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An improved kinetic fluorometric enzymatic coupled assay for the determination of galactose 1-phosphate uridylyltransferase in erythrocytes.

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AN IMPROVED KINETIC FLUOROMETRIC ENZYMATIC COUPLED ASSAY FOR THE DETERMINATION OF GALACTOSE 1-PHOSPHATE URIDYLYLTRANSFERASE IN ERYTHROCYTES

by

PETER CATOMERIS

A THESIS

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ABSTRACT

AN IMPROVED KINETIC FLUOROMETRIC ENZYMATIC COUPLED ASSAY FOR THE DETERMINATION OF GALACTOSE 1-PHOSPHATE URIDYLYLTRANSFERASE IN ERYTHROCYTES

by

PETER CATOMERIS

An improved method for the determination of uridylyltransferase (GALT) in erythrocytes has been developed to aid in the diagnosis of "classical" galactosemia. The method utilizes the following enzymatic system:

$$\text{Galactose 1-Phosphate} + \text{UDPG} \xrightarrow{\text{GALT}} \text{Glucose 1-Phosphate} + \text{UDPGal}$$

$$\text{Glucose 1-Phosphate} \xrightarrow[\text{Glucose 1,6-Diphosphate}]{\text{Phosphoglucomutase}} \text{Glucose 6-Phosphate}$$

$$\text{Glucose 6-Phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+$$

$$\text{6-Phosphogluconate} + \text{NADP}^+ \xrightarrow{\text{6-PGADH}} \text{Ribulose 5-Phosphate} + \text{NADPH} + \text{H}^+$$

$$2\ \text{Resazurin} + 2\text{NADPH} + 2\text{H}^+ \xrightarrow{\text{Diaphorase}} 2\ \text{Resorufin} + 2\text{NADP}^+ + 2\text{H}_2\text{O}$$

In the above scheme, UDPGal, G6PDH and 6-PGADH represent uridine 5'-diphosphogalactose, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, respectively.

In the last reaction, the non-fluorescent resazurin is converted into the highly fluorescent resorufin. Theoretically, two molecules of resorufin are produced per molecule of UDPG/galactose 1-phosphate consumed. However, there is only about 40% efficiency in converting the NADPH equivalents to resorufin.

Due to the high native fluorescence of resorufin (100-fold greater than that of NADPH), low levels of GALT activity can be measured continuously and quickly. This has a distinct advantage over UDPG-consumption and radiochemical methods, which require three steps, and other kinetic methods which require a long pre-incubation period (15 to 20 minutes) to generate enough product to be measured. In contrast the present coupled assay has a lag phase of only 3 to 4 minutes.

GALT activity at pH 8.7, 37°C was determined, using a 10-μL sample of hemolysate, by monitoring the rate of increase in fluorescence ($\lambda_{excitation}$ = 520 nm, $\lambda_{emission}$ = 585 nm) with time.

The present method correlated well (r = 0.977) with the reference UDPG-consumption method for GALT, and clearly differentiated between deficient and normal samples. GALT activities for normal patients ranged from 240-456 U/kg hemoglobin.

Also described is the optimization of the resazurin/diaphorase reaction for the determination of dehydrogenase activity. Under the new conditions, improved standard curves were generated and lower levels of dehydrogenase activity were determined.

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I would first like to thank my supervisor, Dr. Roger Thibert, for giving me the opportunity to be in the Clinical Chemistry Programme and to work with him. At several times during my M.Sc., he displayed great patience and was constantly encouraging.

I would also like to express my appreciation to other members of the committee (Drs. Draisey, Mutus and Virgo) for taking the time to critique this paper.

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To members of the faculty, staff and students, I thank you for your suggestions, help and friendship.

Lastly, I would like to say that none of this would have been possible if not for my parents, who constantly stressed the importance of education to me. I hope that this makes them as proud of me as I am of them.

DEDICATION

Στον Παππού Μιλτιάδη

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LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ACS	American Chemical Society
app	apparent
BSA	bovine serum albumin
Δ	change in
$\Delta F/\Delta t$	change in fluorescence/change in time
CV	coefficient of variation
DCPIP	2,6-dichlorophenol-indophenol
DTT	dithiothreitol
λ_{emit}	emission wavelength
EDTA	ethylenediaminetetraacetic acid
λ_{exc}	excitation wavelength
GAL1P	galactose 1-phosphate
GALT	galactose 1-phosphate uridylyltransferase
G1,6DP	glucose 1,6-diphosphate
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
G6PDH	glucose 6-phosphate dehydrogenase
Hb	hemoglobin
K_m	Michaelis constant

min	minute
NAD^+	β-nicotinamide adenine dinucleotide, oxidized form
NADH	β-nicotinamide adenine dinucleotide, reduced form
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
PGM	phosphoglucomutase
6-PGA	6-phosphogluconic acid
6-PGADH	6-phosphogluconic acid dehydrogenase
RFI	relative fluorescence intensity
S.D.	standard deviation
U	units
UDPGal	uridine 5'-diphosphogalactose
UDPG	uridine 5'-diphosphoglucose
UDPG-DH	uridine 5'-diphosphoglucose dehydrogenase
UDPglucuronate	uridine 5'-diphosphoglucuronic acid

CHAPTER 1

INTRODUCTION

1.1 Galactose 1-Phosphate Uridylyltransferase

1.1.1 Role in Galactose Metabolism

Galactose is primarily obtained by the hydrolysis of lactose (the sugar of milk) in the intestine and converted to glucose in the liver (1). The pathway by which this is done involves three steps and is shown in Figure 1.

In the first reaction, galactokinase transfers a phosphate from ATP to galactose to produce galactose 1-phosphate. GALT hydrolyzes UDPG, liberating glucose 1-phosphate, and transfers the uridylyl moiety to galactose 1-phosphate, forming UDPGal. It is in this nucleotide-containing form that galactose is converted to glucose, using UDPGal 4-epimerase. The glucose is eventually released in the form of glucose 1-phosphate, after incorporation into glycogen and phosphorolysis.

It should be noted that the third reaction is freely reversible, allowing the conversion of glucose into galactose (1). For this reason, galactose is not essential in the diet.

1.1.2 Galactosemia

1.1.2.1 Prevalence

Galactosemia is the general term which refers to the inability to metabolize galactose, and cases have been reported involving deficiencies of each of the three enzymes discussed (2-4).

FIGURE 1

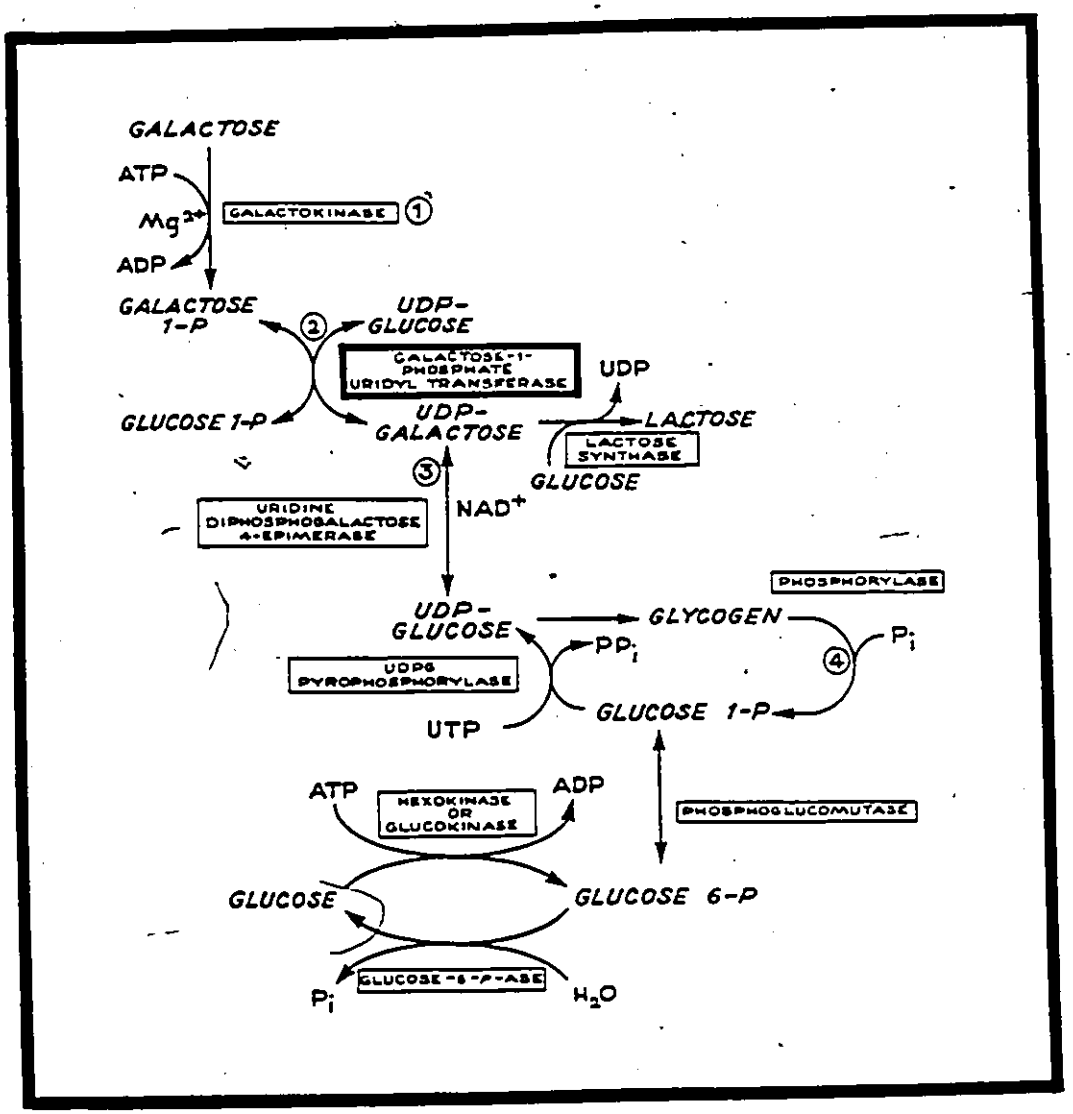
PATHWAY OF GALACTOSE METABOLISM

Legend

The key steps in converting galactose into glucose are those marked ①, ②, and ③, involving the enzymes galactokinase, GALT, and UDPGal 4-epimerase. Deficiency of any of these three enzymes leads to the condition known as galactosemia, or the inability to metabolize galactose.

(Borrowed, without permission, from Harper's Review of Biochemistry, 19th edition (1983) Martin, D.W. Jr., Mayes, P.A. and Rodwell, V.W. (editors). Lange Medical Publications, Los Altos.)

FIGURE 1


GALACTOSE
ATP
Mg2+
ADP
GALACTOKINASE
1
GALACTOSE 1-P
2
UDP-GLUCOSE
GALACTOSE-1-PHOSPHATE URIDYL TRANSFERASE
UDP
GLUCOSE 1-P
UDP-GALACTOSE
LACTOSE
LACTOSE SYNTHASE
GLUCOSE
3
NAD+
URIDINE DIPHOSPHOGALACTOSE 4-EPIMERASE
PHOSPHORYLASE
UDP-GLUCOSE
GLYCOGEN
PPi
Pi
4
UDPG PYROPHOSPHORYLASE
UTP
GLUCOSE 1-P
ATP
HEXOKINASE OR GLUCOKINASE
ADP
PHOSPHOGLUCOMUTASE
GLUCOSE
GLUCOSE 6-P
Pi
GLUCOSE-6-P-ASE
H2O

The transferase (GALT)-deficient or "classical" galactosemia is the most common of the three. The prevalence of the disease has varied from 1:18,000 to 1:81,600 depending on the population studied (5-6). A more comprehensive study (7) involving several countries, indicates that the prevalence is about 1:75,000 overall.

