 0.56 mg protein, Lot No. 81C-8500

d. Boehringer-Mannheim (BMC) .... rabbit muscle, 400 U/ml at 25°C, 2.0 mol/liter $(NH_4)_2SO_4$ at pH 6.0, less than 0.01% each of ALD, GAPDH, LDH and PK

22. Glucose Standard (200 mg/100 ml in 0.25% Benzoic acid) was obtained from the College of American Pathologists Chicago, Ill. 60601

23. BSP dye, Bromsulphthalein (phenoltetrabromphthalein-disodium sulfonate), 50 mg/ml, was purchased from Hyson, Westcott, and Dunning, Inc., Baltimore, Md. 21201

All chemical reagents were Analytical Reagents or equivalent grades.

METHODS

A. An Enzymatic Triglyceride Assay using GK-PK-LDH Method:

An enzymatic triglyceride assay was essentially the procedure of Bucolo and David (100) using Calbiochem Triglyceride Stat-Pack with the following modification: 15.5 ml of deionized water was placed in a vial-C containing lipase and NADH. The content of the vial-C was transferred into vial-A, which contained 0.1 mol/liter phosphate buffer (pH 7.1 $\pm$ 0.1), magnesium, phosphoenolpyruvate, LDH, ATP, pyruvic kinase, and α-chymotrypsin. The vial-B containing glycerokinase was reconstituted with 0.5 ml deionized water. All reagents were made just before use. The serum triglycerides were assayed by adding 1.2 ml of the substrate (vial-A) in a cuvette (1-cm light paths), which was then warmed to 30°C. Twenty µl of a serum sample was added and the content was incubated at 30°C for 10 minutes. At the end of 10-minute incubation, the first absorbance reading was taken (A_o). The first absorbance (A_o) was multiplied by 0.98 to correct for a volume change (A_c). The second absorbance (A_{10}) was read after adding 0.020 ml glycerokinase and re-incubated at 30°C for additional 10 minutes. The blank rate was determined for each serum specimen by re-incubating at 30°C for another 10 minutes and the absorbance reading was recorded. This absorbance value was subtracted from the second absorbance (A_{10}) to obtain a blank absorbance (A_{blank}). The difference between the first (A_o) and second absorbance (A_{10}) gives the total absorbance

(A_{total}) due to glycerol and non-glycerol reactions. In order to correct for the non-glycerol rate, the blank absorbance (A_{blank}) of each serum specimen was subtracted from the total absorbance (A_{total}) to give a corrected absorbance (A_{corr}). The corrected absorbance (A_{corr}) was multiplied by 9.968 to give a serum triglyceride concentration in mmol/liter.

$$\text{mmol/liter} = \frac{A \times 1.24 \text{ ml}}{6.22^{*} \times 0.02 \text{ ml}} = A \times 9.968$$

* 6.22×10^3 is the molar extinction coefficient of NADH at 340 nm and 6.22 is the millimolar extinction coefficient.

B. Fluorometric Autoanalyzer Method using Hantzsch Condensation Reaction for Measuring Serum Triglycerides

This method is essentially the method of Kessler and Lederer (46). Acetyl-acetone (2,4 pentanedione), isopropanol and ammonium acetate (2 mol/liter) were made fresh daily. The extraction of triglycerides was made by mixing 0.5 ml serum sample and 9.5 ml isopropanol. The triolein standards, 50, 100, 150, 200, 250 and 300 mg/100 ml, were processed in the same manner. After mixing a serum sample with isopropanol for 15 seconds using a Vortex Mixer, the tubes were allowed to stand at room temperature for 15 minutes. Two grams of the zeolite mixture, consisted of zeolite, Lloyd reagent, cupric sulfate and calcium hydroxide, was added to each extraction tube and vigorously mixed for 30 seconds using a Vortex Mixer. The content was allowed to stand at room temperature for 30 minutes

with occasional mixing. After centrifuging the test tube for 10 minutes at 2500 rpm, the supernatant was decanted into a clean screw-capped test tube. On the Autoanalyzer the isopropanol triglyceride extract was mixed with an air-segmented alkaline reagent, and triglycerides were saponified in the 50°C heating bath. The liberated glycerol was then oxidized to formaldehyde after periodic acid was added. The 2,4-pentanedione with ammonium acetate was also added at the same time as the periodic acid for condensation with formaldehyde to give the fluorescent product (3,5-diacetyl-1,4-dihydrolutidine). The oxidation and condensation reactions were carried out simultaneously during passage through the 50°C heating bath. As the reaction mixture entered the fluorometer, air was removed and the fluorescence activated. The base line was adjusted to 3 - 4 per cent with the blank control to correct for a reagent fluorescence. A 400 nm primary interference filter and 485 nm sharp cut off secondary filter were used in the fluorometer.

C. A U.V. Kinetic Measurement of α-chymotrypsin:

The kinetic assay of α-chymotrypsin is essentially the method of Schwert and Takenaka (138) and the modified method of Hummel (139). N-benzoyl-L-tyrosine ethyl ester (BTEE) was used as a substrate and the rate of hydrolysis of BTEE was kinetically assayed at 256 nm.

One unit is equivalent to one micromole of BTEE hydrolyzed per minute per milligram of protein at pH 7.8 and 30°C under the specified condition of this procedure.

The buffer solution of 0.08 mol/liter Tris (hydroxymethyl)

aminomethane and 0.1 mol/liter $CaCl_2 \cdot 2H_2O$ (140) was adjusted to pH 7.8 with 1.0 mol/liter HCl. The methanol solution (50%, w/w) was made by mixing 63.5 ml of methanol and 50 ml of deionized water. The substrate reagent was made by dissolving 33.6 mg N-benzoyl-L-tyrosine ethyl ester (BTEE) in 100 ml of 50 per cent methanol. Accurately weighed α-chymotrypsin was dissolved in a known quantity of the following solutions: 0.001 mol/liter HCl; deionized water; 0.05 mol/liter Tris·HCl buffer, pH 7.6; and 0.1 mol/liter Tris·HCl buffer, pH 7.6. Into each above solution, 1.0 mol/liter $CaCl_2 \cdot 2H_2O$ was added, having the final concentration of $CaCl_2$ as 0.005 mol/liter. Each solution was then divided into two equal volumes. One set was stored at 0 - 4°C and the other set at 30°C. The enzyme activity at 0 - 4°C and at 30°C was assayed at various time intervals to determine its stability under the varying temperatures and solution conditions.

A Beckman DU Spectrophotometer fitted with Gilford attachments and temperature controlled bath (30°C) was used. The enzyme assay was made using the matched quartz cells with 1-cm light paths. Into each of two matched cuvettes, 1.5 ml of the buffer solution and 1.4 ml of substrate (BTEE) were pipetted. To the blank cuvette was added 0.1 ml of 0.001 mol/liter HCl and 0.005 ml deionized water, and to the unknown cuvette was added 0.1 ml of 0.001 mol/liter HCl and 0.005 ml sample. The contents of each cuvette were mixed for 5 to 10 seconds with a spatula. Absorbance reading was monitored at 30-second intervals for 5 to 8 minutes at 256 nm. From

the recorder graph an average absorbance change per minute (ΔA/min.) was determined for both blank and unknown. Then, the absorbance difference was calculated by subtracting a blank absorbance from the unknown absorbance. The net absorbance per minute was multiplied by 1247 to obtain units of α-chymotrypsin per mg of protein.

$$\text{Units/mg protein} = \frac{\text{A/min. X 1000 X 3.005 ml}}{\text{964* X mg of protein}}$$

$$= \text{A/min. X 1247}$$

* 964 is the molar extinction coefficient of BTEE at 256 nm.

D. Separation and Identification of Lipid Classes by use of Thin-layer Chromatography:

1. Apparatus:

The TLC plates were initially developed in a chloroform-methanol (2:1, v/v) solvent to remove organic contaminants. The plates were then activated at 150°C for 40 to 60 minutes before use (141).

2. Preparation of lipid standards:

The lipid standards were made up in chloroform-methanol (2:1, v/v) to contain 20 g/liter.

3. Extraction of lipids:

The lipids were extracted according to Folch et al (142) with a following modification: one ml of pooled serum or 14 ml of lipolyzed pooled serum (1 volume of sample plus 13 volumes of

lipolysis reagent mixture) was pipetted in a separatory funnel and 8 ml of chloroform-methanol (2:1, v/v) was added. The lower phase was collected and the solvent was then evaporated at 40°C under a stream of nitrogen gas. For the lipolyzed pooled serum, four additional 20 ml portions of solvent were used to extract the lipids and the solvent was evaporated as above. The residue was redissolved in 0.1 ml of chloroform-methanol (2:1, v/v) before applying on a TLC plate.

4. Thin-layer chromatography:

Five µl of lipid standards and extracted lipids from the pooled serum was applied on appropriate lanes of a silica gel plate using a 10 µl Hamilton Repeating Dispenser Syringe. Each plate was developed in two different solvent systems.

a. Solvent system No. 1:

Diethyl ether:benzene:ethanol:acetic acid (40:50:2:0.2 v/v) mixture was placed in the developing tank. The solvent was then allowed to ascend a distance of about 15 cm from the point of sample application to separate more polar lipids. The TLC plate was removed, air-dried, and placed in the second tank. It was developed in the same direction using the second solvent (141).

b. Solvent system No. 2:

Hexane:diethyl ether:acetic acid (180:30:2) mixture was allowed to reach about 1 cm from the top of the TLC plate

(35, 67 and 68). The TLC plate was then air-dried and charred. In the second solvent system, non-polar lipids are separated.

5. Charring for lipid identification:

A 70 per cent (v/v) sulfuric acid solution saturated with potassium dichromate was made according to Privett and Blank (143). After the TLC plate had been sprayed with this solution, it was charred by heating at 150°C for 60 minutes.

E. Calibration of 340 nm Filter of CentrifiChem Fast Analyzer using Glucose-Hexokinase Method and Estimation of Autopipettor Accuracy using Acid Potassium Dichromate Standards:

Reagent preparation:

1. Glucose standards:

   a. Working glucose standard No. 1 (0.20 g/liter):

      Five ml of 200 mg/100 ml glucose (CAP Clinical Standard) was placed in 50 ml volumetric flask. Deionized water was added to the 50 ml mark.

   b. Working glucose standard No. 2 (0.10 g/liter):

      Twenty ml of the working glucose standard No. 1 and 20.0 ml of deionized water were thoroughly mixed.

   c. Working glucose standard No. 3 (0.05 g/liter):

      Into a 10.0 ml of the working glucose standard No. 2, 10.0 ml of deionized water was added.

2. Glucose assay: (Calbiochem Glucose Stat-Pack)

For calibration of 340 nm filter, 13.65 ml of deionized water was pipetted into vial-B and all contents of vial-B was poured into vial-A. Glucose reagents contained 0.66 U/ml hexokinase, 0.33 U/ml glucose-6-phosphate dehydrogenase, 17.0 mmol/liter magnesium, 0.5 mmol/liter ATP and 0.45 mmol/liter NADP in the final reaction mixture.

Method for calibration of 340 nm filter:

1. Absorbance of reagent blank:

   Three hundred fifty µl of glucose reagent was pipetted into the reagent wells of the transfer disc using a 1.0 ml serological pipette. Fifty µl of deionized water was pipetted into the sample wells of the transfer disc using a 0.2 ml T.D. pipette. Deionized water was placed in the reference well.

   The controls on CentrifiChem Fast Analyzer were set to read as follows: 340 nm, 30°C, T_o delay of 60 seconds, Δ T of 1 minute, 5 prints, Write, Terminal Mode, Operate and Absorbance. The reagent blank was stored in the analyzer after examining the absorbance values.

2. Absorbance calibrating solutions:

   Fifty µl of the working glucose standards and 0.35 ml of glucose reagent were manually pipetted into the appropriate wells of a transfer disc. Deionized water was placed in the reference well. The controls on CentrifiChem Fast Analyzer were set to read: 340 nm, 30°C, T_o delay of 3 seconds, ΔT of 4 minutes, 4 prints, Store, Terminal Mode, Operate and Absorbance. The

absorbance results obtained for each glucose standard were averaged and plotted on a graph, absorbance vs. glucose concentration, to check for linearity. If the results showed linearity, a new absorbance potentiometer setting was calculated using the working glucose standard No. 1 (0.02 g/liter).

$$\text{new absorbance potentiometer setting} = \frac{\text{Expected A} \times \text{Old absorbance potentiometer setting}}{\text{Measured A}}$$

Estimation of autopipettor accuracy:

Potassium dichromate crystals (primary standard) was dried in 140°C oven for two hours. Dehydrated potassium dichromate (898 mg) was dissolved in one liter of 0.01 N H_2SO_4. One hundred ml of 898 mg/liter acid potassium dichromate standard solution and 100 ml of 0.01 N H_2SO_4 were thoroughly mixed to give up 449 mg/liter acid potassium dichromate standard solution.

These two acid potassium dichromate standard solutions were appropriately diluted with 0.01 N H_2SO_4 to give 0.05 g/liter and 0.10 g/liter acid potassium dichromate.

After storing the absorbance of 0.01 N H_2SO_4 in the instrument, approximately 0.4 ml of the 0.05 and 0.10 g/liter acid potassium dichromate standards was manually pipetted into the appropriate reagent wells of a transfer disc using Eppendorf pipette while the reference well contained 0.01 N H_2SO_4. The absorbance results of the two working standards were averaged, which served as the reference values.

The autopipettor accuracy was determined by programming 50 µl of sample volume, 99 µl of sample-diluent volume, and 350 µl of 0.01 N H_2SO_4. The acid potassium dichromate standards, 449 mg/liter and 898 mg/liter, were poured into the sample cups and the cups were placed on a sample holder. The reagent reservoir contained 0.01 N H_2SO_4. The acid potassium dichromate standards and 0.01 N H_2SO_4 reagent were automatically pipetted into appropriate wells of the transfer disc. The control settings of the Centrifi-Chem Fast Analyzer were the same as for the manual pipetting procedure described above. The absorbance values obtained by the reference method and those of the automatic pipetting method were compared to determine the automatic pipetting accuracy.

F. Recommended Routine Procedure for Serum Triglyceride Assay:

1. An enzymatic hydrolysis of serum triglycerides:

Reagent preparation:

1. Bovine serum albumin (12.73 g/liter): Sigma, 35% BSA (3.46 ml) was placed in a 100 ml volumetric flask. Tris-HCl buffer of pH 7.6 and 0.1 mol/liter was added to the 100 ml mark.
2. Gum arabic (127 g/liter):

   Gum arabic (12.7 g) was placed in a 250 ml Erlenmeyer flask. Fifty ml of deionized water and 50 ml of Tris-HCl, pH 7.6 and 0.1 mol/liter, were added. The content was completely dissolved by vigorously shaking for 1 - 2 hours using a reciprocating shaker.

3. Deoxycholic acid (140 mmol/liter): Calbiochem, MW 392.6
   Deoxycholic acid (274.8 mg) was dissolved in 4.3 ml deionized water and 0.7 ml of 1.0 mol/liter NaOH.
4. α-chymotrypsin (700 U/ml at 30°C): Calbiochem, 37 I.U./liter at 30°C.
   α-chymotrypsin (18.9 mg) was dissolved in 1.0 ml of 0.001 mol/liter HCl. Ten µl of 1.0 mol/liter $CaCl_2$ was added to make a final concentration of 0.01 mol/liter.
5. Universal buffer consisted of 9.714 g of sodium acetate and 14.714 g of sodium barbital in 500 ml deionized water. Five ml of this solution and 2.0 ml of 8.5% NaCl were mixed and the volume was adjusted to 25 ml after adjusting the pH to 5.6 with 0.1 N HCl (18).
6. Lipase (Rhizopus delemar, 3500 U/ml at 25°C): Miles Laboratories, Inc., having 7040 U/mg at 25°C
   Rhizopus delemar lipase (2.01 mg) was dissolved in 4.0 ml of universal buffer solution (pH 5.6, ionic strength of 0.16) and 0.02 ml of 1.0 mol/liter $CaCl_2$ was added.

Procedure:

Into a 13 X 100 mm disposable test tube, 0.55 ml of bovine serum albumin (12.73 g/liter), 0.055 ml of gum arabic (127 g/liter), 0.01 ml of deoxycholate (140 mmol/liter), 0.01 ml of α-chymotrypsin (700 U/ml at 30°C), and 0.025 ml of lipase (3500 U/ml at 25°C) were added. Fifty µl of a serum

specimen and 50 µl of deionized water (as a reagent blank) were added to the lipolysis reagent and the contents were gently mixed. The above mixture was then incubated for 15 minutes in a 30°C water bath. The final concentrations of the lipolysis reagents consisted of 10 g/liter BSA, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholic acid, 10 U/ml α-chymotrypsin, and 125 U/ml lipase.

2. An enzymatic glycerol assay using GK-GDH method:

Reagents preparation:

1. Hydrazine-glycine-magnesium buffer solution:

   a. Hydrazine (460.8 mmol/liter): 99%, Mallinckrodt Chemical Works

      Hydrazine hydrate (2.24 ml) was placed in a 100 ml volumetric flask. Density of hydrazine hydrate, 1.03 kg/liter at 21°C, was used.

   b. Glycine (229.7 mmol/liter): 99.5%, ammonium-free, Matheson Coleman and Bell

      Glycine (1.721 g) was added to the above volumetric flask.

   c. Magnesium (3.2 mmol/liter):

      $MgCl_2 \cdot 6H_2O$ (0.32 ml of 1.0 mol/liter) was placed in the above volumetric flask.

   About 90 ml of deionized water was added to the volumetric flask and the contents were dissolved. Using

10 mol/liter KOH, pH was adusted to 9.2 ± 0.1. Then, the final volume was brought to the 100 ml mark with deionized water. The hydrazine-glycine-magnesium reagent was stored in a brown bottle at 0 - 4°C.

2. ATP (40.18 mmol/liter): MW 551.2, Calbiochem

   ATP (1.107 g) was placed in a 50 ml volumetric flask. Forty-five ml of deionized water was added to dissolve the contents and the pH was adjusted to 7.0 with 1.0 mol/liter NaOH. The final volume of 50 ml was adjusted with deionized water. About 5 ml of ATP reagent was placed in a 13 X 100 mm test tube and stored at -20°C.

3. NAD^+ (24.2 mmol/liter): Sigma Chemical Co., β-NAD, MW 663.5

   NAD^+ (802.8 mg) was dissolved in 50 ml deionized water. About 5 ml of the NAD^+ reagent was placed in a 13 X 100 mm test tube and stored at -20°C.

4. DTT (Dithiothreitol, 1.58 mol/liter):

   DTT (243.5 mg) was dissolved in 1.0 ml of deionized water and stored at 0 - 4°C.

5. Bovine serum albumin (35%, Sigma Chemical Co., A5128)

6. Glycerokinase (197 I.U./ml, 30°C, in 2.6 mol/liter $(NH_4)_2SO_4$, Calbiochem)

7. L-Glycerophosphate dehydrogenase (160 U/mg protein, 25°C, 2.0 mol/liter $(NH_4)_2SO_4$, Sigma Chemical Co.)

Enzymatic assay of glycerol:

Just before analysis of glycerol, the following volumes of glycerol measuring reagents were premixed and poured in a reagent tray: 1.0 ml of hydrazine-glycine-magnesium solution, 0.018 ml of 35% BSA, 0.1 ml of ATP, 0.1 ml of NAD^+, 0.005 ml DTT, 0.005 ml of glycerokinase (GK), and 0.005 ml of α-glycerophosphate dehydrogenase (GDH). The final concentrations of these reagents consisted of 290 mmol/liter hydrazine, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4.0 g/liter BSA, 2.5 mmol/liter ATP, 1.5 mmol/liter NAD^+, 5.0 mmol/liter DTT, 0.4 U/ml (30°C) GK, and 4.0 U/ml (25°C) GDH.

1. Loading transfer disc:

   Deionized water and the glycerol measuring reagent trays were placed in the appropriate spaces on the automatic pipettor. The lipolyzed specimens were poured into cups and placed in a sample tray and lipolysis reagent blank was placed at zero or reference position. A magnetic stop was inserted after the last sample. This was to prevent further pipetting of the reagent to the transfer disc. After placing a dry and clean transfer disc inside the sample tray, 50 µl of sample, 49 µl of deionized water (sample diluent volume of 99 µl), and 350 µl of the reagent were automatically pipetted into a transfer disc. The reagent was pipetted into the zero or reference well of the transfer disc by turning off the "Power Switch" at

No. 29 position and then turning on the "Power" to initiate pipetting of the reagent into the reference well and the lipolysis reagent blank into the sample well.

2. Assay of glycerol:

The liberated glycerol was assayed at 30°C, 340 nm and with the following setting of the control panel of CentrifiChem: absorbance potentiometer of 680, T_o delay of 3 seconds, 5 prints, concentration factor of 20.2, ΔT of 4 minutes, Write, Terminal Mode, Operate, and Concentration.

a. Absorbance potentiometer was calibrated monthly using glucose standard (200 mg/100 ml in 0.25% benzoic acid).

b. Concentration factor = 20.2 This is the number by which the absorbance readings are multiplied to yield a result in mmol/liter.

$$\text{mmol/liter} = \frac{A \times 0.70 \text{ ml} \times 0.449 \text{ ml}}{6.22 \times 0.05 \text{ ml} \times 0.05 \text{ ml}} = A \times 20.2$$

RESULTS

INSTRUMENTAL PERFORMANCE

Spectrophotometric Accuracy and Linearity

The calibration of the Beckman DU Spectrophotometer using acid potassium dichromate was essentially the method described by Rand (144). At 350 nm, the expected absorbances of the 0.10 g/liter and 0.05 g/liter working acid potassium dichromate standards should be 1.070 and 0.535, respectively. A cuvette with a 10 mm light path was used for the calibration.

The 1.41 mmol/liter NADH in 0.2 mol/liter, pH 7.6 Tris-HCl buffer solution was serially diluted to give NADH concentrations ranging from 0.022 to 0.705 mmol/liter in the same Tris-HCl buffer solution. The absorbance values of the diluted NADH solutions were obtained at 340 nm using the Tris-HCl buffer solution as a blank. It was found that the spectrophotometric linearity of the Beckman DU Spectrophotometer was good up to approximately 1.5 absorbance unit (Fig. 4).

The calibration of the 340 nm interference filter of the Centrifi-Chem Fast Analyzer was done by using glucose-hexokinase method in which each mole of glucose reduces one mole of $NADP^+$ to yield NADPH.

$$\text{Glucose} + \text{ATP} \xrightleftharpoons{\text{HK}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightleftharpoons{\text{G-6-PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+$$

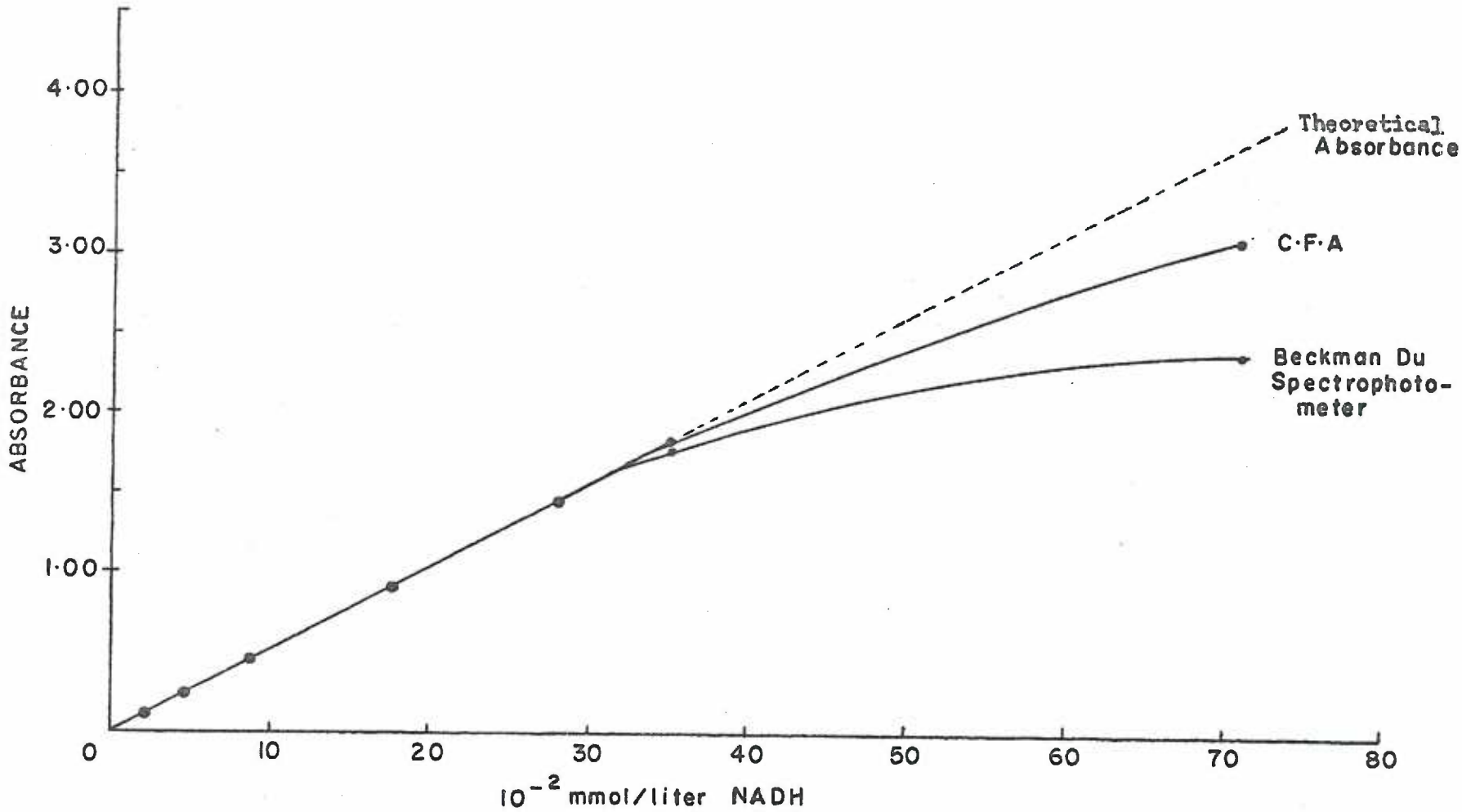


Fig. 4. The absorbance linearity assessment of the 340 nm interference filter of CentrifiChem Fast Analyzer and Beckman DU Spectrophotometer with Gilford Attachment using NADH in PH 7.6 Tris.HCl buffer solution.

After equilibrium was reached, the absorbance potentiometer of the CentrifiChem Fast Analyzer was manually adjusted to give the expected absorbance value. The reason for calibrating the CentrifiChem Fast Analyzer by the glucose-hexokinase method was that this method did not depend on the accuracy of another spectrophotometer. The band width of the 340 nm filter is about 15 nm (Fig. 5) and might be expected to show poorer linearity or yield lower absorbance values than a narrow band pass instrument (145).

After the calibration of the 340 nm interference filter, the same NADH solutions used to measure the absorbance linearity of the Beckman DU Spectrophotometer were analyzed and the mean absorbance results were plotted (Fig. 4). CentrifiChem Fast Analyzer showed higher photometric linearity, 1.8 absorbance units, than the Beckman DU Spectrophotometer, 1.5 absorbance units. A comparison of absorbance values of NADH obtained by the Beckman DU Spectrophotometer and CentrifiChem Fast Analyzer was summarized in Table 2. Both of the instruments showed identical absorbance results up to ca. 1.000.

Accuracy Assessment of the Autopipettor

Four tenths ml of the 0.05 g/liter and 0.10 g/liter acid potassium dichromate standard solutions was manually pipetted into the appropriate wells of the transfer disc and the absorbance values at 340 nm were obtained. This manual technique was used as a reference method to check the precision and the accuracy of the autopipettor. Fifty µl of the acid potassium dichromate standard solutions having 0.449 g/liter

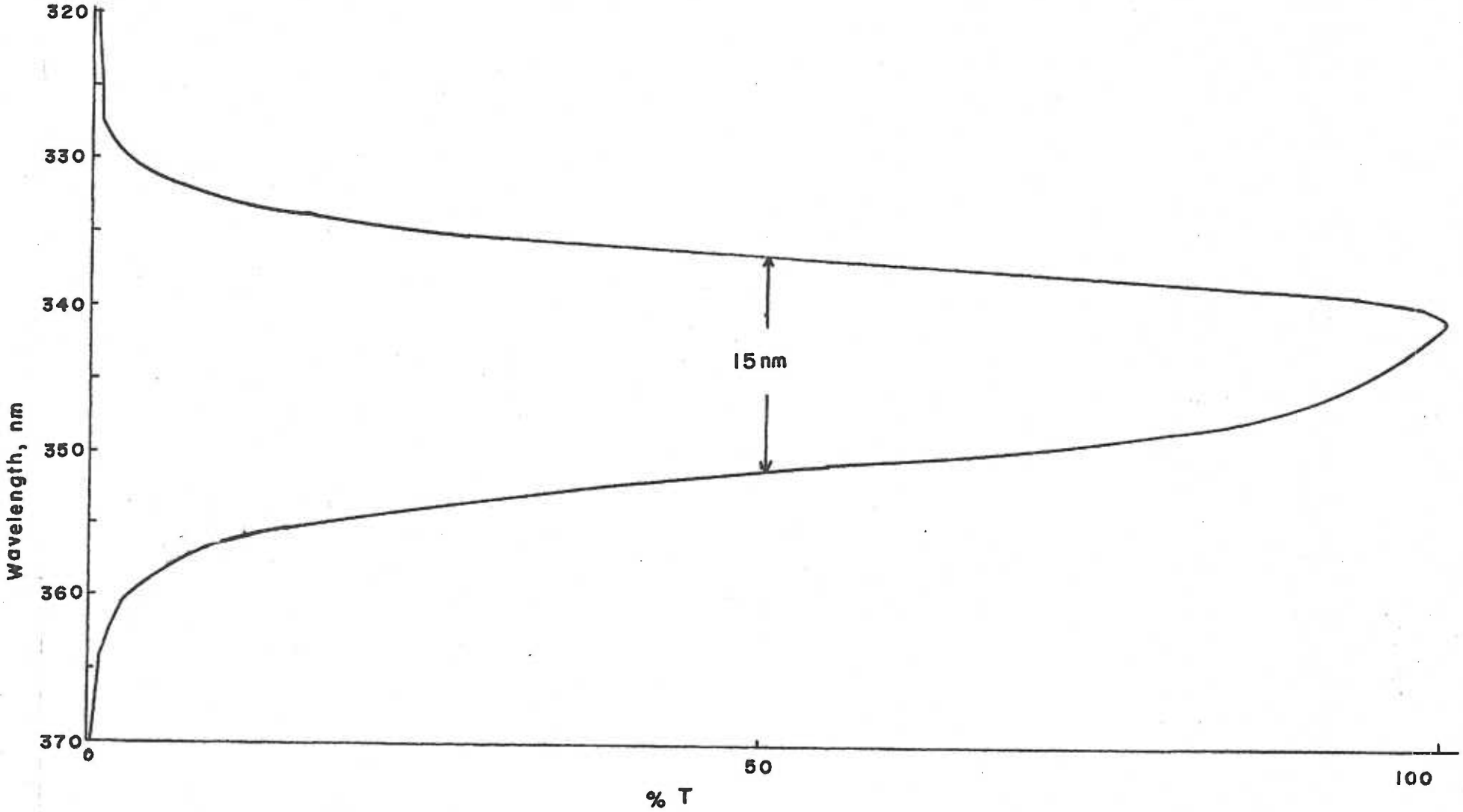


Fig. 5. Estimation of bandwidth of 340 nm interference filter of CentrifiChem Fast Analyzer. Beckman Acta CIII Double Beam Spectrophotometer was used to scan the filter at 1 nm bandwidth.

Table 2. A comparison of absorbance values obtained between Beckman DU spectrophotometer with Gilford attachment and CentrifiChem Fast Analyzer.

Concentration of NADH (Approx. mmol/liter)	Absorbance values obtained				
	Beckman Du with Gilford attachment	CentrifiChem Fast Analyzer			
		$\bar{X}$	S.D.	C.V.(%)	N
0.022	0.126	0.123	0.00185	1.51	29
0.044	0.245	0.243	0.00235	0.96	26
0.088	0.489	0.485	0.00190	0.40	29
0.176	0.960	0.961	0.00230	0.23	28
0.282	1.483	1.502	0.00240	0.16	29
0.352	1.799	1.850	0.00360	0.19	27
0.705	2.399	3.110	0.01020	0.30	28
1.410	2.434	3.379	0.08800	2.60	28

Note: Absorbance potentiometer setting of 673 was used to determine the absorbance values of various NADH solution.

and 0.898 g/liter were automatically pipetted into the appropriate wells of the transfer disc and their absorbance values were determined. The accuracy of the autopipettor technique was expressed as a per cent difference from the mean absorbance of the manual pipetting technique. It was found that using the 0.05 g/liter acid potassium dichromate standard solution, the per cent difference ranged from + 0.56 per cent to + 1.02 per cent while using 0.10 g/liter acid potassium dichromate standard solution, the per cent difference was between + 0.45 per cent and - 1.36 per cent (Table 3). The intra-disc coefficient of variation of the autopipettor using 50 µl of sample, 49 µl of diluent, and 350 µl of 0.01 N H_2SO_4 was calculated to be 0.67 per cent with a mean absorbance of 0.55 while at a mean absorbance value of 1.1, the coefficient of variation was 0.34 per cent. In another experiment 50 µl of BSP dye (Bromosulphthalein) in a pooled serum and 350 µl of pH 10.0 $\pm$ 0.1 alkaline buffer solution were pipetted using the autopipettor. The absorbance values were measured at 550 nm and the coefficient of variation was 0.94 per cent for a mean absorbance of 0.282.

The electronic-optical uncertainty of the analyzer unit was also determined using the measured absorbances of the manually diluted dichromate solutions. Four tenths ml of the diluted standard solutions was manually pipetted into the wells of a transfer disc. Since there was no further dilution or addition of 0.01 N H_2SO_4 or diluent water to these diluted standard solutions, any variation in the mean absorbance values from cell to cell must be caused by electronic-optical noise and inaccuracies of the CentrifiChem Fast Analyzer. The mean

Table 3. Estimation of autopipettor accuracy and electronic-optical uncertainty of CentrifiChem Fast Analyzer using acid potassium dichromate standard solutions.

Acid $K_2Cr_2O_7$	manual pipetting (absorbance unit)		autopipetting (absorbance unit)	
(0.05 gm/liter) N	10	29	10	29
$\bar{X}$	0.5528	0.5602	0.5497	0.5545
SEM	0.00073	0.00037	0.00165	0.00120
1 S.D.	0.0023	0.00199	0.00521	0.00646
C.V.(%)	0.42	0.36	0.95	1.17
	mean = 0.39%		mean = 1.06%	
(0.10 gm/liter) N	10	29	10	29
$\bar{X}$	1.101	1.109	1.116	1.104
SEM	0.0019	0.00044	0.00173	0.00207
1 S.D.	0.0061	0.00237	0.00547	0.01115
C.V.(%)	0.56	0.21	0.49	1.01
	mean = 0.41%		mean = 0.75%	

Difference between manual and autopipettor means

Acid $K_2Cr_2O_7$	manual $\bar{X}$ - autopipettor $\bar{X}$ (absorbance unit)	% Difference from the manual mean
0.05 gm/liter	+0.0031 to -0.0057	+0.56% to +1.02%
0.10 gm/liter	+0.005 to -0.015	+0.45% to -1.36%

electronic-photometric uncertainty was found to be ca. 0.40 per cent (Table 3) for absorbance values of 0.5 and 1.0. When BSP dye was used to assess the electronic-optical uncertainty, it was 0.46 per cent. Statland et al (146) reported that the electronic-optical uncertainty was 0.34 per cent using a different kind of Centrifugal Fast Analyzer.

Effect of Hydrazine on NAD^+ at Alkaline pH

Two solutions containing ca. 0.025 mmol/liter NAD^+ and NADH were separately scanned against a water blank using the Beckman DB Spectrophotometer. A characteristic absorption curve of NAD^+ and NADH were obtained: NAD^+ and NADH showed maximal absorption at 260 nm and only NADH showed an absorption peak at 340 nm (Figs. 6 and 7). The NAD^+ and NADH were dissolved in deionized water. Figure 7 illustrates the absorption curve of the combined NAD^+ and NADH.

When 1.0 mol/liter hydrazine hydrate in 230 mmol/liter glycine and 3.2 mmol/liter magnesium chloride buffer was scanned against a water blank, there was no absorption peak observed near 340 nm (Fig. 8). However, when the same concentrations of hydrazine buffer solution (pH 9.8) was mixed with 1.8 mmol/liter NAD^+ and scanned against a water blank, the absorption curve did not resemble those of Figures 6 or 8. Instead, at 340 nm, the absorbance value was increased from 0.0 to 0.5 (Fig. 9-A). When the same hydrazine-NAD^+ solution was scanned against a blank solution containing an equal amount of NAD^+ (1.8 mmol/liter) in deionized water, the difference spectrum showed a peak absorption of the hydrazine-NAD^+ at 310 nm (Fig. 9-B). The same

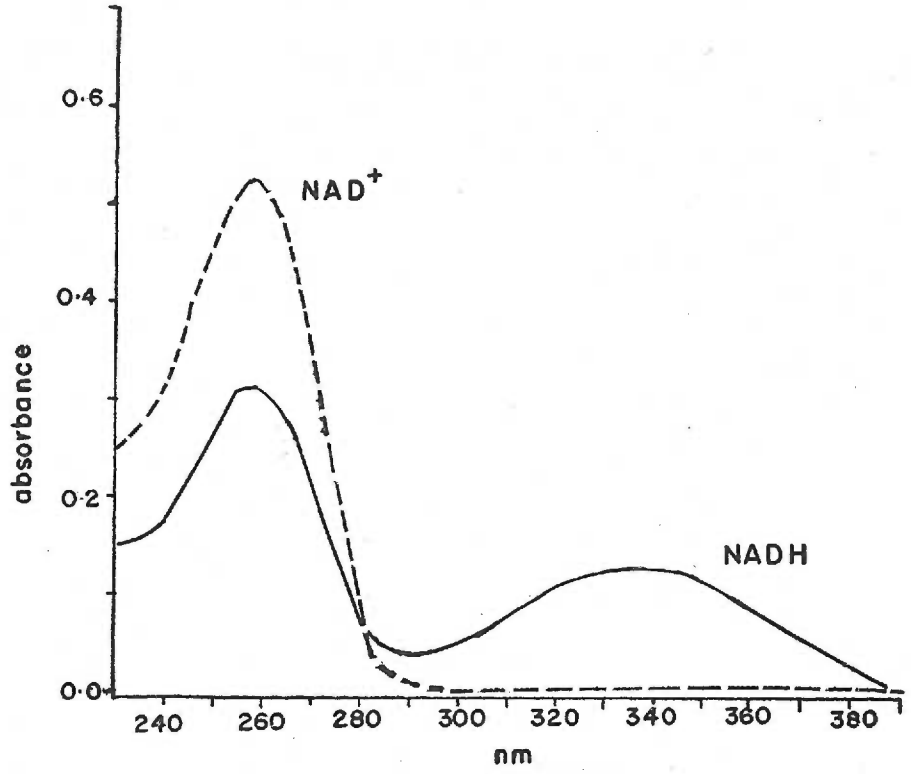


Fig. 6. Absorption curves of NAD^+ and NADH which were separately scanned against water blank. The concentrations of NAD^+ and NADH were ca. 0.025 mmol/liter, dissolved in deionized water.