1.1.2.2 Clinical Symptoms and Biochemical Basis

Symptoms of the disease appear within a few days of the birth of the child, usually a few days after its first feedings of milk (2-5). The child develops diarrhea, vomiting and dehydration and hyperbilirubinemia is seen for several days. If not diagnosed early and treated properly, the child develops cataracts, renal and hepatic dysfunction, and mental retardation. In severe cases, the outcome is fatal.

Due to the deficiency of GALT, there is an accumulation of galactose 1-phosphate in the lens, liver, kidney and brain. It is believed that this causes depletion of inorganic phosphate in these tissues, causing their dysfunction (1,4). In addition, galactitol, the sugar alcohol of galactose, accumulates in the lens, drawing water into it, and causes the formation of cataracts.

1.1.2.3 Laboratory Tests, Diagnosis and Treatment

Galactosemia should be considered in cases where the neonate develops jaundice, diarrhea, vomiting and failure to thrive while receiving lactose in the diet. A preliminary test on the urine will show high levels of galactose. However, it is not uncommon to find galactosuria in newborns with low weight, who have immature renal glucose-galactose transport systems (4).

Diagnosis of GALT-deficient galactosemia can, therefore, only be made definitively by assay of the enzyme itself. Although GALT is present in a variety

of cells and tissues (8), for clinical purposes, it is measured most often in red blood cells. Several workers (9-11) have also assayed for the enzyme in cultured amniotic cells, for use in the prenatal diagnosis of galactosemia.

There is effective treatment, i.e., removal of galactose from the diet, for the disease. In GALT-deficient patients, the epimerase is present in sufficient amounts to provide the necessary quantity of galactose required by the body. Because of this fact, and because of the severity of the disease, it is important that a proper diagnosis be made early in the patient's life.

1.1.3 Properties of the Erythrocyte Enzyme

The enzyme is composed of two similar or identical subunits with combined molecular weight of 88,000 (11). Kinetic studies (12) have shown that the reaction catalyzed by GALT proceeds by a ping-pong mechanism, in which a covalent uridylyl-enzyme intermediate is formed.

GALT, in general, can be stabilized and activated by the thiol reagents: 2-mercaptoethanol, cysteine, and dithioerythritol/dithiothreitol (12). The effect is more pronounced in leukocytes than erythrocytes, which have relatively high concentrations of reduced glutathione to stabilize the enzyme (13).

The reported pH optimum of the enzyme ranges from 8.5 (2,12) to 8.7 (8,14,15). The pH most commonly used for the assay of GALT is 8.7 at 25°C.

Due to the low activity of GALT in erythrocytes, it is assayed at 37°C.

Chloride ions have been shown to inhibit GALT (14). Tris-acetate and glycine-NaOH solutions are generally accepted as the buffers of choice.

One study (16) has reported that GALT is also inhibited by glucose 1-phosphate, glucose 6-phosphate, UDPG, ATP and ADP, but this has not been

confirmed by other workers.

1.1.4 Genetic Variants

GALT-deficient galactosemia is an autosomal recessive disorder, in which the affected individual is completely devoid of, or markedly deficient in, GALT activity. The basis of the abnormality is believed to be a structural gene mutation, resulting in the replacement of an amino acid at or near the catalytic site (17,18).

A variant of galactosemia has been described (19) in which GALT was almost completely inhibited by glucose 1-phosphate at a concentration within the range of stationary G1P concentration in the liver. It is suggested that the product inhibition is responsible, in this case, for the impaired galactose metabolism in the patient.

Several other catalytically impaired variants, in which GALT activity is 30-65% of normal, have also been described (5). Patients with the more severe of these variants present the clinical symptoms related to classical galactosemia.

On the other hand, the Los Angeles variant (20) is associated with greater than normal GALT activity and is not linked to any clinical disorder.

1.1.5 Methods of Analysis

The reaction catalyzed by GALT is:

UDPG + Galactose 1-Phosphate (GAL1P) → UDPGal + Glucose 1-Phosphate (G1P)

The methods of analysis can be summarized as the UDPG-consumption methods, those based on the generation of G1P, and the radiochemical methods.

1.1.5.1 UDPG-Consumption Method

The UDPG-consumption assay is the method selected by the American Association for Clinical Chemistry for the determination of GALT in erythrocytes (5), and the assay was first described by Anderson et al. (21).

UDPG and GAL1P are first allowed to incubate in presence of GALT for a fixed period of time, after which the reaction is stopped. After centrifugation, the amount of UDPG remaining is determined using uridine 5'-diphosphoglucose dehydrogenase (UDPG-DH) in the following system:

$$\text{UDPG} + 2\text{NAD}^+ \xrightarrow{\text{UDPG-DH}} \text{UDPglucuronate} + 2\text{NADH} + 2\text{H}^+$$

The increase in absorbance at 340 nm is measured and is proportional to the amount of UDPG present. When subtracted from the amount of UDPG originally added (determined from the blank), the UDPG consumed is determined and is porportional to the GALT activity present.

The main problem associated with this method is the fact that the concentration of UDPG used is a compromise (5,14). Early versions of the assay (21,22) used low concentrations of UDPG, and suffered from the fact that the relationship between GALT activity and rate of UDPG-consumption departed from linearity. This is a result of the depletion of UDPG in the latter portion of the incubation period, leading to slower reaction rates. As a consequence, the procedures could not differentiate between carriers and normals with any degree of certainty (14).

Later versions of the assay (5,14) have increased the amount of UDPG used to improve linearity, but have the disadvantage of producing high absorbance values for tests and blanks. GALT activity must be calculated from two large numbers, differing only slightly from one another. This has led to

a procedure which is less precise.

From a practical point of view, the reference UDPG-consumption method has several drawbacks in that it requires:

a. a 15-minute incubation period;

b. that the reaction to be stopped by placing the assay mixture in a boiling water bath;

c. a 20-minute centrifugation step;

d. an end-point assay for UDPG which is lengthy (20 to 30 minutes).

It should also be noted that UDPGal 4-epimerase, if active, interferes with this test, by providing a means for UDPGal to be converted back to UDPG (23). Thus, it is necessary to inactivate the enzyme by heating the sample (hemolysate) to destroy NAD^+, a co-factor for the enzyme.

1.1.5.2 Methods Based on the Generation of Glucose 1-phosphate

These methods monitor the amount of G1P formed using the coupled assay below (Scheme 1):

$$G1P \xrightarrow{PGM} \text{Glucose 6-Phosphate (G6P)}$$

$$G6P + NADP^+ \xrightarrow{G6PDH} \text{6-Phosphogluconate(6-PGA)} + NADPH + H^+$$

$$6\text{-PGA} + NADP^+ \xrightarrow{6\text{-PGADH}} \text{Ribulose 5-Phosphate} + CO_2 + NADPH + H^+$$

A multitude of assays for GALT have been developed, based on the above principle.

A manometric technique (24) incorporates methylene blue to transport electrons from NADPH to molecular oxygen, and GALT is measured as the rate of oxygen consumption. The method, however, is tedious and requires large amounts of blood.

Fluorometric assays, based on the fluorescence of NADPH, have been used for qualitative (5) and quantitative (6) determination of GALT. These require incubation periods of 1 to 2 hours.

Spectrophotometric assays, of note, are those of Pesce et al. (25) and Schutgens et al. (26), which both follow the increase in absorbance at 340 nm. The first involves only one step, but requires a 20-minute incubation period to generate enough product to be measured and the increase in absorbance must be followed for 9 minutes. In addition, it requires a spectrophotometer with extremely high sensitivity, since the observed absorbance changes range from 0.0005 to 0.006 per minute. The latter assay produces greater absorbance changes, but requires the same basic steps (incubation, stopping reaction, centrifugation, and end-point assay) associated with UDPG-consumption methods.

1.1.5.3 Radiochemical Methods

A number of radioisotopic methods have been developed, using either [^{14}C]GAL1P (23,27) or [^{32}P]GAL1P (28). The assays are sensitive, but require an addditional step to separate substrate from product before measurement.

1.2 Purpose of Project

We set out to develop a sensitive fluorometric, continuous enzymatic coupled assay for GALT in which the electrons from NADPH, generated as in Scheme 1, are transferred to resazurin, forming a fluorescent product, resorufin.

The aim was to eliminate the drawbacks of other methods discussed previously, namely:

1. large sample volumes;

2. separation steps, associated with UDPG-consumption and radiochemical methods;

3. long incubation periods.

The first is an important consideration, since the patients affected by this disease are neonates, from whom very little blood may be drawn. The second and third are important from a practical point of view, i.e., reduction of technologist time required to perform the assay.

1.3. The Resazurin/Diaphorase System

Electrons from NADPH (or NADH) are enzymatically trasnferred to non-fluorescent resazurin in the presence of diaphorase to produce resorufin, a highly fluorescent molecule (Figure 2).

The use of the resazurin/diaphorase system for the determination of dehydrogenase activity was first proposed by Guilbault and Kramer (29). They indicated that as low as 10^{-4} units of enzyme activity could be determined, making this procedure several orders of magnitude more sensitive than spectrophotometric procedures (30). In addition, enzyme activity can be determined rapidly and directly by this method, which is a distinct advantage over the procedure of Lowry et al. (31,32). The latter assay, based on the fluorescence of NAD^+, requires that the NAD^+ be heated with 6N sodium hydroxide for 5 to 30 minutes, before a measurement can be made.

Due to its sensitivity and simplicity, the resazurin/diaphorase system has been exploited in a number of assays (31-37) which generate NADH or NADPH. For these same reasons, we proposed to use it for the determination of GALT activity.