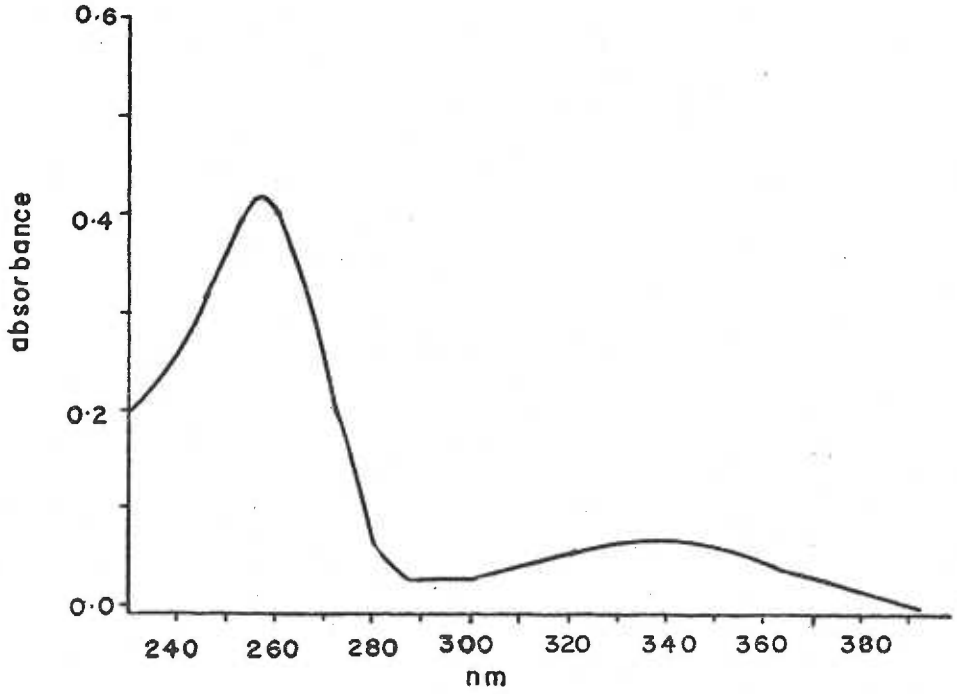


Fig. 7. Absorption curve of the combined NAD^+ and NADH solution (0.025 mmol/liter) which was scanned against a water blank.

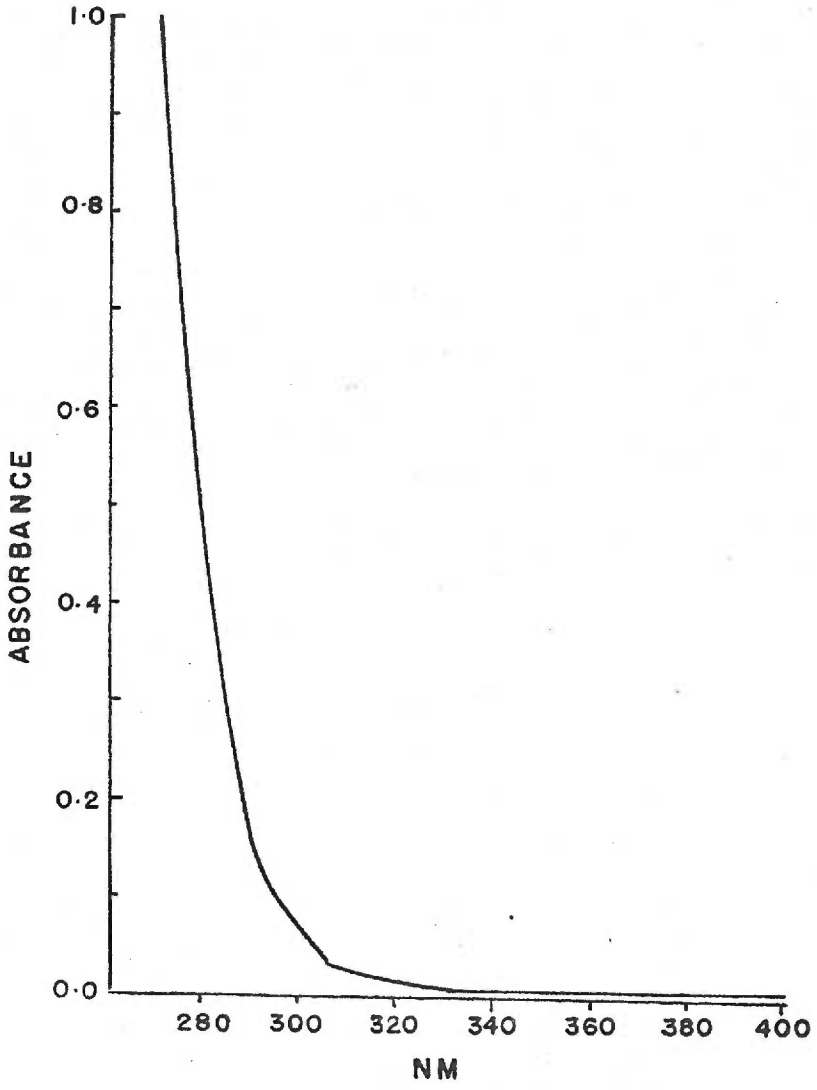


Fig.8. 1.0 mol/liter hydrazine in the buffer solution was scanned against water blank. The hydrazine buffer solution contained 230 mmol/liter glycine and 3.2 mmol/liter magnesium.

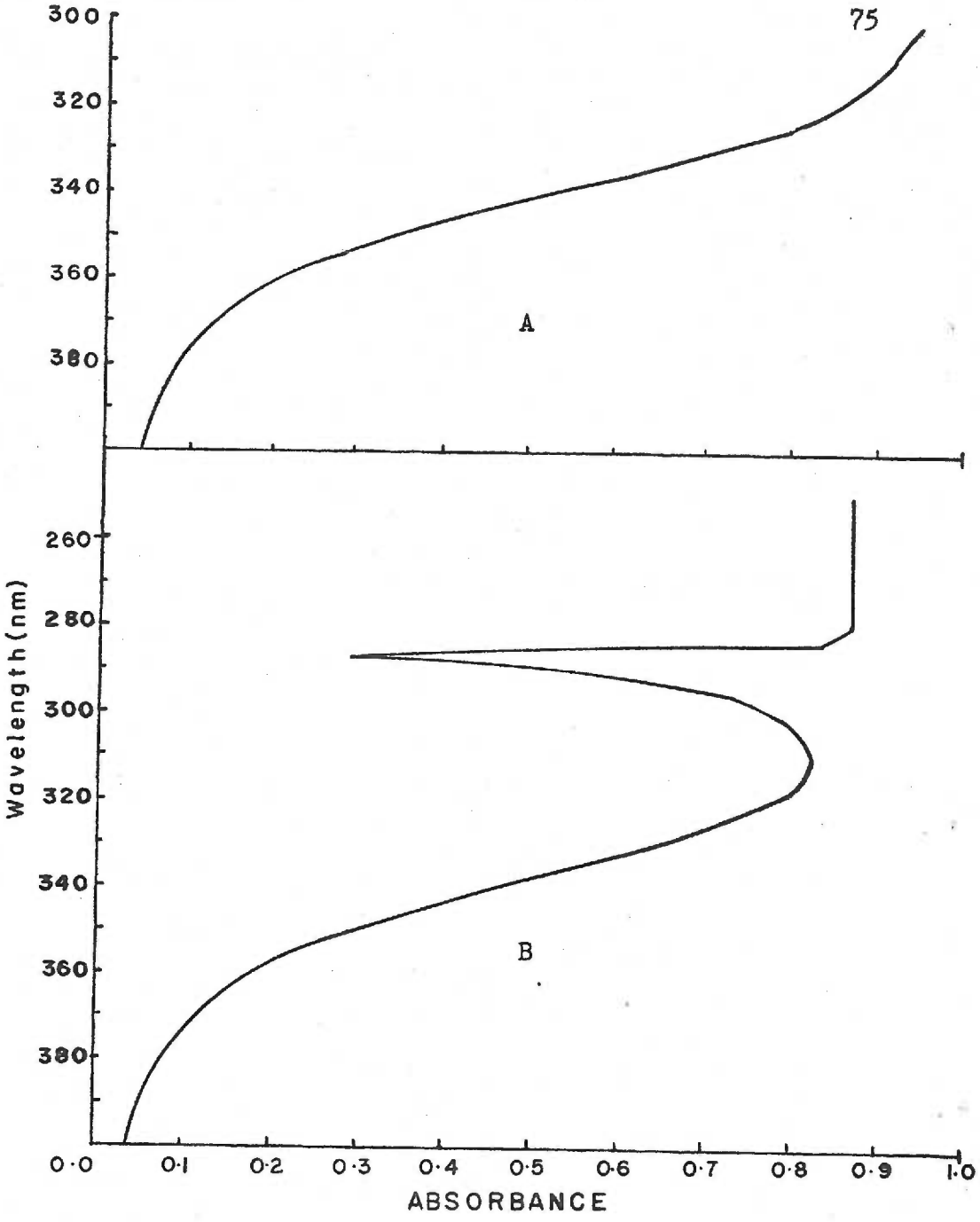


Fig. 9. 1.0 mol/liter, pH 9.8 hydrazine·H_2O and 1.8 mmol/liter NAD^+ were scanned against water (A) and against water containing 1.8 mmol/liter NAD^+ blank (B).

concentrations of glycine with NAD^+ showed essentially no increase in absorbance at 340 nm and the absorption curve was identical to the Figure 8. Therefore, it appeared that NAD^+ molecules had been altered by hydrazine, giving a new maximal absorption at 310 nm (Fig. 9-B).

When 0.1 mmol/liter NADH was added to glycerol measuring reagent containing 1.0 mol/liter hydrazine buffer, 5.0 mmol/liter ATP, glycerokinase, and glycerophosphate dehydrogenase without NAD^+, the maximum absorbance was at 340 nm (Fig. 10). When 0.025 mmol/liter NADH in hydrazine buffer was scanned against water containing the same concentration of NADH, the maximal absorption at 340 nm as seen in Figure 10 disappeared as one expected (Fig. 11). After incubating NADH in the glycerol measuring reagent without NAD^+ for 20 minutes in a 30°C water bath, the identical absorption curve as in Figure 10 was obtained. Since the proposed glycerol measuring technique utilizes hydrazine buffer in which NAD^+ is reduced to NADH by glycerophosphate dehydrogenase in the presence of α-glycerophosphate, it is extremely important that the reduced NADH must not be altered by hydrazine. To substantiate that the reduced NADH is stable in the hydrazine buffer, NADH concentrations of 0.016 and 0.070 mmol/liter in 1.0 mol/liter hydrazine buffer containing 5.0 mmol/liter ATP, glycerokinase, and glycerophosphate dehydrogenase were incubated in a 30°C water bath. The change in absorbance at 340 nm was monitored for 20 minutes using the Beckman DU Spectrophotometer. Table 4 shows the results of this experiment in which there was insignificant change in absorbance: 0.016 mmol/liter NADH gave 0.105 $\pm$ 0.003 absorbance while 0.070 mmol/liter NADH gave

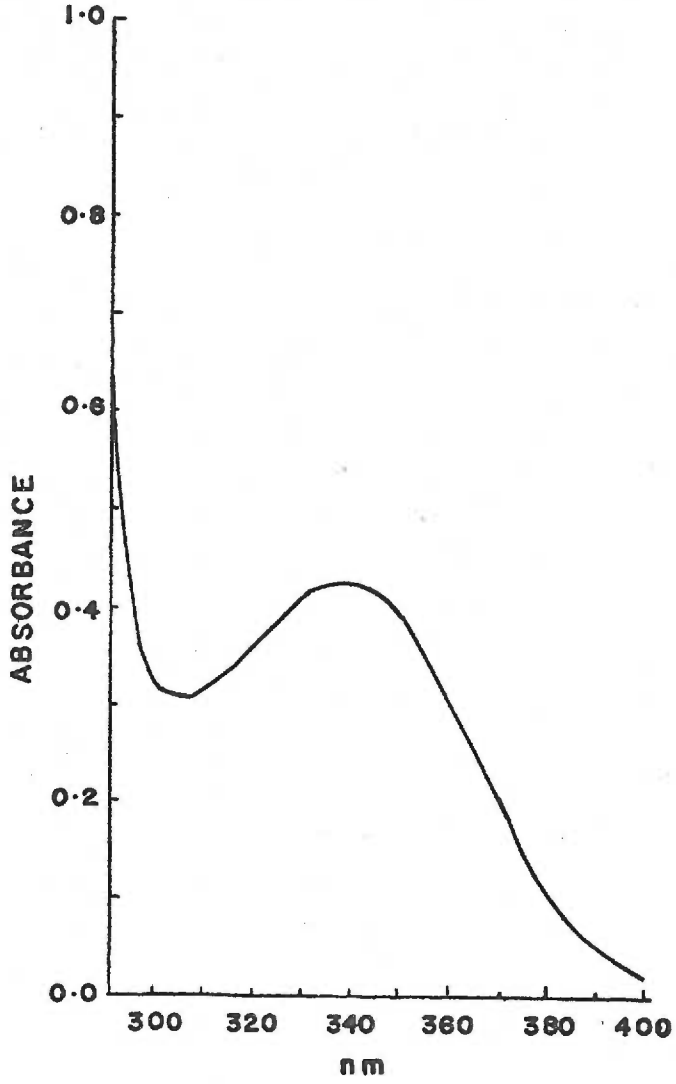


Fig.10. 0.1 mmol/liter NADH in hydrazine buffer, 5.0 mmol/liter ATP, GK, and GDH were scanned against the hydrazine buffer blank.

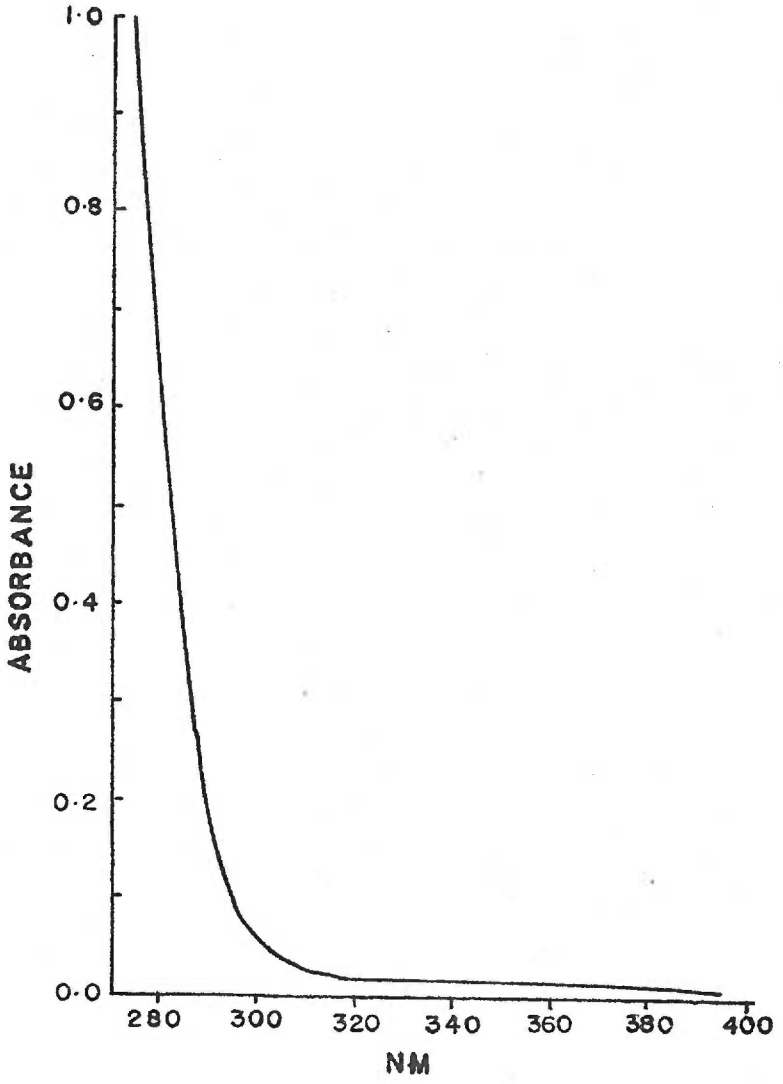


Fig. 11. 0.025 mmol/liter NADH and hydrazine buffer were scanned against water containing 0.025 mmol/liter NADH.

Table 4. Stability of NADH in 1.0 mol/liter hydrazine buffer, 5.0 mmol/liter ATP, GK, and GDH glycerol measuring reagent incubated at 30°C.

Time of incubation (min.)	Absorbance values obtained by Beckman DU Spectrophotometer with Gilford Attachments	
	0.016 mmol/liter NADH	0.070 mmol/liter NADH
1	0.100	0.441
2	0.106	0.439
3	0.110	0.437
4	0.108	0.436
5	0.108	0.436
6	0.108	0.435
7	0.105	0.434
8	0.105	0.435
9	0.103	0.431
10	0.103	0.431
15	0.101	0.431
20	0.101	0.430
$\bar{X}$	0.105	0.435

0.435 ± 0.003 during the 20-minute observation.

The glycerol measuring reagent containing hydrazine buffer, ATP, NAD^+, glycerokinase, glycerophosphate dehydrogenase, and 0.05 umole of glycerol standard solution was incubated in a 30°C water bath for 15 minutes and the reaction mixture was scanned against a water blank (Fig. 12-A). As observed in Figure 9-A, the peak absorption was at 310 nm. However, when the same reaction mixture was scanned against the glycerol measuring reagent without the glycerol standard, the maximal absorption was at 340 nm (12-B). This experiment indicates that the glycerol measuring reagent blank should be simultaneously run along with the unknown in order to correct for the erroneous absorption curve superimposed on the NADH absorbance.

The effect of hydrazine concentration on the absorbance of the NAD^+-hydrazine complex was examined. Glycine buffer containing 0.30 mol/liter glycine and 3.2 mmol/liter magnesium chloride was used to make appropriate dilutions of hydrazine hydrate. The pH of the diluted hydrazine-glycine-magnesium buffer solutions was adjusted to 9.8 with 10 mol/liter KOH. Then, hydrazine-glycine-magnesium buffer and NAD^+ were gently mixed to give final concentrations of 145 mmol/liter glycine, 2.0 mmol/liter magnesium, and 1.5 mmol/liter NAD^+ in the reaction mixture. A total of 99 µl of deionized water and 350 µl of the glycerol measuring reagents containing 0.04 to 2.86 mol/liter hydrazine hydrate were automatically pipetted into the appropriate wells of a transfer disc. The absorbance change between 3 seconds and 15 minutes after mixing was measured. It was found that the increase in

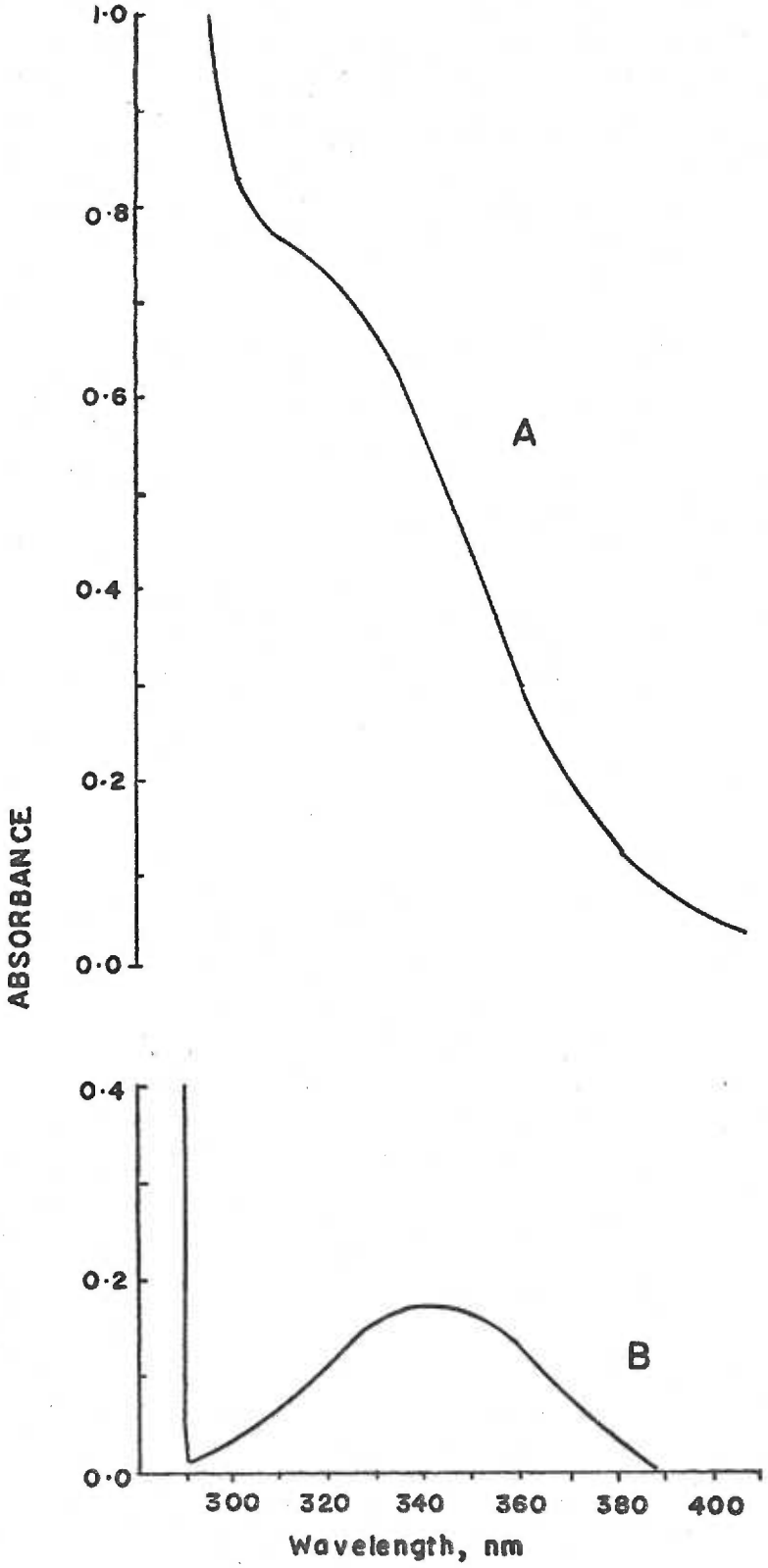


Fig.12. The glycerol measuring reaction mixture containing hydrazine buffer, ATP, NAD^+, GK, GDH, and 0.05 µmol glycerol standard per assay was incubated in 30°C water bath and scanned against water blank(A) and against the glycerol measuring reagent balnk without glycerol standard (B).

hydrazine concentration with a constant concentration of NAD^+ rendered a linear absorbance increase (Fig. 15-H_2O). The hydrazine of 2.86 mol/liter with 1.5 mmol/liter NAD^+ showed an absorbance value of 0.10 while 0.25 mol/liter hydrazine gave 0.02 A. Absorbance changes prior to 3 seconds were not measured.

Effect of Various pHs of Hydrazine Buffer on NAD^+

We examined the extent of absorbance change when varying pHs of hydrazine-glycine-magnesium buffer solutions was reacted with NAD^+ containing BSA, ATP, GK, and GDH. Hydrazine-glycine-magnesium buffer solutions were adjusted to pH 8.8, 9.0, 9.2, 9.4, 9.6 and 9.8 with 10 mol/liter KOH. The reaction mixture contained 0.290 mol/liter hydrazine hydrate, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 1.5 mmol/liter NAD^+, 2.5 mmol/liter ATP, 4.0 g/liter BSA, 0.4 U/ml GK, and 4.0 U/ml GDH. Fifty µl of the NAD^+ solution, 50 µl of deionized water and 350 µl of hydrazine-glycine-Mg^{+2} buffer solution were manually pipetted into the appropriate wells of a transfer disc. The reference cuvette contained 350 µl of hydrazine-glycine-Mg^{+2} buffer (pH 8.8) and 100 µl of deionized water. No NAD^+ solution was added to this cuvette. After storing a water blank, final absorbance values were taken when absorbance values of each measurement were stabilized. Figure 13 represents the results of replicate analyses. With an increasing pH of the hydrazine-glycine-Mg^{+2} buffer, the NAD^+ showed increasing absorbance values at 340 nm, especially for pH values above 9.4.

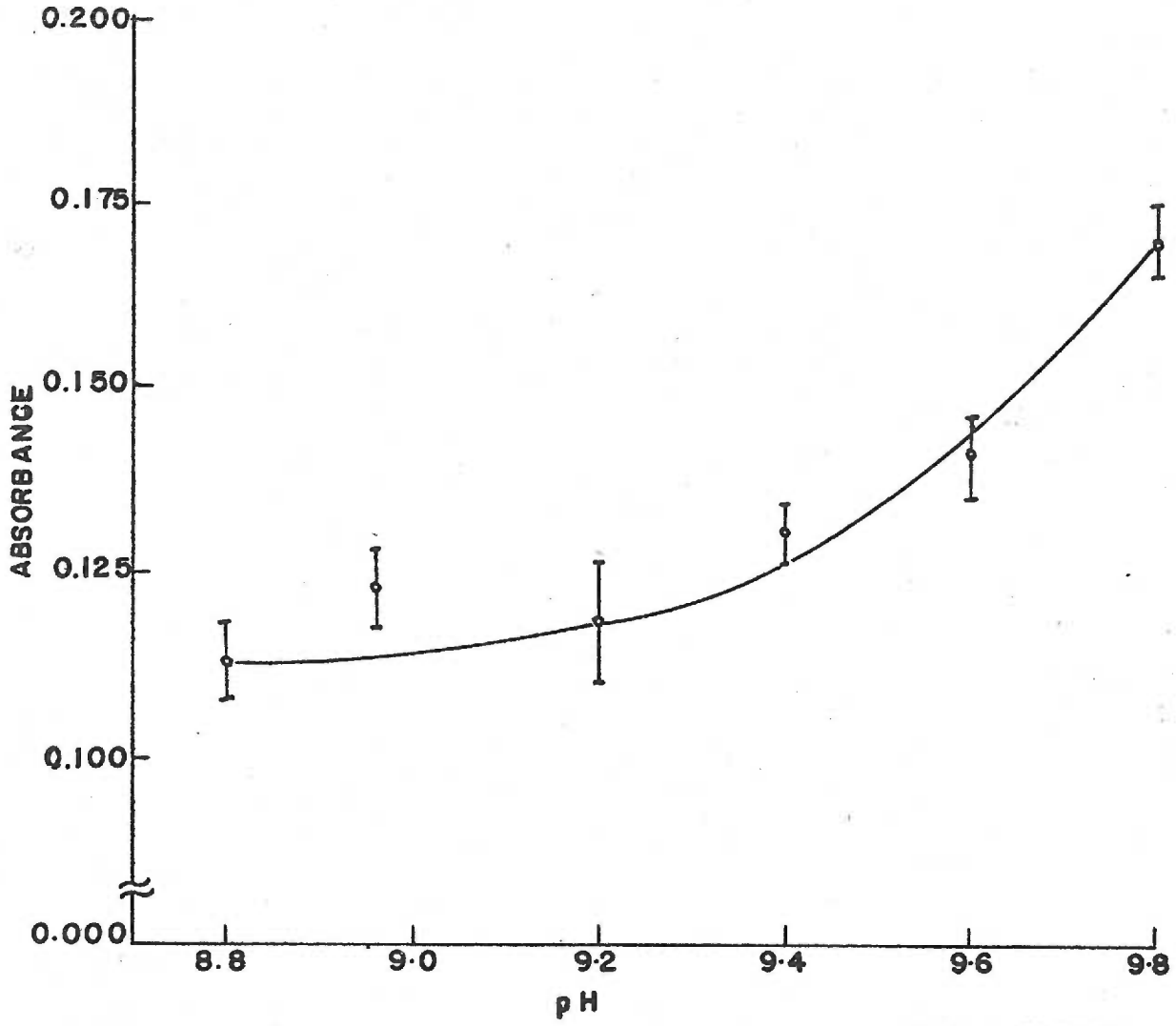


Fig. 13. The absorbance change at 340 nm with increasing pH after the glycerol measuring reagents were simultaneously mixed and assayed. Each bar is a mean ± 1 S.D.

Effect of KOH Concentration on NAD^+

Thus far, we have observed that increasing the hydrazine concentration while keeping NAD^+ constant showed a linear increase in absorbance (Fig. 15). When the pH of the hydrazine-glycine-magnesium-NAD^+ solution is increased the absorbance values above pH 9.4 increase appreciably (Fig. 13). It was of interest to examine the effect of KOH on NAD^+ since hydrazine-glycine-magnesium buffer solutions contained KOH to adjust the pH to 9.8. Potassium hydroxide solutions containing 0.1 to 10.0 mmol/liter were prepared and 350 µl was pipetted into the reagent wells of a transfer disc. Fifty µl of 13.47 mmol/liter NAD^+ and 50 µl of deionized water were placed in the sample wells of the transfer disc. The reference cuvette contained deionized water. After storing a water blank, changes in absorbance values were recorded. Figure 14 illustrates the findings. The KOH solutions without NAD^+ showed zero absorbance. However, increasing the concentration of KOH with 1.5 mmol/liter NAD^+ rendered increasing absorbance values. The KOH with 0.1 mmol/liter gave 0.021 absorbance and 1.0 mmol/liter KOH showed 0.032 A. At the higher KOH (10.0 mmol/liter), it was 0.122 A. KOH concentrations above 2.0 mmol/liter showed a linear increase in absorbance with 1.5 mmol/liter NAD^+.

Spcetral Effect of Varying the Concentration of NAD^+ in the Hydrazine Buffer Mixture at pH 9.8

Final NAD^+ concentrations between 0.0 and 3.0 mmol/liter were added to the glycerol measuring reagent (pH 9.8) containing

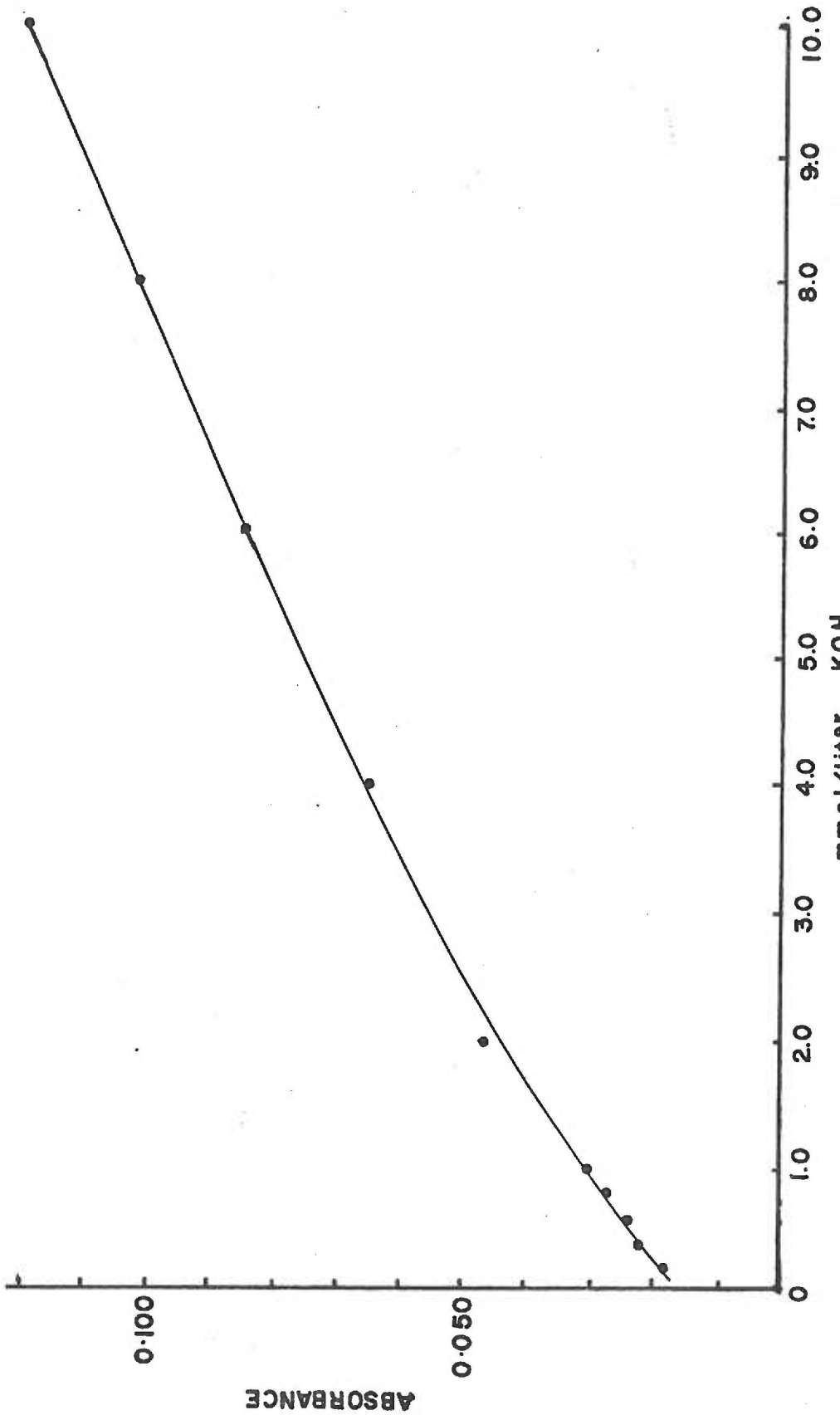


Fig. 14. Absorbance at 340 nm vs. increases in KOH concentration with 1.5 mmol/liter NAD^+.

0.290 mol/liter hydrazine, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4.0 g/liter BSA, 2.5 mmol/liter ATP, 0.4 U/ml GK and 4.0 U/ml GDH. Approximately 10 minutes later, 50 µl of hydrolyzed pooled serum containing 6.7 mmol/liter glycerol, 49 µl of deionized water, and 350 µl of the glycerol measuring reagents with varying concentrations of NAD^+ were pipetted into the appropriate wells of a transfer disc. The glycerol measuring reagent without NAD^+ and but with the lipolysate was placed in the reference cuvette. The absorbance value in this reference cuvette was automatically subtracted from those of the measuring components. The absorbance values are plotted in Figure 19. The NAD^+ concentrations of 1.0, 2.0, and 3.0 mmol/liter gave 0.1, 0.2, and 0.3 absorbance, respectively. At 1.5 mmol/liter NAD^+, the absorbance value was 0.15.

GLYCEROKINASE-GLYCEROPHOSPHATE DEHYDROGENASE METHOD FOR ASSAYING GLYCEROL

Optimal Concentration of Hydrazine

Final hydrazine hydrate concentrations ranging from 0.04 to 2.86 mol/liter in the glycerol measuring reaction mixture were used to determine the optimal concentration needed to quantitatively assay glycerol. The glycine buffer containing 0.30 mol/liter glycine and 3.2 mmol/liter magnesium chloride was used to make appropriate dilutions of hydrazine hydrate. The pH of the diluted hydrazine-glycine-magnesium buffer solutions was adjusted to 9.8 with 10 mol/liter KOH. The final reaction mixtures contained 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 2.5 mmol/liter ATP, 4 g/liter BSA, 1.5 mmol/liter NAD^+,

0.4 U/ml GK and 4.0 U/ml GDH. The pooled sera marked "c" and "2" (Fig. 15) were subjected to enzymatic hydrolysis. Then, 50 µl of the hydrolyzed pooled serum, 49 µl of deionized water, and 350 µl of glycerol measuring reagent were placed in the appropriate wells of a transfer disc. Final absorbance values were taken when there was no further increase in absorbance. When the glycerol content of the lipolysates was assayed in the varying amounts of hydrazine hydrate, the optimal concentrations were found to be between 0.15 and 0.50 mol/liter. The hydrazine concentrations above 0.50 mol/liter showed a decreased recovery of glycerol (Fig. 15, pooled sera c and 2). The optimal concentration of hydrazine hydrate selected for the glycerol measuring method was 0.290 mol/liter.

Effect of Bovine Serum Albumin on Glycerol Measuring Reagent

Stability of absorbance was noted when albumin was present in the glycerol measuring reagent. To verify this phenomenum, 1.0 to 10.0 g/liter bovine serum albumin was added to the final reaction mixture containing hydrazine-glycine-Mg^{+2} buffer and ATP. Fifty µl of 13.47 mmol/liter NAD^{+}, 50 µl of deionized water, and 350 µl of the hydrazine-glycine-Mg^{+2} buffer (pH 9.8) with ATP and varying concentrations of BSA were manually pipetted into the appropriate wells of a transfer disc. After storing a water blank, the change in absorbance was monitored with the analyzer settings as follows: T_o of 3 second, ΔT of 1 minute, 340 nm, 30°C, Store, Terminal Mode, Operate, and Absorbance. In the absence of albumin after 12 minutes, the absorbance changed at the rate of 0.001 per minute (Fig. 16). With 1.0 g/liter and 2.0 g/liter of BSA, 11 and 10 minutes

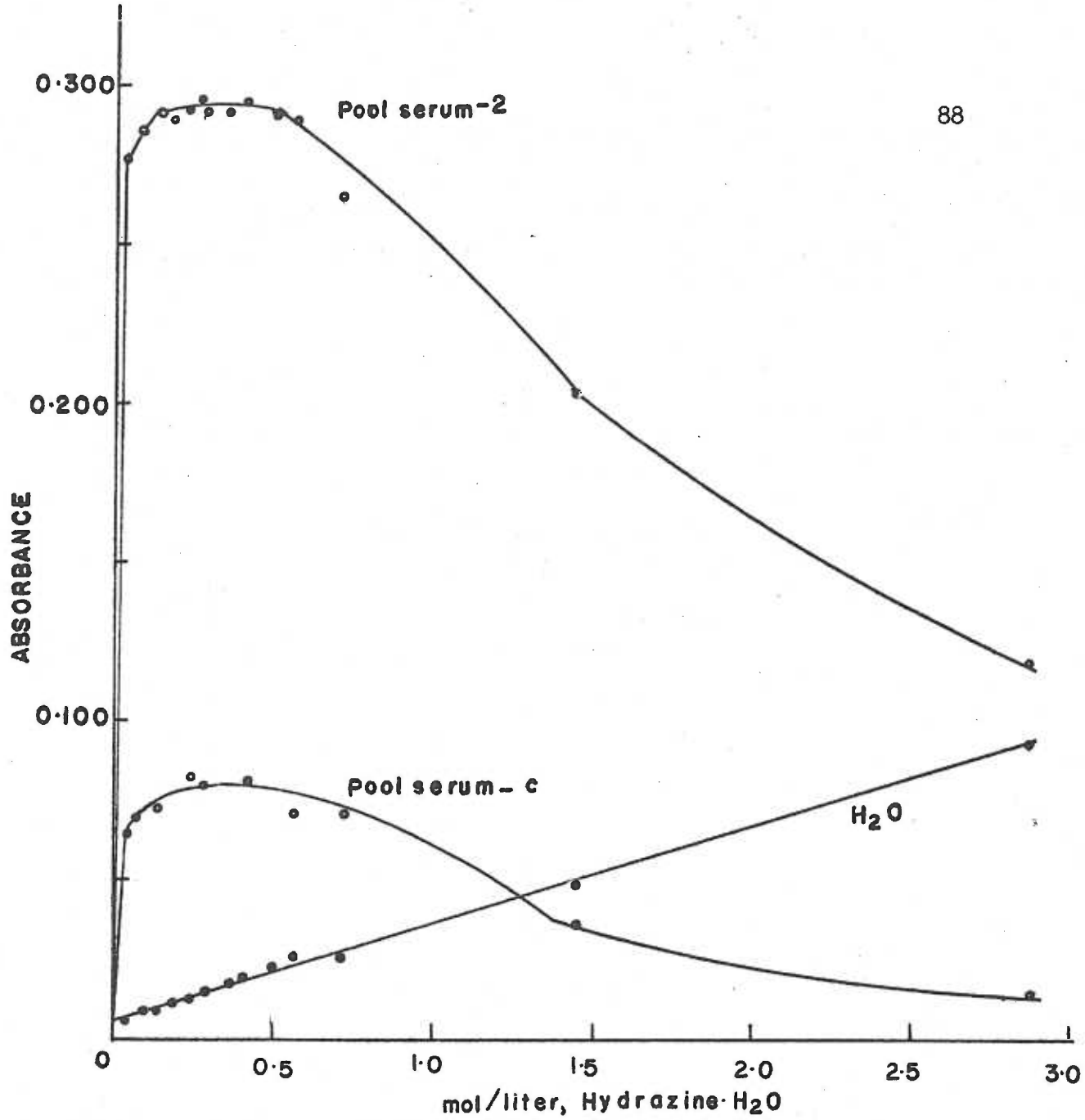


Fig. 15. The optimal concentration of hydrazine·H_2O in the glycerol measuring system (pH9.8); and the increase in absorbance with increase in concentration of hydrazine at pH 9.8(•——•). The concentrations of other glycerol measuring reagents were held constant, and the absorbance values were measured at 340 nm.

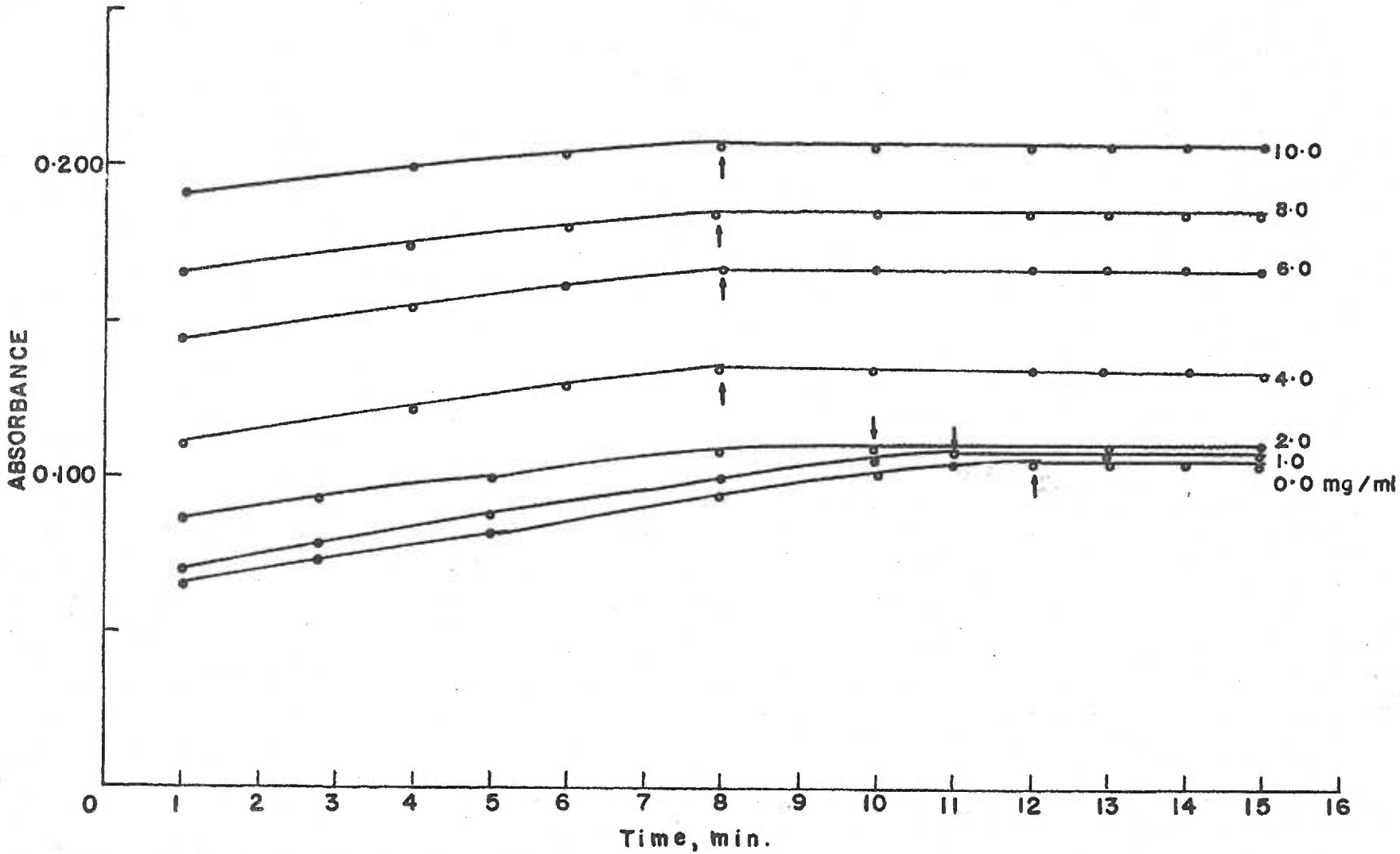


Fig. 16. The effect of a Bovine Serum Albumin on the stability of an absorbance at 340 nm when hydrazine (0.357 mol/liter) and NAD^+ (1.5 mmol/liter) were reacted. The arrows indicate a stability of absorbance at that point.

were required to reach stable absorbance, respectively. BSA concentrations ranging from 4.0 to 10.0 g/liter stabilized the absorbance change at 8 minutes and no further increase in absorbance was noted. The final concentration of BSA in the glycerol measuring reaction mixture was chosen as 4.0 g/liter.

Optimal Magnesium Concentration

Final glycerol measuring reaction mixtures containing 0.0 to 5.98 mmol/liter magnesium chloride were used to determine the optimal magnesium concentration for glycerol assay. A pooled serum was subjected to enzymatic hydrolysis. Fifty µl of the lipolyzed pooled serum and 50 µl of deionized water were placed in sample wells of a transfer disc. Three hundred fifty µl of the glycerol measuring reagent (pH 9.8) containing varying magnesium concentrations was pipetted into the reagent wells of a transfer disc. The absorbance changes were monitored as described in the previous section. Figure 17 shows the absorbance results at 1 minute (A) and at equilibrium (B). Magnesium concentrations above 0.62 mmol/liter showed the same triglyceride results. However, at 1 minute reading, the reaction mixture with magnesium concentration of 0.62 mmol/liter gave lower absorbance value than that of higher concentrations. The optimal concentration of magnesium in the glycerol measuring method was selected as 2.0 mmol/liter.

Optimal ATP Concentration

Varying amounts of 78.62 mmol/liter ATP were pipetted into the test tubes containing the hydrazine-glycine-magnesium buffer (pH 9.8), NAD^+,

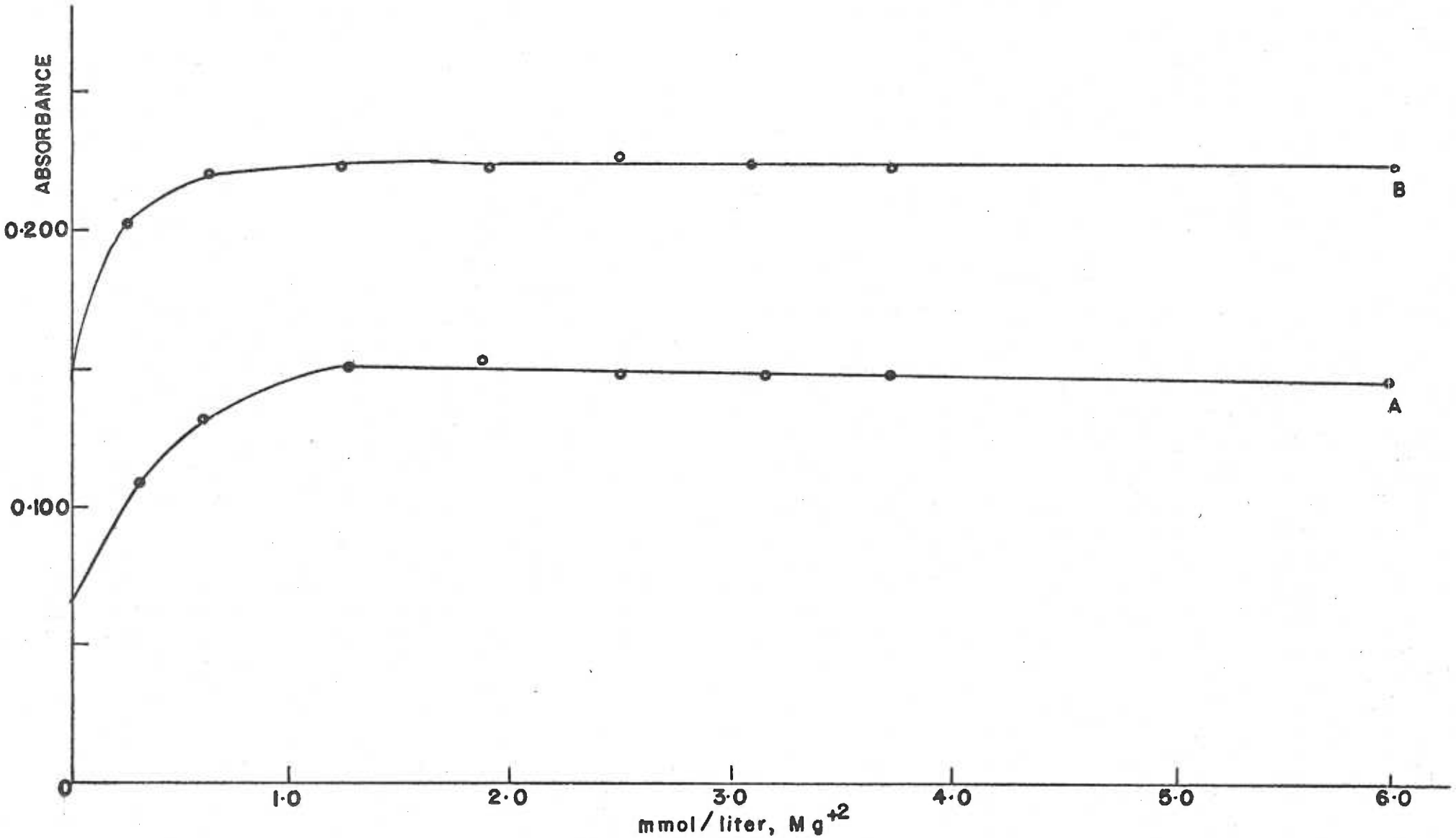


Fig. 17. The optimal concentration of Mg^{+2} for the glycerol measuring system. The concentration of other glycerol measuring reagents were held constant. Absorbance values were read at 340 nm.

BSA, GK, and GDH. Fifty µl of two lipolysates, 50 µl of deionized water, and 350 µl of the above reagents were manually pipetted into the appropriate wells of a transfer disc. Final concentrations of the glycerol measuring reagents were 0.290 mol/liter hydrazine, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4.0 g/liter BSA, 1.5 mmol/liter NAD^+, 0.4 U/ml GK and 4.0 U/ml GDH. The ATP concentrations in the final reaction mixture consisted of 0.0 to 5.0 mmol/liter. It was observed that all ATP concentrations above 1.0 mmol/liter gave essentially the same triglyceride result (Fig. 18). The time required to reach equilibrium with ATP concentrations above 1.0 mmol/liter was about the same. The excess ATP of 2.5 mmol/liter in the final reaction mixture was used for the proposed GK-GDH method.

Optimal NAD^+ Concentration

NAD^+ concentrations ranging from 0.0 to 3.0 mmol/liter in the final reaction mixture were mixed with the glycerol measuring reagents. The reaction mixture contained 0.290 mol/liter hydrazine, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4 g/liter BSA, 2.5 mmol/liter ATP, 0.4 U/ml GK, and 4.0 U/ml GDH. The NAD^+ in the glycerol measuring reagents was left at ambient temperature for approximately 10 minutes. The pooled serum containing 6.7 mmol/liter triglycerides was subjected to the enzymatic hydrolysis. This lipolyzed pooled serum was reacted with the above glycerol measuring reagent. Fifty µl of the lipolysate, 49 µl of deionized water and 350 µl of the glycerol measuring reagents (pH 9.8) containing varying amounts of NAD^+ were pipetted into the

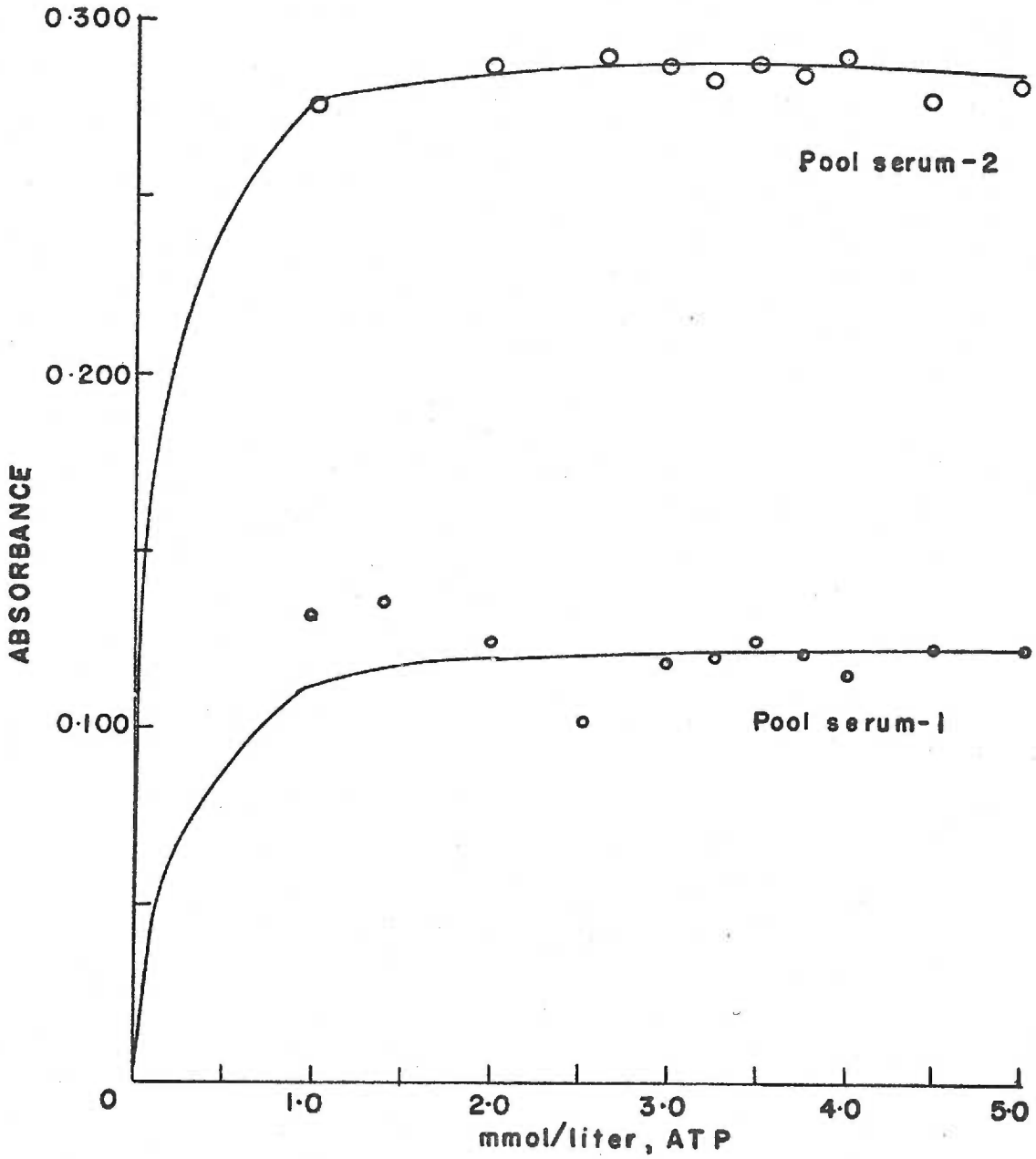


Fig. 18. The optimal concentration of ATP for the glycerol measuring system. The concentrations of other glycerol measuring reagent were held constant.

appropriate wells of a transfer disc. The glycerol measuring reagent without NAD^+ and the lipolysate were placed in the reference cuvette. The absorbance value of the glycerol measuring reagent without NAD^+ was subtracted from those with varying quantities of NAD^+.

When the lipolysate of the pooled serum was assayed, NAD^+ concentrations above 0.9 mmol/liter showed essentially the same recovery of triglycerides (Fig. 19). However, the higher NAD^+ concentrations gave slightly lower triglyceride results. Similar results were observed with lower serum triglycerides (1.0 mmol/liter). The optimal NAD^+ concentration was chosen as 1.5 mmol/liter since higher NAD^+ concentrations caused excessive blank absorbance values.

Optimal Activity of Glycerophosphate Dehydrogenase

From 0.0 to 30 µl of 1008 U/ml GDH were added to the glycerol measuring reagents. The final concentrations of the glycerol measuring reagents were 0.290 mol/liter hydrazine, 145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4 g/liter BSA, 1.5 mmol/liter NAD^+, 0.4 U/ml GK, and 2.5 mmol/liter ATP. Two lipolysates were simultaneously assayed for the liberated glycerol with 0.0 to 24.0 U/ml GDH in the reaction mixture. Figure 20 shows the reaction rates between absorbance values and the amounts of enzyme. With increasing amounts of GDH equilibrium was reached more rapidly. The GDH concentrations less than 2.4 U/ml took longer than 11 minutes to reach an equilibrium with the pooled serum-d; however, the GDH concentrations above 4.0 U/ml took about 8 minutes (Table 5). With 0.80 U/ml GDH, 95 per cent of the glycerol was

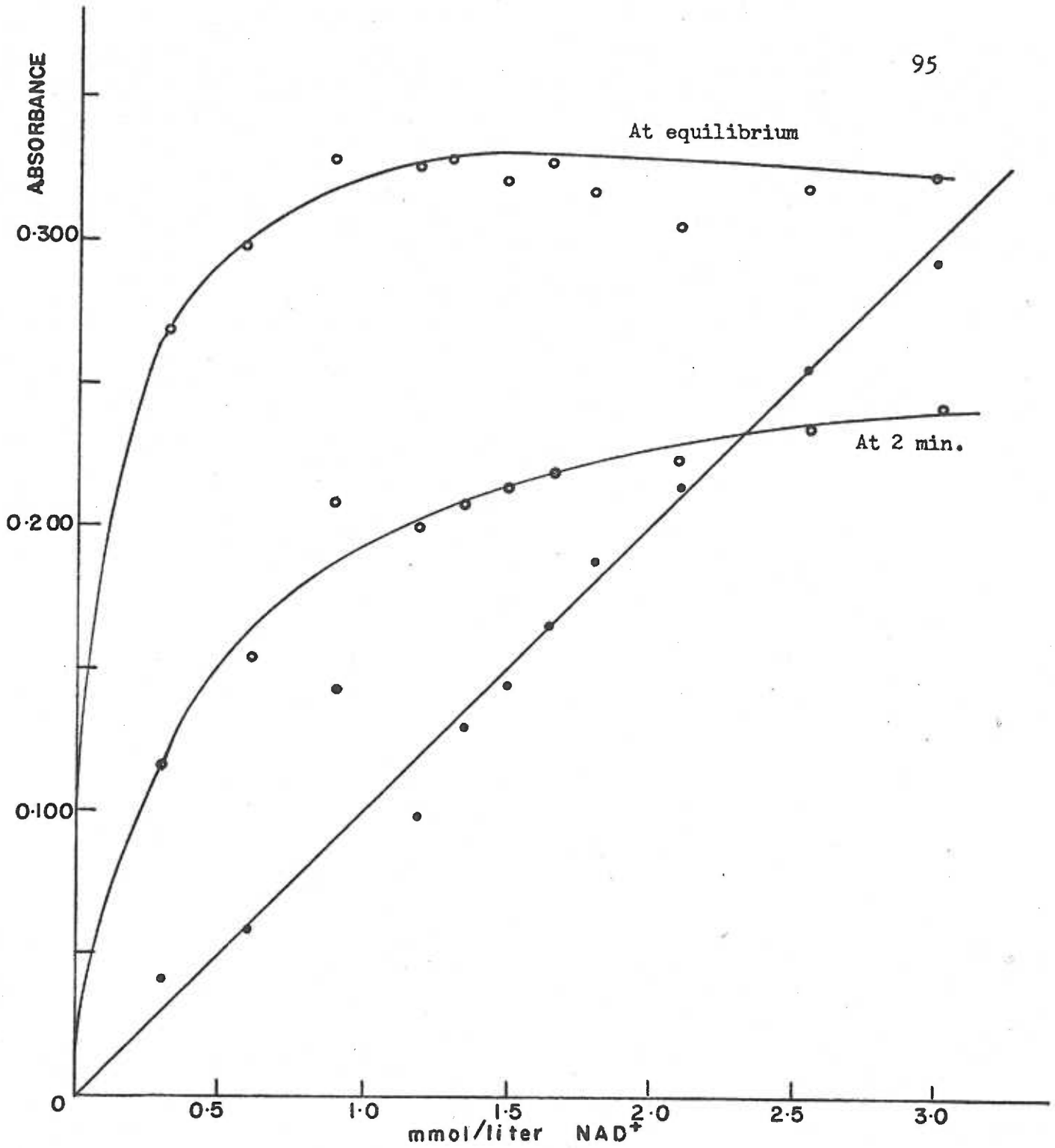


Fig. 19. The optimal concentration of NAD^+ in the glycerol measuring system (o—•); and the increase in absorbance with increasing amounts of NAD^+ in the glycerol measuring reagents without the presence of glycerol (•—•).

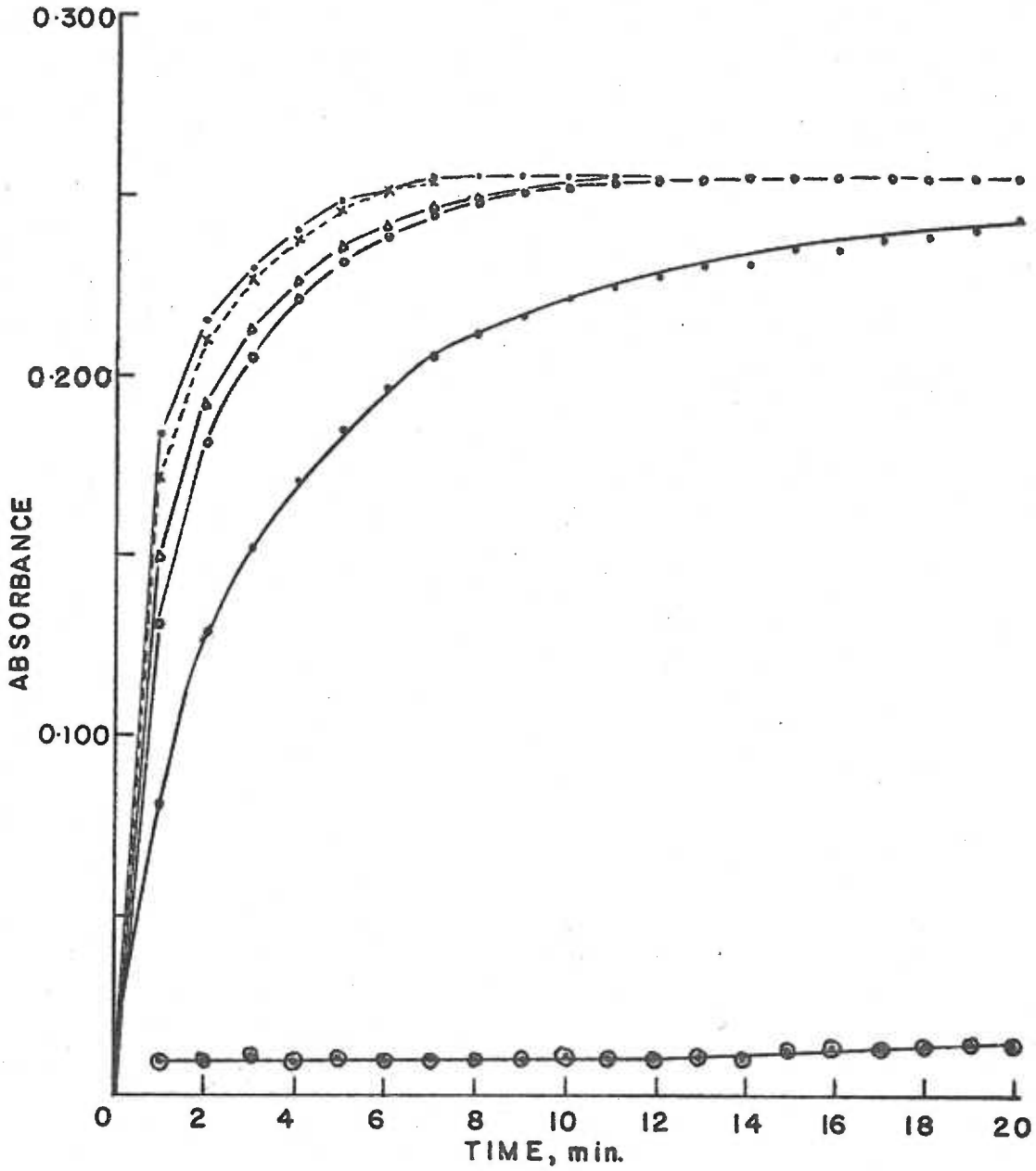


Fig. 20. The reaction rate with varying activities of glycerophosphate dehydrogenase (GDH) in the glycerol measuring system. The other concentrations of the glycerol measuring reagents were kept constant.
⊙—⊙ Blank rate, no GDH added; •—• 0.8 U/ml; ◦—◦ 1.6 U/ml; △—△ 2.4 U/ml; ×·····× 4.0 U/ml; and •—• 4.9.

Table 5. The time required to reach equilibrium with varying activities of glycerophosphate dehydrogenase (GDH) in the glycerol measuring system. The concentrations of other glycerol measuring reagents were kept constant.

GDH	pooled serum-c		pooled serum-d	
U/ml, 25°C	mmol/liter	Time (min)	mmol/liter	Time (min)
0.0	0.10	1	0.20	2
0.8	1.31	6	4.93 ↑	20
1.6	1.29	5	5.15	13
2.4	1.35	3	5.10	11
4.0	1.37	2	5.17	8
4.9	1.39	2	5.16	8
5.6	1.37	2	5.13	8
8.0	1.43	3	5.15	8
12.0	1.39	2	5.10	8
20.0	1.31	3	5.00	8

converted to NADH at 20-minute reaction time. However, with 1.6 U/ml of GDH, the reaction was completed at 13 minutes. The pooled sera "c" and "d" showed 0.10 and 0.20 mmol/liter glycerol, respectively, without added GDH. The manufacturer stated that this GK preparation contained 0.0015 per cent GDH. The glycerol measuring reagent without GK and GDH showed the final absorbance values between 0.002 to 0.003. Figure 21 shows the typical curves of this experiment. The optimal activity of GDH was determined to be 4.0 U/ml (25°C).

Optimal Activity of Glycerokinase

Glycerokinase (197 U/ml) was diluted in deionized water to give 49.25 U/ml. Final activities of GK ranging from 0.0 to 0.92 U/ml were made up in the glycerol measuring reaction mixture. Glycerokinase values less than 0.15 U/ml gave slightly lower triglyceride recovery (Fig. 22). The plateau was reached with glycerokinase activities greater than 0.31 U/ml. In the glycerol measuring reagent with no glycerokinase, an average increase in absorbance was ca. 0.001 for pooled serum-1 and 0.005 for pooled serum-2. At 2 minutes the pooled serum-1 gave 0.24 mmol/liter glycerol while the pooled serum-2 gave 0.44 mmol/liter at the end of 5 minutes (Table 6). The manufacturer indicated that this GDH preparation contained 0.003 per cent GK. The presence of a small amount of GK in GDH reagent is responsible for increases in absorbance values when no glycerokinase was added to the reaction mixture. The activity of GK (Calbiochem) selected for this GK-GDH method was 0.4 U/ml.

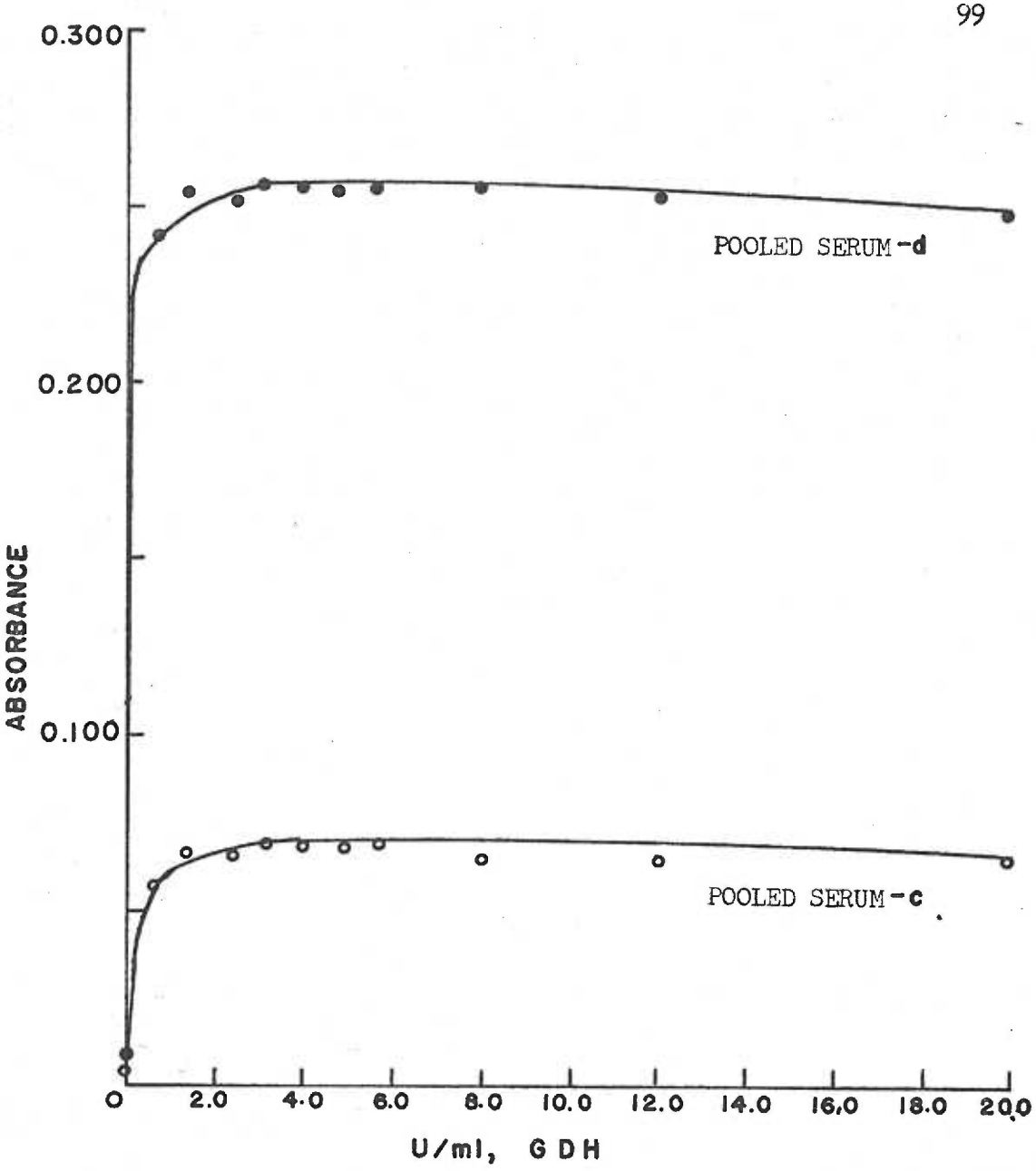


Fig. 21. Absorbance as a function of glycerophosphate dehydrogenase (GDH) concentrations in two different pooled sera. The concentrations of other glycerol measuring reagents were held constant.

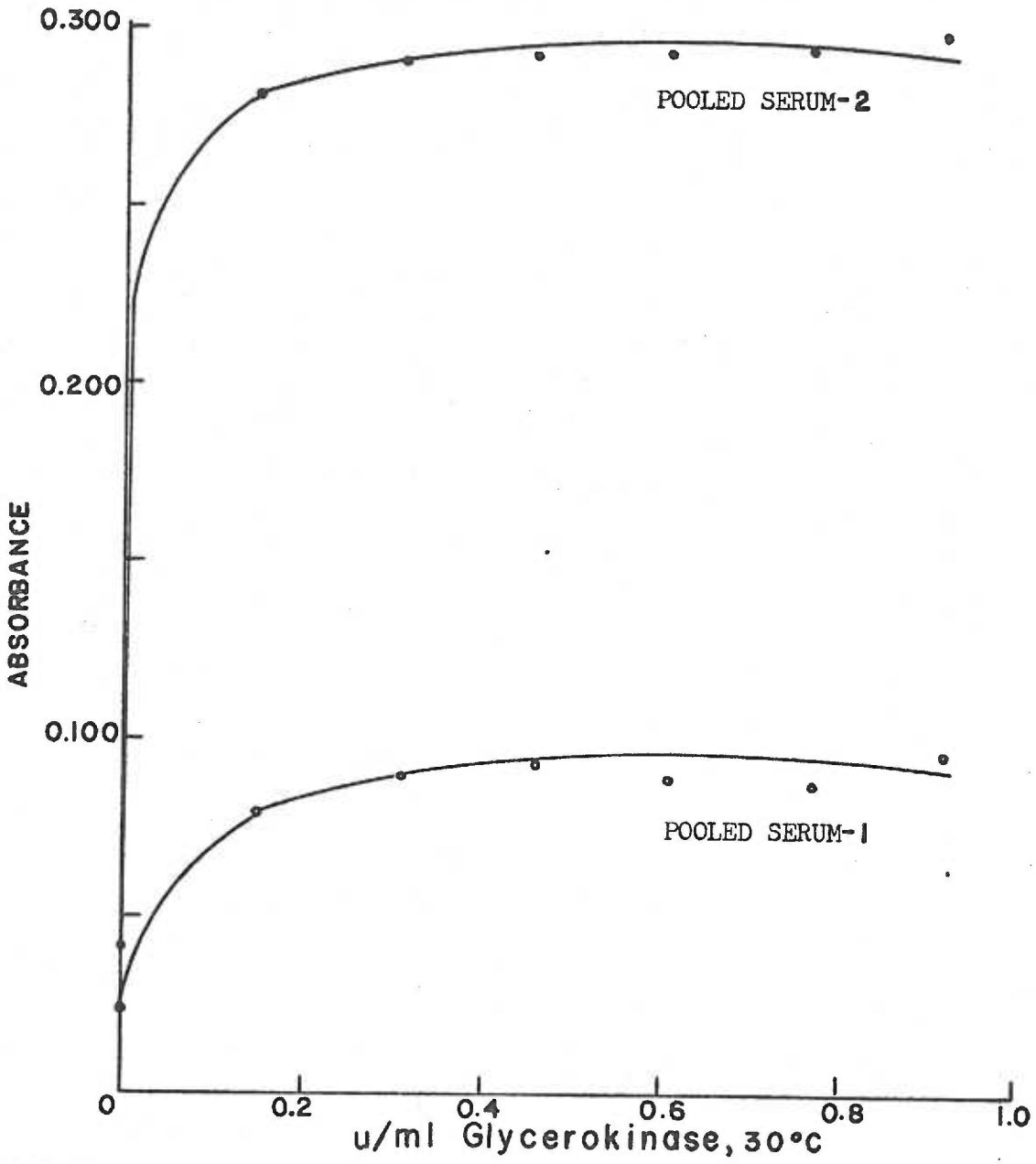


Fig. 22. Absorbance as a function of glycerokinase (GK) concentrations in two different pooled sera. The concentrations of other glycerol measuring reagents were kept constant.

Table 6. The optimal time required to reach equilibrium with varying activities of glycerokinase (GK) in the glycerol measuring system. The concentrations of other glycerol measuring reagents were held constant.

Glycerokinase	pooled serum-1		pooled serum-2	
U/ml, 30°C	mmol/liter	Time(min)	mmol/liter	Time(min)
0.0	0.24	2↑[a]	0.44	5↑[a]
0.15	1.60	6	5.72	16
0.31	1.80	5	5.89	15
0.46	1.88	5	5.90	16
0.16	1.75	5	5.94	16
0.77	1.74	6	6.15	16
0.92	1.96	5	6.20	15

a. The absorbance was slowly changing while those GK units greater than 0.31 U/ml had reached an equilibrium.

Effect of Sulfhydryl Reagents on GK and GDH

Cysteine, glutathione, BAL (2,3-dimercaptopropanol), dithiothreitol (DTT) and mercaptoethanol are commonly used sulfhydryl reagents which maintain sulfhydryl bonds in a reduced state. Since glycerophosphate dehydrogenase (GDH) contained 15 SH groups per mole (103) and GDH had been reported to be susceptible to hydrazine inhibition, it was of interest to investigate the effect of SH reagents on the glycerol measuring enzymes. A sulfhydryl reagent was added to the glycerol measuring reagent and they were incubated in a 30°C water bath for 4 hours. Two lipolyzed pooled sera were used to assay the 4-hour old glycerol measuring reagents. The final concentrations of the reaction mixture were 19.0 mmol/liter SH reagents, 0.4 U/ml GK and 4.0 U/ml GDH. Figure 23 shows the absorbance changes vs. time. The glycerol measuring reagent containing BAL formed a pink color immediately after mixing and ca. one hour later turned turbid, giving an absorbance value above 2.0 at 340 nm. Thus, it was excluded from the experiment. The glycerol measuring reagents without a sulfhydryl reagent and with cysteine showed lower reaction rates than those with glutathione, mercaptoethanol, and dithiotheritol (Fig. 23). Cysteine has been known to form cystine by air oxidation in an alkaline solution. Glutathione formed a white precipitate while mercaptoethanol gave a repugnant odor which caused us to choose dithiothreitol in the GK-GDH method. Dithiothreitol (DTT); 2,3-dihydroxy-1,4-dithiolbutane, has hydroxy groups on the middle carbons, which make this compound water soluble. It has little odor and little tendency to be oxidized by air (147). For these reasons, DTT was selected

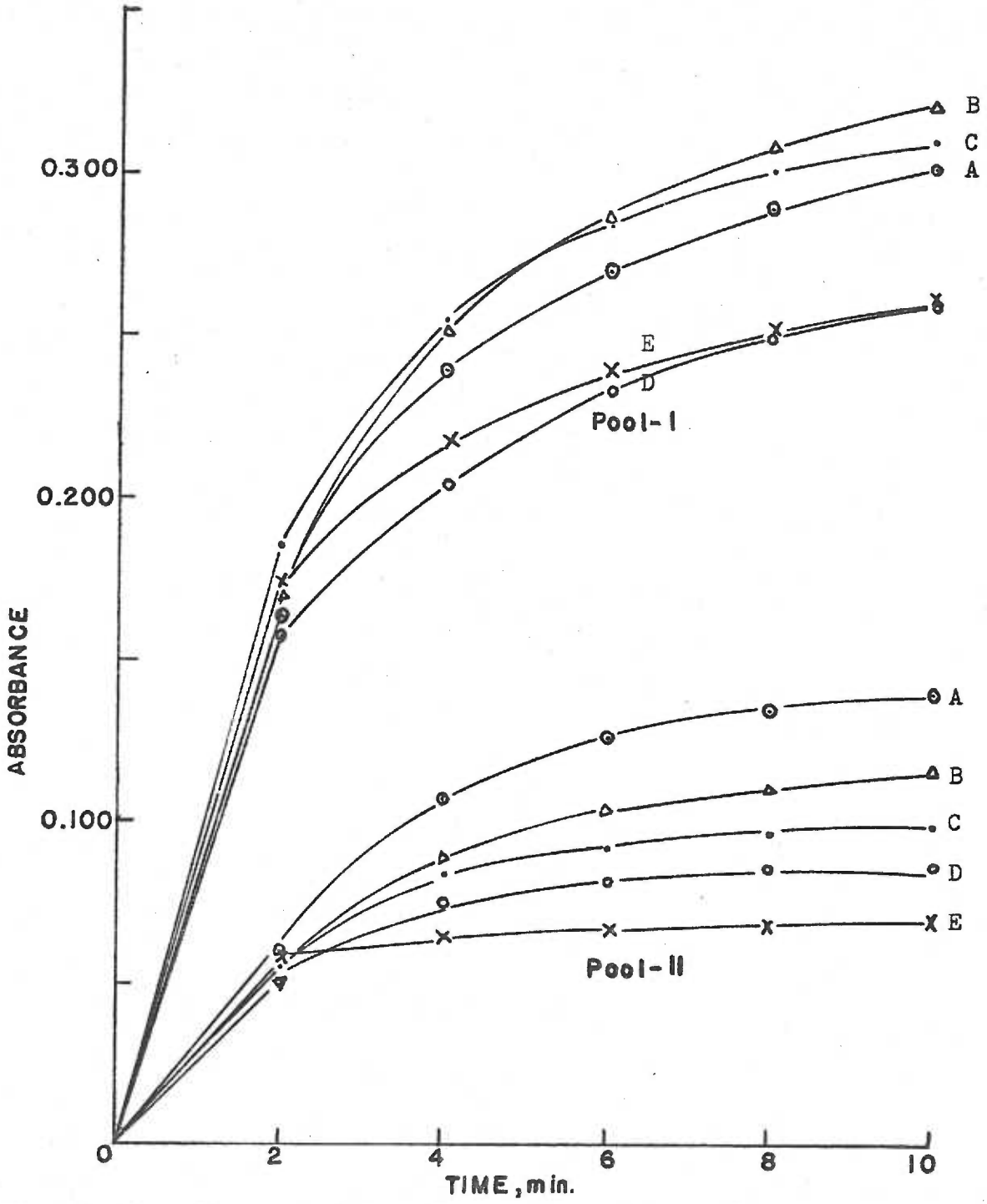


Fig. 23. The effect of sulfhydryl reagents on GK and GDH after incubating a SH agent with glycerol measuring reagent for 4 hours at 30°C. The glycerol measuring reagent, thus, contained hydrazine, glycine, Mg^{+2}, BSA, ATP, NAD^{+}, GK, GDH, and 19 mmol/liter SH agent. The final concentrations of the glycerol measuring reagent were the same as the proposed concentrations. A=Dithiothreitol (DTT), B=Glutathione, C=Mercaptoethanol, D=No SH agent, and E=Cysteine.

for use in the GK-GDH method.