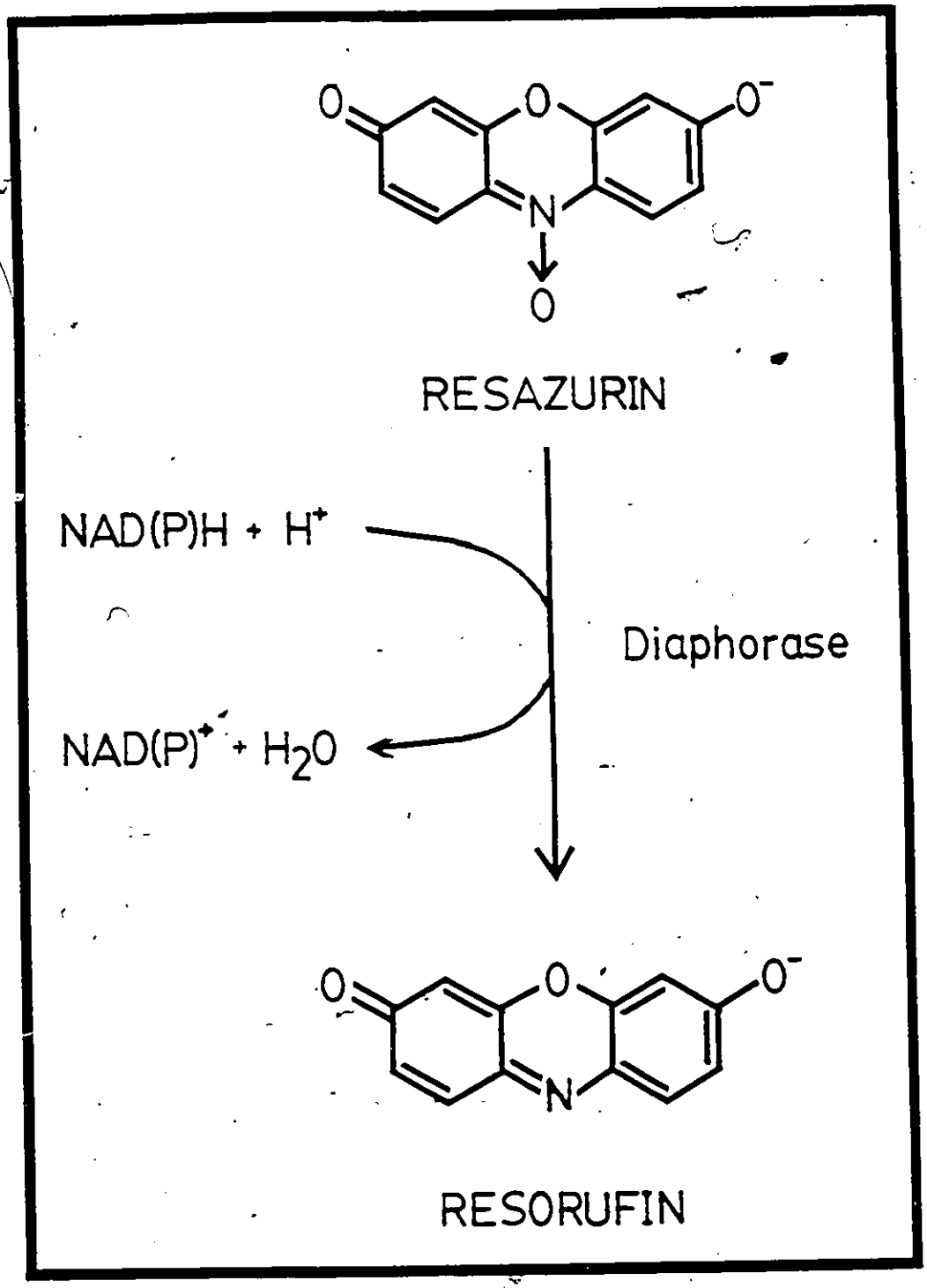
FIGURE 2

DIAPHORASE-MEDIATED REDUCTION OF RESAZURIN

Legend

Non-fluorescent resazurin is enzymatically reduced to the highly fluorescent resorufin, using diaphorase and NADH or NADPH.

FIGURE 2



CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Bovine serum albumin (Fraction V), citric acid (free acid), 2,6-dichlorophenol-indophenol, DL-dithiothreitol, flavin mononucleotide (sodium salt), glucose 1,6-diphosphate [tetra(cyclohexylammonium)salt], glucose 1-phosphate (disodium salt), glucose 6-phosphate (monosodium salt), glycine (free base), NAD^+ (free acid), NADH (disodium salt), $NADP^+$ (sodium salt), NADPH (tetrasodium salt), 6-phosphogluconic acid (tri(cyclohexylammonium)salt), Trizma base, Triton X-100 and UDPG (sodium salt) were purchased from Sigma Chemical Company (St. Louis, MO).

Galactose 1-phosphate (dipotassium salt) was generously donated by Biosynth Ag (Skokie, Ill.), courtesy of Dr. C. Patel.

All of the following enzymes were purchased from Sigma Chemical Company:

Diaphorase [NADH: lipoamide oxidoreductase (EC 1.6.4.3) from *Clostridium kluyveri*].

Galactose 1-phosphate uridylyltransferase [UDPG: α-D-galactose 1-phosphate uridylyltrasferase (EC 2.7.7.12) from galactose adapted yeast].

Glucose 6-phosphate dehydrogenase [D-glucose 6-phosphate: $NADP^+$ oxidoreductase (EC 1.1.1.49) from Bakers yeast].

Phosphoglucomutase [α-D-glucose 1,6-diphosphate: α-D-glucose 1-phosphate phosphotransferase (EC 2.7.5.1) from rabbit muscle].

6-Phosphogluconic dehydrogenase [6-phospho-D-gluconate: $NADP^+$ oxidoreductase (EC 1.1.1.44) from yeast].

UDPG dehydrogenase [UDPG: NAD^+ 6-oxidoreductase (EC 1.1.1.22) from bovine liver].

Unless otherwise indicated, the unit definitions of the enzymes used throughout this thesis are those quoted in Table I. The enzymes were assayed using the manufacturer's bulletins (38-42) to ensure that the activities were those claimed.

GALT Deficient Control and Hemoglobin Standard were purchased from Sigma Chemical Company.

Resazurin (sodium salt) and resorufin were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI).

Acetic acid, ethoxyethanol, hydrochloric acid and sulphuric acid were all ACS grade and purchased from BDH Chemicals (Toronto, Ontario).

EDTA (disodium salt), magnesium chloride hexahydrate, potassium cyanide, potassium ferricyanide, potassium phosphate monobasic, quinine sulphate, sodium chloride and sodium hydroxide were purchased from Fisher Scientific Co. (Canada) and were of analytical grade.

Distilled de-ionized water was obtained by passing water from a distillation apparatus through a reverse osmosis Xenopure® system.

2.2 Apparatus

A fluorescence spectrophotometer (Model 204) equipped with a xenon power supply (Model 150) and a recorder (Model 56), all manufactured by Perkin Elmer Corporation (Norwalk, Conn.), was used for all fluorometric studies. The cuvette holder was connected to a water bath (Haake Corp.; Berlin, Germany) for

TABLE I

UNIT DEFINITIONS OF ENZYMES

Enzyme	Unit definition
Diaphorase	One DCPIP unit will oxidize 1.0 μmole of β-NADH per minute at pH 7.5 at 25°C, with the corresponding reduction of 2,6-dichlorophenol-indophenol.
Diaphorase	One resazurin unit will convert 1.0 μmole of resazurin to resorufin per minute in the presence of NADPH at pH 8.7 at 25°C.
Galactose 1-phosphate uridylyltransferase	One unit will form 1.0 μmole resorufin per minute at pH 8.7 and 37°C.
Glucose 6-phosphate dehydrogenase	One unit will oxidize 1.0 μmole of glucose 6-phosphate to 6-phosphogluconate per minute in the presence of $NADP^+$ at pH 7.4 at 25°C.
Phosphoglucomutase	One unit will convert 1.0 μmole of glucose 1-phosphate to glucose 6-phosphate per minute at pH 7.4 at 30°C.
6-Phosphogluconate dehydrogenase	One unit will oxidize 1.0 μmole of 6-phosphogluconate to D-ribulose 5-phosphate and CO_2 per minute at pH 7.4 and 37°C.

experiments performed at 37°C. Unless otherwise indicated, a 1.0-mL fluorometric quartz cuvette (1.00-cm pathlength), from Hellma (Canada) Limited (Concord, Ontario), was used. A small plastic platform was placed in the cuvette holder to elevate the cuvette.

Ultraviolet/visible readings and recordings, taken at room temperature, were obtained using a Shimadzu spectrophotometer (Model UV-240) and printer (Model PR-1) made by Tekscience (Oakville, Ontario). A Model 35 recording spectrophotometer, with temperature-controlled cuvette holder, (Beckman Instruments, Inc.; Palo Alto, California) was used for experiments requiring temperatures higher than 25°C.

Other equipment included:

1. TJ-6 table-top centrifuge, with TH-4 rotor and TJ-R refrigeration unit (Beckman Instruments Inc.);
2. Thermolyne Dri-bath heater (Sybron, Boston, MA);
3. Mettler P1000 balance for masses greater than one gram and Type H16 balance for less than one gram masses (Fischer Scientific Co.; Toronto, Ontario);
4. Gilson Pipetman variable-volume pipetters (Mandel Scientific Co., Ltd.; Ville St. Pierre, Quebec) and Oxford fixed-volume pipettors and pipette tips (Canadian Laboratory Supplies, Ltd.; Toronto, Ont.);
5. Fisher Accumet® pH meter (Fisher Scientific Co.; Toronto, Ont.);
6. Borosilicate glass culture tubes (Fisher Scientific Co.; Toronto, Ont.);

2.3 Methods

2.3.1 Study of Resazurin/Diaphorase System

2.3.1.1 Reagents

1. Glycine Buffer (100 mM), pH 8.7 at 25°C: Glycine (3.8 grams) was dissolved in about 450 mL of water and pH was adjusted to 8.7 at 25°C with NaOH. The solution was diluted to 500 mL with water and stored at 4°C.
2. BSA Solution (1% w/v): BSA (100 mg) was dissolved in 10.0 mL of glycine buffer.
3. Diaphorase: Solutions were prepared fresh on day of use by dissolving material in 1% BSA.
4. Resorufin: Solutions were prepared fresh by dissolving appropriate amounts of material in water.
5. Resazurin: Solutions were prepared fresh by dissolving appropriate amounts of material in water.
6. NADPH: Solutions were prepared fresh by dissolving appropriate amounts of material in water. Exact concentrations were determined using a molar extinction coefficient of $6.3 \times 10^{3} cm^{-1} \times M^{-1}$ at 340 nm (43).
7. Glucose 6-Phosphate Dehydrogenase: Solutions were prepared when required by dissolving material in 1% BSA solution.
8. $NADP^{+}$ Solution (0.03 M): $NADP^{+}$ (8.3 mg) was dissolved in 500 µL water. Exact concentration was determined using a molar extinction coefficient of $17.4 \times 10^{3} cm^{-1} \times M^{-1}$ at 340 nm (44).

9. Glucose 6-Phosphate Solution (0.034 M): G6P (6.0 mg) was dissolved in 500 µL of water.

10. Quinine Sulphate Solution (1.0 µg/mL): Quinine sulphate (0.100 g) was dissolved in 1.0 L of 0.1 N H_2SO_4 solution. This solution (10 mL) was then diluted to 1.0 L with 0.1 N acid solution (45).

2.3.1.2. Procedures

a. Generation of Fluorescence Spectra

The procedure followed was that outlined in the manual (46) which accompanies the fluorescence spectrophotometer. The emission spectra were generated first by exciting with light of all wavelengths. The wavelength giving the highest emission, was then used to generate the excitation spectra.

b. Fluorescence Coefficient of Compounds

Appropriate solutions of resorufin and NADPH, in glycine buffer, and quinine sulphate were made and their relative fluorescence intensity (RFI) were measured at the corresponding λ_{exc} and λ_{emit} for each compound. The settings on the fluorometer at which each measurement was made was also noted.

For the determination of the fluorescence coefficient of resorufin in the presence of resazurin, the following procedure was followed: Solutions of varying resazurin concentration were made by serially diluting a stock 2.0 mM solution with water. A resorufin solution was made to be 3.1 µM. Solutions were made up as described below and their RFI measured.

	3.0-mL cuvette		1.0-mL cuvette	
	Blank	Sample	Blank	Sample
Buffer	2.90 mL	2.90 mL	0.48 mL	0.48 mL
H_2O	0.05 mL	-	0.01 mL	-
Resazurin	0.05 mL	0.05 mL	0.01 mL	0.01 mL
Resorufin	-	0.05 mL	-	0.01 mL

A control was prepared, in each case, by replacing resazurin with water.

c. Determination of Diaphorase Activity

Diaphorase activity, using DCPIP as a substrate, was determined according to the manufacturer's bulletin (38).

Diaphorase activity, using resazurin as a substrate, was determined by monitoring the rate of increase in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm) at 25°C. The general procedure was to add resazurin and diaphorase to glycine buffer, and initiate the reaction with NADPH. The non-enzymatic reduction of resazurin by NADPH, in all cases was determined by replacing the diaphorase with an appropriate aliquot of 1% BSA.