Optimal DTT Concentration

Varying amounts of 1.58 mol/liter dithiothreitol (DTT) were added to the glycerol measuring reagents to make final DTT concentrations of 4.9, 9.8 and 19.6 mmol/liter. The glycerol measuring reagent without DTT was also prepared. Final concentrations of the glycerol measuring reagents were the same as previously used. All reagents were then incubated in a 30°C water bath for 5 hours. Using the same lipolysate of a pooled serum, the glycerol assay was monitored at 1 minute intervals. The glycerol measuring reagent with 4.9 mmol/liter DTT showed the highest rate of absorbance change while the glycerol measuring reagent without DTT rendered the lowest rate (Fig. 24). At the same time, the high concentrations of DTT in the glycerol measuring reagent gave a proportional decrease in the rate of absorbance change. When two lipolysates of the pooled sera A and B were assayed with the 5-hour old glycerol measuring reagents, the reaction mixture without DTT took the longest time to reach an equilibrium (16 minutes for the pooled serum A and 24 minutes for the pooled serum B) while the reagents containing higher concentrations of DTT required a slightly longer time to complete the reaction than those with lower DTT (Table 7). The final reaction mixture containing ca. 5.0 mmol/liter DTT was chosen as an optimal level.

Optimal pH of the Glycerol Measuring System

The pH of the hydrazine-glycine-Mg^{+2} buffer solution was adjusted

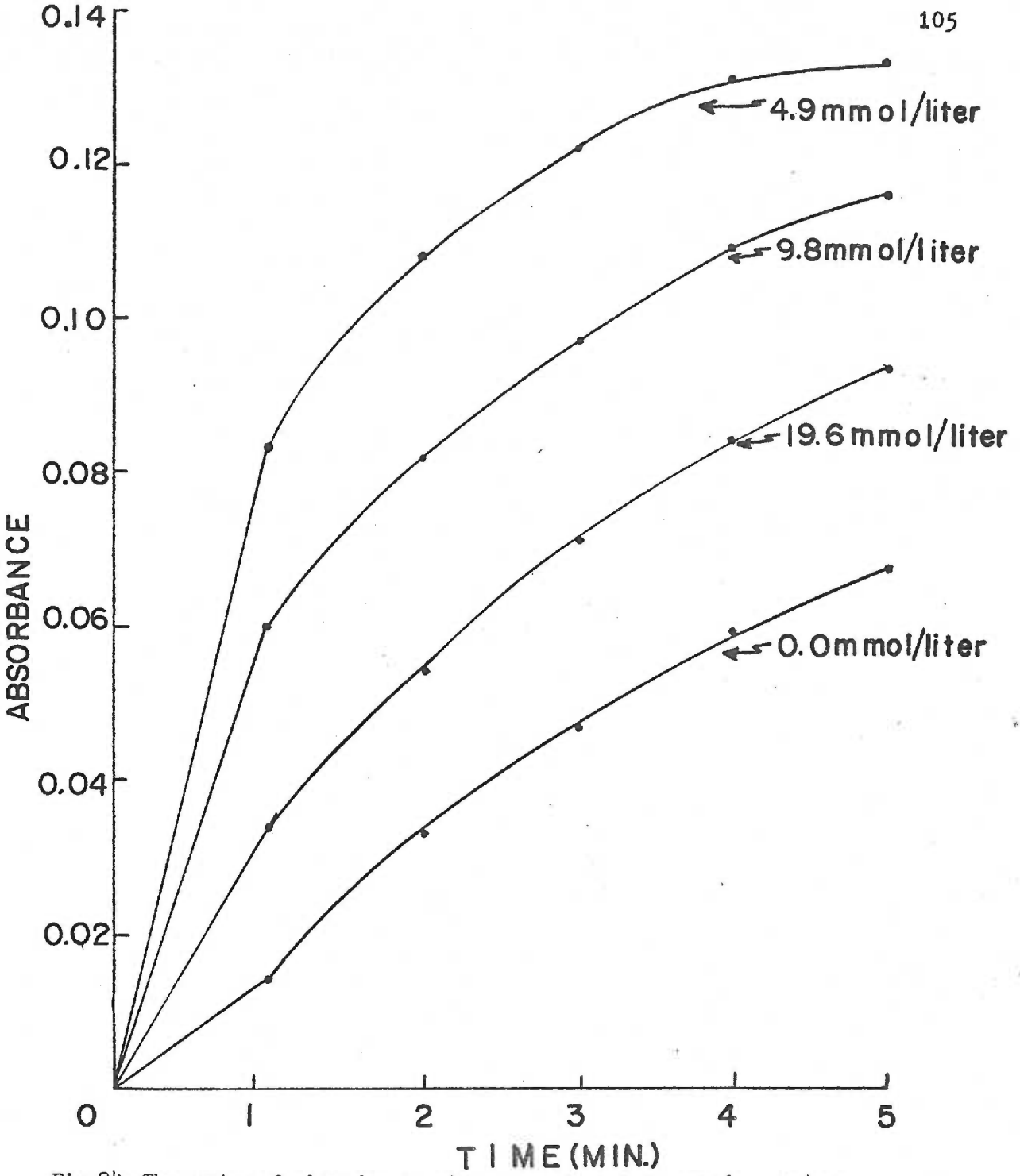


Fig.24. The rate of absorbance change with 0.0 to 19.6 mmol/liter dithiothreitol (DTT) in the glycerol measuring reagent. The GK and GDH in the glycerol measuring reagent were incubated at 30°C water bath for 5 hours with varying concentrations of DTT. Then, using the same concentration of serum triglycerides which had been hydrolyzed, the glycerol contents were assayed.

Table 7. The optimal time required to reach equilibrium with 0.0 to 19.6 mmol/liter dithiothreitol (DTT).

DTT	pooled serum-A		pooled serum-B	
(mmol/liter)	mmol/liter	Time (min)	mmol/liter	Time (min)
0.0	1.53	16	2.93	24
4.9	1.53	4	2.83	11
9.8	1.53	4	2.89	10
19.6	1.52	6	2.83	12

to 8.8, 9.0, 9.2, 9.4, 9.6 and 9.8. Then the optimal concentrations of BSA, ATP, NAD^+, GK and GDH were added to the buffer solutions. After three different pooled sera were subjected to enzymatic hydrolysis, the three lipolysates were simultaneously assayed for glycerol with the above reagents. Although the triglyceride values appeared to decrease at the lower and higher pHs, the values were nearly constant from pH 8.8 to 9.8 (Fig. 25). The reaction time required to reach equilibrium did not vary in this pH range. As previously described the higher pH caused an increase in reagent absorbance values (Fig. 13). Hydrazine-glycine-Mg^{+2} buffer with pH 9.2 was selected as the optimized condition of the glycerol measuring method.

Effect of Various Albumin Preparations on the GK-GDH Method

Six commercially available albumin preparations were used in this experiment. Two mmol/liter glycerol standard was added to portions of the lipolysis reagent containing Bacto BSA, 35% BSA (Sigma), Metrix BSA, Pentex HSA, Factor V BSA (Sigma), or FFA free BSA (Sigma). The same glycerol standard was also placed in the lipolysis reagent containing no albumin. Then they were incubated in a 30°C water bath for 40 minutes before assaying for glycerol. The glycerol measuring reagent contained 4.0 g/liter BSA (35%, Sigma). Figure 36 illustrates the results of the effects of various commercial albumin preparations on the enzymatic hydrolysis of serum triglycerides and on the glycerol measuring method. Each bar graph indicates the final results obtained in glycerol concentrations in which bar No. 1 represents both the pooled

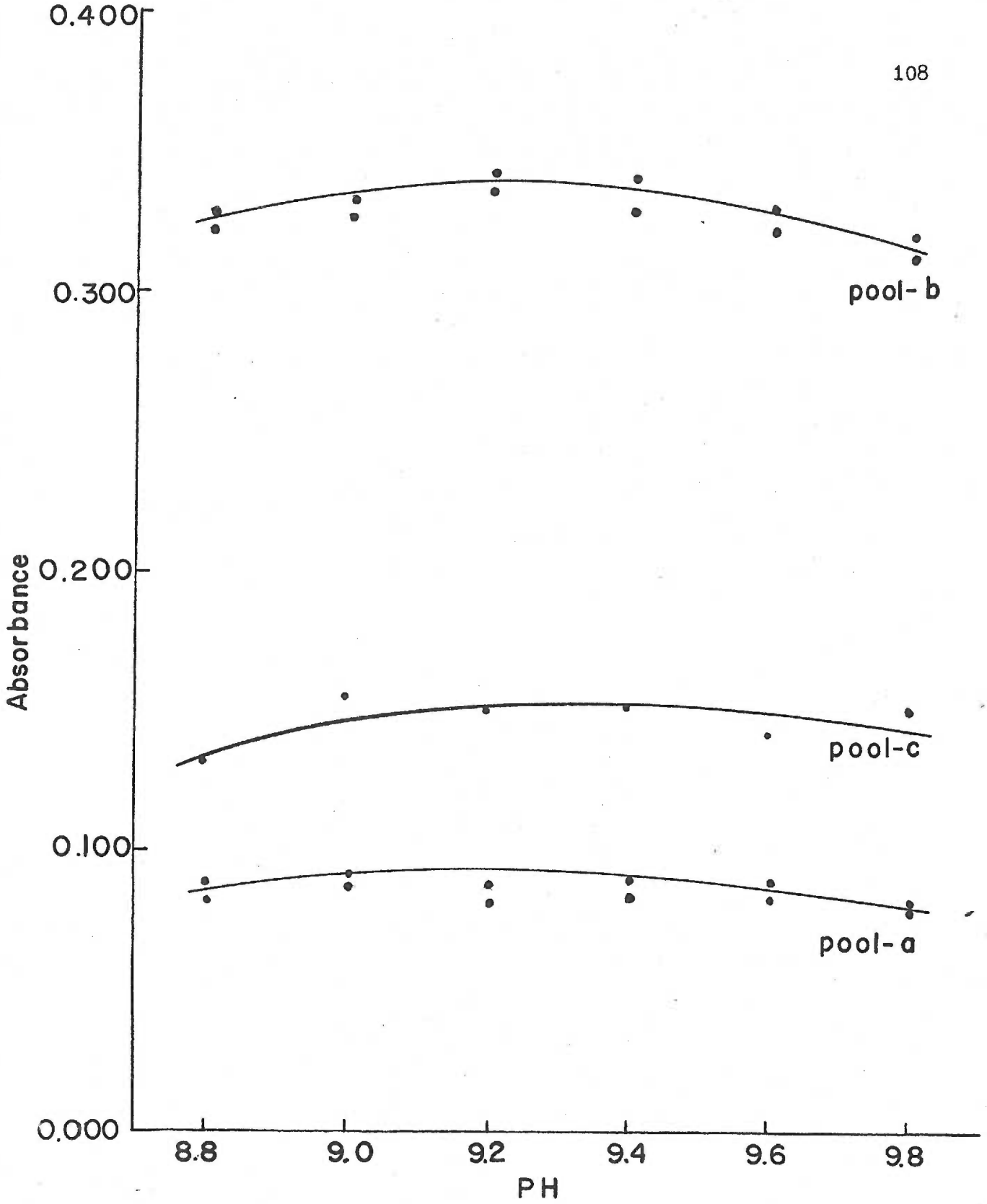


Fig. 25. The optimal pH of the glycerol measuring system.

serum and albumin in the lipolysis reagent without lipase and α-chymotrypsin present; bar No. 2 shows 2.0 mmol/liter glycerol standard in the lipolysis reagent; bar No. 3 is the lipolysis reagent pre-incubated in a 30°C water bath for 40 minutes before addition of a sample; and bar No. 4 depicts the lipolysis reagent without pre-incubation. It was found that 2.0 mmol/liter glycerol standard in the lipolysis reagent containing no albumin yielded 1.99 mmol/liter glycerol. The lipolysates containing Pentex Human Serum Albumin and Fraction V BSA (Sigma) gave much less glycerol recovery: 1.46 and 1.34 mmol/liter glycerol, respectively. The lipolysates with Sigma BSA (35%), Metrix BSA, and FFA free BSA (Sigma) showed 1.95, 2.08, and 1.90 mmol/liter glycerol recoveries, respectively. Two additional experiments repeated at different days showed similar results as shown in Figure 36. Bacto BSA, Pentex HSA, and Fraction V BSA (Sigma) were excluded since they appeared to interfere with the glycerol measuring method.

Effect of Various Trapping Agents for Dihydroxyacetone Phosphate

The possible use of other ketone trapping agents was investigated. Final concentrations ranging from 0.2 to 0.8 mol/liter phenylhydrazine, hydroxlyamine, semicarbazide, and (aminooxy) acetic acid hemihydrochloride (0.1 and 0.2 mol/liter only) were made in pH 9.2 glycine-magnesium buffer solution. The concentrations of other glycerol measuring reagents were held constant. Hydrazine with sulfate and hydrochloride was also used. Table 8 summarizes the findings in which the results are expressed in mmol/liter triglycerides obtained. A pooled

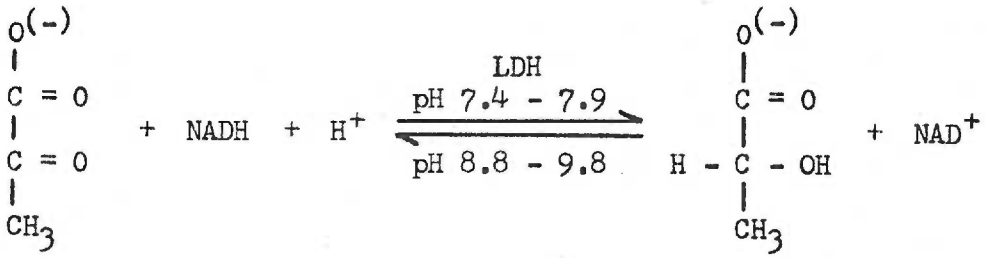
Table 8. Triglyceride concentrations (mmol/liter) measured in a pooled serum with various trapping agents at several concentrations.

Trapping agents	Triglyceride measured, mmol/liter and time to reach equilibrium				
	Final concentration in the reaction mixture(mol/liter)				
	0.1	0.2	0.3	0.4	0.8
Hydrazine·H_2O	-	-	4.04 (8 min)	-	-
Hydrazine·SO_4^{-2}	-	3.5 (20 min)	2.9 (20 min)	2.9 (20 min)	High absorbance
Hydrazine·HCl	-	3.6 (10 min)	-	3.4 (20 min)	1.7 (10 min)
Phenylhydrazine	-	0.7 (10 min)	1.0 (8 min)	1.1 (8 min)	1.6 (8 min)
Hydroxylamine	-	0.6 (1 min)	-	0.8 (2 min)	1.0 (3 min)
Semicarbazide·HCl	-	0.3 (1 min)	-	0.5 (2 min)	0.3 (1 min)
(aminooxy) acetic acid hemihydrochloride	0.6 (1 min)	1.0 (2 min) High absorbance	-	-	-

serum containing 4.05 mmol/liter serum triglycerides was used. The glycerol measuring reagent containing hydrazine·HCl gave a result comparable to that for hydrazine hydrate. However, the hydrazine·SO_4^{-2} reagent mixture took longer than 20 minutes to give 3.5 mmol/liter triglyceride value with 0.2 mmol/liter hydrazine·SO_4^{-2}. Hydrazine sulfate concentrations at 0.3 and 0.4 mol/liter showed lower recovery of triglycerides. Hydrazine·SO_4^{-2} at 0.8 mol/liter in the glycerol measuring reagent rendered too high a reagent blank absorbance to be useable. The glycerol measuring reagents containing phenylhydrazine, hydroxylamine, semicarbazide, and (aminooxy) acetic acid hemihydrochloride were found to be inefficient trapping agents for dihydroxyacetone phosphate under the condition of this experiment.

Effect of Lactic Acid on the Glycerol Measuring Method

Equilibrium of the LDH catalyzed reaction is strongly in the direction of lactate production at pH 7.4 to 7.9. The maximal rate of pyruvate reduction (pH 7.4 - 7.9) is ca. three times faster than the maximal rate of oxidation of lactate (pH 8.8 - 9.8).



In the proposed GK-GDH method, NAD^+ is present in an excess amount (1.5 mmol/liter in the final reaction mixture). Although the stability

of LDH in the glycerol measuring reagent is unknown, this enzyme and ca. 0.6 to 1.0 mmol/liter lactate (148) are present in serum samples. The presence of LDH and lactate in a serum specimen may be a possible interference in the glycerol measuring method by reducing NAD^+ to NADH. To substantiate lactate interference, 1.0 to 5.0 mmol/liter lactate were added to a pooled serum. A lipolysis reagent containing 0.55 mmol/liter NAD^+ was prepared. Fifty µl of a pooled serum with 1.0 to 5.0 mmol/liter lactate was pipetted into a lipolysis reagent containing NAD^+ and without NAD^+. The lipolysis reaction mixtures were incubated in a 30°C water bath for 15 minutes. At the end of a 15-minute incubation, absorbance values of the lipolysates with NAD^+ and without NAD^+ were compared. It was found that the absorbance results of two lipolysates were identical. When glycerol was assayed, lipolysates with NAD^+ gave a mean of 1.23 mmol/liter while those without NAD^+ were 1.21 mmol/liter (Table 9). The pooled serum without lactate was 1.218 $\pm$ 0.027 mmol/liter triglycerides. When serum samples containing 125 to 250 I.U./liter LDH were hydrolyzed and subsequently assayed for glycerol as described above, the results showed no significant difference between two means (Table 9).

ATP and GK in Lipolysis Reagent vs. in Glycerol Measuring Reagent

It was of interest to incorporate ATP and GK in the lipolysis reagent and to measure L-glycerophosphate by GDH and NAD^+ in the glycerol measuring reagent. During the enzymatic hydrolysis, glycerol would be phosphorylated to L-glycerophosphate in the presence of ATP

Table 9. The effect of lactic acid on the glycerol measuring system. The varying amounts of lactic acid, 1.0 to 5.0 mmol/liter, were added to a serum pool. 0.05 ml of the serum pool was then added to the lipolysis reagent with and without NAD^+ and incubated at 30°C for 15 minutes.

amount of lactate (mmol/liter)	lipolysis reagent with NAD^+ (mmol/liter)	lipolysis reagent without NAD^+ (mmol/liter)
1.0	1.21	1.15
2.0	1.25	1.23
3.0	1.23	1.21
4.0	1.23	1.23
5.0	1.23	1.21
mean triglycerides obtained =	1.23	1.21
pooled serum without lactate	1.218 ± 0.027	($\bar{X} \pm$ I.S.D., N = 10)

serum samples without added lactic acid

No.	LDH[a] I.U./liter		
A	125	1.15	1.11
B	152	1.23	1.23
C	165	1.84	1.74
D	148	2.30	2.12
E	250	3.76	3.68

a. LDH activity was measured using pyruvate as a substrate at 30°C, pH 7.5 and the normal range was estimated as 0-270 I.U./liter.

and GK. Fifty µl of a pooled serum was added to lipolysis reagent containing ATP and GK and to the lipolysis reagent without ATP and GK. At the end of a 15-minute incubation at 30°C, the lipolysates with ATP and GK were assayed by the glycerol measuring reagent containing no ATP and GK. The lipolysates without ATP and GK were analyzed by the proposed GK-GDH method. The final concentrations of all reagents used were the same in both systems. The lipolysate with ATP and GK contained 2.5 mmol/liter ATP and 0.4 U/ml GK. The lipolysis reagents containing ATP and GK took longer to reach equilibrium than those without ATP and GK (Table 10).

Validity of 3-second Time Delay

This experiment tested the validity of taking initial absorbance readings at 3 seconds to serve as the specimen blanks for the glycerol assay. If the reaction rates were too rapid, a significant amount of NAD^+ would be reduced by glycerol at 3 seconds and triglyceride values would be too low. To investigate this three separate analytical methods were used. Glycerol standard solutions having 0.5 to 4.0 mmol/liter were added to a pooled serum. The same concentrations of glycerol measuring reagents were used in all three experiments. In the first experiment method, 50 µl of the pooled serum containing 0.0 to 4.0 mmol/liter glycerol standards, 49 µl of deionized water, and 350 µl of the reagent were pipetted into the appropriate wells of a transfer disc. Three hundred fifty µl of glycerol measuring reagent and 99 µl of deionized water were pipetted into the reference well to serve as a

Table 10. A comparison of the results between ATP and GK in the lipolysis reagent and ATP and GK in the glycerol measuring reagent, using the proposed enzymatic triglyceride assay technique.

Serum pool	ATP and GK in the glycerol measuring reagent		ATP and GK in the lipolysis reagent	
	mmol/liter[a]	Time (min)[b]	mmol/liter[a]	Time (min)[c]
A	0.95	5	0.79	>10
B	0.75	4	0.73	>10
C	1.29	8	1.09	>16
D	2.54	10	0.93	>15
E	2.46	10	1.01	>15
F	2.74	10	2.70	>20
G	2.85	12	2.54	>20

a. mmol/liter triglycerides obtained.

b. Time required to reach equilibrium.

c. The equilibrium had not been reached at the given assayed time and the absorbance was changing when the results were being taken.

reagent blank. Fifty µl of the pooled serum with 0.0 to 4.0 mmol/liter glycerol standards and a total of 399 µl of deionized water were also pipetted into the same transfer disc. These were used as specimen blanks. After storing water, the total absorbance values at equilibrium were obtained. Then the absorbance values of reagent blank and specimen blank were subtracted from the reaction absorbance results. Thus, the net absorbance values were attributed only by the glycerol. The reagent blank in a sample well of the transfer disc should be 0.000 ± 0.002 since the reference well also contained the same volume and concentration of reagent. The second experimental method used was to store in the instrument the absorbance values of the appropriate dilutions of pooled serum containing varying amounts of glycerol standards. Then glycerol was assayed as described above. In this method, sample absorbance were automatically subtracted from the reaction absorbance values. The third method was to use 3-second "Time Delay" in which neither water nor sample absorbances were stored. Instead, after samples and reagents were quantitatively transferred to the cuvettes and mixed, the absorbance values at exactly 3 seconds were taken. The 3-second absorbance values were automatically subtracted from all subsequent readings. We found that the results obtained by the three methods described above showed no significant difference (Table 11). When the results were statistically analyzed by the method of analysis of Variance (ANOV), at the alpha level of 0.05 there was no significant variability .. observed F was 2.68 while Table F was 3.49 (Table 12). Therefore, the use of 3-second Time Delay for assay-

Table 11. The Validity and the use of 3-second "Time Delay" in the glycerol measuring system.[a]

Amount of glycerol standards in the pooled serum (mmol/liter)	mmol/liter glycerol obtained		
	Storing water	Storing pooled serum	T_o = 3 sec. delay
0.5	0.55	0.45	0.50
1.0	1.06	1.02	0.99
2.0	1.98	1.88	2.00
3.0	3.17	3.02	3.12
4.0	4.02	3.98	4.07

a. The absorbance values due to 340 nm absorbing substance(s), endogenous free glycerol, and L-glycerophosphate in the pooled serum had been subtracted.

Table 12. Analysis of Variance (ANOV) of four observations: mmol/liter glycerol standards added to a pooled serum, measuring those added glycerol contents by storing water blank, by storing the pooled serum blank, and by taking Time Delay of 3 seconds.

	SS	df	MS	F
SS total	33.57	19		
SS between	33.52	4		
SS within	0.05	15		
SS treatment	0.02	3	0.0067	2.68
SS residual	0.03	12	0.0025	

At 0.05, Table F = 3.49

Since the observed F, 2.68, is less than the Table F, 3.49, there was no significant variability at alpha level of 0.05.

ing glycerol by the proposed GK-GDH method is valid. The final absorbance results obtained by the three methods showed a comparable linear relation. Figure 26 shows the mean absorbance values obtained by the three methods plotted along the expected absorbance line. Each bar represents a mean ± 1 S.D.

ENZYMATIC HYDROLYSIS OF SERUM TRIGLYCERIDES

pH Optimum of Rhizopus Delemar Lipase

The variation of Rhizopus delemar lipase activity with pH was determined by using three different buffer solutions: 0.2 mol/liter Imidazole buffer (pH 6.2 to 7.8), 0.2 mmol/liter Tris-HCl buffer (pH 7.2 to 8.2), and Michaelis universal buffer (pH 2.50 to 9.96) having ionic strength of 0.16. The final concentrations of the lipolysate were 11.0 mmol/liter calcium, 99.2 U/ml lipase, and 0.1 ml of a pooled serum in 0.665 ml reaction mixture. The pooled serum and lipolysis reagent were incubated for 30 minutes in a 30°C water bath and assayed for liberated glycerol by the GK-GDH method. The same lipolysates were subjected to additional incubation, a total of 90 minutes, at 30°C and glycerol was assayed. Figure 27 shows the results of the above experiment in which two pH optima were observed, one at ca. pH 5.6 and another at ca. pH 7.5 to pH 7.9. The lipase activity was greatly decreased below pH 5.6 and above pH 7.9. An increase in 0.1 pH unit from pH 7.9 showed a sharp decrease in the lipolytic activity while the decrease in enzyme activities near pH 5.6 was gradual. Table 13 summarizes the enzymatic hydrolysis of the serum triglycerides obtained by incubating the pooled serum

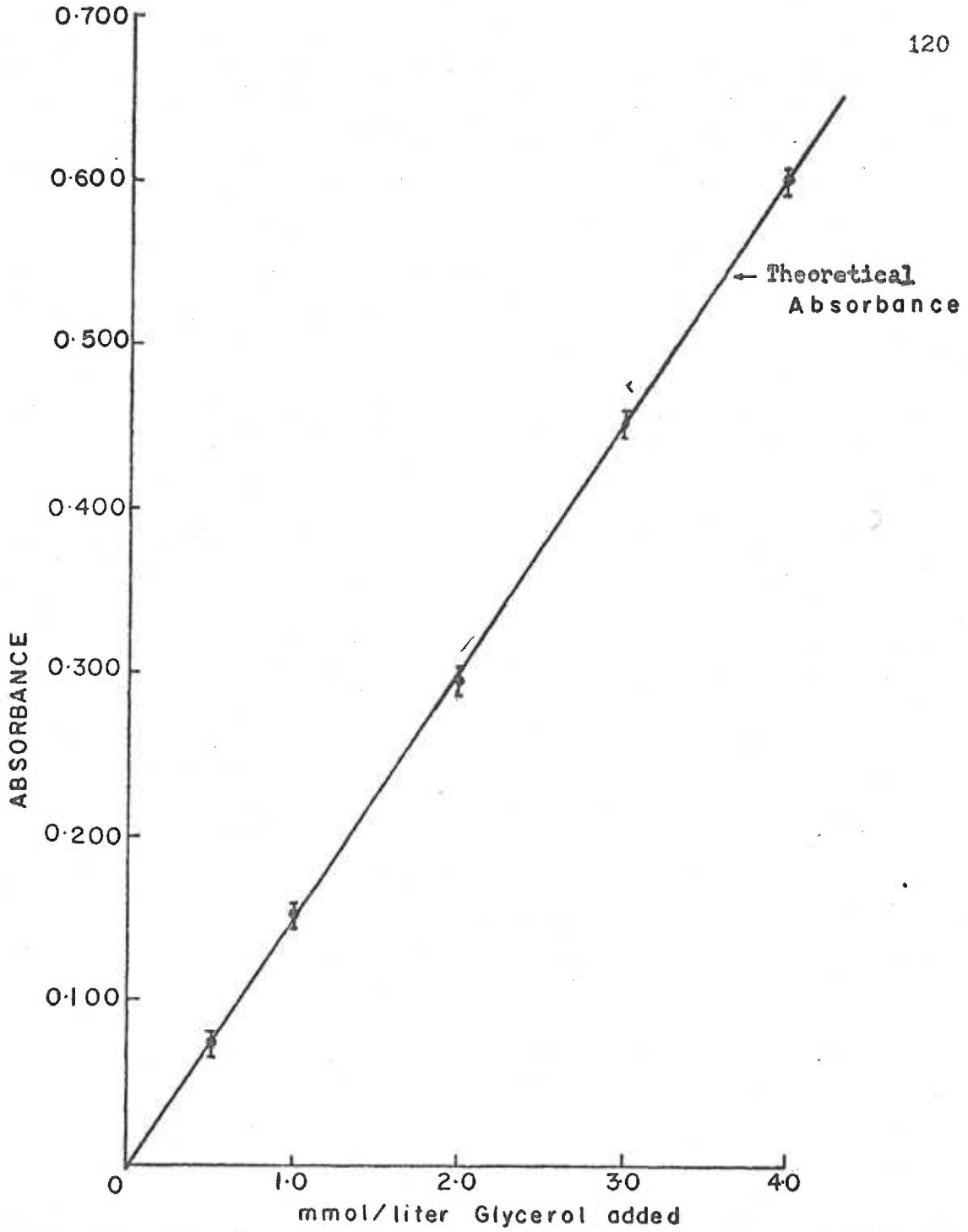


Fig. 26. Mean absorbance values with varying concentrations of glycerol standards in the pooled serum measured by storing water, by storing saline diluted pooled serum, and by taking T_o 3-second Time Delay. Each bar shows a mean $\pm$ 1 S.D.

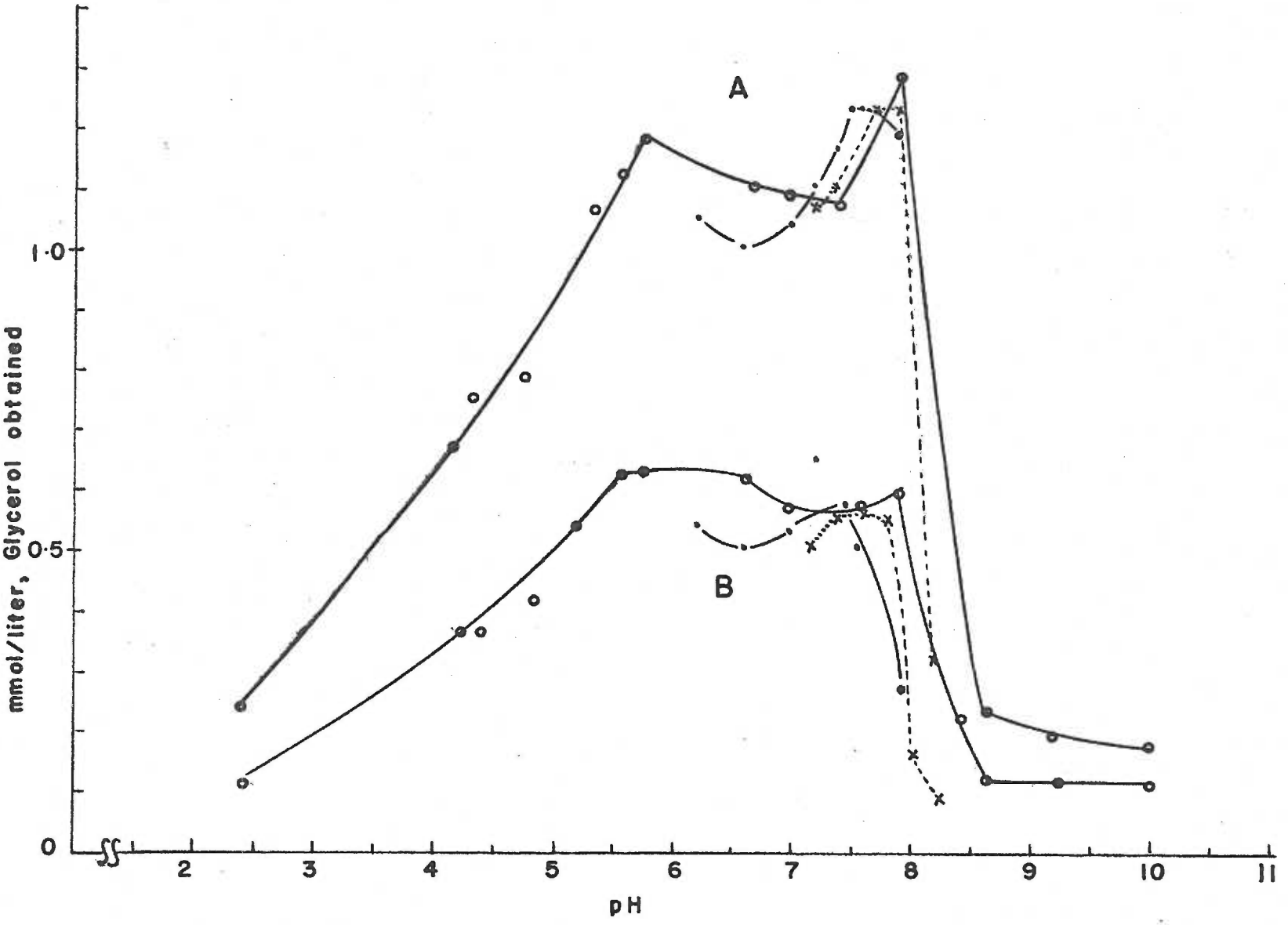


Fig. 27. The pH optimum of Rhizopus delemar lipase activities in three different buffer solutions. The final concentrations of the lipolysate contained 11 mmol/liter Ca^{+2}, 99.2 U/ml lipase, and 0.1 ml serum pool in 0.665 ml reaction mixture. o—o Universal buffer(Michaelis), μ=0.16; •—• Imidazole buffer, 0.2 mol/liter; x---x Tris.HCl buffer, 0.2 mol/liter; A = 90 min. incubation at 30°C; B = 30 min. incubation at 30°C.

Table 13. The lipolytic activities of Rhizopus delemar lipase in Imidazole (0.2 mol/liter), Michaelis Universal (μ=0.16), and Tris-HCl (0.2 mol/liter) buffer solutions at 30 minutes, 90 minutes, and 17-hour incubation in 30°C water bath.

pH of buffers	Duration of incubation in 30°C water bath			Final pH of lipolysates
	30 min.	90 min.	17 hours	
	mmol/liter Glycerol obtained			
Imidazole buffer:				
pH 6.2	0.54	1.06	0.46	6.30
6.6	0.51	1.01	1.33	6.68
7.0	0.53	1.05	1.76*	7.10
7.2	0.66*	1.11	1.83*	7.30
7.4	0.58*	1.20*	1.83*	7.50
7.6	0.61*	1.24*	1.76*	7.70
7.8	0.28	1.20*	1.69	7.96
Tris-HCl buffer:				
pH 7.2	0.52	1.08	1.45	7.26
7.4	0.56*	1.12	1.62	7.45
7.6	0.57*	1.16	1.74*	7.60
7.8	0.56*	1.24*	1.74*	7.80
7.9	0.42	1.24*	1.71*	7.93
8.0	0.17	1.04	1.53	8.04
8.2	0.10	0.33	1.67	8.25
Universal buffer:				
pH 2.50	0.12	0.27	0.81	
4.17	0.37	0.68	1.59	
4.35	0.37	0.75	1.65	
4.80	0.42	0.79	1.57	
5.36	0.55	1.07	1.70	
5.60	0.64*	1.15*	1.82*	
5.77	0.64*	1.19*	1.85*	
6.65	0.63	1.12	1.74*	
7.00	0.58	1.10	1.74*	
7.36	0.56	1.08	1.66	
7.90	0.64*	1.29*	1.68*	
8.55	0.14	0.28	1.57	
8.64	0.12	0.24	1.66	
9.27	0.12	0.20	-	
9.96	0.14	0.18	0.6	

asterisk (*) indicated the highest glycerol recovered from the lipase hydrolysis of serum triglycerides at the given incubation times and at the given pHs.

in three different buffer solutions for 30 minutes, 90 minutes, and 17 hours in a 30°C water bath. The final pH of the lipolysates was measured to confirm the buffering effects. The 0.2 mol/liter Tris-HCL buffer solutions seemed to show better buffering capacity at all pH ranges than the 0.2 mol/liter Imidazole buffer solution. The final pHs of lipolysates using Imidazole buffer solution were ca. 0.1 pH unit higher than the original pHs. At 30-minute incubation, pHs between 7.2 and 7.6 gave the highest liberation of glycerol by the Imidazole buffer; however, using the Tris-HCl buffer, pH 7.4 to 7.8 showed the highest activities. At 90-minute incubation, the highest lipolytic activities were similar to those observed at 30 minutes; however, at 17-hour incubation, the optimal pH ranged from 7.0 to 7.6 for the Imidazole buffer; 7.6 to 7.9 for the Tris-HCl; and 5.60 to 7.00 and 7.90 for the universal buffer solutions.

Since the proposed glycerol measuring method requires alkaline pH, the pH range between 7.4 and 7.9 was chosen for the enzymatic hydrolysis of serum triglycerides. A pooled serum having 1.65 mmol/liter triglycerides as determined by the Automated Fluorometric Hantzsch Condensation method was used to more carefully assess the pH optimum of Rhizopus delemar lipase activity in the pH 7 - 9 range. Final lipolysis mixtures containing 102 U/ml lipase, 7.4 mmol/liter calcium, and 0.1 ml of the pooled serum in a final volume of 0.7 ml was incubated in a 30°C water bath for 60 minutes and 120 minutes. It was also desired to determine that the glycerol measuring system would not be affected by the molar concentration of Tris-HCl buffer as well as by the changes in pH.

Thus, the 2.0 mmol/liter glycerol standard was included in the lipolysis reagent at various pH's of Tris-HCl buffer and glycerol was assayed by the GK-GDH method. The optimal pH using 0.1 mol/liter Tris-HCl buffer during 60 minutes incubation at 30°C was found to be 7.6 (Fig. 28). After 120-minute incubation, the optimal pH range between 7.2 and 8.0. At values above 8.0, the enzyme activities were greatly decreased at 60-minute incubation; however, after incubating for 120-minute, the liberation of glycerol was considerably increased at all pH ranges. At pHs above 8.8, there was no significant lipolysis of serum triglycerides. The measurement of 2.0 mmol/liter glycerol standard solution in the lipolysis reagent was apropos. In the same experiment using higher concentration, 0.5 mol/liter, of Tris-HCl buffer, the optimal pH was also found at 7.6 although the lipolytic activity was less than that of using 0.1 mol/liter Tris-HCl.