The study of the effect of resazurin on diaphorase activity was carried out in a 3.0-mL cuvette. The assay mixture consisted of 2.88 mL of glycine buffer, 20 µL diaphorase (1.7 U/mL) 50 µL of resazurin (ranging from 51 µM up to 2.0 mM), and 50 µL of NADPH (4.7 mM).

The study of the effect of NADPH on diaphorase activity was carried out in a 1.0-mL cuvette, using two different concentrations of resazurin. The assay mixture consisted of 470 µL of glycine buffer, 10 µL of diaphorase (0.9 U/mL), 10 µL of resazurin (0.34 mM or 1.7 mM) and 10 µL of NADPH (ranging

from 47 μM up to 1.5 mM).

The "linearity of diaphorase activity" study was carried out in a 1.0 mL cuvette. Serial two-fold dilutions of a stock diaphorase solution (3.2 DCPIP U/mL) were made with 1% BSA, and assayed. The assay mixture consisted of 470 μL of glycine buffer, 10 μL of diaphorase, 10 μL of resazurin (1.7 mM) and 10 μL of NADPH (1.1 mM).

d. Determination of Glucose 6-phosphate Dehydrogenase Activity

G6PDH activity at 25°C was first determined spectrophotometrically by monitoring the rate of increase in absorbance at 340 nm, due to the reduction of $NADP^+$ to NADPH. The reaction was initiated by adding 20 μL of G6PDH to a solution containing 940 μL of glycine buffer, 20 μL of $NADP^+$ (0.03 M) and 20 μL of glucose 6-phosphate (0.034 M).

Serial two-fold dilutions of the G6PDH solution were made and then assayed, using the resazurin/diaphorase system, by monitoring the rate of increase of fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm) at 25°C. The reaction was initiated by adding 10 μL of G6PDH to a solution containing 450 μL of glycine buffer, 10 μL of $NADP^+$ (0.03 M), 10 μL of G6P (0.034 M), 10 μL of resazurin and 10 μL diaphorase. G6PDH was assayed in this way under two different conditions, in which the working solutions of resazurin and diaphorase were: i) 1.7 mM and 16.5 DCPIP U/mL, respectively; ii) 0.34 mM and 0.67 DCPIP U/mL, respectively.

e. Stability of Resazurin

A solution of resazurin (0.85 mM) was made by dissolving 11.0 mg in 50.0 mL of water and kept at room temperature in an amber glass bottle. The absorbance of a dilute solution (50 μL of stock solution added to 5.0 mL of water) was read (in duplicate) at 600 nm on 11 occasions over a period of 40

days.

2.3.2 Measurement of GALT in Hemolysates

2.3.2.1 Reagents

1. NaCl Solution (0.9% w/v): NaCl (9.0 mg) was dissolved in 1.0 L of water. The solution was stored at 4°C.
2. Modified Drabkin's Reagent: Potassium ferricyanide (200 mg), potassium cyanide (500 mg), potassium phosphate monobasic (140 mg), and Triton X-100 (1.0 mL) were dissolved in water and diluted to 1.0 L. The solution was kept in an amber glass bottle in the dark at room temperature and was stable for several months.
3. Hemoglobin Standard: Lyophilized powder was reconstituted with 50.0 mL of Drabkin's reagent. Concentration of hemoglobin in this solution was the equivalent of making a 251-fold dilution of 18 g Hb/100 mL with Drabkin's reagent.
4. Glycine Buffer (100 mM) with EDTA (0.5 mM), pH 8.7 at 25°C: Glycine (3.75 g) and EDTA (92.5 mg) were dissolved in about 450 mL of water and the pH was adjusted to 8.7 with NaOH. The solution was diluted to 0.50 L and kept at 4°C.
5. BSA Solution (1% w/v): BSA (50 mg) was dissolved in 5.0 mL of buffer.
6. Phosphoglucomutase (15 U/mL): On day of assay, a PGM solution (15 U/mL) was made by an appropriate dilution of stock PGM with 1% BSA.
7. Glucose 6-Phosphate Dehydrogenase (200 U/mL): Lyophilized powder was reconstituted with sufficient 0.005 M citrate buffer, pH 7.5 to make a 200 U/mL solution. Aliquots were kept at -20°C.

8. Diaphorase (8.9 U/mL): Solution was prepared fresh on day of assay by dissolving appropriate amounts of material in 450 µL of 1% BSA.

9. UDPG Solution (27.5 mM): UDPG (27 mg) was dissolved in 1.0 mL of water. The absorbance of a 1000x dilution, in water, was read at 260 nm. Based on the extinction coefficient of $9.9 \times 10^{3} cm^{-1} \times M^{-1}$ (47), the exact concentration of the solution was calculated and adjusted, if necessary, by dilution with water. Aliquots were stored at -20°C.

10. Galactose 1-Phosphate Solution (0.17 M): GAL1P (73 mg) was dissolved in 1.0 mL of water. Aliquots were stored at -20°C.

11. $NADP^{+}$ Solution (35.2 mM): $NADP^{+}$ (28 mg) was dissolved in 1.0 mL of water. Aliquots were stored at -20°C.

12. Glucose 1,6-Diphosphate Solution (1.0 mM): G1,6DP (1.5 mg) was dissolved in 2.0 mL H_2O. Aliquots were stored at -20°C.

13. Resazurin Solution (0.85 mM): Resazurin (10.9 mg) was dissolved in 50.0 mL water. The solution was kept at room temperature in an amber glass bottle and was stable for about one month.

14. Resorufin Solution (0.21 mM): On day of assay, about 5.0 mg (exact mass was noted) of resorufin were dissolved in 100 mL water.

15. GALT: Solutions were prepared fresh as needed by dissolving appropriate amounts of material in 1% BSA.

16. GALT Deficient Control: Lyophilized powder was reconstituted, as needed, with 500 µL of water.

2.3.2.2 Procedures

a. Preparation of Hemolysates

1. On the day of collection, venous blood samples in EDTA Vacutainers® were transferred to 13 x 75 mm plastic tubes and washed as described by Ibbott (5).

2. Hemolysates of the packed erythrocyte were made by adding an equal volume of cold water and freeze/thawing in a solid CO_2/acetone bath. The freeze/thaw procedure was repeated three times as recommended by Schutgens et al. (26).

3. For those samples to be used in the comparison of methods study, the hemolysates were incubated at 37°C for ten minutes (5).

4. Hemoglobin concentrations in hemolysates were determined by method described below.

5. Hemolysates were stored at 4°C until assayed.

b. Determination of Hemoglobin Concentration

Hemoglobin concentration was determined with the use of the Hemoglobin Standard by the cyanmethemoglobin method recommended by the International Committee for Standardization in Hematology (48,49).

c. Coupled Assay of GALT

1. 410 µL of buffer solution were pipetted into 10 x 75 mm glass tubes (6 for each sample to be analyzed). Test tubes were covered with parafilm and placed in a 37°C dry-bath heater.

2. PGM (15 U/mL), diaphorase (8.9 U/mL) and resorufin (0.21 mM) were prepared.

3. "Enzyme" solution was prepared by adding 25 µL of PGM and 25 µL of G6PDH (200 U/mL) to the diaphorase solution.

4. "Substrate" solution was prepared by mixing 75 µL each of GAL1P (0.17 M), $NADP^+$ (35.2 mM) and G1,6DP (1.0 mM).

5. To the buffer (at 37°C) were added 10 µL of hemolysate, 20 µL of resazurin (0.85 mM), 20 µL of UDPG (27.5 mM), 20 µL of "enzymes" and 20 µL of "substrates". The solution was transferred to a 1.0-mL cuvette and allowed to incubate at 37°C for three minutes, after which the increase in fluorescence (λ_{exc} = 520 nm, λ_{emit} = 585 nm) was recorded for three minutes.

6. Step 5 was repeated for the blank with the exception that 20 µL of water were used instead of UDPG.

7. An internal standard was set up for each run in the following manner: To 410 µL of buffer solution were added 10 µL of hemolysate, 20 µL resazurin and 50 µL of water. The solution was transferred to a 1.0-mL cuvette and allowed to incubate at 37°C for three minutes. Its RFI was adjusted to read "zero" at the same settings used for steps 5 and 6. 10 µL of resorufin (0.21 mM) were added; the solution was mixed (by inversion, using parafilm) and its RFI was recorded.

8. Steps 5, 6 and 7 were performed twice for each sample assayed.

d. UDPG-Consumption Assay

For the comparison of methods study, hemolysates were also analyzed using the reference UDPG-consumption method of Ibbott (5). For this study, some samples were prepared as described in "Preparation of Sample" (Section 2.3.2.2 a., p. 23), while others were produced by mixing normal samples with GALT Deficient Control in various ratios.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Introduction

In kinetic enzymatic coupled assays, the enzymes of the auxiliary and indicator reactions must be present in sufficient amounts in order to not be rate-limiting (50-52). Our preliminary investigation into the resazurin/diaphorase system suggested that the amount of diaphorase used by Guilbault and Kramer (29,31) would always be rate-limiting in their kinetic assay for the determination of dehydrogenases. These initial observations led us to investigate the resazurin/diaphorase system more thoroughly.

3.2 The Resazurin/Diaphorase System

3.2.1 Fluorescence of Resorufin

The excitation and emission wavelengths for resorufin were found to vary between 565-570 nm and 580-585 nm, respectively (Figure 3).

Relative fluorescence intensity (RFI) was proportional to resorufin concentration up to about 5×10^{-6} M (Table II) with an average "fluorescence coefficient" of 8.4×10^{6}. Fluorescent coefficients were calculated by dividing the corrected RFI and dividing by the molar concentrations. Resorufin has a native fluorescence on the same order as quinine sulphate and an order of 100 times that of NADPH. It is obvious then that assays based on the fluorescence of resorufin are much more sensitive than those based on NADPH fluorescence.

FIGURE 3

FLUORESCENCE SPECTRA OF RESORUFIN

Legend

Shown are the excitation and emission spectra of resorufin at pH 8.7 at 25°C. The emission spectrum was generated first by using exciting light 568 nm. The excitation spectrum was then generated by monitoring the fluorescence at 582 nm (wavelength of peak fluorescence).

FIGURE 3

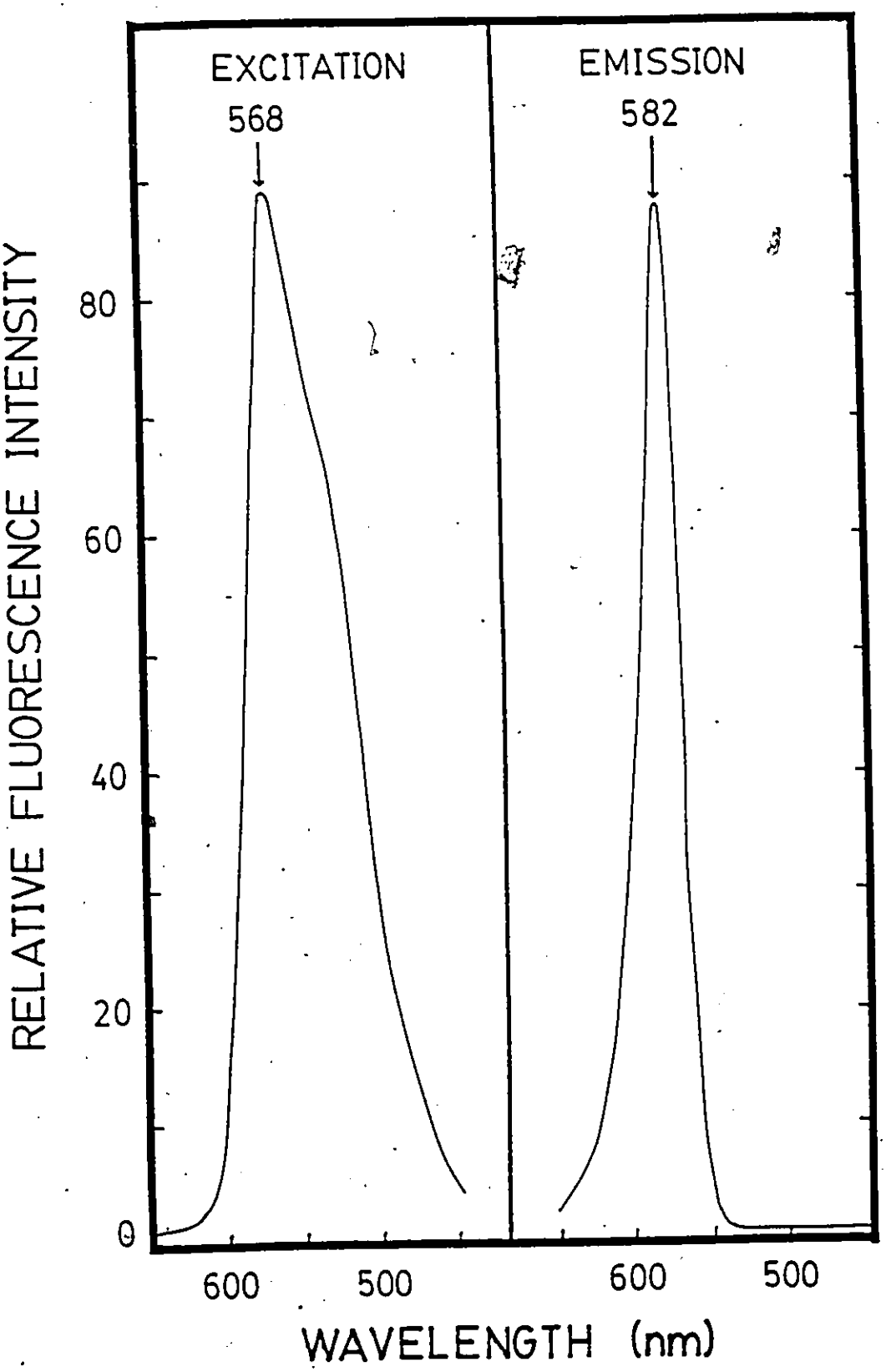

EXCITATION
568
EMISSION
582
RELATIVE FLUORESCENCE INTENSITY
80
60
40
20
0
600
500
600
500
WAVELENGTH (nm)

TABLE II

FLUORESCENCE COEFFICIENTS OF RESORUFIN, QUININE SULPHATE AND NADPH

Compound[a]	Concentration (moles/litre)	Fluorescence coefficient[b]
Resorufin	1.11×10^{-4}	2.7×10^{4}
(pH 8.7, 25°C)	3.70×10^{-5}	1.2×10^{6}
	1.23×10^{-5}	5.2×10^{6}
	4.12×10^{-6}	8.4×10^{6}
	1.37×10^{-6}	9.0×10^{6}
	4.57×10^{-7}	9.9×10^{6}
	1.52×10^{-7}	9.4×10^{6}
	5.08×10^{-8}	8.7×10^{6}
	1.69×10^{-8}	7.5×10^{6}
Quinine sulphate	1.28×10^{-6}	1.4×10^{7}
(in 0.1N H_2SO_4, 25°C)	0.64×10^{-6}	1.4×10^{7}
NADPH	8.7×10^{-7}	1.2×10^{5}
(pH 8.7, 25°C)	4.4×10^{-7}	1.2×10^{5}

(a) λ_{exc} and λ_{emit} were respectively: 568 nm, 582 nm (resorufin); 350 nm, 450 nm (quinine sulphate); 365 nm, 450 nm (NADPH).

(b) These were determined by correcting the observed fluorescence intensities to a common setting of 1 (sensitivity control), x 1 (selector control) [see APPENDIX A1 and dividing by the molar concentration.

3.2.2 Preliminary Studies

Our preliminary studies indicated that the specific activity of diaphorase, using resazurin (6.7 μM) as a substrate, was only about 2×10^{-3} that using 2,6-dichlorophenol-indophenol (DCPIP) as a substrate. This sharply differs from the findings of Guilbault et al. (53), who found the "resazurin specific activity" to be about 5×10^{-2} that of the "DCPIP specific activity".(a) Although they used NADH as the redcing agent for resazurin, while we used NADPH, we have found that the rates of reactions are only slightly slower when using NADPH, and this would not account for the huge discrepancy between our findings. One important point which they neglected to mention is that the non-enzymatic rate of reduction of resazurin by NADH or NADPH is significant when these are present in concentrations greater than 1×10^{-5} M (Guilbault et al. (53) used 2×10^{-4}M NADH).

In their assay for dehydrogenase, Guilbault et al. (29) claim that the rate of reaction is dependent only upon the dehydrogenase concentration, between 4.7×10^{-4} and 0.4 units/mL. In the assay, diaphorase is present in 0.08 "old" DCPIP units/mL, which according to our results, is only about 3×10^{-5} resazurin units/mL. It seems, therefore, that diaphorase should always be rate-limiting in their assay. This was supported by the finding that in other assays (32,33),

---

(a) The "new" DCPIP unit of diaphorase activity is given in Table I. It should be noted that in the papers of Guilbault et al. (29,31,53), the old definition of a DCPIP unit was used, in which "one unit equals a decrease in absorbance of 1.00/min, using the dye DCPIP and the coenzyme NADH, at 25°C". Six "old" DCPIP units are equal to one "new" DCPIP unit.

calibration curves were generated in which rates of reaction were proportional to the logarithm of analyte concentration, not the analyte concentration itself.

It seemed obvious that procedure of Guilbault and Kramer (29,31) could be improved by ensuring that the indicator reaction (resazurin/diaphorase) is not rate-limiting.

3.2.3 Reaction Kinetics of Diaphorase/Resazurin System

Guilbault et al. (53) show a Lineweaver-Burk plot which passes through the origin. Concentrations of resazurin up to about 5.0×10^{-6}M were used, indicating that the K_m of this substrate must be much higher than 5.0×10^{-6}M. Considering this fact, it was not clear why Guilbault et al. chose to use a resazurin concentration of 6.6×10^{-6}M in their assays for the determination of dehydrogenase activity.

The effect of resazurin concentration on diaphorase activity was, therefore, studied and the results are shown in Figure 4. Maximum signal ($\Delta F/\Delta t$) was obtained at concentration of resazurin between 6.7×10^{-6} and 13.4×10^{-6}M and a decrease in signal was seen at concentrations above that.

It had been noted in our studies, however, that resazurin had an absorption maximum (600 nm) near the excitation and emission wavelengths of resorufin and quenched the fluorescence signal of resorufin. The quenching effect of resazurin on the fluorescence of resorufin is shown in Figure 5.

Diaphorase activities were calculated by dividing the $\Delta F/\Delta t$ (from Figure 4) by the corresponding fluorescence coefficient of resorufin in the presence of different amounts of resazurin. The data is shown in Figure 6 as a Lineweaver-Burk plot, and indicates that diaphorase activity is first order with respect to resazurin concentration up to 34 μM.

FIGURE 4

EFFECT OF RESAZURIN ON RATE OF REDUCTION (OBSERVED Δ FLUORESCENCE SIGNALS) USING DIAPHORASE AND NADPH

Legend

The reduction of resazurin by NADPH was followed by monitoring the change in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm). Reactions were carried out in a 3.0-mL cuvette at pH 8.7 and 25°C, using 0.011 DCPIP U/mL diaphorase and 7.8 x 10^{-5}M NADPH. Non-enzymatic rate of reduction of resazurin by NADPH was followed using 1% BSA instead of diaphorase and was substracted from the total rate (enzymatic + non-enzymatic).

FIGURE 4

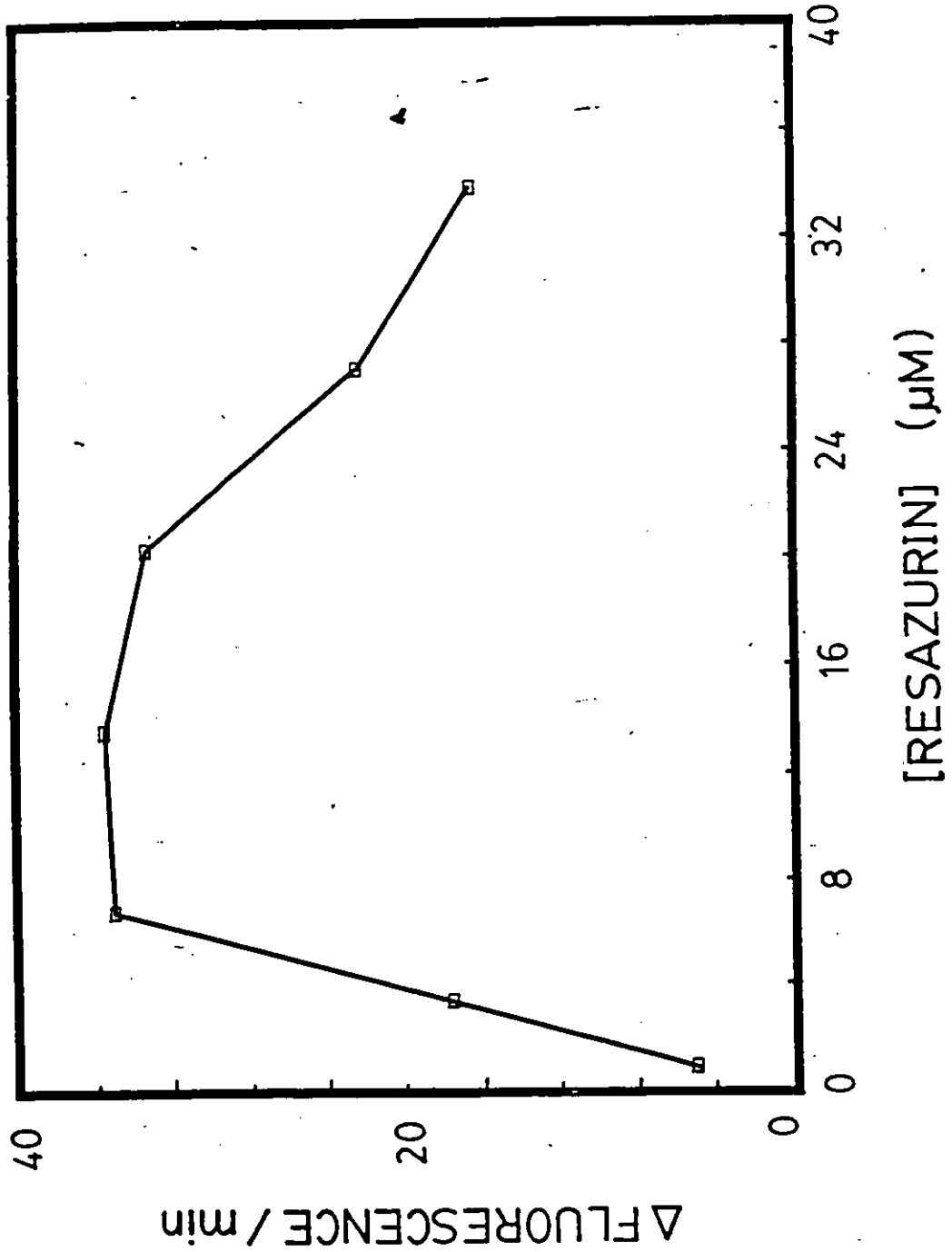


FIGURE 5

QUENCHING OF FLUORESCENCE OF RESORUFIN BY RESAZURIN

Legend

The relative fluorescence intensity of resorufin (3.1×10^{-6}M) was measured in duplicate in the presence of various amounts of resazurin, and compared to that in the absence of resazurin. Blank values (RFI of resazurin in absence of resorufin) were subtracted. Measurements were made in a 3.0-mL (□) and 1.0 mL (△) cuvette, at 25°C, pH 8.7.