Optimal Molar Concentration of Tris-HCl

The criteria for choosing an optimal molar concentration of Tris-HCl should be that it will give the highest lipolytic activities and have adequate buffering effect. Tris-HCl buffer solutions ranging from 0.05 to 0.5 mol/liter with 35.6 mg/ml albumin were adjusted to pH 7.6. Pooled serum (0.1 ml) and the final concentration of 7.6 mmol/liter calcium were added to these solutions. Glycerol was assayed at 15, 30, and 60 minutes. Figure 29 shows that the higher the molar concentrations of Tris-HCl buffer, the poorer the recovery of glycerol, especially at the beginning of the lipolysis. However, at 60-minute incubation at

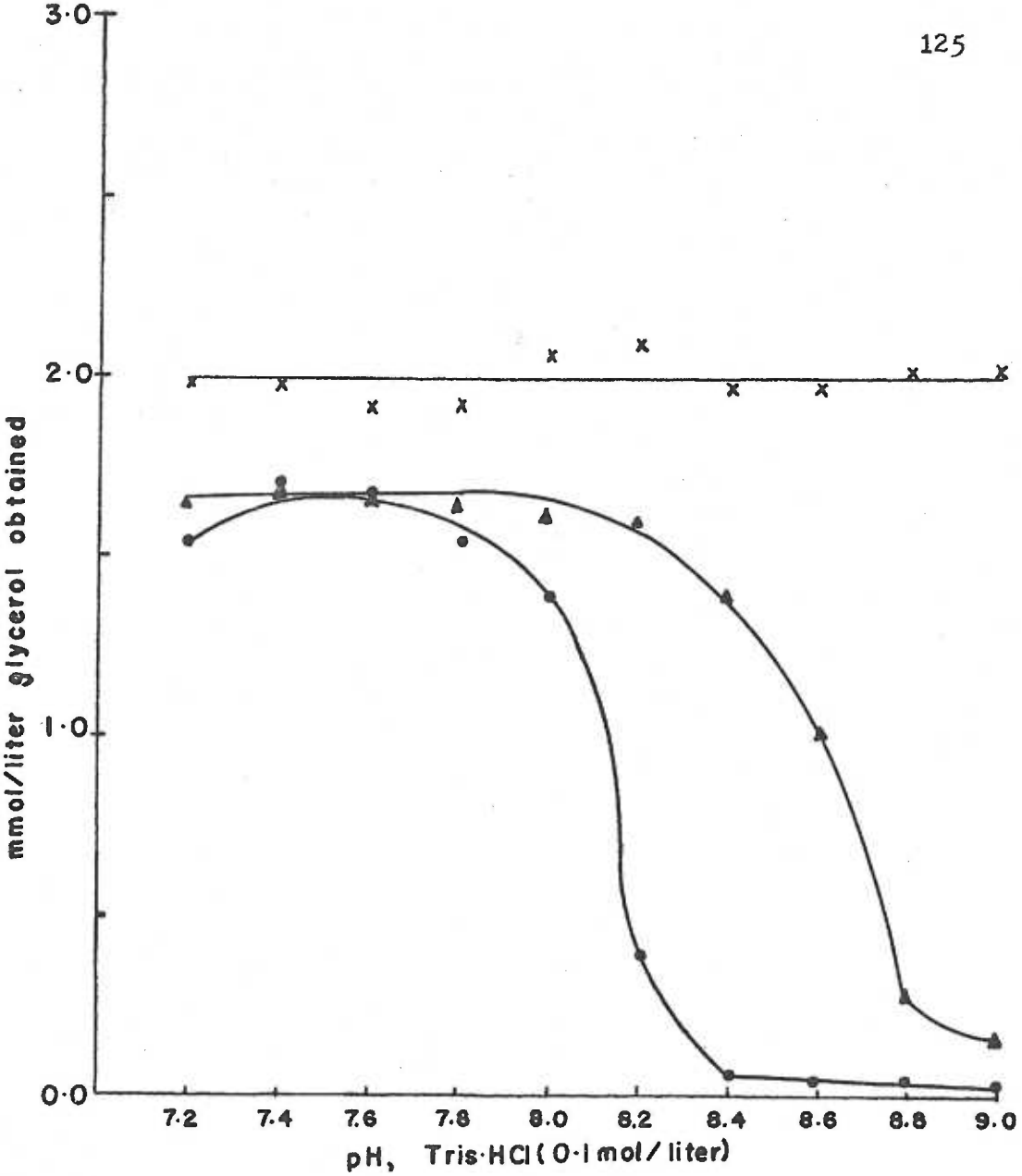


Fig.28. The pH optimum for Rhizopus delemar lipase activities using 0.1 mol/liter Tris.HCl buffer . The final concentrations of the lipolysis reaction mixture were 102 U/ml lipase, 7.4 mmol/liter Ca^{+2}, and 0.1 ml of a serum pool in the total volume of 0.7 ml. 2.0 mmol/liter glycerol standard was added to the lipolysis reagent at various pH and the glycerol contents were assayed by the GK-GDH Method. A serum pool and glycerol standard in the lipolysis reagent were incubated for 60 and 120 minutes in 30°C water bath before assayed for the glycerol moiety.

x——x 2 mmol/liter glycerol standard
•——• 60 min. at 30°C
▲——▲ 120 min. at 30°C

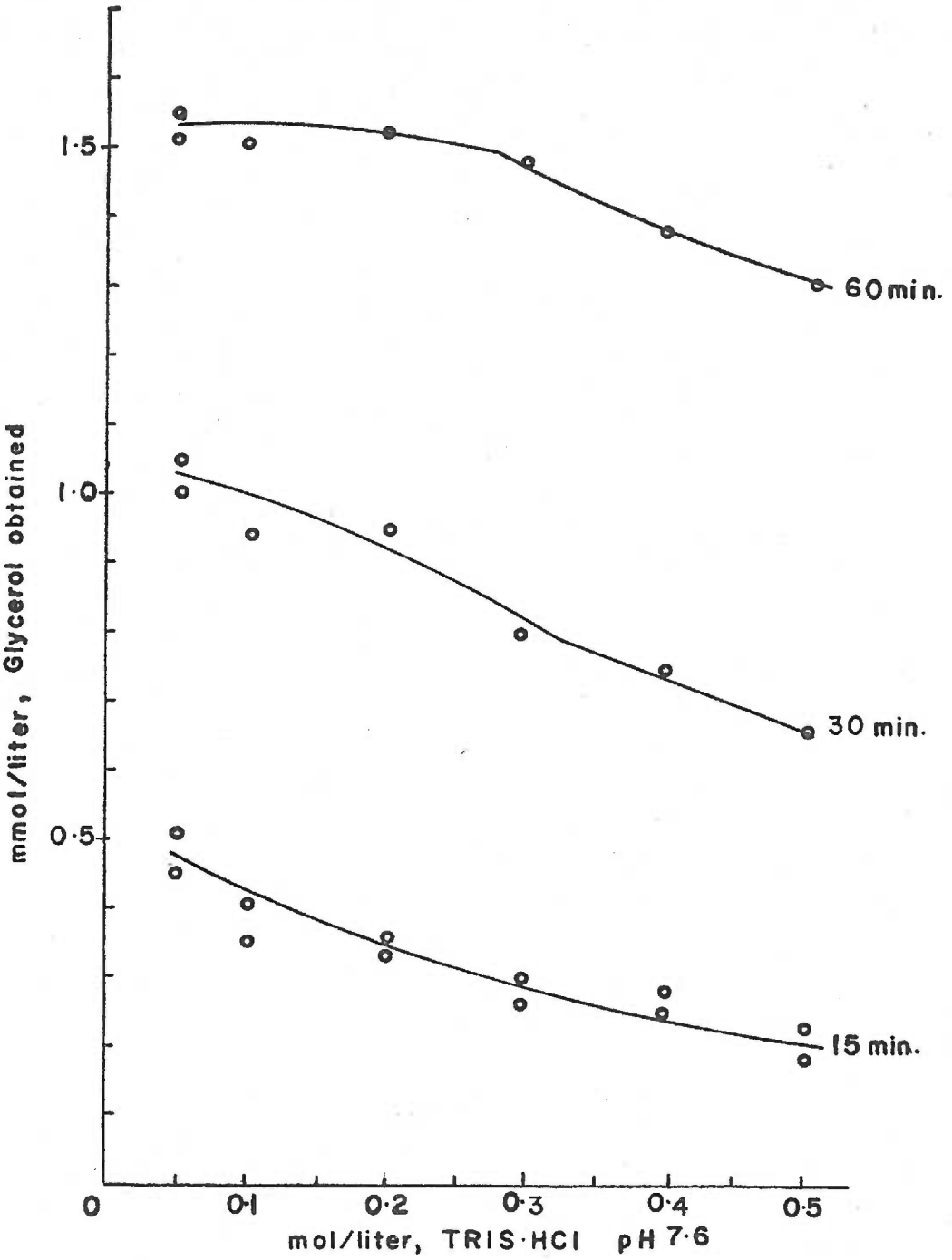


Fig. 29. Glycerol liberated from serum triglycerides by lipase as a function of buffer concentration at three time intervals. The final concentrations of the lipolysis reaction mixture were 7.6 mmol/liter Ca^{+2}, 35.6 g/liter albumin, 100 U/ml lipase, and 0.1 ml pooled serum in 0.66 ml.

30°C, 0.5 to 0.2 mol/liter Tris-HCl buffer solutions gave the same results. Table 14 summarizes the lipolytic activities of Rhizopus delemar lipase in various molar concentrations of Tris-HCl and Imidazole buffer solutions at pH 7.6. The pooled sera, A and B, in the lipolysis reagent were incubated in a 30°C water bath for 30 minutes and the liberated glycerol content was assayed by GK-GDH method at the end of incubation. The highest lipolytic activity in Tris-HCl buffer was at 0.05 mol/liter while the highest liberation of glycerol in Imidazole buffer was at 0.05 and 0.10 mol/liter. Calculating the mmol/liter of glycerol obtained in 0.05 mol/liter Tris-HCl buffer as 100 per cent, Tris-HCl buffer with 0.1 and 0.2 mol/liter was 97.5 and 95.0 per cent for the pooled serum A, respectively. With higher concentrations of serum triglycerides, pooled serum B, the lipolytic activities in 0.1 and 0.2 mol/liter Tris-HCl buffer were 90.5 and 87.0 per cent respectively. However, the lipolytic activities were reduced by approximately 25 per cent in 0.5 mol/liter Tris-HCl and Imidazole buffer solutions.

The final pH of the lipolysates was the same as the original buffer when molar concentrations greater than 0.2 mol/liter Tris-HCl buffer were used. With 0.1 mol/liter Tris-HCl buffer, the final pH was 7.66; however, with 0.05 mol/liter, the final pH was 7.70. The Imidazole buffer showed the final pHs ranged from 0.05 to 0.16 higher than that of the original pH of the buffer. Thus, Tris-HCl buffer with 0.1 mol/liter of pH 7.6 was used in the enzymatic hydrolysis reagent. The recovery of the glycerol standard, which had been added to the

Table 14. The effect of different molar concentration of Tris·HCl and Imidazole buffers at pH 7.6 on the enzymatic hydrolysis and the effect of Tris·HCl buffer on the glycerol measuring system. The lipolysates were incubated in a 30°C water bath for 30 minutes and assayed for liberated content by the GK-GDH technique.

Buffers	mmol/liter glycerol obtained		% activity of 0.05 mol/liter		Final pH of lipolysates	Recovery of added glycerol[a]	
	pooled serum-A	pooled serum-B	pooled serum-A	pooled serum-B		mmol/liter	% recovered
Tris·HCl (mmol/liter)							
0.05	1.25	1.80	100	100	7.70	1.78	100
0.10	1.22	1.63	97.5	90.5	7.66	1.77	100
0.20	1.19	1.57	95.0	87.0	7.58	1.75	99
0.30	1.02	1.41	81.5	78.5	7.59	1.66	94
0.40	0.95	1.40	76.0	78.0	7.60	1.77	100
0.50	0.91	1.42	73.0	79.0	7.64	1.79	101
Imidazole: (mol/liter)							
0.05	1.79		100		7.76	-	-
0.10	1.79		100		7.75	-	-
0.20	1.61		90.0		7.71	-	-
0.30	1.61		90.0		7.68	-	-
0.40	1.42		79.5		7.64	-	-
0.50	1.33		74.5		7.65	-	-

a. 1.77 mmol/liter glycerol standard was added to varying molar concentrations of Tris·HCl and subsequently assayed for glycerol.

various molar concentrations of Tris-HCl buffer solutions, ranged from 94 to 100 per cent.

Optimal Activity of α-chymotrypsin

The stability of the reconstituted α-chymotrypsin in a solution was investigated by dissolving ca. 43 units (BTEE units at 30°C) of α-chymotrypsin crystals in 0.001 mol/liter HCl, deionized water, 0.05 mol/liter and 0.10 mol/liter Tris-HCl (pH 7.6) buffer solutions. Each solution contains 0.005 mol/liter calcium chloride. Each solution was divided into two equal parts and one part was stored at 0 to 4°C while the other was put in a 30°C water bath. We also examined the stability of this enzyme in the enzymatic hydrolysis reagent containing 0.1 mol/liter Tris-HCl (pH 7.6), 100 U/ml lipase and 14.0 mmol/liter calcium chloride incubated in a 30°C water bath. At various time intervals, the activity of α-chymotrypsin was assayed by the method of Hummel (139) using N-benzoyl-L-tyrosine ethyl ester (BTEE) as a substrate at 30°C. Figure 30 shows the results of the findings.

When α-chymotrypsin dissolved in deionized water, 0.05 mol/liter and 0.10 mol/liter Tris-HCl (pH 7.6) buffers was assayed at the end of 100-hour incubation at 30°C, the enzyme activities were decreased by ca. 50 per cent while, storing at 0 - 4°C, 28 per cent decrease in activities was observed. The results obtained with these three solutions were essentially the same so that all results were averaged and plotted on a graph (Fig. 30). α-chymotrypsin in 0.001 mol/liter HCl with 0.01 mmol/liter calcium chloride showed considerably better stability than in deionized water, 0.05 mol/liter or 0.10 mol/liter Tris-HCl

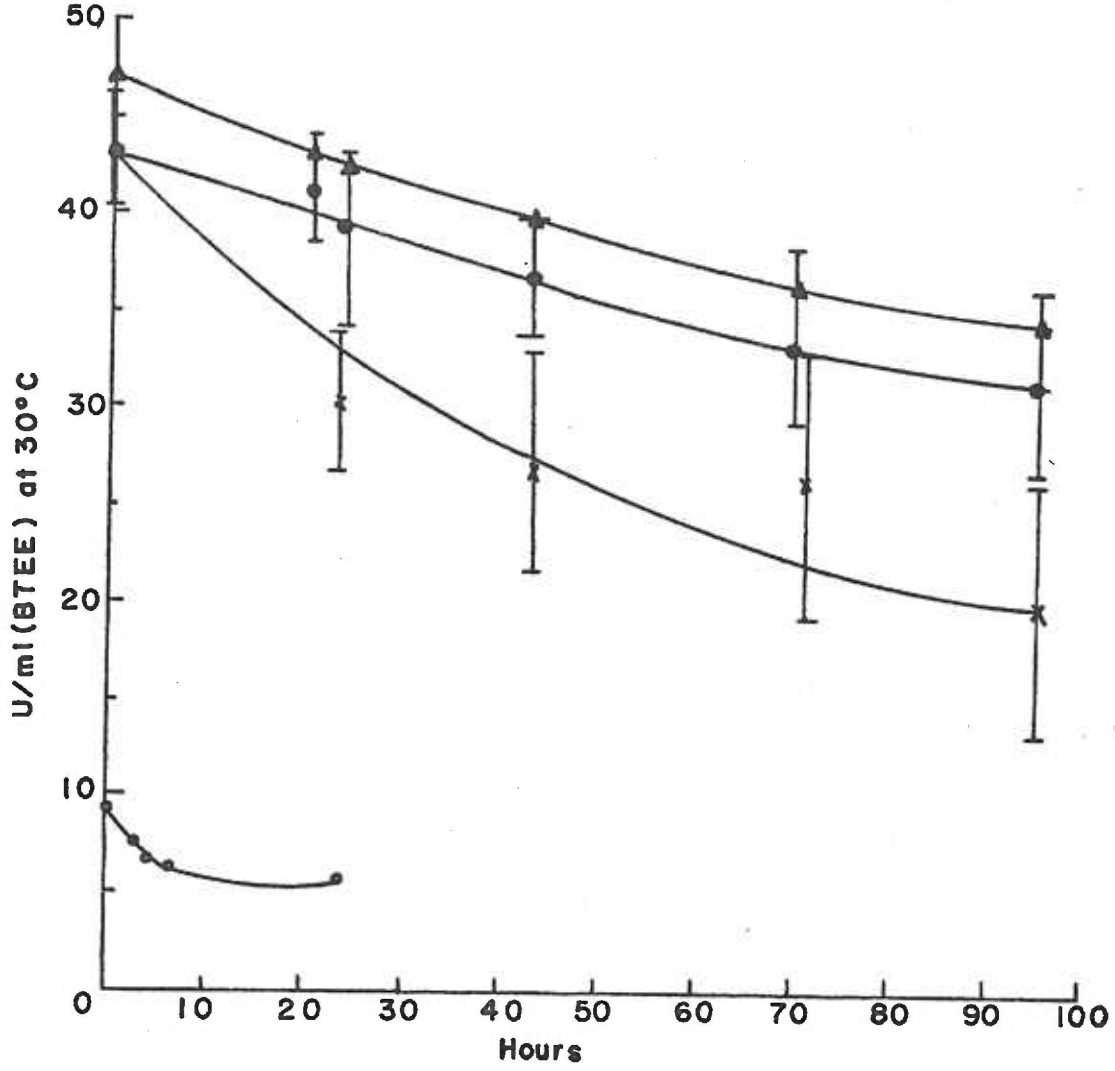


Fig. 30. Stability and activity of α-chymotrypsin at 0-4°C storage and 30°C incubation in 0.001 mol/liter HCl; deionized water; 0.05 mol/liter pH 7.6 Tris·HCl; and 0.1 mol/liter, pH 7.6, Tris·HCl. Each bar represents a mean ± 1 S.D.

•——• 0-4°C storage
×——× 30°C incubation
▲——▲ 0.001 mol/liter HCl with 0.01 mmol/liter Ca^{+2} at 0-4°
•——○ 9.3 U/ml α-chymotrypsin in the lipolysate which was kept in 30°C water bath. Then, the enzyme activity was determined at varying time intervals.

(pH 7.6) buffer solutions. The decrease in activities at the end of 100-hour incubation at 30°C was ca. 28 per cent. At the end of 24-hour incubation at 30°C, α-chymotrypsin in deionized water, 0.05 mol/liter and 0.10 mol/liter Tris-HCl (pH 7.6) buffer solutions showed a decrease in enzyme activity by ca. 23 per cent. However, α-chymotrypsin in the lipolysis reagent decreased by ca. 45 per cent. At the end of 2-hour incubation at 30°C, there was ca. 20 per cent decrease in the enzyme activity. When α-chymotrypsin in 0.001 mol/liter HCl with 0.01 mmol/liter calcium chloride was stored at 0 - 4°C, it was stable for approximately one week.

The optimal activity of α-chymotrypsin required to complete enzymatic hydrolysis of serum triglycerides was determined by adding varying amounts of α-chymotrypsin in 0.1 mol/liter, pH 7.6 Tris-HCl buffer containing 71.5 U/ml and 100 U/ml lipase, 14.0 mmol/liter calcium chloride, and 0.1 ml pooled serum in a total volume of 0.71 ml of lipolysis reaction mixture. The reaction mixture was incubated in a 30°C water bath for 30 minutes and glycerol was assayed by the GK-GDH method. During a 4-day period four different pooled sera were used to assess the optimal activity of α-chymotrypsin. The α-chymotrypsin dissolved in 0.001 mol/liter HCl with 0.01 mmol/liter calcium chloride was used for this experiment during which the α-chymotrypsin reagent was stored at 0 - 4°C. It was found that the optimal amount of α-chymotrypsin required to complete enzymatic hydrolysis ranged from 8.0 to 14.0 U/ml (Fig. 31). Two of the pooled sera, C and D, gave a slight decrease in recovery of triglycerides as the activity of α-chymotrypsin increased

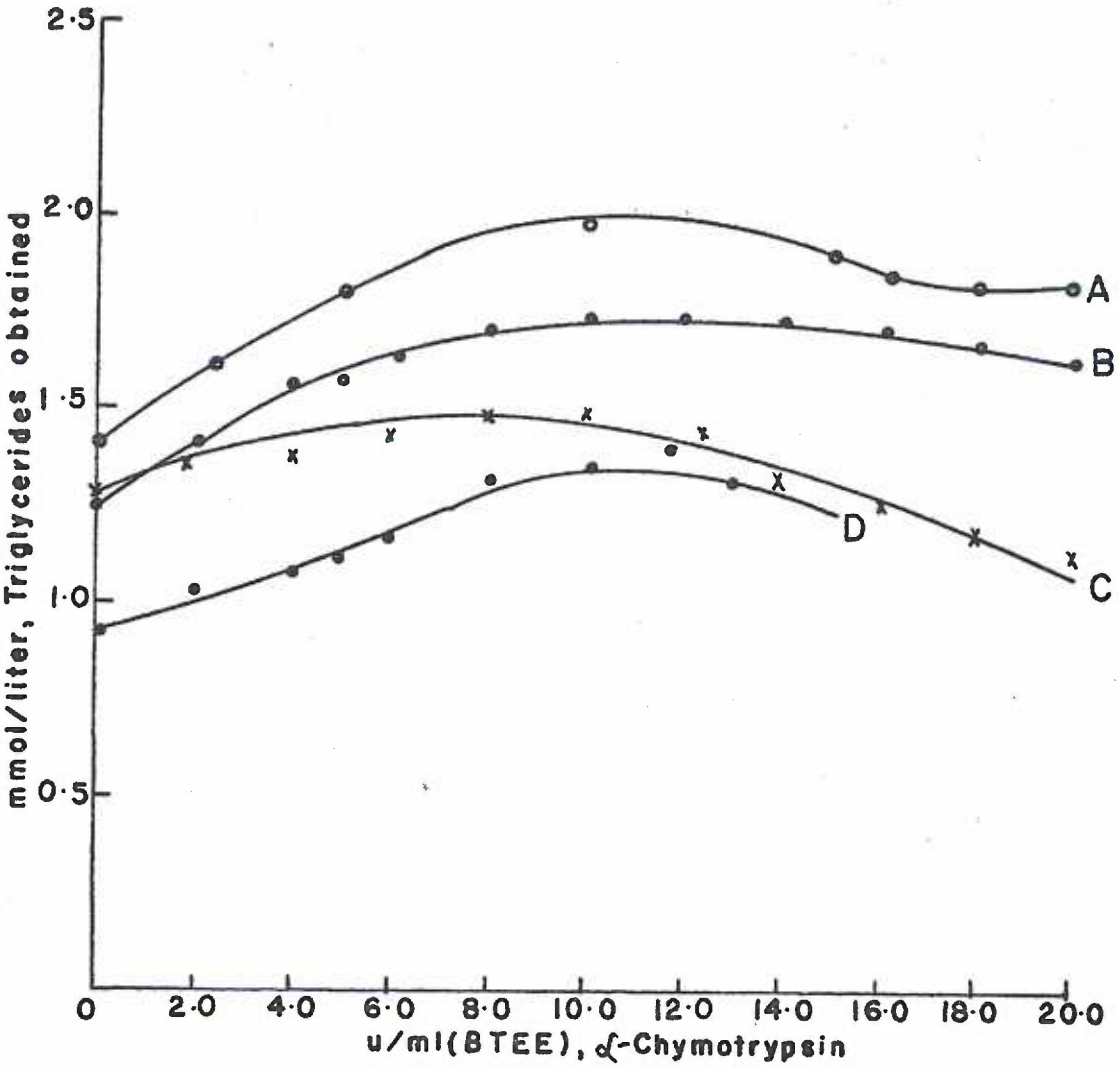


Fig. 31. The optimal activity of α -chymotrypsin for the enzymatic hydrolysis of serum triglycerides using four different pooled sera (A to D).

A = 30 min., 30°C, 100 U/ml lipase
B = 15 min., 30°C, 100 U/ml lipase
C = 60 min., 30°C, 71.5 U/ml lipase
D = 30 min., 30°C, 71.5 U/ml lipase

above 14.0 U/ml with 71.5 U/ml lipase. However, with 100 U/ml lipase, there was no appreciable decrease in lipolytic activity - pooled sera A and B. This phenomenum is probably caused by inactivation of lipase by α-chymotrypsin resulting in inadequate amount of lipase to complete hydrolysis of serum triglycerides during 30-minute incubation at 30°C. Ten U/ml α-chymotrypsin in the final lipolysis reaction mixture was chosen as the optimal activity.

Optimal Concentration of Bovine Serum Albumin

The optimal concentration of bovine serum albumin in the lipolysis reaction mixture was determined by adding varying quantities of albumin to the lipolysis reagent containing 100 U/ml lipase and 10 U/ml α-chymotrypsin in 0.1 mol/liter pH 7.6 Tris-HCl buffer. One set of the lipolysis reagents containing 0.0 to 24.0 g/liter albumin was preincubated in a 30°C water bath for 40 minutes before adding pooled serum. The second set of lipolysis reagents with 0.0 to 24.0 g/liter albumin was not preincubated at 30°C for 40 minutes. The results of the experiment are shown in Figure 32. A pooled serum containing 2.2 mmol/liter triglycerides as determined by Automated Fluorometric Hantzsch Condensation method was added to preincubated and non-preincubated sets of lipolysis reagent and they were incubated in a 30°C water bath for 120 minutes. At the end of 120-minute incubation, the liberated glycerol was assayed by the GK-GDH method. At the end of 120-minute incubation at 30°C the enzymatic hydrolysis seemed to be completed when the lipolysis reagent containing more than 8.0 g/liter bovine serum albumin and

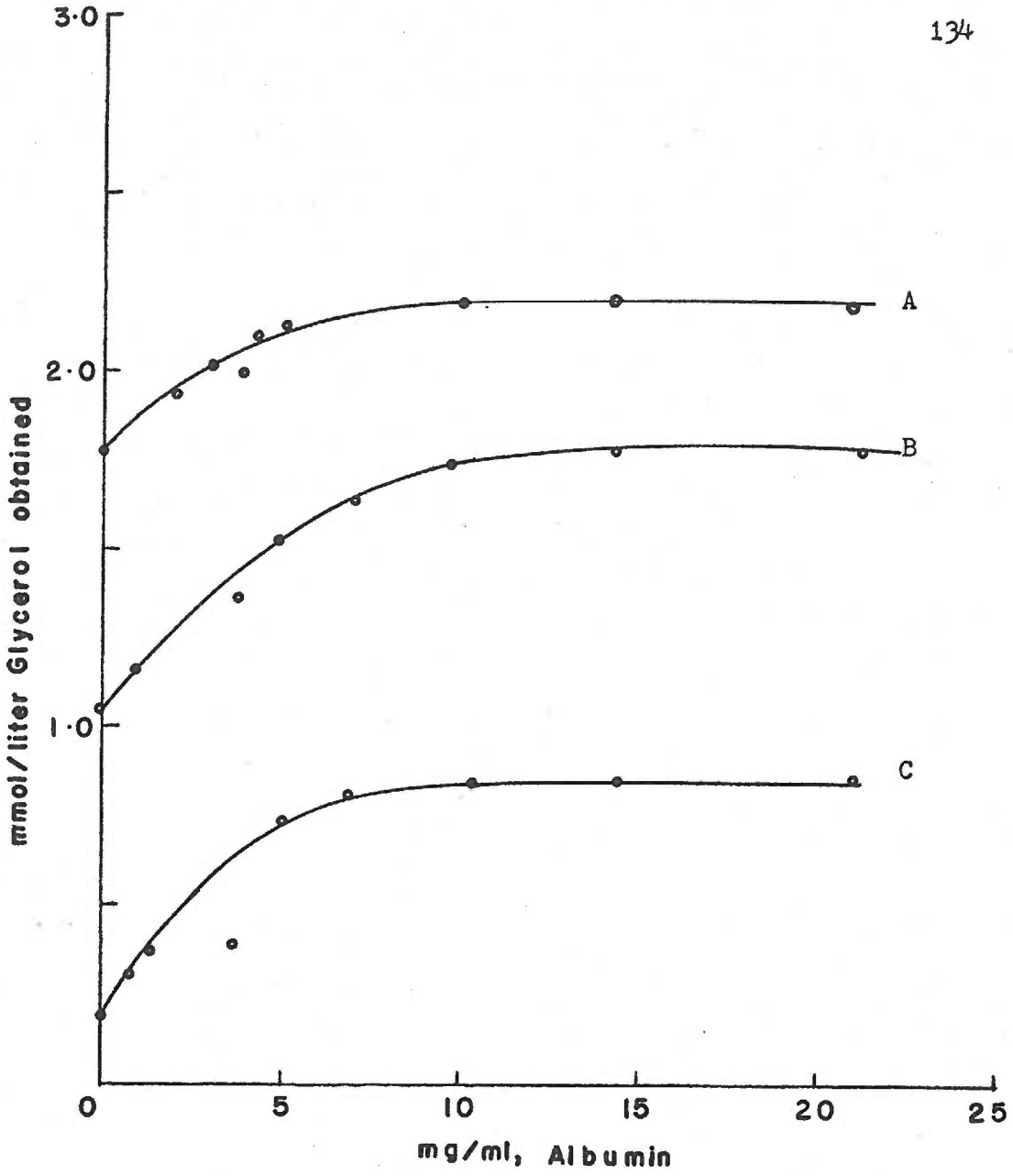


Fig. 32. The optimal concentration of Sigma Bovine Serum Albumin (35%) in the lipolysis reagent.

A = No pre-incubation at 30°C (serum pool having 2.2 mmol per liter triglycerides)

B and C = Pre-incubation of the lipolysis reagent for 40 minutes in 30°C water bath before adding a pooled serum having 2.2 (B) and 0.85 (C) mmol/liter serum triglycerides.

the lipolysis reagent had not been preincubated (Fig. 32-A). However, when the same lipolysis reagent was preincubated at 30°C for 40 minutes, the final triglyceride values were ca. 18 per cent lower than the non-preincubated reactions with more than 8.0 g/liter albumin (Fig. 32-B). The lipolysis reagent with less than 8.0 g/liter albumin showed poorer lipolytic activities in all three curves, A, B, and C. This study reveals that albumin is necessary for complete enzymatic hydrolysis of serum triglycerides. The optimal concentration chosen was 10.0 g/liter bovine serum albumin. The effects of other sources and preparations of albumin on the stability of lipolytic enzymes and on the enzymatic hydrolysis are discussed later.

Optimal Concentration of Gum Arabic

Two concentrations of lipase, 62.5 and 125.0 U/ml, were used to determine the optimal concentration of gum arabic. The final lipolysis reagent contained 0.1 mol/liter, pH 7.6 Tris-HCl buffer with 10.0 U/ml α-chymotrypsin, and 10.0 g/liter bovine serum albumin. Gum arabic concentration ranging from 0.0 to 29.0 g/liter were added to the lipolysis reagent. Fifty µl of a pooled serum was added to the lipolysis reagent and the mixture was incubated for 60 minutes in a 30°C water bath. Glycerol was assayed by the GK-GDH method immediately after the 60-minute incubation (Fig. 33). When the lipolysis time was reduced and glycerol was measured at the end of 15 and 30 minutes incubation, the final lipolysis reagent containing more than 10 g/liter gum arabic gave the highest lipolytic activity (Table 15). The lipolysis reaction mixture without gum arabic showed lower liberation of glycerol, e.g. at 30

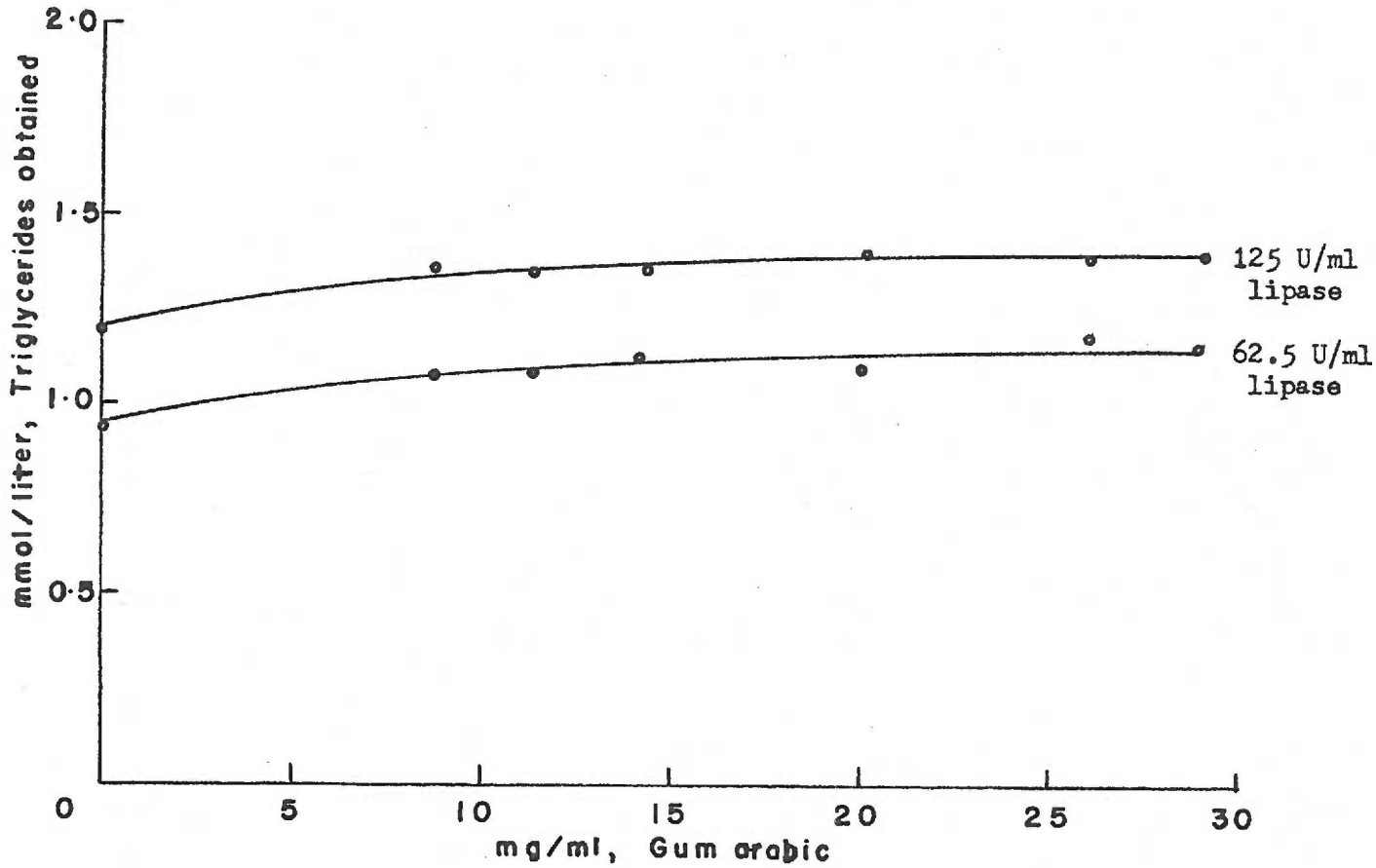


Fig. 33. The optimal concentration of Gum arabic for the enzymatic hydrolysis of serum triglycerides while maintaining the concentrations of other lipolysis reagents constant. The results were taken at 2 minutes after the glycerol measuring reaction.

Table 15. Effect of Gum Arabic on lipolysis of serum triglycerides. The liberated glycerol was assayed at the end of 15 and 30 minutes incubation at 30°C. A serum pool containing 1.85 mmol/liter triglycerides was used.

Gum Arabic (mg/ml)	15 minutes (mmol/liter)	30 minutes
0.0	1.10	1.43
4.76	1.07	1.53
7.14	1.15	1.55
10.7	1.27	1.67
11.9	1.27	1.68

minutes, 0.0 g/liter gum arabic gave 1.43 mmol/liter glycerol while the lipolysis reaction mixture with 10.7 g/liter gum arabic gave 1.67 mmol/liter. The optimal concentration of gum arabic, therefore, was selected to be ca. 10 g/liter.

Optimal Concentration of Deoxycholate

Deoxycholate concentrations in the final lipolysis reaction mixture ranging from 0.0 to 4.25 mmol/liter were analyzed for the optimal amount needed to complete enzymatic hydrolysis of serum triglycerides. The concentrations of the other lipolysis components were 100 U/ml lipase, 10 U/ml α-chymotrypsin, 10 g/liter BSA, and 10 g/liter gum arabic in 0.1 mol/liter Tris·HCl (pH 7.6). Fifty µl of a pooled serum and the lipolysis reagent were incubated for 15 and 28 minutes in a 30°C water bath. Then glycerol was assayed at the end of 15 and 28 minutes using the GK-GDH method. It was found that 2.0 mmol/liter deoxycholate in the final lipolysis reaction mixture showed a maximal lipolytic activity during 15-minute incubation (Fig. 34). When the lipolysates were analyzed at the end of 28-minute incubation, 2.0 mmol/liter deoxycholate gave the same result (2.1 mmol/liter triglyceride value) as observed in the 15-minute incubation. Thus, the optimal concentration of deoxycholate required to complete hydrolysis of serum triglycerides at the 15-minute lipolysis time was found to be 2.0 mmol/liter. At the higher concentrations of deoxycholate (2.5 mmol/liter), the lipolytic activities were poorer as in the lower concentrations (Fig. 34). Three separate experiments using the

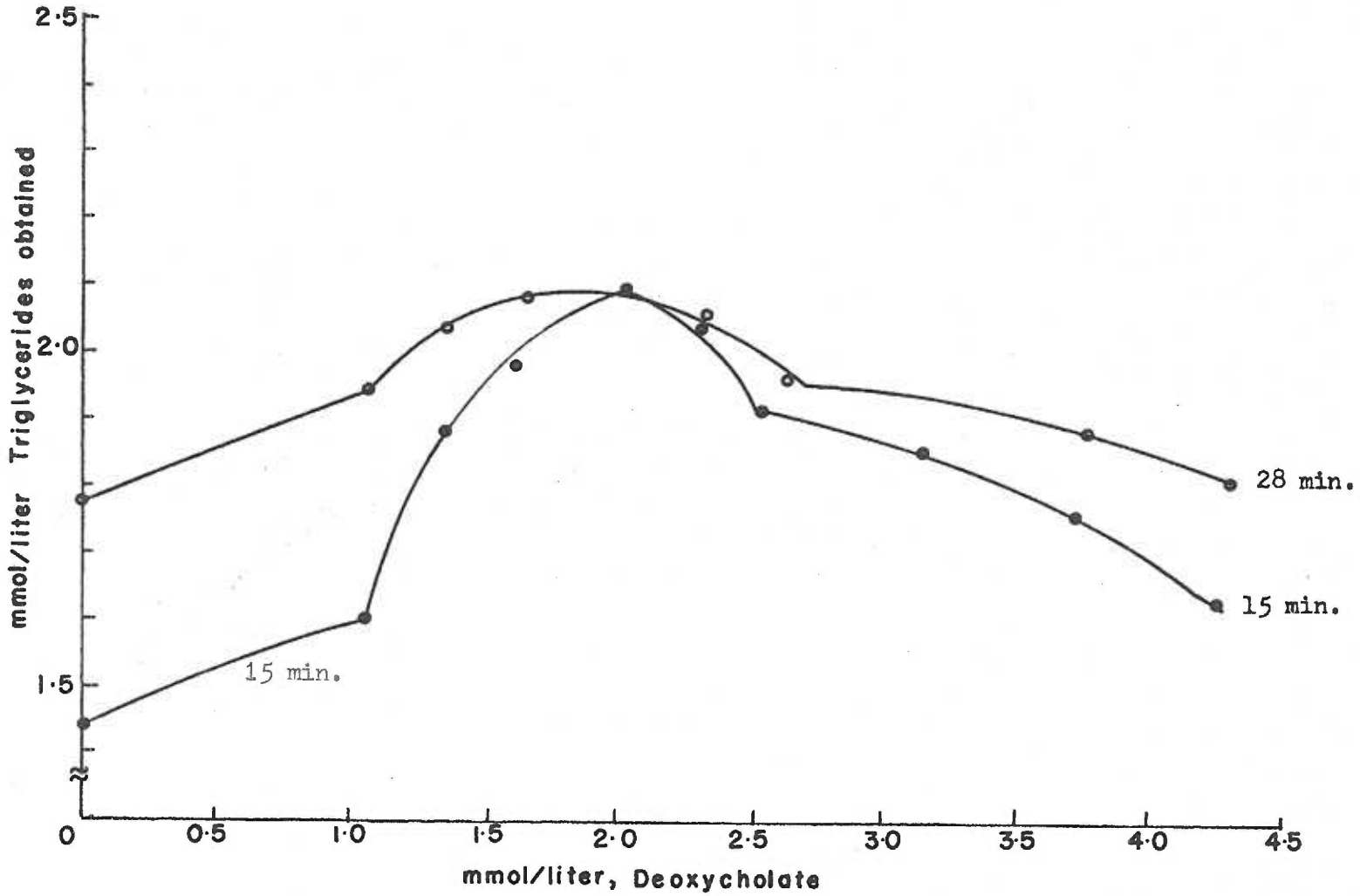


Fig. 34. The optimal concentration of deoxycholate for the enzymatic hydrolysis of serum triglycerides at 30°C. A pool serum with varying concentrations of deoxycholate and the constant concentrations of other lipolysis reagents was incubated for 15 min. and 28 min. Then, the liberated glycerol was assayed by the GK- GDH Method.

different pooled serum over the 3-day period showed similar curves as in Figure 34 with an optimal concentration of deoxycholate of 2.0 mmol/liter.

Optimal Activity of Rhizopus Delemar Lipase

Six different amounts of lipase ranging from 25 to 150 U/ml were used for this experiment. A pooled serum containing 2.0 mmol/liter serum triglycerides as measured by the Automated Fluorometric Hantzsch Condensation method and the lipolysis reagent mixtures containing varying amounts of lipase were incubated in a 30°C water bath for 15 minutes. Then the liberated glycerol was assayed by the GK-GDH method. Figure 35 shows the typical curve of the lipase activity. The lipase activities greater than 100 U/ml showed complete hydrolysis of serum triglycerides. The same lipolysates stood at ambient temperature for an additional 15 minutes and they were re-assayed for glycerol. Seventy-five U/ml lipase in the final lipolysis reaction mixture gave 2.0 mmol/liter triglycerides. α-chymotrypsin and lipase were added to the lipolysis reagent just prior to adding a pooled serum for enzymatic hydrolysis. Even though 100 U/ml of lipase in the final reaction mixture was shown to be sufficient in completing hydrolysis of serum triglycerides at 30°C for 15 minutes incubation, 125 U/ml was chosen for the enzymatic hydrolysis.