FIGURE 5

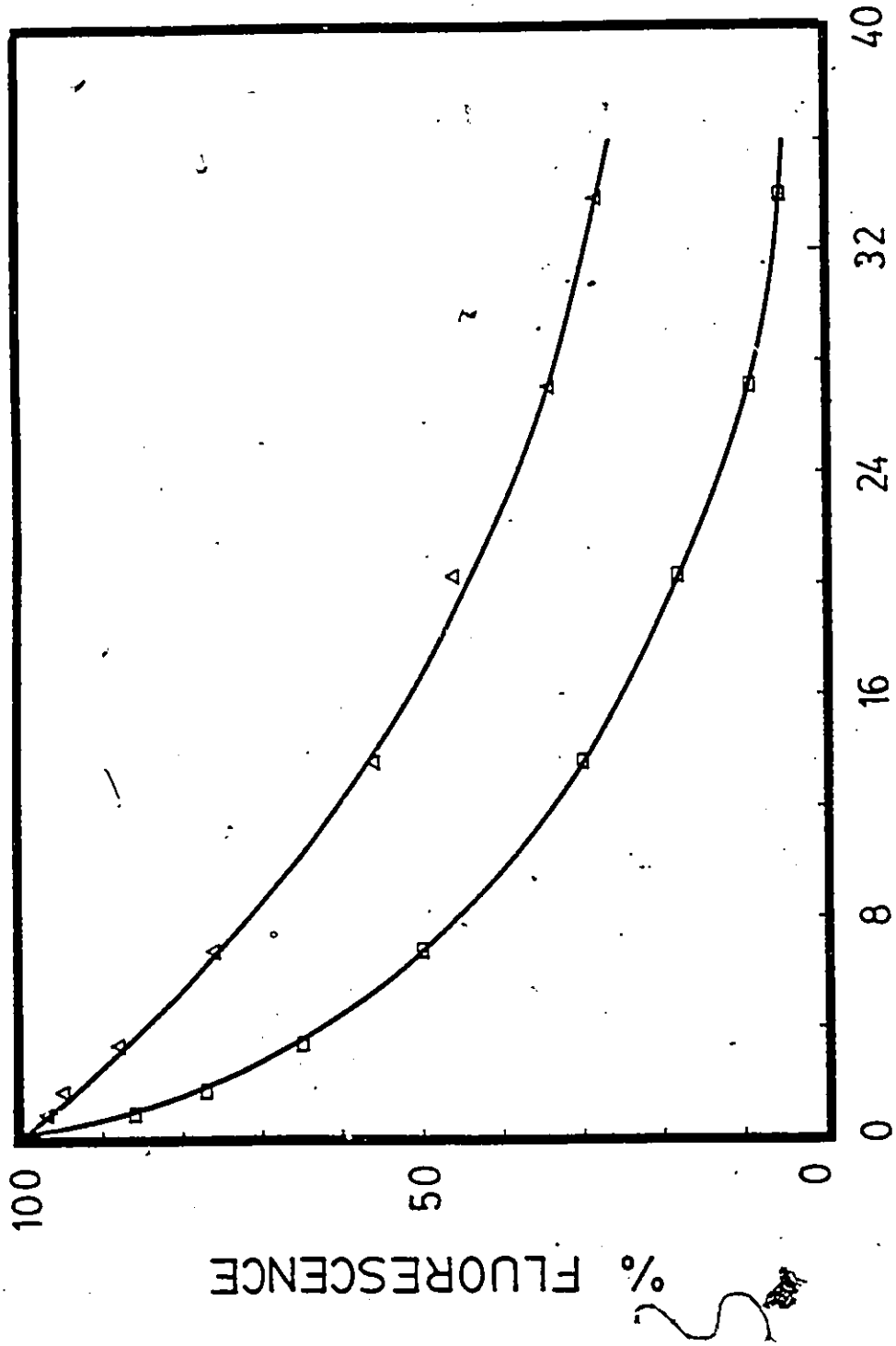


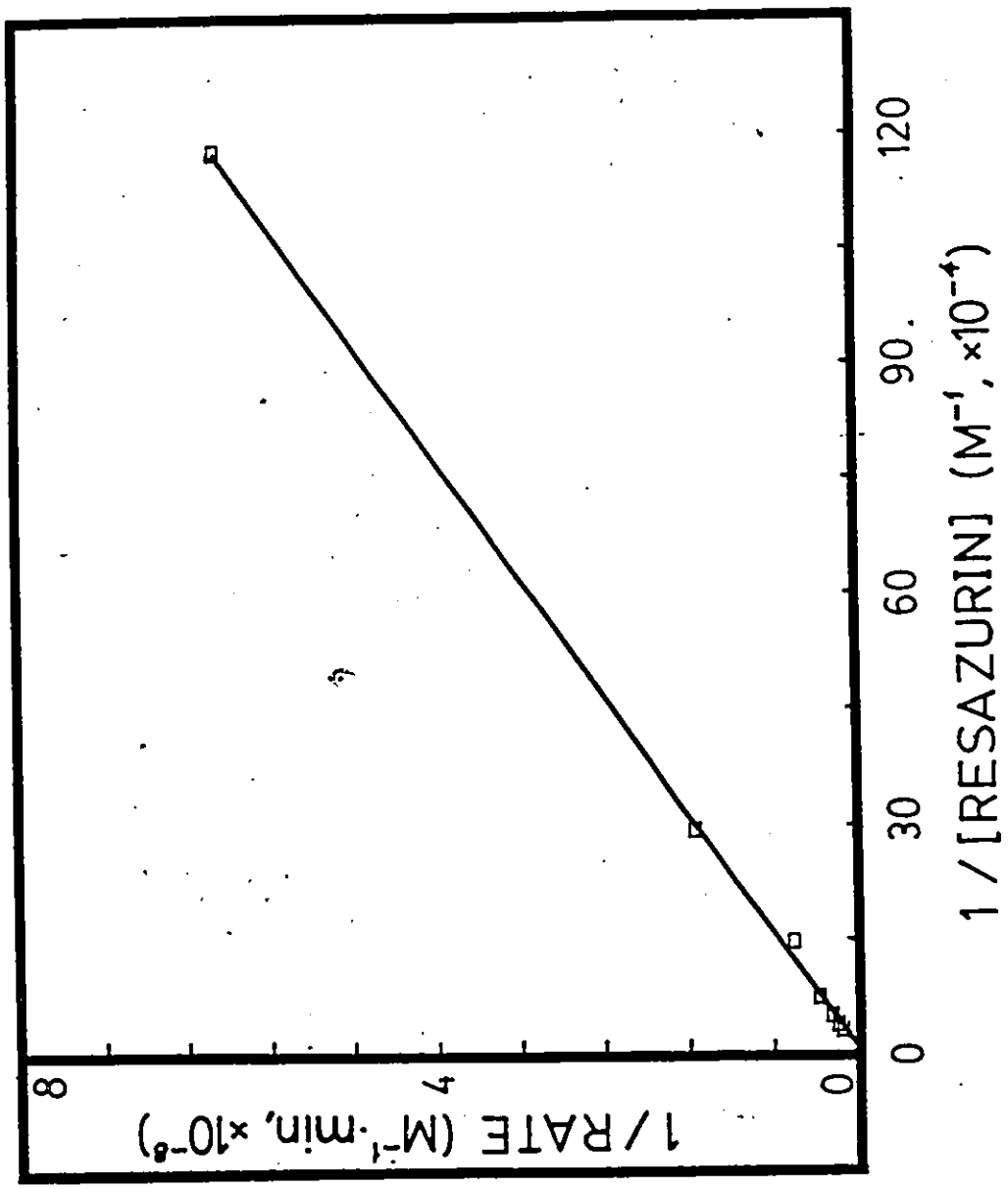
FIGURE 6

LINEWEAVER-BURK PLOT OF DIAPHORASE ACTIVITY WITH RESPECT TO RESAZURIN

Legend

Rates of reaction were followed by monitoring change in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm). Reactions were carried out in a 3.0-mL cuvette at pH 8.7 and 25°C, using 0.011 DCPIP U/mL diaphorase and 7.8 x 10^{-5}M NADPH. Non-enzymatic rate of reduction of resazurin by NADPH was followed using 1% BSA instead of diaphorase, and was subtracted from the total rate (enzymatic + non-enzymatic).

FIGURE 6



The lower signals at high concentrations of resazurin (Figure 4) can be explained in terms of increased rates of reaction and increased quenching as the amount of resazurin is increased. When a 1.0-mL cuvette was used, however, the quenching effect was reduced (see Figure 5). As a result, higher $\Delta F/\Delta t$ signals were observed at 34 μM resazurin than at 6.8 μM. The use of higher resazurin concentrations can, therefore, be justified by this fact.

The effect of NADPH concentration on diaphorase activity was studied at two different concentrations of resazurin. From the Hanes-Woolf plot (Figure 7), the $(K_m)_{app}$ of NADPH were found to be 1.31 (± 0.04) μM and 0.78 (± 0.04) μM at 34 μM and 6.8 μM resazurin, respectively. This differs from the findings of Guilbault et al. (53) who found diaphorase activity to be independent of NADH concentration at 6.8 μM resazurin. They, however, worked in an NADH concentration range, approximately 8 to 80 times the K_m we determined, i.e., zero order with respect to substrate.

Diaphorase activity, at 34 μM resazurin, was linear up to 1.4×10^{-3} units/mL (Figure 8). In this range, the specific activity using resazurin is on the order of 100 times less than that using DCPIP as a substrate.

3.2.4 Calibration Curves

Curve B of Figure 9 is a calibration curve of $\Delta F/\Delta t$ versus glucose 6-phosphate dehydrogenase (G6PDH) activity, using the conditions suggested by Guilbault et al (29,31) i.e., 6.8 μM resazurin and diaphorase at 0.013 DCPIP units/mL. In no part of the graph is the $\Delta F/\Delta t$ directly proportional to the amount of G6PDH.

Curve A is a second calibration curve using higher amounts of both resazurin and diaphorase (34 μM and 0.33 DCPIP units/mL, respectively). This curve gives a better separation of points and is clearly proportional up to 6×10^{-4} units/mL of G6PDH. In addition, the higher signals make the assay more

FIGURE 7

HANES-WOOLF PLOT OF DIAPHORASE ACTIVITY WITH RESPECT TO NADPH

Legend

Rates of reaction were followed by monitoring change in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm). Reactions were carried out in a 1.0-mL cuvette at pH 8.7 and 25°C, using 0.018 DCPIP U/mL diaphorase and two different concentrations of resazurin [6.7 µM (□) and 34 µM (Δ)]. Non-enzymatic rate of reduction of resazurin by NADPH was followed using 1% BSA instead of diaphorase, and was subtracted from the enzymatic rate.

FIGURE 7

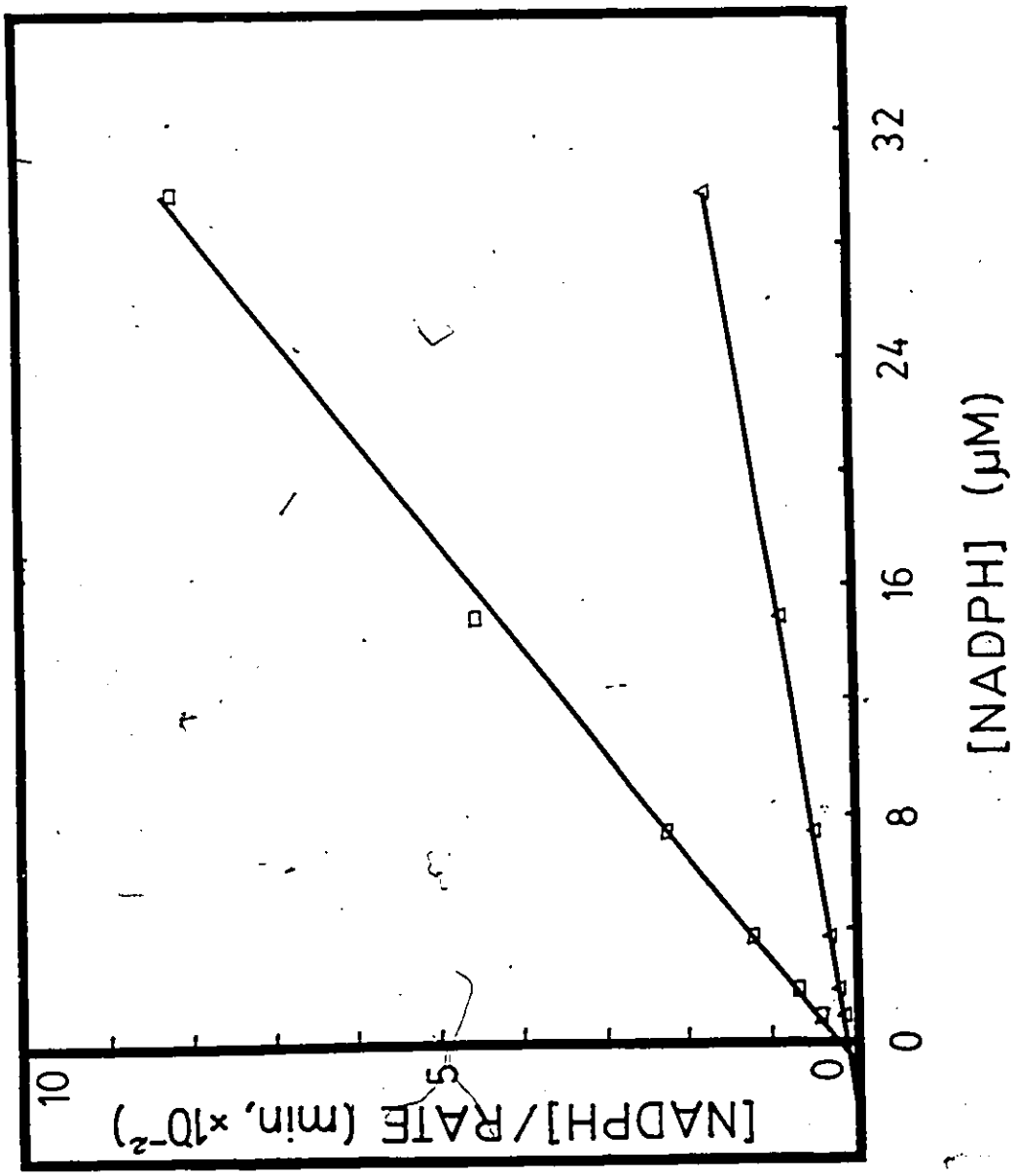


FIGURE 8

COMPARISON OF RESAZURIN AND DCPIP AS SUBSTRATES FOR DIAPHORASE

Legend

Diaphorase activity using DCPIP as a substrate was determined by following decrease in absorbance at 600 nm. Reactions were carried out at pH 7.5 and 25°C, using 39 μM DCPIP and 0.2 mM NADH. Non-enzymatic rates of reduction were subtracted.

Diaphorase activity using resazurin as a substrate was determined by monitoring the increase in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm). Reactions were carried out at pH 8.7 and 25°C, using 34 μM resazurin and 22 μM NADPH. Non-enzymatic rates of reduction were subtracted.

FIGURE 8

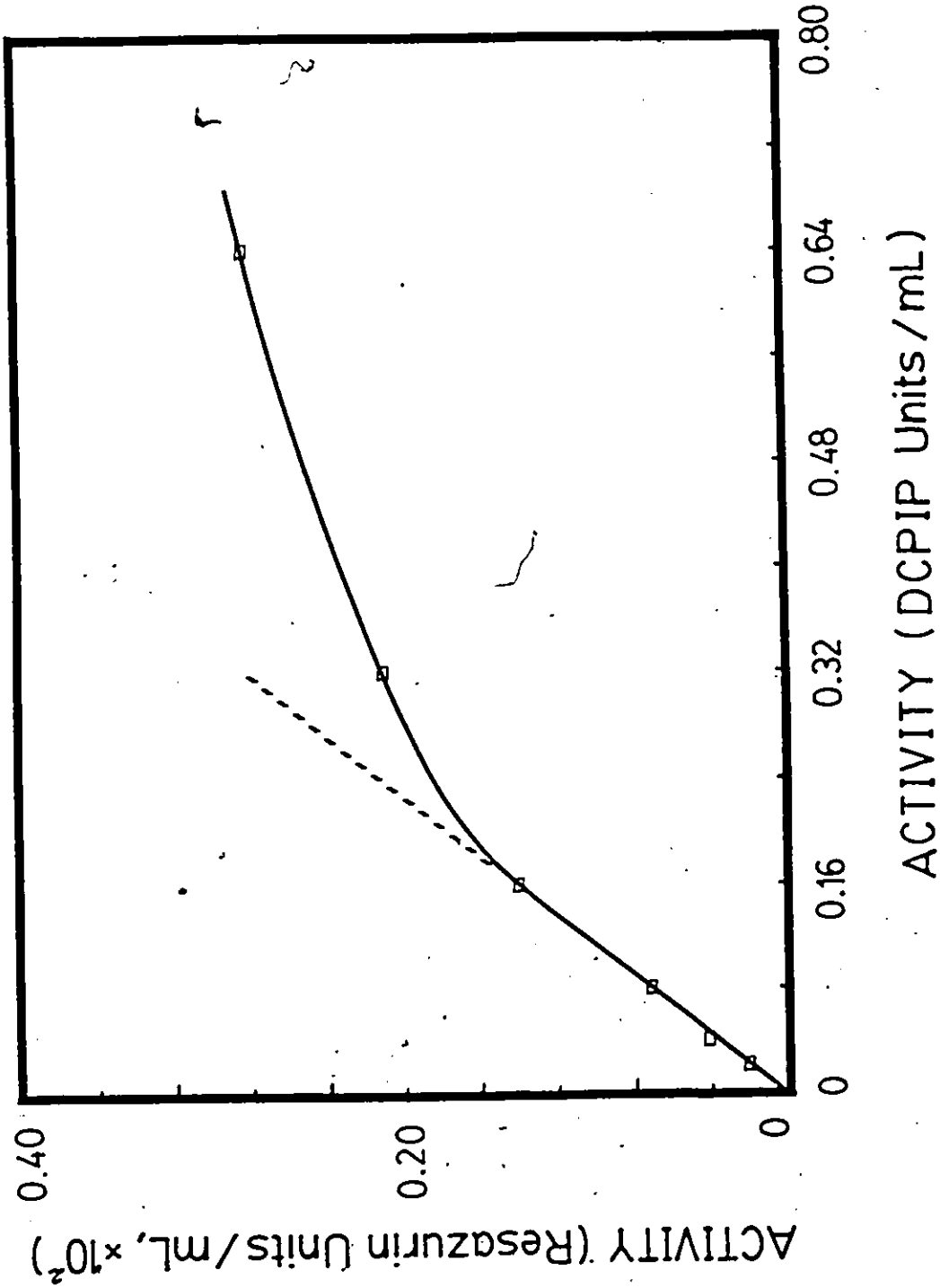

ACTIVITY (Resazurin Units/mL, ×10²)
0.40
0.20
0
ACTIVITY (DCPIP Units/mL)
0
0.16
0.32
0.48
0.64
0.80

FIGURE 9

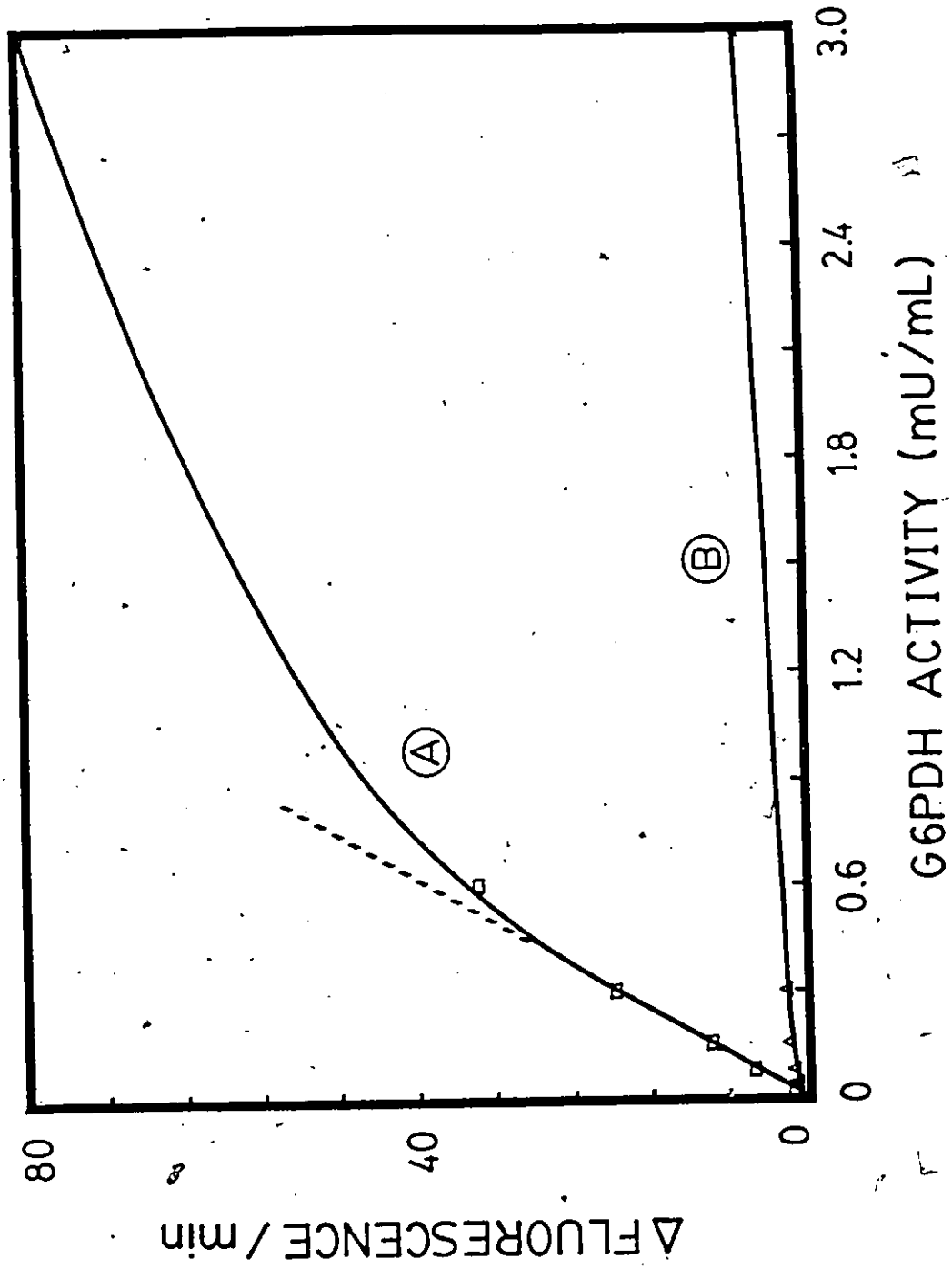
CALIBRATION CURVE OF Δ FLUORESCENCE/MIN VERSUS GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY

Legend

Dilutions of G6PDH were made in 1% BSA and assayed by monitoring the increase in absorbance at 340 nm. Reactions were carried out at pH 8.7 and 25°C, using 0.6 mM $NADP^+$ and 0.68 mM glucose 6-phosphate.