Effect of Calcium Ion on Enzyme Hydrolysis

It was of interest to determine whether or not more than ca. 0.3 mmol/liter calcium ion, which was an average amount present in the

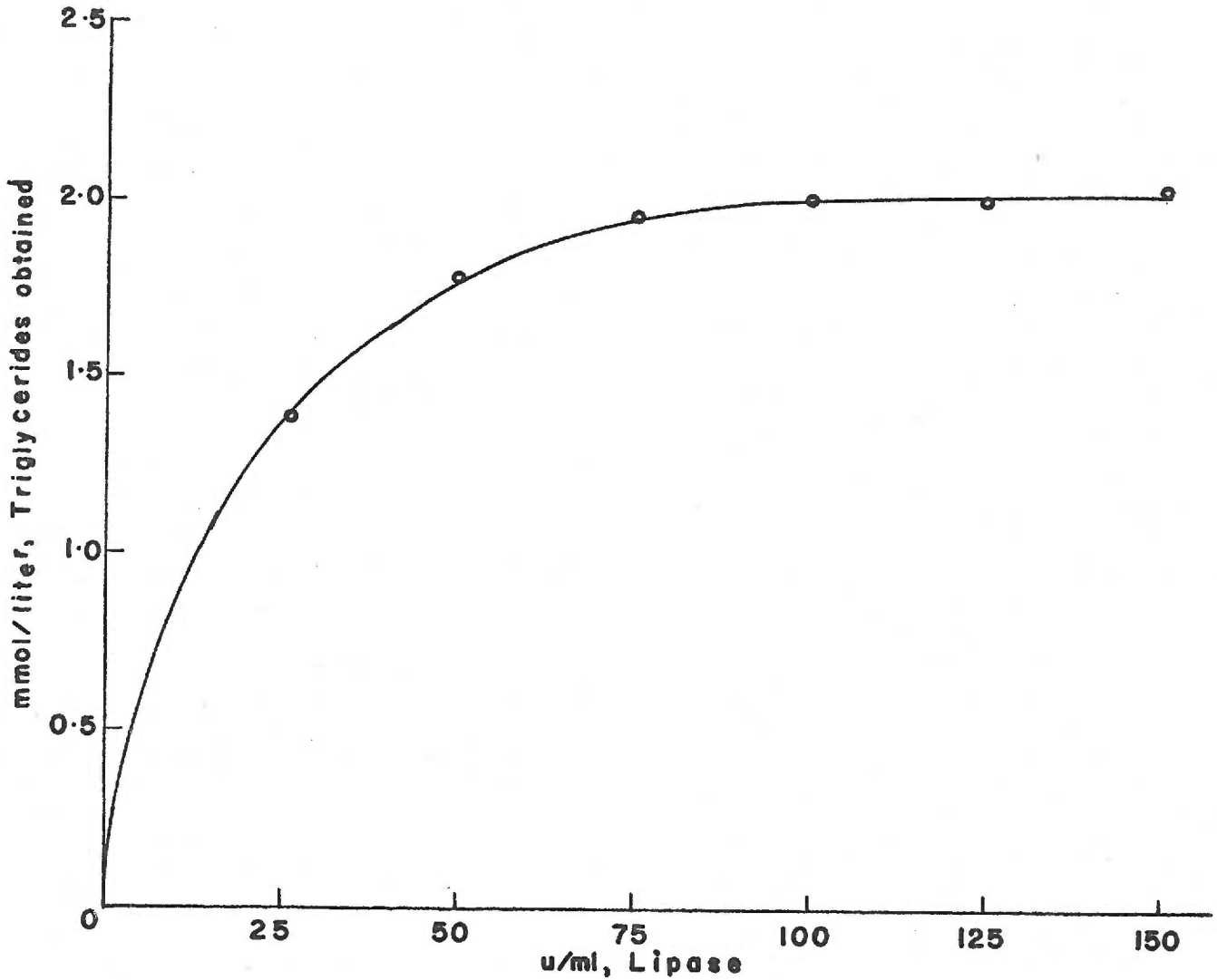


Fig. 35. The optimal activity of Rhizopus delemar lipase needed to reach equilibrium when the concentrations of other lipolysis reagents were held constant.

lipolysate, would be needed for the enzymatic hydrolysis of serum triglycerides. The mean calcium ion of ca. 0.3 mmol/liter in the final lipolysis reaction mixture came from endogenous sources such as a serum sample, α-chymotrypsin and lipase stock reagents which contained calcium to stabilize the enzymes during the storage at 0 - 4°C. To substantiate the need for an additional calcium ion, calcium concentrations ranging from 0.0 to 2.14 mmol/liter were added to the lipolysis reagent containing 10 g/liter BSA, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate, 125 U/ml lipase and 10 U/ml α-chymotrypsin in the final reaction mixture. The lipolysates were assayed for glycerol according to the GK-GDH method. When two pooled sera with triglyceride values of 1.78 and 2.06 mmol/liter were subjected to enzymatic hydrolysis for 15 minutes at 30°C, the results showed no significant difference whether 0.0 mmol/liter calcium ion or 2.14 mmol/liter calcium ions was added to the lipolysates (Table 16). Therefore, no additional calcium ion was added to the lipolysis reagent.

Time Required to Complete Enzymatic Hydrolysis

Serums with 0.40 to 5.40 mmol/liter triglycerides were assayed by the proposed enzymatic hydrolysis method. The lipolysis reagent contained 10 g/liter BSA, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate, 10 U/ml α-chymotrypsin and 125 U/ml lipase. The liberated glycerol was analyzed at 10, 15, 30, and 60 minutes intervals to determine the necessary incubation time required to complete hydrolysis of the triglycerides. Table 17 depicts the results of this experiment. It appeared

Table 16. The effect of calcium ion on enzyme hydrolysis of serum triglycerides.

Ca^{+2}	pooled serum - l	pooled serum - h
(mmol/liter)	mmol/liter	
0.00	1.78	2.06
0.07	-	2.02
0.14	1.76	2.06
0.21	1.76	2.06
0.29	1.76	2.10
0.36	1.80	2.10
0.43	1.70	2.02
0.71	1.78	1.98
1.07	1.78	1.98
1.43	1.72	2.02
1.79	-	2.00
2.14	1.70	2.10

Note: A total of 0.316 mmol/liter Ca^{+2}, from a pooled serum and α-chymotrypsin, was present in the lipolysate before addition of varying amounts of calcium.

Table 17. The optimum time required to complete enzymatic hydrolysis of serum triglyceride.

Serum triglycerides (mmol/liter)	Duration of hydrolysis at 30°C			
	10 min.	15 min.	30 min.	60 min.
		(mmol/liter)		
0.40	0.43	0.43	0.45	0.46
0.78	0.69	0.77	0.79	0.71
1.60	1.60	1.62	1.58	1.51
2.95	3.03	2.92	2.98	2.79
5.40	3.75	5.22	5.42	5.47

that enzymatic hydrolysis was completed with serum triglyceride levels of ca. 3.0 mmol/liter during 10-minute incubation at 30°C. The serum triglyceride level of 5.40 mmol/liter was not quite completely hydrolyzed at the end of 15 minutes incubation, but it was completed at 30 minutes.

Twenty-seven serum samples were incubated with the lipolysis reagents for 15 minutes in a 30°C water bath and glycerol was assayed by the GK-GDH method. To verify that the lipolysis was completed at the end of 15-minute incubation and there was no further increase in triglyceride values, the same lipolysates incubated for 15 minutes were subjected to an additional 30 minutes incubation. Serum specimens with triglyceride values ranging from ca. 0.73 to 4.80 mmol/liter were used for this study. It was found that the enzyme hydrolysis was completed at the end of 15-minute incubation in a 30°C water bath and there was no further change in the triglyceride values when the same lipolysates used in the 15-minute incubation were assayed at the end of 45 minutes (Table 18). The mean of triglyceride results obtained between 15 and 45 minutes incubation showed no significant difference at 95 per cent level .. the calculated t (0.085) was less than the Table - t (2.056) at df = 26. The correlation coefficient was 0.9938 with regression line of $y = 0.9655X + 0.044$ in which X was 15-minute and y was 45-minute incubation.

Effect of Various Albumin Preparations on Enzymatic Hydrolysis

In the early part of this investigation, ca. 36 g/liter human serum

Table 18. Comparison of triglyceride glycerol liberated from 27 serum samples at the end of 15 minutes and 45 minutes lipolysis at 30°C.

Sample	At the end of 15 min. at 30°C (mmol/liter)	45 min. later at 30°C
1	1.15	1.11
2	1.36	1.19
3	1.19	1.17
4	1.32	1.22
5	1.13	0.97
6	4.65	4.26
7	4.32	4.57
8	4.08	4.14
9	1.28	1.36
10	1.24	1.26
11	1.98	1.96
12	1.94	1.90
13	3.64	3.58
14	3.50	3.66
15	3.46	3.42
16	1.03	1.03
17	4.80	4.40
18	3.21	3.03
19	2.81	2.69
20	2.40	2.36
21	1.58	1.60
22	0.73	0.85
23	1.24	1.36
24	1.07	1.07
25	1.22	1.26
26	1.32	1.30
27	2.40	2.45

Calculated t = 0.085
df = 26
Table t = 2.056 and α = 0.05
Y = 0.9655X + 0.044 in which y = 45 minutes later at 30°C
and X = 15 minutes at 30°C.
Correlation coefficient = 0.9938
r^2 = 99%

albumin (Pentex) was added to the lipolysis reagent to stabilize lipolytic enzymes (lipase and α-chymotrypsin) and to remove free fatty acids that formed during enzymatic hydrolysis of serum triglycerides. When the lipolysis reagent was made immediately before adding a serum sample, the enzymes seemed to be completely hydrolyzing serum triglycerides (ca. 1.0 to 2.0 mmol/liter) within 90 minutes at 30°C incubation. When the lipolysis reagent was preincubated in a 30°C water bath for 40 minutes before adding serum, the subsequent incubation for 90 minutes produced no glycerol. It appeared that the lipolytic enzymes were completely inactivated during preincubation in a 30°C water bath for 40 minutes. After this discovery, it was of interest to investigate the effect of other albumin preparations. When bovine serum albumin (Sigma, 35%) was used in the lipolysis reagent, the preincubation of the reagent did not totally abolish lipolytic activity. After the optimal conditions and concentrations of the enzymatic hydrolysis reagents were established, six different albumin preparations were added to the lipolysis reagent in which one set was preincubated at 30°C for 40 minutes and the other set was made just before use. The final concentrations of the lipolysis reagents were 10 g/liter albumin, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate, 10 U/ml α-chymotrypsin, and 125 U/ml Rhizopus delemar lipase in 0.1 mol/liter of Tris·HCl buffer (pH 7.6). At the end of a 40-minute preincubation at 30°C, 50 µl of a pooled serum was simultaneously pipetted into both preincubated and non-preincubated lipolysis reagents and they were re-incubated for an additional 15 minutes in a 30°C water bath. The pooled serum

contained 2.24 mmol/liter triglycerides. Fifty µl of 2.0 mmol/liter glycerol standard was also added to the lipolysis reagents containing various albumin preparations. The reagent-sample blank was prepared by adding 50 µl of a pooled serum to the lipolysis reagent containing neither lipase nor α-chymotrypsin. At the end of a 15-minute incubation at 30°C, the lipolysates, the lipolysis reagents with glycerol standard, and the reagent-sample blank were simultaneously assayed for the glycerol by the GK-GDH method. The results are illustrated in Figure 36 as a bar graph.

When there was no albumin present in the lipolysis reagent, preincubated reagent rendered lower glycerol results and non-preincubated reagent also showed a result which was less than quantitative. Although the non-preincubated lipolysis reagent with Metrix BSA gave glycerol value equivalent to those with Sigma BSA (35%) and FFA free BSA (Sigma), the preincubated lipolysate showed poor glycerol recovery. On the other hand, the preincubated lipolysates of Sigma BSA (35%) and FFA free BSA (Sigma) yielded the highest glycerol values. The preincubated lipolysis reagents containing Bacto BSA and HSA (Pentex) were about equal and less than quantitative. The lipolysis reagent with Fraction V BSA (Sigma) resulted in the lowest liberation of glycerol after preincubation and it appeared to affect the glycerol measuring method in addition to having an effect on lipolysis. The non-preincubated lipolysate also gave lower results. Thus, in the reaction mixtures with HSA (Pentex) and Fraction V BSA (Sigma), albumin seemed to interfere with both enzymatic hydrolysis and the GK-GDH Method. Among six albumin preparations

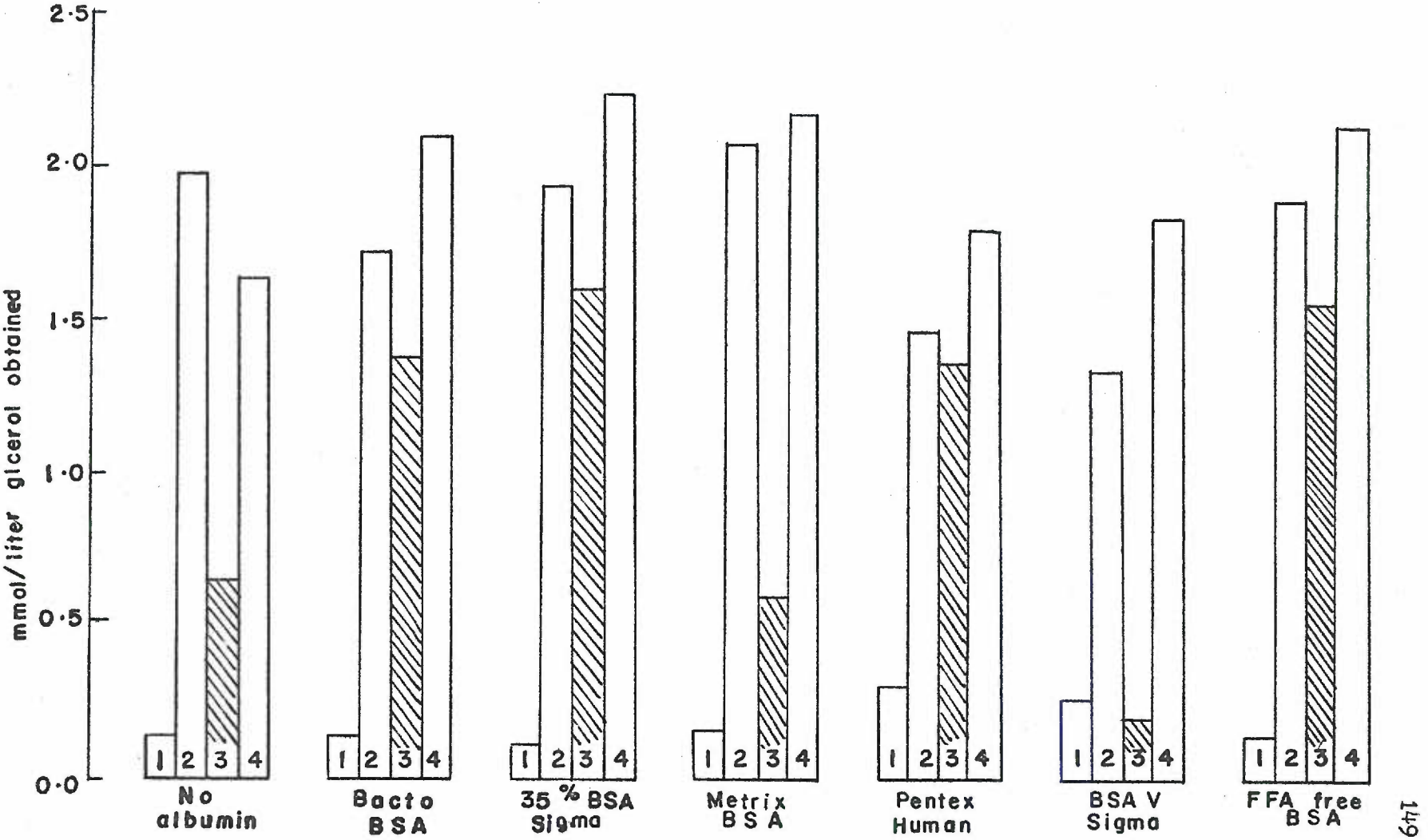


Fig. 36. Effect of various commercial preparations of albumin on lipolysis and glycerol measuring system. Pre-incubation refers to incubating lipolysis reagents with albumin for 40 min. in 30°C water bath before adding a serum pool. 1 = blank of serum pool; 2= 2.0 mmol/liter glycerol standard; 3 = pre-incubation of lipolysis reagent; and 4 = No pre-incubation.

studied, the albumins of choice in the suggested triglyceride method are Sigma BSA (35%) and FFA free BSA (Sigma).

Effect of Removing Various Components from the Lipolysis Reaction Mixture

It had been demonstrated thus far that the optimal conditions for triglycerides hydrolysis was incubation for 15 minutes at 30°C in a reaction mixture containing 10 g/liter bovine serum albumin (Sigma, 35%), 2.0 mmol/liter deoxycholate, 10 g/liter gum arabic, 10 U/ml α-chymotrypsin, and 125 U/ml lipase in 0.1 mol/liter Tris·HCl, pH 7.6. To examine the effect of each constituent of the lipolysis reagent in a different way, a pooled serum was reacted with lipolysis reagent that was missing one or more components. The pooled serum lipolysis-reagent mixtures were analyzed for glycerol after 15-minutes incubation in a 30°C water bath. The same lipolysis reagents were also preincubated at 30°C for 30 minutes before adding a pooled serum.

The pooled serum contained 2.0 mmol/liter triglyceride and the results, Figure 37, are expressed as a per cent of the 2.0 mmol/liter value determined using the lipolysis reagent containing all components without preincubation. In both experiments (preincubation and non-preincubation), the presence of bovine serum albumin is sine qua non. The lipolysis reagent without BSA depicted the lowest lipolytic activity. The addition of both deoxycholate and gum arabic enhanced the recovery of triglycerides.

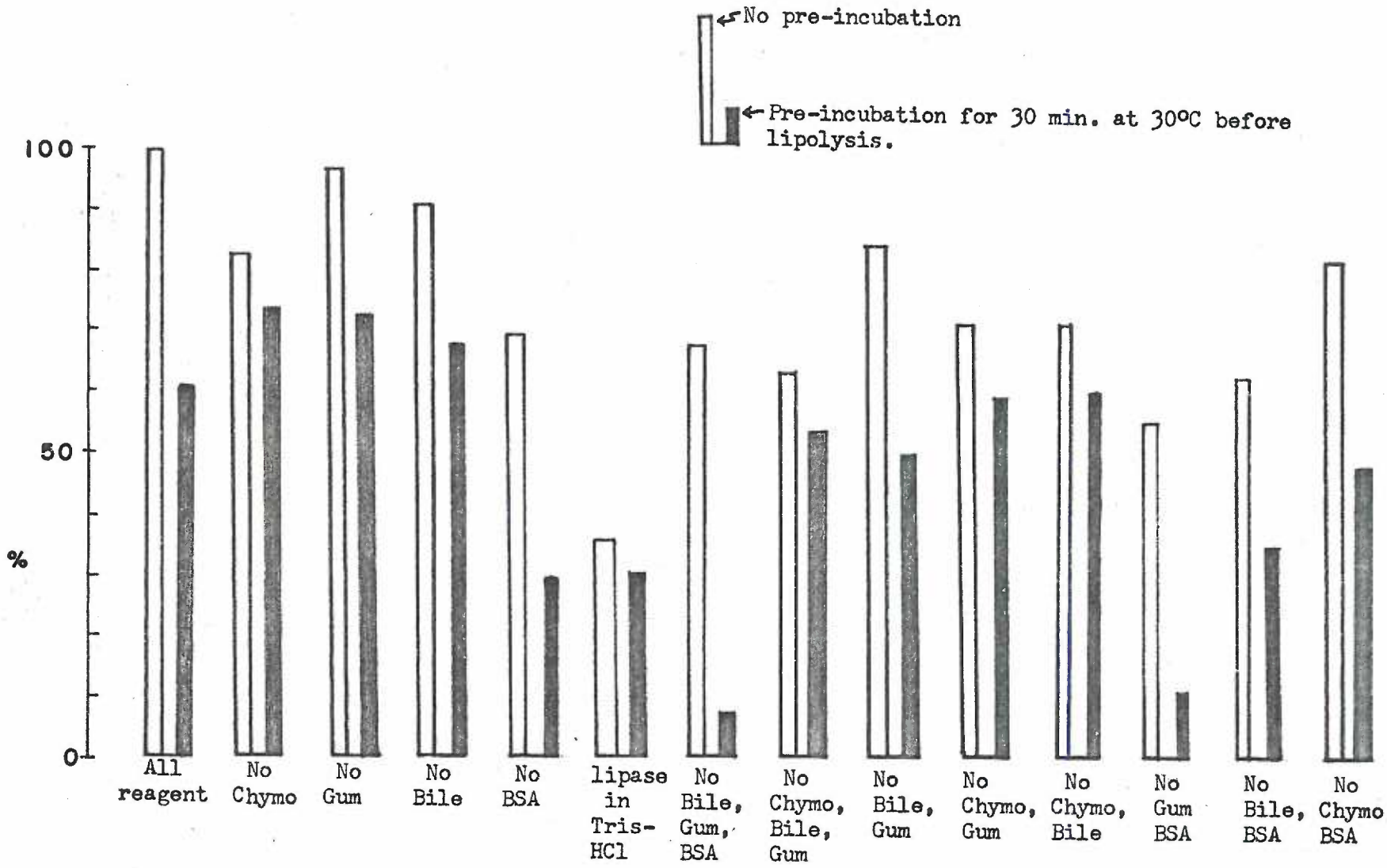


Fig. 37. Effect of lipolytic activity with and without various components of the lipolysis reagent. Chymo = α - chymotrypsin, Bile = deoxycholate, Gum = Gum arabic, BSA = bovine serum albumin.

Effect of α-chymotrypsin on Hydrolysis of Serum Triglycerides in Absence of Lipase

A final lipolysis reaction mixture containing 0.0 to 13.5 U/ml (BTEE units) α-chymotrypsin without added lipase was used to determine, the extent of triglyceride hydrolysis by α-chymotrypsin in the absence of lipase. The lipolysis reagent contained 10 g/liter BSA, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate in 0.1 mol/liter Tris·HCl of pH 7.6 buffer with varying amounts of α-chymotrypsin. The lipolysis reagent was mixed with 50 µl of a pooled serum, incubated for 15 minutes in a 30°C water bath, and glycerol was assayed by the GK-GDH method. The results are listed in Table 19. The pooled serum marked "a" showed 0.00 to 0.06 mmol/liter of glycerol while the pooled serum marked "b" was ca. 0.11 mmol/liter. The lipolysates containing neither α-chymotrypsin nor Rhizopus delemar lipase showed 0.06 and 0.12 mmol/liter glycerol in two pooled sera labelled "a" and "b", respectively. Since the other lipolysates containing 5.4 to 13.5 U/ml α-chymotrypsin yielded similar glycerol results as observed with 0.0 U/ml α-chymotrypsin, α-chymotrypsin was not responsible for the hydrolysis of all three ester bonds of serum triglycerides. The results of 0.06 and 0.12 mmol/liter are most likely the free glycerol that is present in the serum. However, it is possible that if a β-monoglyceride is present in those pooled sera, α-chymotrypsin may hydrolyze the β-ester bond to produce glycerol. Nevertheless, the pooled sera with and without α-chymotrypsin showed no significant difference in measured glycerol thus, the presence of

Table 19. Effect of α-chymotrypsin on lipolysis of serum triglycerides without the presence of lipase in the lipolysis reagent.

α-chymotrypsin (U/ml)	pooled serum-a mmol/liter glycerol obtained	pooled serum-b mmol/liter glycerol obtained
0.0	0.06	0.12
5.4	0.05	0.10
8.1	0.05	0.10
10.8	0.05	0.11
13.5	0.00	0.11

β-monoglycerides in the serum cannot be the origin of the glycerol which was measured here.

Enzyme Hydrolysis of Phospholipids

Three phospholipids, 2.7 mmol/liter phosphatidylcholine, 2.7 mmol/liter sphingomyelin, and 3.4 mmol/liter phosphatidylethanolamine, were tested to see if they would interfere with the triglyceride assay. To investigate the extent of interference or conversely to study the specificity of the lipolysis enzymes, three phospholipids in deionized water and also in pooled serum were analyzed in the proposed triglyceride method. The phospholipids in deionized water gave 0.006 to 0.008 mmol/liter glycerol equivalent results (Table 20). The pooled serum with a mean triglyceride value of 1.03 mmol/liter was hydrolyzed with and without the phospholipids and the difference in triglyceride results obtained between the presence and absence of phospholipids ranged from zero to 0.07 mmol/liter. The higher recovery may be responsible for the activity of a phospholipid enzyme such as lethicinase in a serum sample. Nonetheless, the hydrolysis of these phospholipids was insignificant to cause interference with triglyceride measurement. The concentrations of these phospholipids used were much higher than those found in plasma. The concentrations of phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine in plasma were ca. 0.092 mmol/liter, 0.026 mmol/liter, and 0.017 mmol/liter, respectively (148).

Table 20. The enzyme hydrolysis of phospholipids as determined by this enzyme method. Two and seven-tenths mmol/liter phosphatidylcholine, 2.7 mmol/liter sphingomyelin, and 3.4 mmol/liter phosphatidylethanolamine were assayed either in 0.05 ml deionized water or in 0.05 ml pooled serum.

phospholipids (mmol/liter)	mmol/liter of glycerol found			
	with D.I. water	with serum	serum only	Difference between phospholipids with serum and serum only
Phosphatidycholine (2.7)	0.008	0.93 1.05	0.95 0.99	-0.02 +0.06
Sphingomyelin (2.7)	0.006	0.97 1.03	0.90 1.03	+0.07 0
Phosphatidylethanolamine (3.4)	0.006	1.03 1.04	1.03 1.02	0 +0.02

Lipolytic Activities of Other Lipase Preparations

Thusfar, lipase used in the hydrolysis of serum triglycerides was Rhizopus delemar lipase. It was of interest to look for other sources of lipase for a possible use in the assay system. Equal activities of lipase from hog pancreas, Candida cylindracea and Rhizopus delemar were added to portions of the lipolysis reagent. After 15-minute incubation in a 30°C water bath, glycerol was assayed by the GK-GDH method. Table 21 lists some of the findings. Taking 0.95 mmol/liter and 3.11 mmol/liter triglyceride results obtained from the pooled serum A and B, respectively, as 100 per cent activity, hog lipase showed the lowest lipolytic activity. The pooled serum A, which contains 0.95 mmol/liter triglycerides, with hog lipase gave 68.5 per cent lipolytic activity under the condition of this assay system while that with Candida lipase was 94 per cent. However, when higher serum triglycerides (pooled serum B) were hydrolyzed by the hog and yeast lipases, per cent activity was much lower (19.6 per cent and 42.4 per cent, respectively). Under this assay conditions, Rhizopus delemar lipase gave the highest activity.

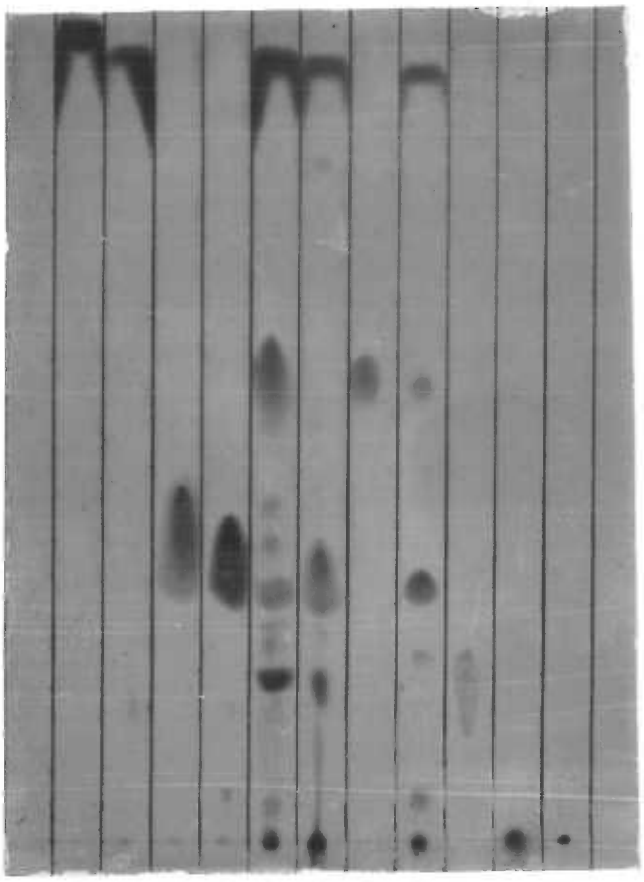
Confirmation of Enzyme Hydrolysis Using Thin-Layer Chromatography

The completeness of the enzymatic hydrolysis of serum triglycerides was demonstrated by extracting the hydrolyzed pooled serum and non-hydrolyzed pooled serum in methanol-chloroform solvent, chromatographying on TLC, and identifying the charred spot with various lipid standards. Figure 38 shows the photographed TLC plate with charred spots. Figure 38 E is the non-hydrolyzed pooled serum while F is the lipolyzed pooled

Table 21. The lipolytic activities of various sources of lipase preparations in the proposed assay condition: pH 7.6, 0.1 mol/liter Tris·HCl, 10 g/liter BSA, 10 g/liter gum arabic, 2 mmol/liter deoxycholate, 10 U/ml α-chymotrypsin, and 125 U/ml lipase.

Lipase sources	pooled serum-A		pooled serum-B	
	mmol/liter	% Activity	mmol/liter	% Activity
R. delemar	0.95	100%	3.11	100%
Hog pancreas	0.65	68.5%	0.61	19.6%
Candida	0.89	94%	1.32	42.4%

Fig. 38. A confirmation of enzyme hydrolysis of serum triglycerides using Thin-Layer Chromatography.



A B C D E F G H I J K

A = Cholesterol oleate
B = Cholesterol linoleate
C = Oleic acid
D = Linolenic acid
E = Pooled serum
F = Lipolyzed pooled serum
G = Triolein
H = Mixture of lipid standards containing cholesterol linoleate, triolein, linolenic acid, dipalmitin, monopalmitin, and phosphatidylcholine.
I = Dipalmitin
J = Phosphatidylcholine
K = Sphingomyelin

serum. The spot appearing next to the triolein spot in E must be the serum triglycerides. After enzymatic hydrolysis, the same spot seen in non-hydrolyzed pooled serum disappeared completely and the free fatty acid (FFA) spot showed intense charring as compared to the non-hydrolyzed FFA spot. A mixture of lipid standards containing cholesterol linoleate, triolein, linolenic acid, dipalmitin, monopalmitin, and phosphatidylcholine showed up at the appropriate spots on TLC compared to the individual lipid standards. The enzymatic hydrolysis of serum triglycerides appeared to be completed at the end of 15-minute incubation in a 30°C water bath. The pooled serum used in this experiment contained 1.0 mmol/liter triglyceride.

Linearity of the Suggested Glycerol Measuring Method

Varying quantities of glycerol standards, 0.05 to 10.0 mmol/liter, were added to the lipolysis reagent and glycerol was assayed by the GK-GDH method. The results are plotted on a graph (Fig. 39) which shows mmol/liter glycerol standards vs. absorbance values obtained. The GK-GDH method was linear up to 10.0 mmol/liter glycerol, which gave ca. 0.50 absorbance units.

Linearity of the Proposed Enzymatic Hydrolysis of Serum Triglycerides in a 30°C Water Bath for 15 minutes

Varying amounts of a serum sample containing 7.0 mmol/liter triglycerides were pipetted into portions of lipolysis reagent. The lipolysates were incubated at 30°C for 15 minutes before assaying for glycerol. The triglyceride assay was linear (Fig. 40) up to 35 µl on this specimen

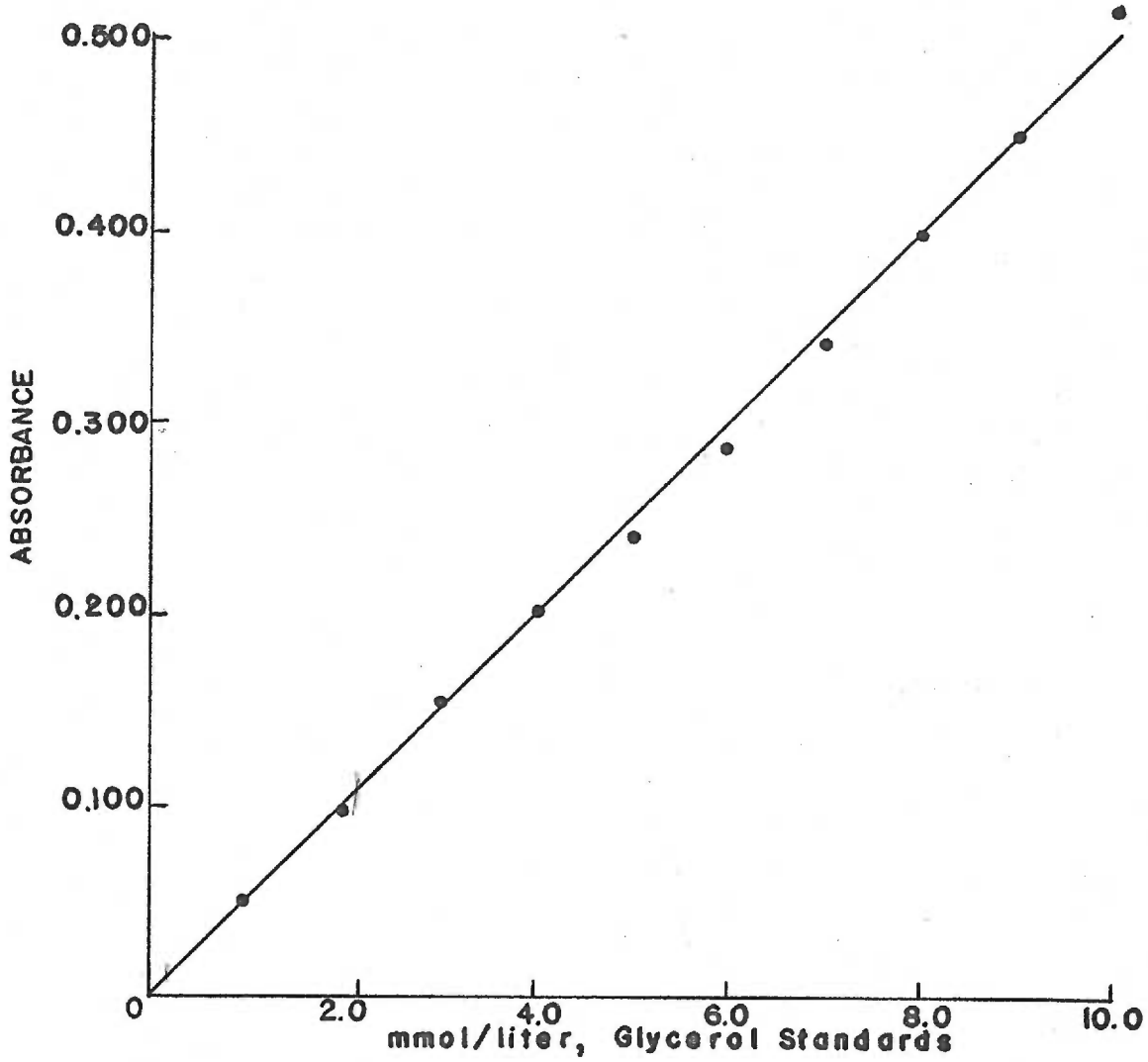


Fig. 39. The linearity study of the glycerol measuring system with varying concentrations of the glycerol standards. The glycerol standards ranging from 0.05 to 10.0 mmol/liter were added to the lipolysis reagent and the glycerol contents were assayed by the proposed GK-GDH Method.

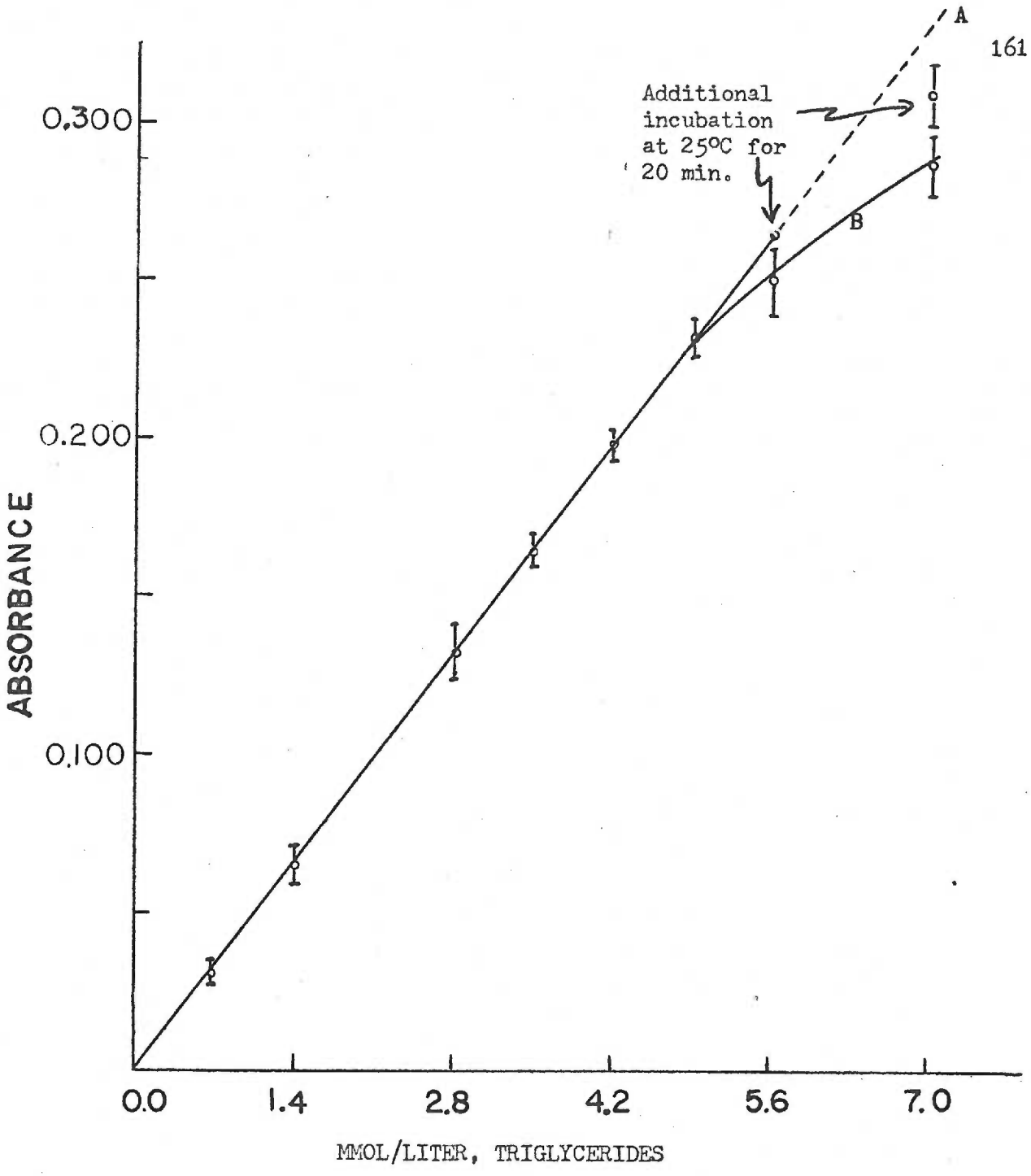


Fig. 40. Absorbance as a function of serum triglyceride concentration. Varying amounts of 7.0 mmol/liter serum triglycerides were added to a lipolysis reagent and the liberated glycerol was assayed according to the proposed GK-GDH Method. The lipolysates were incubated for 15 min. at 30°C.

A = Theoretical absorbance

B = 15 min. incubation at 30°C.

Each bar represents a mean ± 1 S.D.

which corresponds to 4.9 mmol/liter using the normal procedure (50 µl of specimen). When the lipolysates with higher serum triglyceride concentrations remained at ambient temperature for an additional 20 minutes and were re-assayed for the glycerol, serum triglyceride level up to 5.6 mmol/liter was completely hydrolyzed (Fig. 40). However, levels above 5.6 mmol/liter serum triglycerides required further incubation in order to complete enzymatic hydrolysis.

Recovery Study of Glycerol in a Pooled Serum

Glycerol standards ranging from 0.5 to 6.0 mmol/liter were added to a pooled serum and glycerol was assayed according to the recommended GK-GDH method. Table 22 shows the results of this experiment in which the mean recovery was 99.9 per cent. The per cent recovery ranged from 92.0 to 103.7 per cent. The coefficient of variation of the replicate analyses of glycerol was between 0.24 to 7.61 per cent.

Recovery Study Using Triolein in Lipolysis Reagent

Varying concentrations of triolein standards, 0.5 to 3.0 mmol/liter, were pipetted into ca. 0.3 ml of isopropanol. After isopropanol was evaporated by a stream of nitrogen gas, either 50 µl deionized water or 50 µl of a pooled serum was added to the test tube. Then, the lipolysis reagent was added to the test tubes. After incubating all test tubes in a 30°C water bath for 15 minutes, glycerol in the lipolysates containing deionized water and a pooled serum were simultaneously assayed by the GK-GDH method. The endogenous level of serum triglycerides of the pooled serum was determined by carrying out the enzymatic hydro-

Table 22. Recovery of glycerol added to a pooled serum.

Amount of glycerol added (mmol/liter)	N	Recovered from pooled serum[a]	
		mmol/liter $\bar{X} \pm$ I.S.D.	% found
6.0	2	5.950 ± 0.0141	99.2
5.0	4	4.995 ± 0.0619	99.9
4.0	4	4.035 ± 0.0985	100.8
3.0	2	3.105 ± 0.0778	103.5
2.0	5	2.004 ± 0.0456	100.2
1.0	6	1.0367 ± 0.0320	103.7
0.5	6	0.4583 ± 0.0349	92.0
			mean recovery = 99.9%

a. Varying concentrations of glycerol standard were added to a pooled serum. The endogenous glycerol and L-glycerophosphate values in a pooled serum, which were determined as 0.0875 ± 0.00957 mmol/liter, were subtracted from the original results to show the amount of glycerol recovered.

lysis without adding triolein standard. This endogenous triglyceride value was subsequently subtracted from those of added triolein standards so that the net results would be the added quantities of triolein. The results of the findings are listed in Table 23. The triolein standards in deionized water averaged 97.0 per cent recovery while the mean recovery of triolein in a pooled serum was 98.6 per cent.

When 0.5 and 1.0 mmol/liter triolein standards were added to a series of serums in another experiment, the mean recovery using 0.5 mmol/liter triolein was 106.8 per cent while adding 1.0 mmol/liter gave 106.4 per cent recovery (Table 24).

Accuracy Assessment of Three Triglyceride Methods Using CDC Reference Sera

Two lyophilized sera furnished from the U.S. Public Health, Communicable Disease Center, Atlanta, Georgia, were analyzed by the enzymatic hydrolysis with the GK-PK-LDH method (Calbiochem Triglyceride Stat-Pack), Automated Fluorometric Hantzsch Condensation method, and the suggested enzymatic hydrolysis with the GK-GDH method. The mean triglyceride values of the specimens A and B were 1.18 $\pm$ 0.11 mmol/liter and 1.74 $\pm$ 0.14 mmol/liter, respectively. The acceptable ranges given by CDC, were between 1.07 and 1.29 mmol/liter for the specimen A and 1.60 and 1.88 mmol/liter for the specimen B. Lofland's chromotropic acid procedure was used as the reference method for measuring serum triglycerides at the Communicable Disease Center. Serum triglyceride method using the GK-PK-LDH technique showed 1.22 and 1.94 mmol/liter for

Table 23. Recovery of added triolein to deionized water and to a pooled serum.

Amount of Triolein added (mmol/liter)	Amount of Triolein Recovered					
	from D.I. water[a]			from a pooled serum[b]		
	N	$\bar{X}$	%	N	$\bar{X}$	%
0.5	3	0.457	91.4	2	0.440	88
1.0	2	1.000	100	2	0.975	97.5
2.0	1	1.90	95	1	1.92	96
3.0	1	3.05	101.7	1	3.39	113
	mean recovery = 97.0%			mean recovery = 98.6%		

a. Varying amounts of triolein standards were added to 0.3 ml of isopropanol and isopropanol was evaporated under a stream of nitrogen gas. Deionized water (0.05 ml) was added to each test tube.

b. The procedure was the same as (a), except 0.05 ml of a pooled serum was added instead of deionized water. Then, the endogenous triglyceride results were subsequently subtracted from the total triglyceride values to correct for the amounts of triolein recovered as shown above.

Table 24. Recovery of triolein added to serum specimens with low and normal triglyceride levels. The triolein standards, 0.5 and 1.0 mmol/liter, were added to the serum samples and assayed for the liberated glycerol after enzyme hydrolysis.

Concentration of serum triglycerides without triolein (mmol/liter)	Amount of triolein recovered					
	With 0.5 mmol/liter			With 1.0 mmol/liter		
	Total conc.	Diff[a]	% found[b]	Total conc.	Diff	% found
0.57	1.11	0.54	108.0	1.42	0.85	85.0
1.05	1.58	0.53	106.0	2.08	1.03	103.0
1.85	2.30	0.45	90.0	2.87	1.02	102.0
2.33	3.01	0.68	136.0	3.47	1.14	114.0
2.36	2.83	0.47	94.0	3.64	1.28	128.0
	mean recovery = 106.8%			mean recovery = 106.4%		

a. The difference (Diff) reflects the amount of triolein recovered after subtracting the concentration of serum triglycerides without triolein from the total concentration of triglycerides obtained.

b. $$\% \text{ found} = \frac{\text{Diff.}}{\text{amt. of triolein added}} \times 100$$

the specimens A and B, respectively (Table 25). The results of 1.94 mmol/liter was outside the acceptable range set by CDC. The Automated Fluorometric Hantzsch Condensation method gave mean triglyceride results of 1.13 and 1.79 mmol/liter for the specimens A and B, respectively. Both triglyceride values were within the appropriate ranges. During the 7-day assay, the mean triglycerides were 1.08 $\pm$ 0.13 and 1.70 $\pm$ 0.19 mmol/liter for the specimens A and B, respectively, by the proposed enzymatic triglyceride method.

Reproducibility of Glycerol Measuring Method

Glycerol standards ranging from ca. 1.9 to 5.2 mmol/liter were pipetted into portions of the lipolysis reagent and assayed according to the suggested GK-GDH method. Each lipolysis reagent-glycerol standard mixture was automatically pipetted into about 15 wells of a transfer disc and glycerol was analyzed. Table 26 lists the findings in which the coefficient of variation within a run ranged from 2.8 to 5.4 per cent. A glycerol concentration of ca. 5.19 mmol/liter took 10 to 14 minutes to reach an equilibrium while those concentrations below 2.2 mmol/liter were finished within 8-minute assay time.

Within-Day Reproducibility of Serum Triglycerides Assay

Four pooled sera having triglyceride levels from 0.7 to 3.6 mmol/liter were subjected to the recommended enzymatic hydrolysis. Replicate analyses, 9 or 16 test tubes containing the pooled serum and lipolysis reagents, were set up to assess the within day precision of the suggested enzymatic hydrolysis of various concentrations of serum triglycerides.

Table 25. An accuracy assessment of three triglyceride methods using the CDC reference sera. The enzyme hydrolysis and GK-PK-LDH method (Calbiochem Triglyceride Stat-Pack), Automated Fluorometric Hantzsch Condensation, and this author's method using enzyme hydrolysis and GK-GDH technique.

Triglyceride Methods	Mean ± 1 S.D.(mmol/liter)	
	A	B
CDC values (N = 20)[a]	1.18 ± 0.11	1.74 ± 0.14
Enzyme hydrolysis and GK-PK-LDH method (N = 2)	1.22	1.94
Enzyme hydrolysis and GK-GDH method (N = 7)	1.08 ± 0.13	1.70 ± 0.19
Automated Fluorometric Hantzsch Condensation method (N = 2)	1.13	1.79

a. Lofland's colorimetric chromotropic acid method, analyzed at Communicable Disease Center, Atlanta, Georgia.

Table 26. Reproducibility of the intra-run glycerol measuring system utilizing GK-GDH method.

Glycerol	N	$\bar{X}$ (mmol/liter)	SEM (mmol/liter)	S.D. (mmol/liter)	C.V.(%)	Time required to reach equilibrium
A	15	1.864	0.0135	0.052	2.8	5 - 6 min.
B	15	1.936	0.0281	0.109	5.4	5 - 8 min.
C	15	2.158	0.0152	0.059	2.7	6 - 8 min.
D	11	4.014	0.0375	0.124	3.1	8 - 14 min.
E	29	5.187	0.0343	0.184	3.6	10 - 14 min.

Table 27. Within-Day reproducibility of the triglyceride assay.

Pooled Sera	N[a]	$\bar{X}$ (mmol/liter)	SEM (mmol/liter)	S.D. (mmol/liter)	C.V.(%)
I	16	0.712	0.0079	0.0317	4.4
II	9	1.034	0.0162	0.0488	4.7
III	16	1.261	0.0132	0.0528	4.2
IV	16	3.651	0.0222	0.0888	2.4

a. Nine or sixteen test tubes containing 0.65 ml of lipolysis reagent and 0.05 ml pooled serum I, II, III, or IV (inter-run) were incubated at 30°C for 15 minutes. The liberated glycerol was simultaneously measured by GK-GDH method (intra-run).

It was found that the coefficient of variation was between 4.2 and 4.7 per cent at the triglyceride level of 0.71 to 1.26 mmol/liter. At the triglyceride of 3.65 mmol/liter, the C.V. was 2.4 per cent (Table 27).

Day-to-Day Precision

During 25 days, reproducibility of the proposed enzymatic triglyceride method was determined by using two pooled sera. The coefficient of variation with the mean triglyceride of 1.00 mmol/liter was 8.4 per cent while the mean of 3.6 mmol/liter was 2.7 per cent (Table 28).

The precision of the automated fluorometric method was 16.7 per cent (C.V.) with the mean triglycerides of 70 mg/100 ml (Table 29).

Correlation Study Between the Proposed Enzymatic Triglyceride Method and Two Other Triglyceride Methods

The automated fluorometric method and the proposed enzymatic triglyceride method were compared using 92 serum samples. Figure 41 illustrates the scatter plot of the two methods in which the product-moment correlation coefficient was 0.9763 or the coefficient of determination was 95.3 per cent. The linear regression equation was $y = 1.04X - 0.15$ in which y is the proposed enzymatic triglyceride method and X is the automated fluorometric method.

When the enzymatic triglyceride method using the GK-PK-LDH technique (Calbiochem Triglyceride Stat-Pack) was compared with the recommended enzymatic triglyceride method using the GK-GDH technique,

Table 28. Day-to-day reproducibility of the proposed enzymatic triglyceride method.

	Days	$\bar{X}$	SEM (mmol/liter)	S.D.	C.V.(%)
Pool-1	25	1.064	0.0180	0.0898	8.4
Pool-2	25	3.608	0.0198	0.0988	2.7

Table 29. Day-to-day reproducibility of the Automated Fluorometric Hantzsch Condensation Method.

	Days	$\bar{X}$	SEM mg/100 ml	S.D.	C.V.(%)
QAC	25	69.96	2.34	11.706	16.7

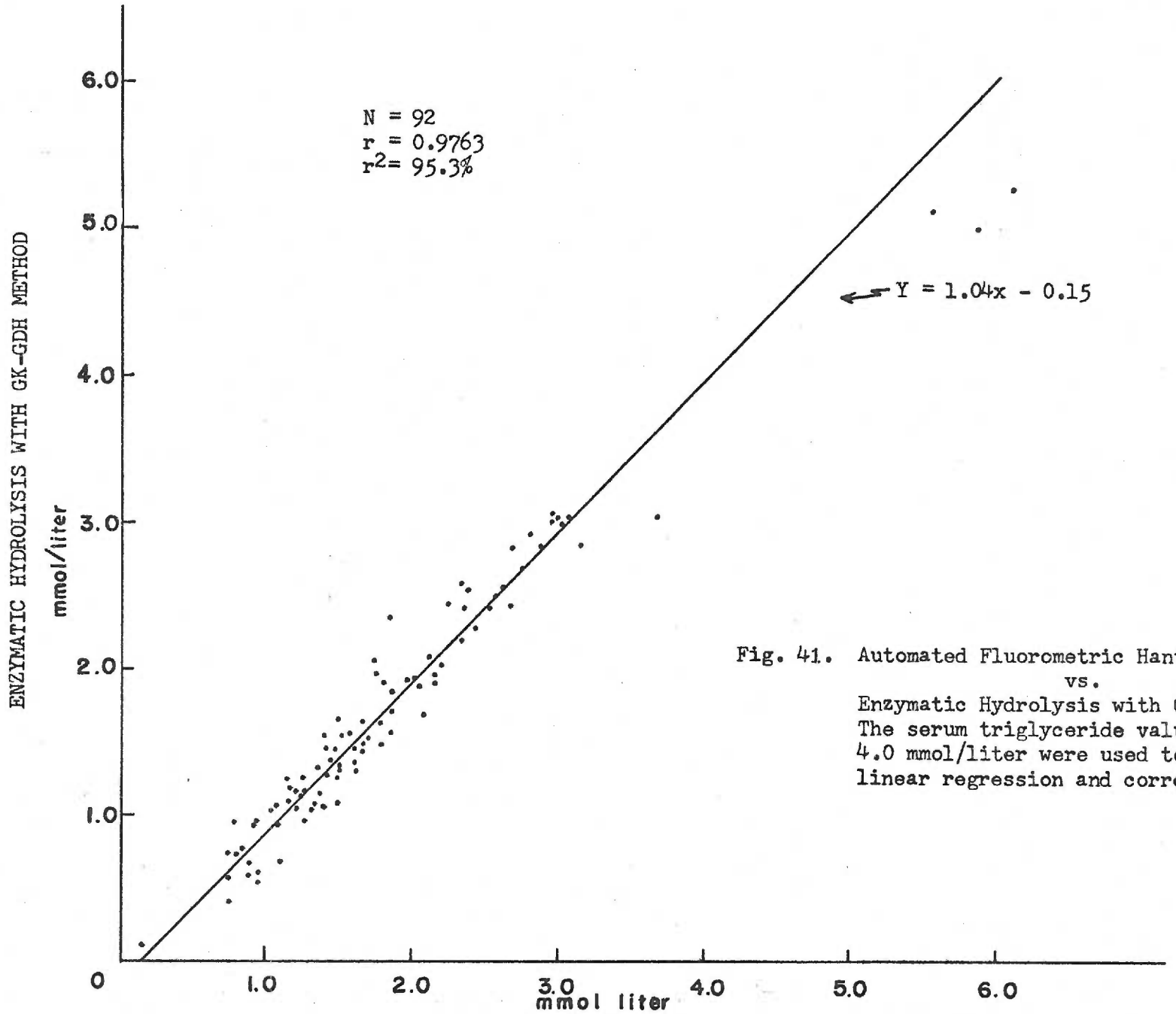


Fig. 41. Automated Fluorometric Hantzsch Condensation
vs.
Enzymatic Hydrolysis with GK-GDH Methods.
The serum triglyceride values less than
4.0 mmol/liter were used to calculate
linear regression and correlation coefficient.

the correlation coefficient was 0.9906 with a coefficient of determination of 98.1 per cent (Fig. 42). The linear regression equation was $y = 0.98 + 0.02$ where X is the Calbiochem triglyceride method. The serum triglyceride results up to 4.0 mmol/liter were used to calculate the linear regression.

Blank Rate of Serum Samples Measured by GK-PK-LDH Method

As mentioned in the introduction, ADP, pyruvate, and phosphatases which are present in a serum specimen are some of the potential sources of error in this assay. In order to assess the magnitude of these interferences, an additional 10-minute absorbance reading was taken after recording the absorbance values of the glycerol measuring reaction. The Calbiochem Triglyceride Stat-Pack was used. The absorbance values measured during 10-minute were converted to appropriate concentrations of serum triglycerides. Among 228 serum samples analyzed, the mean equivalent serum triglyceride was 0.38 mmol/liter in which the maximum and minimum ranges were 3.23 to 0.04 mmol/liter, respectively (Table 30).

The effects of the blank rate which may be caused by phosphatases were investigated. Sixteen serum samples having alkaline phosphatase between 19 and 77 U/liter were assayed before heat denaturation. The same serum specimens were incubated in a 56°C water bath for 5 minutes and they were assayed by the GK-PK-LDH method. After heat denaturation, alkaline phosphatase was 0.0 U/ml. The alkaline phosphate method was that of phenolphthalein monophosphate as described by

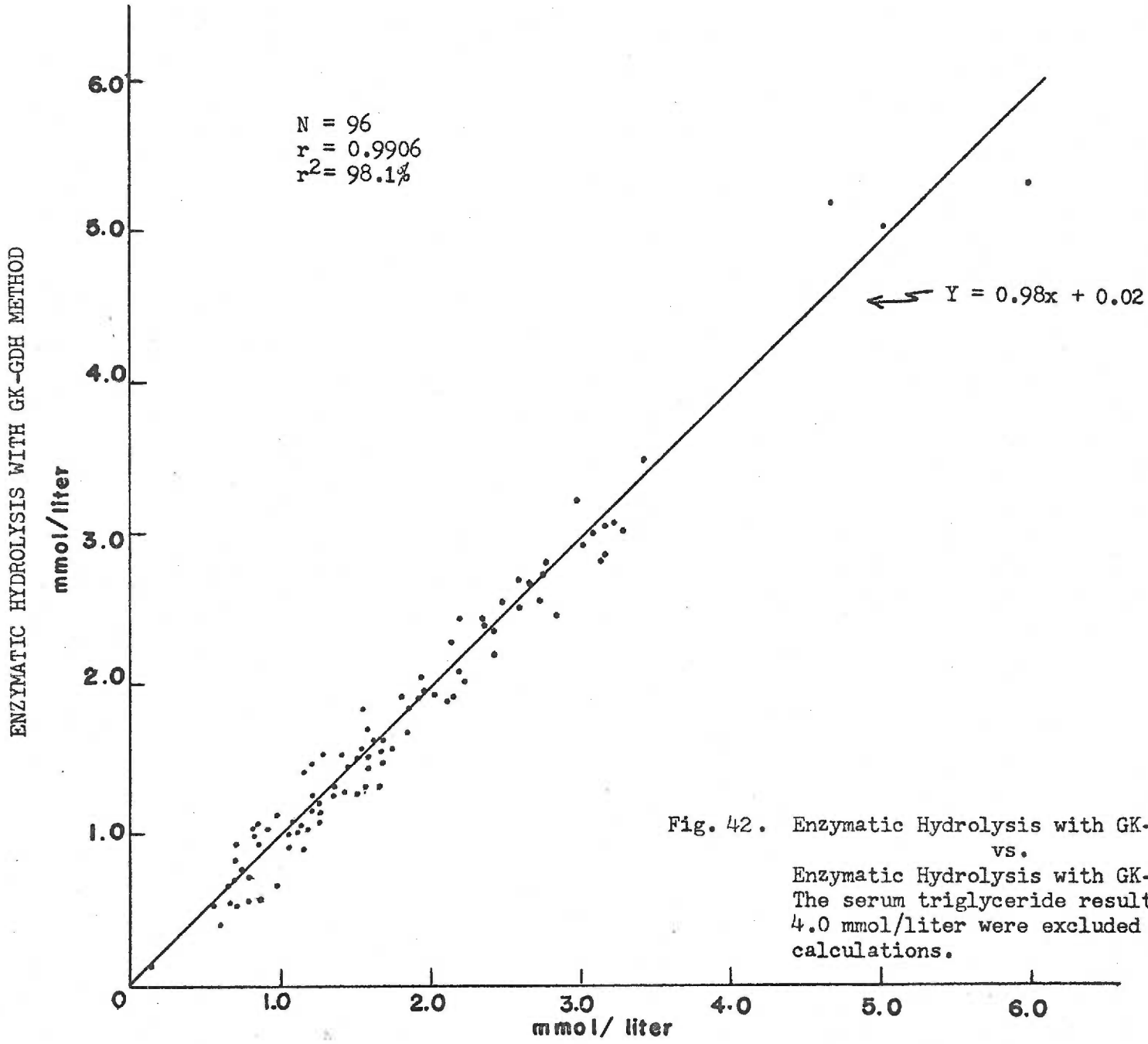


Fig. 42. Enzymatic Hydrolysis with GK-PK-LDH vs. Enzymatic Hydrolysis with GK-GDH Methods. The serum triglyceride results above 4.0 mmol/liter were excluded from calculations.

Table 30. Blank rate of serum samples measured by GK-PK-LDH method; and blank rate of serum samples before and after heating the serum samples for 5 minutes in 56°C water bath.

	Absorbance/10 min.	Equivalent mmol/liter triglycerides
N	228	-
$\bar{X}$	0.038	0.38
SEM	0.00227	0.02
1 S.D.	0.0343	0.34
C.V.	90%	-
maximum value	0.324	3.23
minimum value	0.004	0.04
Range	0.320	3.19

	Before heating		After heating	
	A/10 min.	mmol/liter	A/10 min.	mmol/liter
N	16	-	16	-
$\bar{X}$	0.0496	0.49	0.0349	0.35
SEM	0.0064	0.06	0.0023	0.02
1 S.D.	0.0256	0.25	0.0093	0.09
C.V.	51.6%	-	26.5%	-
maximum value	0.132	1.31	0.050	0.50
minimum value	0.032	0.32	0.004	0.04
Range	0.100	1.00	0.046	0.46

Babson (149) and the normal range is up to 35 I.U./liter. It was found that the mean blank rate before heat denaturation of alkaline phosphatase was 0.0496 absorbance units and after heat denaturation was 0.0349 (Table 30). When the mean absorbance value was converted to appropriate serum triglyceride results, there was a decrease of 0.14 mmol/liter after heat treatment. The highest blank value of 1.31 mmol/liter before denaturation was decreased to 0.50 mmol/liter and the lowest 0.32 mmol/liter to 0.04 mmol/liter.

DISCUSSION

NAD^+ in Hydrazine Buffer at Alkaline pH

After NAD^+ was added to pH 9.8 hydrazine buffer solution, an increase in absorbance value was observed at 340 nm (Fig. 9-A). When the NAD^+-hydrazine buffer was scanned against the same concentration of NAD^+ in water, the difference spectrum showed an absorption peak at 310 nm (Fig. 9-B). The glycerol measuring reagent containing hydrazine-glycine-Mg^{+2} buffer, ATP, NAD^+, GK and GDH was mixed with glycerol standard and they were incubated in a 30°C water bath for 15 minutes. In this reaction mixture, the added glycerol should be phosphorylated to α-glycerophosphate by the catalytic reaction of glycerokinase and ATP, and α-glycerophosphate with NAD^+ to form dihydroxyacetone phosphate and NADH by glycerophosphate dehydrogenase. At the end of 15-minute incubation at 30°C, the reaction mixture was scanned against a water blank (Fig. 12-A). The maximal absorption was at ca. 310 nm. When this reaction mixture was scanned against the same concentrations of the glycerol measuring reagent without the glycerol standard, an absorption peak was found at 340 nm (Fig. 12-B). Therefore, it is important that a reagent blank be included in the glycerol measuring system to correct for the NAD^+-hydrazine buffer induced absorbance values.

Since the quantity of NADH produced in the glycerol measuring reaction is directly related to the amount of glycerol present, this NADH must not be altered by the hydrazine buffer. Two concentrations

of NADH (0.016 and 0.070 mmol/liter) were added to the hydrazine buffer (pH 9.8) and change in absorbance was measured at 340 nm (Table 4). During 20-minute observation, there was no change in absorbance values and an absorption spectrum rendered a maximal peak at 340 nm (Fig. 10). Thus, NADH in hydrazine buffer is stable. Colowick et al (150) reported that NADH was unaffected by cyanide, but NAD^+ reacted with cyanide to form NAD-CN complex which fluoresced.

When increasing concentrations of hydrazine hydrate (pH 9.8) were mixed with a fixed amount of NAD^+, the increases in absorbance values were linear (Fig. 15-H_2O). Since a T_o "Time Delay" of 3-second was used, the initial absorbance values were automatically substracted from the final absorbance results.

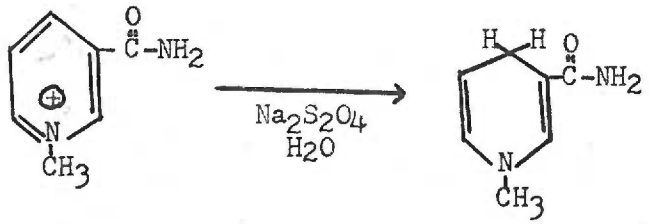
After the pH of the hydrazine buffer solutions was adjusted with KOH to range from 8.8 to 9.8, the NAD^+ solution containing ATP, BSA, GK and GDH was mixed with the buffer solutions with varying pHs. Figure 13 shows that there was a gradual increase in absorbance values between pH 8.8 to 9.4 and a sharp absorbance increase above pH 9.4. The pH 9.8 solution showed a reagent blank of ca. 0.175 absorbance. The reaction mixture containing varying concentrations of KOH and an equal quantity of NAD^+ was analyzed. Increases in KOH showed increasing absorbance values (Fig. 14). Thus, NAD^+ appears to react with KOH to give higher absorbance values at 340 nm.

When varying quantities of NAD^+ were reacted with hydrazine-glycine-Mg^{+2} buffer mixture (pH 9.8) containing BSA, ATP, GK and GDH,

linear increases in absorbance values were observed (Fig. 19). The NAD^+ concentration selected (1.5 mmol/liter in the final reaction mixture) for the GK-GDH method gave the reagent absorbance blank value of ca. 0.15.

In 1965 Kaplan et al (151) isolated α-isomer of NAD^+ having nicotinamide riboside in the α-linkage which reacted with cyanide to give a maximum absorption at 325 nm. This α-isomer was reported to be inactive with the alcohol dehydrogenase and LDH catalyzed reactions and unable to serve as substrate for NADase. Biologically, it does not promote the growth of Neurospora. This isomer has a positive optical rotation (+14°) while β-NAD^+ has a negative optical rotation (-35°).

Mauzerall and Westheimer (152) confirmed the observation of Karrer et al that hydrogen was being directly transferred from the substrate to the pyridine ring of NAD^+ by the action of alcohol dehydrogenase or lactic dehydrogenase. According to Karrer's observation, N-methyl-nicotinamide is also reduced to the corresponding dihydro compound, which in turn reduces methylene blue to a leuco base. $Na_2S_2O_4$ reduces pyridinium salts to the 1,4-isomers (153).



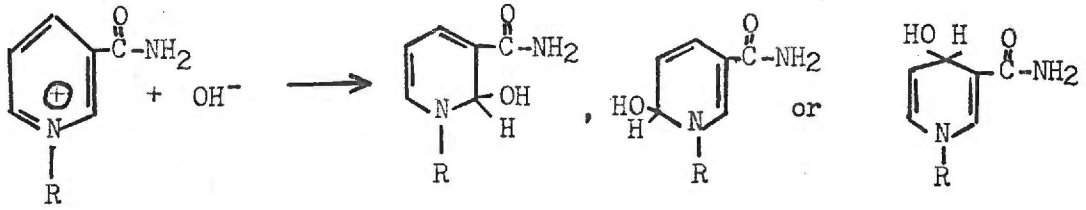
Mauzerall and Westheimer (152) prepared 1-benzyldihyronicotinamide, which was subsequently used to reduce malachite green. The mechanism of this reaction was demonstrated by the use of deuterium as a tracer.

Colowick et al (150) confirmed the observation of Meyerhof et al (1938) that cyanide or bisulfite reacted with NAD^+ to form a complexes, which fluoresced.

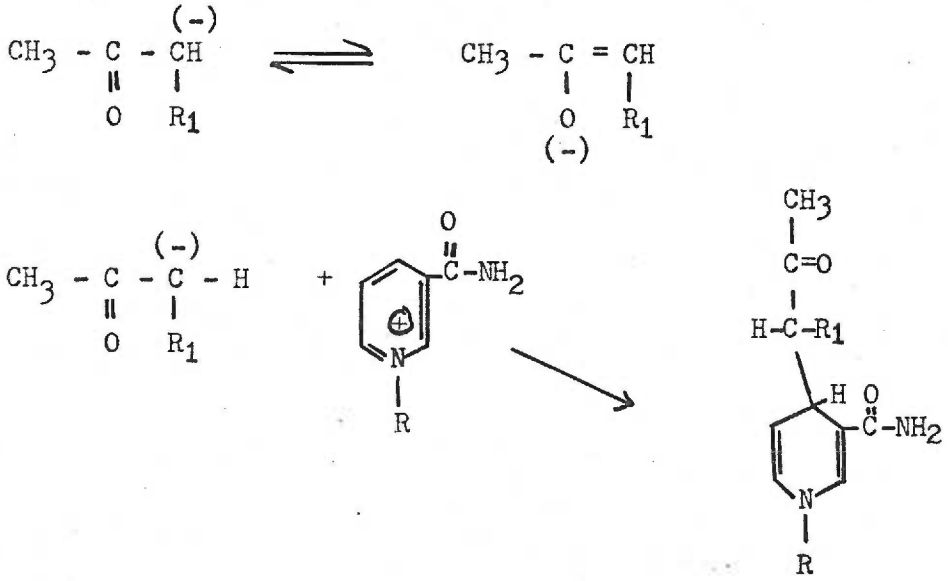
Cyanides altered the absorption spectrum of NAD^+, resulting in appearance of a new peak absorption at 325 nm. The reduced NADH was reported to be unaffected by cyanide. The NAD-CN complex was instantaneously destroyed by acidification. When varying amounts of NAD^+ were added to KCN solution, they observed a linear relationship between NAD^+ concentration and absorbance. Acetone added to NAD^+ also showed an absorption spectrum similar to that of NADH.

Kaplan et al (154) reported that oxidized NAD^+ was destroyed in dilute alkali (0.1 to 1.0 N). The site of cleavage was at the nicotinamide-ribose linkage followed by release of adenylic acid. On the other hand, strong alkali (5N) produced a stable fluorescent product without splitting of the nicotinamide-ribose linkage and it had an absorption maximum at 360 nm. After addition of the strong alkali to the NAD^+, a maximum absorbance was reached in 3 minutes and

then decreased. They proposed that hydroxyl ion was directly added to the double bond of nicotinamide in NAD^+ to yield a pseudobase in a manner identical to the addition of cyanide.



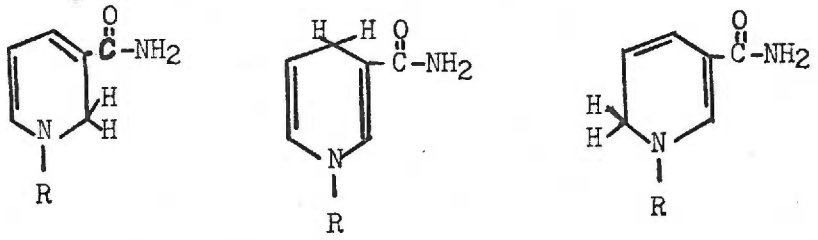
Burton and Kaplan (155) reported that dihydroxyacetone (DHA) reacted with NAD^+ and hydroxyl ions to produce an absorption spectrum similar to NADH. The NAD-DHA complex was enzymatically inactive and stable in strong bases and acids and had an absorption maximum at 290 nm. They also reported that DHA reacted only with pyridine compounds that had a quaternary nitrogen and an amide group. They proposed that the carbonyl compound ionized under the influence of a base and reacted with NAD^+ according to the following reaction:



In 1954, Pullman et al (156) reported that the actual site of NAD^+ reduction was at para position of the nicotinamide ring (position 4 of the pyridine ring). Brown and Mosher (157) showed that reduction of NAD^+ by dithionite was identical to enzymatic reduction at the 4 position and not at either the 2 or 6 position.

The reaction of the ortho and para carbon atom of the NAD^+ is explained by the electron-deficient nature of these carbon atoms which allow the addition of nucleophilic agents.

Wallenfels (153) explained that N-substituted nicotinamide derivatives are reduced to the dehydropyridine by $NaBH_4$ forming three compounds that are dissimilar in chemical and spectral properties:

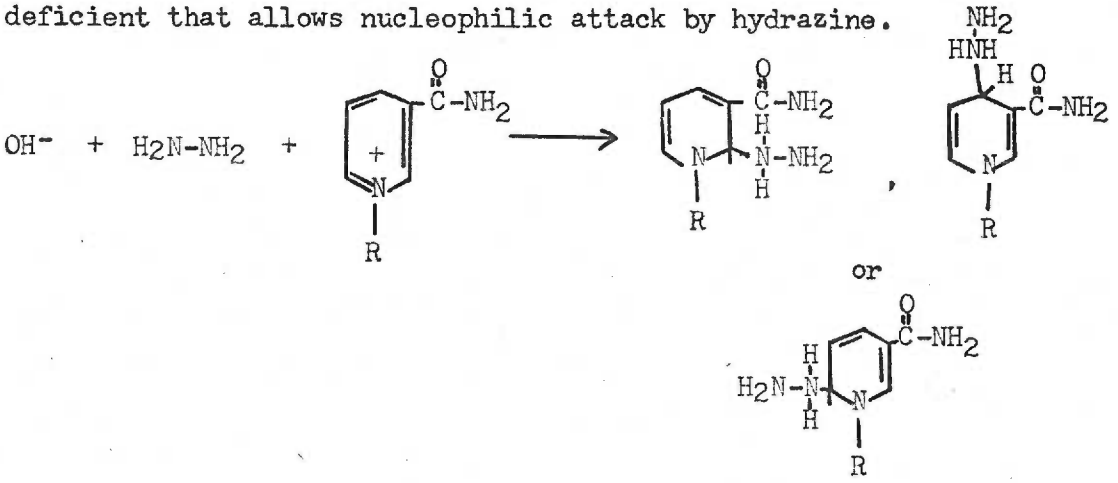


(i) (ii) (iii)

Kosower (158) reported a shift of absorption peak of NADH from 340 to 325 nm when alcohol dehydrogenase and NADH were complexed. He postulated that an ϵ-amino group of lysine was involved in the mechanism of the enzyme. He proposed that the positively charged amino group was located 3A° from the nitrogen of the dihydropyridine ring which changed the electron distribution in the ring and produced the absorption shift.

In our experiment, one possibility of the increases in absorbance after hydrazine and NAD^+ were mixed together is that in strongly

alkaline solution the nicotinamide moiety of NAD^+ becomes electron-deficient that allows nucleophilic attack by hydrazine.



or

Another possible reaction is that of Rafter and Colowick(159) in which the electron-deficient carbon atoms allow the addition of a hydroxyl ion to the nicotinamide ring at one of the ortho positions to form a pseudobase intermediate (154).

According to Kaplan et al (154), the pseudobase intermediate produced in strong alkaline solution had an absorption peak at 360 nm. In out experiment, the maximum absorption was found at 310 nm. Since hydrazine hydrate is strongly alkaline and KOH is added to hydrazine-glycine-Mg^{+2} buffer to adjust pH to 9.8, the maximum absorption at 310 nm may represent a combination of two spectra: NAD-hydrazine complex and pseudobase intermediate.

The Suggested Glycerol Measuring Method

Table 31 summarizes the concentrations of glycerol measuring reagents in the final reaction mixture and the conditions of the GK-GDH method employed by the various authors.

Use of hydrazine in the GK-GDH method was first reported by

Table 31. The final concentrations and conditions of the glycerol measuring system using GK-GDH Technique reported by various authors.

Authors	Hydrazine (mol/liter)	Glycine (mmol/liter)	Mg^{+2} (mmol/liter)	ATP (mmol/liter)	NAD^{+} (mmol/liter)	GK	GDH	Temperature (°C)	pH	-SH reagent (mmol/liter)	Albumin gm/liter	Assay time (min)	Sample size (ml)	Final Volume (ml)	Comments
Bublitz and Kennedy(1954)	-	33.3	1.33	11.7	-	-	-	37	9.4	10 2,3-dimercapto-propanol	-	-	--	-	Assaying GDH
Bublitz and Kennedy(1954)	0.50	-	3.00	6.0	0.75	100U/ml	15µl	37	9.4	Cysteine 20	-	30	?	1.0	-
Wieland(1957)	0.93	90.0	1.85	1.13	0.50	0.4 U/ml	2µg/ml	18	9.8	-	-	60	0.01-0.04	2.0	-
Bublitz and Wieland(1962)	0.90	180.0	1.80	1.88	0.50	0.3-0.6 U/ml	70µg/ml	25	9.8	-	0.001	-	0.05	2.0	-
Boltralik and Noll (1960)	0.40	-	1.00	10.0	10.00	100 U/ml	50µg/ml	37	9.1	-	1.25	60	?	1.0	-
Ciaccio(1962)	0.66	120.0	12.00	-	3.30	-	17µg/ml	-	-	-	-	-	0.2	3.0	Assaying L-GP
Vaughan(1962)	0.36	-	5.2	0.83	1.13	2.08µg/ml	5.95 µg per ml	37	9.4	Cysteine 8.3	-	30	0.5	2.4	-
Shafrin and Gorin(1963)	0.50	500.0	2.00	1.36	0.45	5µg/ml	50µg/ml	25	9.8	-	-	20	0.1-0.3	1.0	-
Wieland(1963)	0.69	140.0	1.40	1.25	0.50	0.3-0.6 U/ml	70µg/ml	-	9.8	-	-	-	0.5	2.0	-
Spinella and Hager(1966)	0.50	-	1.00	1.00	0.75	10µl	10µl	30	9.4	Cysteine 0.01	-	10	1.0	3.0	-
Laurell and Tibbling(1966)	0.03	-	0.045	0.086	0.13	0.04 µg/ml	2.2 µg per ml	Room Temp.	9.4	Cysteine 0.86	-	60	0.2	2.3	-
Parij's et al (1968)	0.46	92.0	9.20	0.84	0.33	3.3µg/ml	66µg/ml	Room Temp.	9.8	-	-	60	1.0	3.02	-
Ko and Royer (1968	0.22	-	6.80	1.23	0.68	0.45 µg/ml	9.08 µg/ml	37	9.0	-	-	-	0.46	2.18	-
Dryer(1970)	0.76	150.0	0.38	3.77	1.50	2.26 U/ml	264 µg/ml	Room Temp.	9.8	-	-	5-10	0.05	2.65	-
Lowry and Passonneau (1972)	0.35	-	-	-	2.00	-	20µg/ml	?	9.2	-	-	10-20	-	-	-
This author's method(1973)	0.29	145.0	2.00	2.50	1.50	0.40 U/ml	4.0 U/ml	30	9.2	DTT 5.0	4.42	5-10	0.05	0.449	-

Bublitz and Kennedy (83 and 84). It functions both as a ketone trapping agent and a buffer. Varying hydrazine concentrations in the glycerol measuring method had been used which ranged from 0.03 mol/liter (125) to 0.93 mol/liter (102). Bublitz and Kennedy (84) used 0.5 mol/liter hydrazine without glycine. Under the proposed condition of the GK-GDH method, the optimal hydrazine concentration were determined to be between 0.15 and 0.50 mol/liter (Fig. 15). Hydrazine levels above 0.50 mol/liter showed decreased recovery of glycerol. This poor recovery of glycerol is probably caused by inactivation of glycerol measuring enzymes, since Boltralik and Noll (104) demonstrated that, in the presence of 2.44 g/liter albumin, 50 per cent of glycerophosphate dehydrogenase was inactivated in the hydrazine within 6.5 minutes at 37°C. The proposed GK-GDH method contained 4.0 g/liter bovine serum albumin in the reaction mixture, which adequately protected the glycerol measuring enzymes. The other cause for the lower recovery of glycerol at the higher hydrazine concentration (above 0.5 mol/liter) might be that a large per cent of the 1.5 mmol/liter NAD^+ added to the glycerol measuring reagent was converted into the pseudobase intermediates and NAD-hydrazine complexes at higher hydrazine concentrations (Fig. 14 and 19). At pH 9.8, the NAD^+ concentration of 1.5 mmol/liter with 0.29 mol/liter hydrazine gave a reagent blank of 0.15 absorbance at 340 nm. If one assumes the NAD^+ complex is enzymatically inactive, then the availability of intact β-NAD^+ needed for the reaction is sub-optimal for quantitatively measuring glycerol. The high absorb-

ance of the reagent blank was found to be contributed by an increase in pH of the hydrazine buffer (Fig. 13), molar concentration of KOH (Fig. 14), concentration of hydrazine (Fig. 15), and NAD^+ concentrations (Fig. 19). To prepare the higher pH of the hydrazine buffer required an increase in amounts of KOH, which resulted in a high reagent absorbance (Fig. 13 and 14). Since the hydrazine optimum was between 0.15 and 0.50 mol/liter, ca. 0.3 mol/liter was chosen for the GK-GDH method. As to the selection of optimal pH for the glycerol measuring method, Figure 25 illustrated that pH optimum was between 8.8 and 9.8. At a higher pH of hydrazine buffer, the reagent absorbance was also increased. Thus, pH 9.2 was used in the suggested glycerol method. In order to prepare pH 9.8 hydrazine-glycine-Mg^{+2} buffer, a large quantity of KOH must be added, which results in higher reagent absorbance as shown in Figure 14.

Final reaction mixture containing 72.5, 145 and 290 mmol/liter glycine and hydrazine hydrate (290 mmol/liter) were used to determine the over-all recovery of the glycerol. There was no difference whether 72.5 or 290 mmol/liter glycine was present in the reaction mixture. Therefore, glycine concentration of 145 mmol/liter was used in the GK-GDH method. Dryer (160) and Wieland (161) used a similar concentration of glycine in the final reaction mixture (Table 31).

The equilibrium constant of the GK-GDH method favors α-glycerophosphate and NAD^+ formation. In order to shift the equilibrium of the reaction to the formation of NADH and dihydroxyacetone phosphate, an excess NAD^+ may be used in addition to other considerations such

as to use hydrazine to trap dihydroxyacetone phosphate to form dihydroxyacetone phosphate hydrazone and alkaline pH to remove a hydrogen ion. As shown before that the use of an excess NAD^+ in the hydrazine buffer caused an increase in absorbance, e.g., 1.0 mmol/liter NAD^+ contributed ca. 0.1 absorbance unit (Fig. 19). Therefore, the final concentration of 1.5 mmol/liter NAD^+ in the suggested GK-GDH method is a compromise between the reagent blank absorbance and the quantity required to reach equilibrium under the condition of this assay technique. Dryer (160), Vaughan (120) and Lowry et al (136) recommended similar concentration of NAD^+ in the final reaction mixture. The pH of the glycerol assay reagent of these three authors was 9.8, 9.4, and 9.2, respectively. Boltralik and Noll (104) used 10 mmol/liter NAD^+ and pH 9.1 in the glycerol measuring technique (Table 31).

The effect of albumin on the proposed GK-GDH method was examined. It appeared that the presence of albumin in the glycerol measuring reagent affected the absorbance change (Fig. 16). Bovine serum albumin concentration between 4.0 and 10.0 g/liter in the reaction mixture stabilized the absorbance change within 8 minutes. BSA concentration of 4.0 g/liter was selected for the glycerol measuring method.

Bublitz et al (105) and Boltralik et al (104) used 0.001 and 1.25 g/liter albumin, respectively, in their GK-GDH methods. Boltralik and Noll (104) reported that addition of albumin delayed the rapid inactivation of GDH by the hydrazine. At 37°C and pH 9.0, an addition of 2.44 g/liter albumin in the reaction mixture had

allowed 6.5 minutes to inactivate 50 per cent of GDH while in the absence of albumin it took less than 0.5 minutes to inactivate 50 per cent of GDH in the hydrazine. Therefore, an inclusion of BSA in the glycerol measuring reagent was necessary to protect GDH from inactivation and to stabilize the creeping absorbance as observed in the glycerol reagent without albumin (Fig. 16). To verify the observation of Boltralik and Noll (104) that albumin in the hydrazine reagent protected GDH, six commercial preparations of bovine and human albumin were examined. At the initial part of the experiment in which Human Serum Albumin (Pentex) was incorporated in the glycerol measuring reagent, it was found that the time required to reach equilibrium of the glycerol measuring reaction was prolonged and also large amounts of GDH were required to complete the reaction within a reasonable time. Glycerol standard added to the lipolysis reagents containing Bacto BSA, Pentex HSA, and Fraction V BSA (Sigma) showed lower recovery of the glycerol (Fig. 36). The lipolysis reagent without albumin gave 99.5 per cent recovery of glycerol. Those reagents containing Metrix BSA, FFA free BSA (Sigma), and Sigma BSA (35%) also rendered a good recovery of glycerol. Thus, it appeared that the use of Bacto BSA, Pentex HSA, and Fraction V BSA (Sigma) was excluded since they had been shown to interfere with glycerol measuring method.

The concentrations of magnesium used by the various authors differ from 0.045 to 12 mmol/liter in the final glycerol measuring mixture (Table 31). The magnesium concentrations above 0.62 mmol/liter in the reaction mixture were found to be sufficient to achieve a reaction

equilibrium. Furthermore, magnesium concentrations up to ca. 6.0 mmol/liter showed no effect on the glycerol measuring system (Fig. 17). The triglyceride results obtained using 0.62 to 6.0 mmol/liter magnesium were the same. In the proposed GK-GDH method, an excess of 2.0 mmol/liter magnesium was added.

ATP used by the various authors also had a wide range: 0.83 to 11.7 mmol/liter (Table 31). Although 1.0 mmol/liter ATP in the glycerol measuring reaction was needed to complete the reaction, an excess of 2.5 mmol/liter ATP was used in the suggested GK-GDH method (Fig. 18). The excess amounts of ATP are used to insure that sufficient quantities of ATP are present, since ATP in the reaction mixture may deteriorate or be hydrolyzed by ATPases.

Wieland (102 and 161) and Bublitz and Wieland (84) used glycerokinase activity between 0.3 and 0.6 U/ml (Table 31). In our proposed GK-GDH method, 0.4 U/ml glycerokinase (Calbiochem) was chosen since the enzyme activities above 0.31 U/ml showed the same recovery of glycerol and time to reach a reaction equilibrium (Table 6). Since glycerophosphate dehydrogenase present in the glycerol measuring reagent is susceptible to hydrazine inactivation, it is preferable to use a large quantity of this enzyme to complete the reaction within a reasonable time. To prevent a rapid inactivation of this enzyme, 4 g/liter bovine serum albumin (Sigma, 35%) was added to the glycerol reagent and the GK-GDH reagent should be prepared just prior to use. The final amount of GDH was 4.0 U/ml in the glycerol measuring reaction mixture (Table 5).

Dithiothreitol (DTT) of varying concentrations was used to establish the reaction rates and time required to complete the glycerol measuring reaction. It was found that the glycerol measuring reagent containing no DTT showed the lowest reaction rate (Fig. 24). However, at the higher concentrations of DTT, the rate was also low and the time to reach equilibrium was slightly longer than for low DTT levels (Table 7). This decrease in enzymatic activities at high DTT levels may be caused by breaking some of the intact disulfide bonds, which are necessary to maintain the enzyme structure for its substrate specificity and activity.

The pooled serum with 4.05 mmol/liter triglyceride was assayed with glycerol measuring reagent containing several concentrations of various trapping agents (Table 8). Among phenylhydrazine, hydroxylamine, semicarbazide, and (aminooxy) acetic acid hemihydrochloride, none of these worked as an adequate agent under the conditions described in this recommended glycerol measuring method. The glycerol measuring reagent containing hydrazine sulfate gave much lower recovery of glycerol: 0.2 mol/liter was 3.5 mmol/liter glycerol, 0.3 and 0.4 mol/liter were 2.9 mmol/liter glycerol. Sellinger and Miller (110) reported that sulfate ion inhibited the activity of dialyzed α-glycerophosphate dehydrogenase. They demonstrated that all of the anions tested inhibited this enzyme. Reaction mixtures containing 4.7×10^{-2} mol/liter phosphate and 6.3×10^{-1} mol/liter sulfate exhibited 52 and 95 per cent inhibition of GDH, respectively, while 1.9×10^{-1} mol/liter glycine and 1.1×10^{-2} mol/liter EDTA showed no

GDH inhibition at pH 7.7 and 24°C. However, this enzyme is entirely stable in ammonium sulfate solution prior to dialysis. They explained this phenomenum that the dialysis exposed active sites on the enzyme surface which caused nonspecific sensitivity to anions. In our GK-GDH method, ca. 2.9 X 10^{-2} mol/liter of ammonium sulfate was present in the final reaction mixture. Baranowski (106) reported that GDH crystals in 50 per cent ammonium sulfate were stable for several weeks in the cold temperature; however, the diluted enzyme lost 90 per cent of its activity in 24 hours at 0°C. Beisenherz (107) reported that GDH in 1.9 mol/liter ammonium sulfate was stable for weeks at 0 - 4°C.

Vijayvargiya and Sighal (134) and Schwark et al (135) also showed that the free fatty acids competitively inhibited this enzyme in vivo. According to Kim and Anderson (133), aliphatic carboxylic acids and n-alkylammonium chlorides were found to competitively inhibit GDH in the oxidation of L-glycerophosphate with NAD^+. They concluded that NAD^+ and fatty acids bind at the same sites on GDH. In the proposed enzymatic triglyceride method, free fatty acids yielded by the lipolytic action of lipase and L-chymotrypsin are probably present as calcium soaps or bound to bovine serum albumin. Thus, GDH might be inhibited by the presence of FFA in the glycerol measuring method. However, even if GDH is being inhibited it is working well enough to produce quantitative triglyceride assays.

Lactic acid ranging from 1.0 to 5.0 mmol/liter was added to the glycerol measuring reagent to determine the extent of lactic acid

interference for our glycerol measuring method. Since a serum specimen (148) contains LDH and lactate (0.6 to 1.0 mmol/liter), the presence of lactate may be a possible error in estimating glycerol if lactate is oxidized by LDH in the presence of NAD^+ to yield pyruvate and NADH. The lipolysis reagent contained 0.55 mmol/liter NAD^+ and various lactate concentrations in the pooled serum were incubated for 15 minutes in a 30°C water bath. The absorbance values obtained by this technique were compared to those lipolysis reagent without NAD^+. If lactate was oxidized and NAD^+ was reduced, the absorbance values of the former would be higher than those of the latter. The pH optimum of LDH catalyzed reaction (lactate to pyruvate) was reported to be 8.8 to 9.8. The GK-GDH method used pH 9.2. The results indicated that there was no increase in absorbance values (Table 9). Five serum samples with LDH values between 125 and 250 (normal ranged from 0 to 270 I.U./liter) rendered no significant difference in the final triglyceride results. Although the stability of LDH in the glycerol measuring reagent was not examined, it appeared that lactate could be excluded as a possible source of interference.

When ATP and glycerokinase were added to the lipolysis reagent and α-glycerophosphate was measured in the glycerol measuring reagent containing GDH and NAD^+, the time required to reach equilibrium was prolonged as compared to the proposed method in which ATP and GK were present in the GK-GDH reagent (Table 10). The pooled sera containing 0.95 to 2.85 triglycerides assayed by the proposed GK-GDH method, reaction was completed between 4 and 12 minutes; however,

when the same pooled sera were assayed by this new technique, the absorbance values continued to change. This is probably caused by inhibition of glycerokinase in lipolysis reagent or possible inactivation of GK by L-chymotrypsin.

Assaying the glycerol with the CentrifiChem Fast Analyzer by taking an initial absorbance values at 3 seconds to serve as the serum blank was shown to be valid (Table 11). There was no difference in the final glycerol results obtained by this technique when it was compared to two other procedures: storing water and storing pooled serum.

The suggested GK-GDH method can assay free glycerol concentration up to 10 mmol/liter since the change in absorbance was linear up to that concentration (Fig. 39).

The recovery of the glycerol from pooled serum was ca. 99.9 per cent and the intra-run reproducibility of the recommended GK-GDH method ranged from 2.8 to 5.4 per cent (Table 26). A glycerol concentration below 2.2 mmol/liter took ca. 8 minutes and 5.19 mmol/liter glycerol produced a stable reading in 10 to 14 minutes.

Enzymatic Hydrolysis of Serum Triglycerides

Bier (162) summarized different assay methods for lipase activity, various sources of lipases, properties, activators and inhibitors. The activity of pancreatic lipase has been well documented by Mattson et al (163) and Desnuelle and Savary (164). The optimum pH for lipolysis of triglycerides with short chain fatty acids was ca. pH 7.0 and

for triglycerides with long chain fatty acids, it was shifted up to 8.8 (169). Desnuelle et al (165 and 166) reported that pancreatic lipase hydrolyzed longer chain fatty acids (C_7 - C_{10}) at higher temperature; however, at low temperatures, it acted as an esterase. According to Fodor (167 and 168), when hog pancreatic lipase was subjected to heat (60°C), alkaline, and trypsin treatments, this enzyme showed greatly decreased activity with monovalent alcohol esters while the enzyme activity with triesters of glycerol was not greatly suppressed. Pancreatic lipase preferentially hydrolyzes the α- and α'-ester bonds (1- and 3-ester bonds) of triglycerides (169). Tattrie et al (170) and Mattson et al (163) also reported that this enzyme randomly hydrolyzed the primary esters of triglycerides. According to Clemont et al (171), after lipolysis of triglycerides by pancreatic lipase, all monoglycerides found were exclusively of β-fatty acid of the original triglycerides. In 1945, Frazer and Sammons (172) reported that no free glycerol was recovered when olive oil was hydrolyzed by pancreatic lipase. During the first 5 hours lipolysis, the only products of olive oil was fatty acids of mono- and diglycerides (172). Mattson and Beck (163) showed that the triglycerides were sequentially hydrolyzed to yield 1,2-diglycerides and to 2-monoglycerides. This route appeared to be the same when the triglycerides were either saturated fatty acids of carbons 16 or 18 or unsaturated fatty acids of 18 carbons (173).

Hydrolysis of β-ester bond to free glycerol is a slow process. Mattson and Bell (174) postulated that a β-ester splitting enzyme or

an isomerase was present in crude pancreatic preparation. In 1967, Morgan et al (175) reported that rat pancreatic juice contained cholesterol ester hydrolase (E.C.3.1.1.13) that hydrolyzed the β-monoglycerides in micellar form. According to Mattson and Volpenhein (174), a sterol ester hydrolase isolated from pancreas hydrolyzed the β-position of glyceride at a very slow rate. This hydrolase was not pancreatic lipase. They stated that ca. 20 per cent of dietary triglycerides was completely hydrolyzed to free fatty acids and free glycerol in the intestine. Desnuelle and Savary (164) reported that production of free glycerol was exclusively (60%) caused by isomerization of unstable β-monoglycerides and 1,2-diglycerides in aqueous alkaline pH in which the β-ester fatty acid was being transferred to an α-positions and thus susceptible to pancreatic lipase.

Desnuelle and Savary (164) extensively reviewed the specificities of pancreatic, plasma, tissue, intestinal, and milk lipases.

Ory et al (176 and 177) reported that castor bean (Ricinus communis) lipase completely hydrolyzed triglycerides to free glycerol in which the reaction rate was first-order.

Alford et al (178) determined the positional and fatty acid specificities of various microbial lipases. They concluded that lipases from Staphylococcus aureus and Aspergillus flavus hydrolyzed the α- and β- position of triglycerides at about the same rate. Lipases isolated from Geotrichum candidum preferentially hydrolyzed the oleate ester regardless of its position, but this positional speci-

ficity was a secondary effect. Geotrichum candidum lipase had a high degree of specificity for unsaturated fatty acids. The findings of Alford et al (178) was confirmed by Marks et al (179) in which equimolar quantities of oleic and linoleic acid were hydrolyzed when triolein and trilinolein were subjected to this lipase. The optimum pH range was 8.1 to 8.5. Stereospecific measurement of this lipase is fully discussed by Sampugna and Jensen (180).

With 125 U/ml of Rhizopus delemar lipase, 10 g/liter bovine serum albumin, 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate and 10 U/ml α-chymotrypsin, a complete hydrolysis of serum triglycerides was obtained within a 15-minute incubation at 30°C. However, without 10 U/ml α-chymotrypsin in the enzymatic hydrolysis reagent mixture, only ca. 60 to 70 per cent serum triglycerides was hydrolyzed within a 15-minute at 30°C incubation (Fig. 36). When the hydrolysis of other lipase preparations was examined under the specified condition of this assay method, the lipolysis reaction mixture containing Candida cylindracea lipase rendered ca. 42.4 per cent hydrolysis of 3.11 mmol/liter pooled serum triglycerides while that of hog pancreatic lipase hydrolyzed ca. 19.6 per cent of the same (Table 21).

Fukumoto et al (181) and Iwai et al (182 and 183) described purification and crystallization method, hydrolysis and esterification, and the effect of calcium ion on the action of crystalline lipase from Aspergillus niger. The maximal hydrolysis activities of this lipase were reported to depend upon the chain length of fatty acids (ca. 8 to 16 carbons). Increasing quantities of glycerol in the

hydrolysis mixture shifted the reaction to esterification while an increase in water contents drove the reaction equilibrium to hydrolysis of triglycerides.

Rhizopus delemar lipase was characterized by Fukumoto et al (184). The effect of pH on lipase activity was done with olive oil incubated at 30°C for 60 minutes in McIlvain buffer with 9 mmol/liter $CaCl_2$. The lipase activity was optimal at pH 5.6 at 35°C and the enzyme was stable in the pH range between 4.0 and 7.0. In an attempt to determine the pH optimum of Rhizopus delemar lipase for hydrolyzing serum triglycerides, we used McIlvain buffer which contained Na_2HPO_4 and citric acid initially. However, the addition of calcium ions caused precipitation of the reagent. Michaelis universal buffer has a wide pH range, does not form insoluble calcium salt, and has a constant ionic strength which is isotonic with whole blood. Thus, it was used to determine the pH optimum of Rhizopus delemar lipase. Using a pooled serum, repeated experiments showed that this enzyme has two pH optima: one at ca. pH 5.6 as observed by Fukumoto et al (184) and the other ca. pH 7.5 to 7.9 (Fig. 27). According to Bragdon and Karmen (185), olive oil contained 67 per cent oleic (18:1), 16 per cent palmitic (16:0), 13 per cent linoleic (18:2), 2.1 per cent stearic (18:0) and 1.8 per cent palmitoleic (16:1) acids. Coconut oil contained 71 per cent lauric (12:0) acids while cod liver oil had 32 per cent oleic acid (18:1), and corn oil consisted of 57 per cent linoleic acid (18:2). Triglyceride molecules with two or three identical chains were rarely found in nature (164). A serum sample

contains various types of triglycerides which differ in chain length and compositions of esterified fatty acids. Therefore, one possibility is that Rhizopus delemar lipase using olive oil (chiefly oleic acid) as a substrate has a pH optimum at 5.6 while another pH optimum at ca. 7.5 to 7.9 appears when serum triglycerides of mixed esterified fatty acids are the substrate. Verger et al (186) used pancreatic lipase to demonstrate the activation of this enzyme with sulfhydryl agents. They found two SH groups near the active site. The first thiol group was on the surface of the protein while the second one was buried in an hydrophobic area. Thus, another possibility for having pH optimum near pH 7.5 to 7.9 may be due to the effect of secondary active site on Rhizopus delemar lipase which is exposed at pH 7.5 to 7.9 to cause hydrolysis of triglycerides. When Tris·HCl and Imidazole buffers were used to assess the pH optimum of this lipase, both buffers rendered the highest liberation of free glycerol at ca. pH 7.6 (Fig. 27 and 28, and Table 13) and the pH of the lipolysates was about the same as the original pH of the buffer. However, the Tris·HCl buffers appeared to be slightly better than the Imidazole buffers (Table 14). Although higher molar concentration of Tris·HCl showed decreased activity of hydrolysis (glycerol measuring method was unaffected), 0.1 mol/liter buffer gave ca. 97.5 per cent recovery of triglyceride with adequate buffering while 0.05 mol/liter Tris·HCl showed pooer buffering capacity of the final lipolysates (Table 14). Thus, Tris·HCl buffer of pH 7.6 and 0.1 mol/liter was selected for this enzymatic hydrolysis method.

Alpha-chymotrypsin dissolved in 0.001 mol/liter HCl with

0.01 mmol/liter calcium chloride was stable for ca. one week at 0 - 4°C. When α-chymotrypsin was added to the lipolysis reagent mixture and incubated for 2 hours at 30°C, the enzyme activity was decreased by ca. 20 per cent. However, when the enzyme was placed in 0.1 mol/liter Tris·HCl buffer (pH 7.6) and incubated at 30°C for 24 hours, the enzyme activity decreased by 23 per cent. It appeared that α-chymotrypsin in the lipolysis reagent was rapidly inactivated at the end of 2 hours incubation at 30°C while the same enzyme in 0.1 mol/liter Tris·HCl buffer (pH 7.6) showed considerably better stability. The cause of this phenomenum is unknown. When the optimum activity of α-chymotrypsin was examined using four different pooled sera in lipolysis reagents, the reaction mixture containing 10.0 U/ml α-chymotrypsin rendered the highest recovery of triglycerides (Fig. 31).

Bovine serum albumin (10.0 g/liter) was chosen for the enzymatic hydrolysis of serum triglycerides (Fig. 32). Gordon et al (187) reported that one mole of albumin was bound to a total of 7 to 8 moles of fatty acids when they were determining the role of albumin in the lipemia clearing reaction. During the hydrolysis of coconut oil, in the absence of albumin lipoprotein lipase activity was found to be inhibited. They concluded that serum albumin entered in the reaction to bind free fatty acids and remove them. Robinson and French (188) found that the extent of clearing reaction was depended upon the concentration of albumin present. Korn (189 and 190) utilized 20 g/liter albumin in the reaction mixture for assaying a heparin-

activated lipoprotein lipase. He also reported that calcium ions were as good a fatty acid acceptor as albumin in the clearing reaction in which albumin stabilized lipoprotein lipase during hydrolysis. Shipe (191) reported that microbial lipases (Aspergillus niger and Penicillium roqueforti) were activated with 0.8 g/liter calcium ion, but the lipase activity declined with higher calcium concentrations. Iwai et al (182 and 183) revealed that up to 1.0 mol/liter calcium ion rendered adequate lipolysis of olive iol by Aspergillus niger lipase, but at higher concentrations the hydrolysis was inhibited. According to Tirunarayanan and Lundbeck (192), 2.0 mmol/liter calcium ion was a better activator than the same quantity of magnesium as fatty acid acceptor using staphylococcal lipase. Desnuelle et al (165 and 166) reported that in the absence of calcium ions mostly diglycerides were produced as end products after hydrolysis, whereas the addition of calcium salts accelerated further hydrolysis by pancreatic lipase to yield monoglycerides. Mattson and Beck (163) showed that esterification of free fatty acids to glycerol was minimized when calcium ions were added to the reaction. They postulated that this minimal esterification was caused by formation of insoluble calcium soap. In the proposed enzymatic hydrolysis reaction mixture, ca. 0.3 mmol/liter calcium ion was present in addition to 10 g/liter bovine serum albumin. When calcium ion ranging from 0.07 to 2.14 mmol/liter was added to the reaction mixture, there was no significant increase or decrease in recovery of serum triglycerides (Table 16). Tauber (193) reported that lima bean inhibitor was a powerful serum lipase activator while hemoglobin was a powerful

lipase inhibitor.

Use of Bacto BSA, Pentex HSA, and Fraction V BSA (Sigma) had been shown to interfere with glycerol measuring method; however, the GK-GDH reaction mixture containing Metrix BSA, FFA free BSA (Sigma) and Sigma BSA (35%) rendered an adequate recovery of glycerol (Fig. 36). When the former three albumin preparations were used in the enzymatic hydrolysis reagent, they also gave poor hydrolysis of serum triglycerides. Among Metrix BSA, FFA free BSA (Sigma) and Sigma BSA (35%) tested in the hydrolysis of serum triglycerides, only FFA free BSA (Sigma) and Sigma BSA (35%) were suitable to use in both enzymatic hydrolysis and glycerol measuring methods. According to a manufacture's information, Sigma BSA (35%) contained no preservative; however, other albumin preparations included varying amounts of both sodium acetyl-tryptophane and sodium caprylate as a stabilizer. Shipe (191) used lipases isolated from Aspergillus niger and Penicillium roqueforte to determine the relative rates of hydrolysis of various substrates. He found that caprylic acid had a high inhibitory effect on both lipases. Hanson and Ballard (194) reported that eleven commercially prepared albumin contained varying concentrations of fatty acids, glucose, and citrate. Human Fraction V (Pentex) contained 760 umoles FFA/mmole of albumin and 3380 umoles citrate/mmole of albumin. This high FFA concentration may be responsible for inhibition of lipase activity.

Emulsifying agents were used in the enzyme hydrolysis of the fatty substrates. Among various emulsifiers available, albumin,

sodium oleate, gum arabic (acacia), benonite and bile salts are commonly used by many authors. Wills (195) described the use of various detergents in lipolysis of triglycerides in which he showed that cholesterol in the lipolysis reaction mixture increased pancreatic lipase activity. Most of the anionic detergents tested were found to inhibit lipase action except 1 per cent (w/v) sodium deoxycholate increased the rate of hydrolysis of triolein by the factor of 7.5. The role of bile salts was to emulsify triglycerides rather than to activate lipase (195). According to Fodor (196), bile salts activated the lipolysis of certain esters of triglycerides by pancreatic lipase, but the hydrolysis of the monoester of methyl-butyrate was strongly inhibited. The lipolysis of methyl laurate was activated in the presence of bile salts in the reaction mixture. Since bile salts are surface-active agent, the primary function is to reduce the tension on the oil-water (ester-water) interface. Desnuelle and Savary (164) claimed that the activity of pancreatic lipase was depended upon the interfacial surface between the substrate and water. This interface determined the enzyme action by adsorbing the enzyme molecules. If no interface were present, lipase would be dissolved in water and inactive. Borgström (197) stated that the presence of bile salts in the lipolysis reaction mixture accelerated the rate of monoglyceride formation. Bile salts, either conjugated or non-conjugated forms, produced mixed micelles of monoglycerides and free fatty acids in which this ionization shifted fatty acids from the oil to the water phase where they were not available for the enzymes. In

the presence of bile salts in alkaline pH, the isomerization of β to α-monoglycerides were reported to be a first-order reaction (197).

In our suggested enzymatic hydrolysis method, the optimum concentrations of gum arabic and deoxycholate were 10 g/liter and 2.0 mmol/liter, respectively. At the lower amounts (< 10 g/liter) of gum arabic in the reaction, the enzymatic hydrolysis was slower (Fig. 33). The concentrations higher than 10 g/liter essentially gave the same results. Final concentration of deoxycholate in the reaction mixture containing ca. 2.0 mmol/liter rendered the highest lipolytic activity at the end of 15-minute incubation at 30°C (Fig. 34). At 28 minutes, 2.0 mmol/liter deoxycholate showed essentially the same triglyceride result as 15 minutes; however, concentration below ca. 1.5 and above 2.5 mmol/liter gave lower recovery of triglycerides. Mattson and Beck (163) reported and it was subsequently confirmed by Benzonana and Desnuelle (198) that higher or lower concentrations of bile salts gave slower rate of hydrolysis in which higher concentration was shown to be inhibitory. According to Desnuelle and Savary (164), the presence of both gum arabic and biles in soluble triglycerides rendered maximal rates of hydrolysis. In 1968, Benzonana (199) reported that pancreatic lipase was inactive without the presence of calcium ions even though deoxycholate was present. Thus, he hypothesized that the chief role of calcium ions was to compensate the electrostatic repulsion existing between the ionized carboxyl groups of deoxycholate and the negatively charged lipase at the interface of the emulsified triglycerides. Calcium ions

appeared to be bound to the enzyme, which provided an ionic bridge with the negatively charged interface.

In 1964, Borgström (197) stated that pancreatic lipase acted at an oil-water interface where the water concentration was very low and allowed lipid-soluble reaction products (FFA) to be removed into the oil phase. Lagocki et al (200) described the action of pancreatic lipase in which the enzymes acted on insoluble monolayers of lipid substrates although calcium ions or emulsifying agents were absent. Brockerhoff (201) showed that water-soluble esters competitively inhibited the hydrolysis of emulsified tripropionin. He proposed a shielding effect of the hydrophilic esters by water molecules at a weakly nucleophilic enzyme. Benzonana and Desnuelle (198) reported that in the absence of bile salts hydrolysis of emulsified long-chain triglycerides was inhibited by the soaps formed during the reaction. They hypothesized the partition effect in which accumulation of long-chain soaps at oil-water interface, where the soaps were partitioned between the water phase of the emulsion and the interface, caused poor diffusion of the soaps into the water. On the contrary, the emulsified short-chain triglycerides incubated with lipase showed no inhibitory effect, which indicated that the short-chain esters were freely diffuse into the water phase. In the presence of bile salts, bile salts and long-chain esters formed water-soluble molecules or micelles as in the intestine where bile salts promote fatty acids and monoglycerides micelles. As shown in Figure 37, the presence of both deoxycholate and gum arabic were necessary to completely hydrolyze all serum

triglycerides to free glycerol. With the presence of bovine serum albumin, the lipolysis enzymes showed the highest hydrolytic activity.

It was observed that lipase was not stable in the lipolysis reagent. Duration of incubation at 30°C has a direct bearing on the activity and stability of this enzyme and α-chymotrypsin. Thus, it is preferable to add lipase and α-chymotrypsin to a lipolysis reagent just prior to use. In the freshly prepared lipolysis reagent, up to ca. 5.0 mmol/liter serum triglycerides was completely hydrolyzed at a 15-minute and 30°C incubation (Fig. 40 and Table 18). The 15 and 45 minutes incubation of serum samples at 30°C showed identical concentrations of serum triglycerides (Table 18). Thus, the enzymatic hydrolysis was completed at the end of 15-minute incubation at 30°C.

In the absence of lipase in the lipolysis reaction mixture, there was no hydrolysis of serum triglycerides by α-chymotrypsin (Table 19). The proposed enzymatic hydrolysis method did not hydrolyze phosphatidylcholine, sphingomyelin or phosphatidylethanolamine whose concentrations tested were ca. 100 fold of the amount present in plasma (Table 20).

After the pooled serum was subjected to the recommended enzymatic hydrolysis method, it was chromatographed on TLC with non-hydrolyzed pooled serum and other lipid standards. The hydrolyzed pooled serum showed no visible triglyceride spot on TLC while the free fatty acid spot was increased in size and intensely charred (Fig. 38-F).

The CDC reference sera were assayed by this method, the results obtained were comparable to the acceptable ranges furnished by the

CDC (Table 25). The recovery experiment using triolein standards in pooled serum and in deionized water rendered 97 to 98.6 per cent (Tables 23 and 24).

Precision of the over-all enzyme triglyceride assay method was adequate. For the glycerol measuring method, the coefficient of variation of intra-run ranged from 2.8 to 5.4 per cent (Table 26). The coefficient of variation of the inter-run (within day) serum triglycerides ranged from 2.4 to 4.7 per cent (Table 27). While the day-to-day was between 2.7 to 8.4 per cent (Table 28).

The correlation coefficient between the suggested enzymatic triglyceride method and the automated fluorometric method was 0.9763 (Fig. 41). and between the suggested method and Calbiochem triglyceride method was 0.9906 (Fig. 42). Calbiochem triglyceride method using the GK-PK-LDH technique to measure free glycerol showed a mean sample blank rates of 0.38 mmol/liter (triglyceride equivalent values). The highest result was 3.23 mmol/liter triglycerides (Table 30). When sixteen serum samples were assayed before and after heat denaturation, the blank rate decreased by the factor of two after heat treatment. The mean difference of 0.14 mmol/liter was probably attributed by the alkaline phosphatase hydrolysis of phosphoenolpyruvate and the ATPase hydrolysis of ATP to form pyruvate and ADP, respectively. After heat treatment of the serum samples, the alkaline phosphatase results were zero.

SUMMARY AND CONCLUSIONS

A simple, rapid, accurate, and precise micromethod for determining serum triglycerides using the CentrifiChem Fast Analyzer is presented. This technique utilized enzymatic hydrolysis of serum triglycerides to free glycerol and the liberated glycerol is assayed enzymatically with glycerokinase (GK) and glycerophosphate dehydrogenase (GDH). The enzymatic hydrolysis takes 15 minutes at 30°C. The loading of lipolysates and glycerol measuring reagents on a transfer disc requires 3 minutes. The analyses of 29 specimens take less than 10 minutes for the normal triglyceride range. Thus, a total time of approximately 30 minutes is needed to complete 29 serum triglyceride assays. Higher serum triglycerides (>5 mmol/liter) can be measured by either standing the same lipolysates at ambient temperature or re-incubating in a 30°C water bath. Then, re-assay for the liberated glycerol by the GK-GDH method. Otherwise, simply use less than 50 µl serum specimen and re-hydrolyze at 30°C.

Optimal conditions and concentrations for the enzymatic hydrolysis of serum triglycerides were 10 g/liter bovine serum albumin (Sigma, 35%), 10 g/liter gum arabic, 2.0 mmol/liter deoxycholate, 10 U/ml α-chymotrypsin, and 125 U/ml Rhizopus delemar lipase in 0.1 mol/liter Tris (hydroxymethyl) aminomethane (Tris·HCl) buffer solution of pH 7.6. Sigma BSA (35%) was selected for this method in order to avoid a possible inhibition of lipase or α-chymotrypsin.

Optimal conditions and concentrations of the GK-GDH method for glycerol assay were determined to be 0.29 mol/liter hydrazine hydrate,

145 mmol/liter glycine, 2.0 mmol/liter magnesium, 4.0 g/liter BSA (Sigma, 35%), 2.5 mmol/liter ATP, 1.5 mmol/liter NAD^+, 5.0 mmol/liter dithiothreitol (DTT), 0.4 U/ml glycerokinase, 4.0 U/ml glycerophosphate dehydrogenase at pH 9.2 and 30°C.

The proposed enzymatic triglyceride method does not require time-consuming extraction of triglycerides, removal of phospholipids, violent alcoholic alkaline saponification, deproteinization, or neutralization with acid. A micro volume (50 µl) serum sample is directly added to the lipolysis reagent. After incubation, the free glycerol is directly added to the GK-GDH reagent and the final triglyceride values are taken when there is no further change in absorbance at 340 nm.

The recovery of the glycerol measuring method was ca. 99.9 per cent and that of triolein was between 97 and 98.6 per cent. When CDC reference sera were assayed by this method, the results obtained were within the acceptable range set by the CDC laboratory.

Reproducibility of the glycerol assay was between 2.8 and 5.4 per cent (C.V.) and reproducibility of the combined enzymatic hydrolysis and glycerol assay was 2.4 to 4.7 per cent (C.V.) for within-day runs and 2.7 to 8.4 per cent (C.V.) for day-to-day assay.

The linearity for measuring glycerol by the GK-GDH method was up to 10 mmol/liter glycerol. Glycerol quantities below 2.2 mmol/liter required ca. 8 minutes to complete the reaction and 10 to 14 minutes was necessary for the glycerol concentration of 5.19 mmol/liter. Serum triglyceride concentrations up to ca. 5 mmol/liter were completely

hydrolyzed at a 15-minute and 30°C incubation.

The proposed enzymatic triglyceride method correlated well with the Automated Fluorometric Hantzsch Condensation method and with a commerical method (Calbiochem) using enzymatic hydrolysis and glycerol assay using glycerokinase, pyruvate kinase and lactate dehydrogenase.

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APPENDIX A

Nonstandard Abbreviations and Trivial Names Used:

1) Alkaline phosphatase (AP) ... E.C.3.1.3.1, Orthophosphoric monester phosphohydrolase.
2) Alpha-chymotrypsin (Chymo) ... E.C.3.4.4.5, Peptidyl-peptide hydrolase.
3) Alpha-glycerophosphate dehydrogenase (GDH) ... E.C.1.1.1.8, L-glycerol-3-phosphate: NAD^+ oxidoreductase.
4) Glucose-6-phosphate dehydrogenase (G6PD) ... E.C.1.1.1.49, D-glucose-6-phosphate: NADP oxidoreductase.
5) Glyceraldehyde-3-phosphate dehydrogenase ... E.C.1.2.1.12, D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase.
6) Glycerokinase (GK) ... E.C.2.7.1.30, ATP: glycerol phosphotransferase.
7) Glutamic-oxaloacetic transaminase (SGOT) ... E.C.2.6.1.1, L-aspartate: 2-oxoglutarate aminotransferase.
8) Glutamic-pyruvic transaminase (SGPT) ... E.C.2.6.1.2, L-alanine: 2-oxoglutarate aminotransferase.
9) Glycerol dehydrogenase (GD) ... E.C.1.1.1.6, Glycerol: NAD^+ oxidoreductase.
10) Hexokinase (HK) ... E.C.2.7.1.1, ATP: D-hexose-6-phosphotransferase.
11) Lactic dehydrogenase (LDH) ... E.C.1.1.1.27, L-lactate: NAD^+ oxidoreductase.

12) Lipase ... E.C.3.1.1.3, Glycerol-ester hydrolase.

13) Pyruvic kinase (PK) ... E.C.2.7.1.30, ATP: pyruvate phospho-transferase.

14) Triose-phosphate isomerase (TIM) ... E.C.5.3.1.1, D-glyceraldehyde-3-phosphate ketal-isomerase.

15) Abn = abnormal

16) ADP = adenosine-5'-diphosphate

17) ATP = adenosine-5'-triphosphate

18) BSA = bovine serum albumin

19) C.F.A. = Centrifugal Fast Analyzer

20) CHO = carbohydrate

21) Chol/TG ratio = cholesterol-triglycerides ratio

22) CRT = Cathode Ray Tube

23) DHAP = dihydroxyacetone phosphate

24) D.I. water = deionized water

25) DTT = dithiothreitol, Cleland's reagent

26) EDTA = ethylene diamine tetracetic acid

27) FFA = free fatty acids

28) GLC = gas liquid chromatography

29) α-GP = alpha-glycerophosphate

30) HDL = high density lipoprotein (α-lipoprotein)

31) HSA = human serum albumin

32) Km = Michaelis constant

33) LDL = low density lipoprotein (β-lipoprotein)

34) LSI = light scattering index

35) MBTH = methyl-benzothiazolone hydrazone

36) N = normal

37) NAD^+ = nicotinamide-adenine dinucleotide, oxidized form

38) NADH = nicotinamide-adenine dinucleotide, reduced form

39) PEP = phosphoenolpyruvate

40) PHLA = plasma heparin lipase activity

41) S_f = Svedberg floatation constant

42) Time delay = A time lag prior to taking the first absorbance readings and the subsequent change in absorbance.

43) Tris = Tris (hydroxymethyl) aminomethane

44) TLC = thin layer chromatography

45) VLDL = very low density lipoprotein (pre- -lipoprotein)

Unit of Measurements

1) A = absorbance

2) nm = nanometer

3) rpm = revolutions per minute

4) μ = micron

5) μM = micrometer

6) mol/liter = mole per liter

7) mmol/liter = millimole per liter

8) μmol/liter = micromole per liter

APPENDIX B

Statistical Definitions

N or n = number of specimens

$\overline{X}$ = mean

SEM = Standard Error of the Mean ..... $\frac{S.D.}{\sqrt{n}}$

C.V. = coefficient of variation ..... $\frac{S.D.}{\overline{X}}$ X 100

r = correlation coefficient or Pearson's r

r^2 = r square, coefficient of determination

Intra-run = using only one transfer disc, which holds 29 samples and assayed simultaneously.

Inter-run = using more than one transfer discs to assay independently within the same day (within-day-run).

Day-to-day = assayed day after day or between days.

t = t-Test

df = dgree of freedom

α = 0.05, alpha level of 5%

μ = ionic strength, $\mu = \frac{\Sigma C_i Z_i^2}{2}$ where Ci is the ion concentration and Zi is the charge on the ion.

F = F test

MS = mean square

SS = sum square

S.D. = standard deviation

ADDENDUM

NORMAL VALUES

The setting of "normal limits" for serum triglycerides and other biological quantities is generally arbitrary. The "normal values" differ with age, sex, seasonal changes, geographic location, diets, and nationality. Thus, the normal values are approximations of norms based upon a "normal and healthy" randomly selected population. Fredrickson's group at NIH Clinical Center screened numerous people and suggested the following normal range for serum triglycerides (2 and 15).

Age	Triglycerides (mg/100 ml, triolein)	mmol/liter
1 - 29	10 - 140	0.11 - 1.58
30 - 39	10 - 150	0.11 - 1.69
40 - 49	10 - 160	0.11 - 1.81
50 - 59	10 - 190	0.11 - 2.14

Blood samples were collected from clinically normal subjects at 10 - 14 hours postprandially. We have selected the Fredrickson's normal limits for our enzymatic triglyceride method since this suggested method correlated well with other two methods and the variability was also within an acceptable limit.