G6PDH solutions were re-assayed in duplicate by coupling the NADPH produced to the diaphorase/resazurin system and monitoring the increase in fluorescence (λ_{exc} = 568 nm, λ_{emit} = 582 nm). Two calibration curves were generated, one using 34 μM resazurin and 0.33 DCPIP U/mL diaphorase (Curve A), and the other using 6.8 μM resazurin and 0.013 DCPIP U/mL (Curve B). Reactions were carried out at pH 8.7 and 25°C, using 0.6 mM $NADP^+$ and 0.68 mM glucose 6-phosphate.

FIGURE 9


ΔFLUORESCENCE / min
80
40
0
0
0.6
1.2
1.8
2.4
3.0
G6PDH ACTIVITY (mU/mL)
A
B

sensitive, i.e., able to detect as low as 2×10^{-5} units/mL. Typically, Guilbault's assay of dehydrogenases (31) can detect on the order of 10^{-4} units/mL.

3.2.5 Stability of Resazurin in Water

Previous workers (29,32) have prepared resazurin solutions in cellosolve. Since this solvent is a teratogen, the possibility of using water as a solvent was investigated. It was first noted that resazurin has a higher solubility in water than cellosolve, allowing the more concentrated solution required for our procedure to be made.

The stability of resazurin in water was then investigated. Figure 10 illustrates the absorption spectra of freshly prepared equimolar (8.5 μM) resazurin and resorufin, and resazurin which had been kept in a brown bottle in the dark for six weeks. Resazurin has a peak at 600 nm, the wavelength chosen for the stability study. It would appear from the spectrum of the old resazurin, that it undergoes a slow conversion to resorufin in solution.

Although a decrease in absorbance of the resazurin solution over time was observed, the conversion of resazurin is slow enough (Figure 11) to allow the solution to be used for up to one month.

3.3 Determination of GALT Activity in Erythrocytes

3.3.1 Preliminary Studies

In the course of preliminary studies of the coupled assay for GALT, a significant reagent blank was discovered. By the process of elimination, it was discovered that it was substantially due to galactose 1-phosphate (GAL1P) and to a lesser extent, glucose 1,6-diphosphate (G1,6DP). Sigma Chemical Company confirmed that their GAL1P was contaminated with glucose 1-phosphate and

FIGURE 10

ABSORPTION SPECTRA OF RESORUFIN AND RESAZURIN

Legend

Spectra were generated using 8.5 μM solutions of resazurin and resorufin in water at 25°C. Freshly prepared resazurin solution (———), six-week old resazurin solution (– – – –) and freshly prepared resorufin solution (– ·– ·–).

FIGURE 10

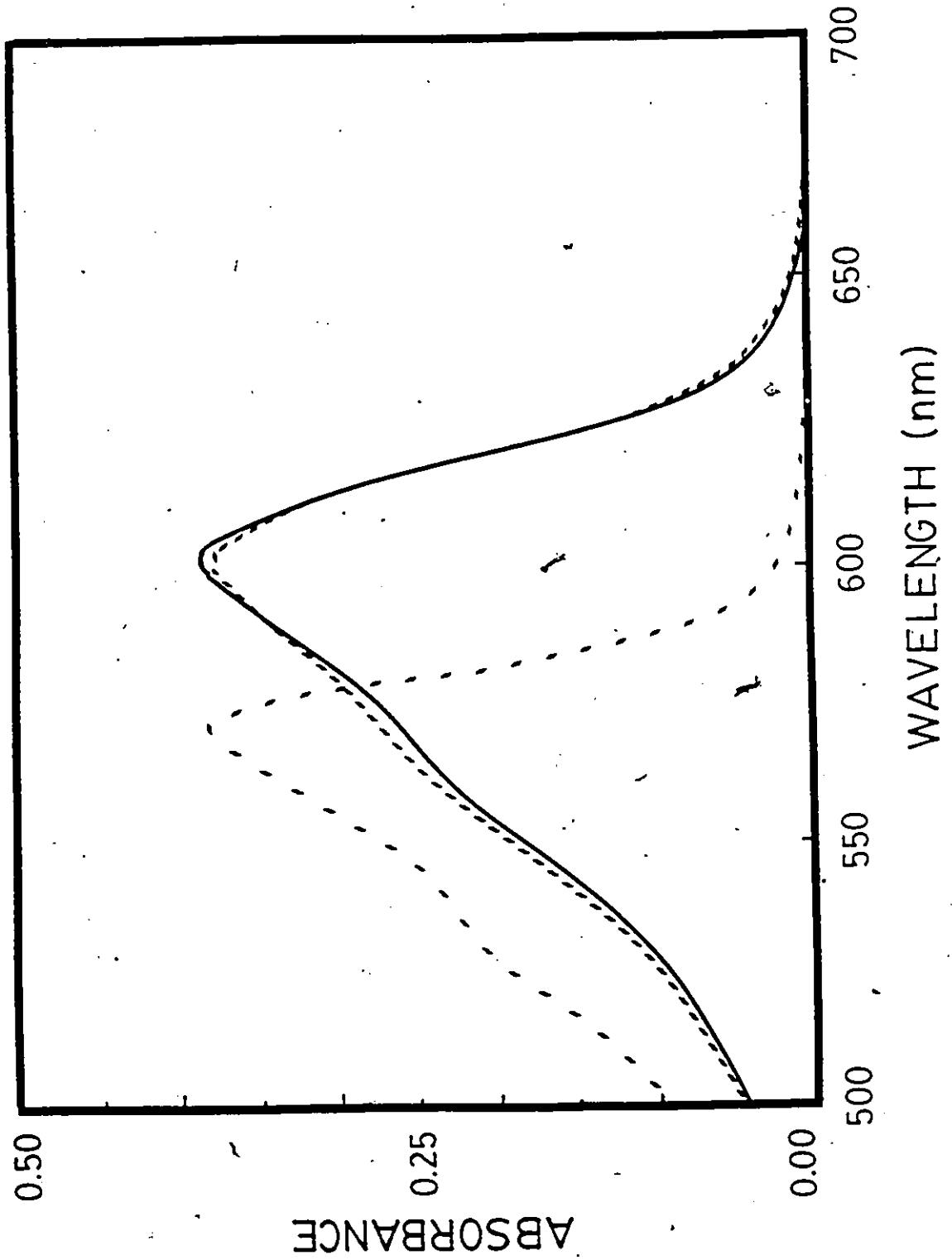


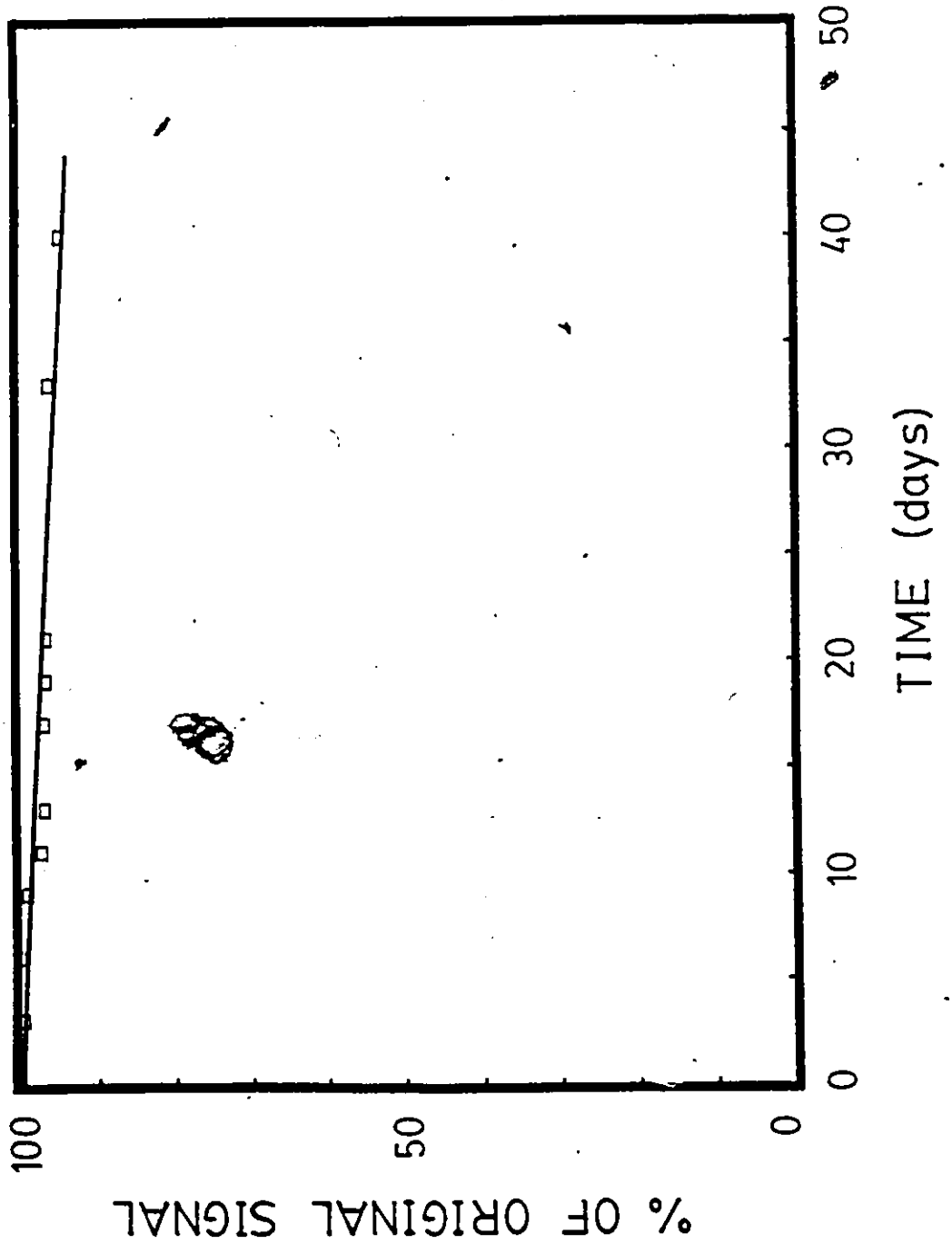
FIGURE 11

STABILITY OF RESAZURIN SOLUTION

Legend

The absorbance of resazurin (50 µL of a 0.85 mM solution added to 5.0 mL of water) was read at 600 nm. Absorbances are the average of duplicate readings and are represented as a percentage of the absorbance reading on the day of preparation of the stock solution.

FIGURE 11



glucose 6-phosphate, which can trigger the second and third reactions of the coupled assay as shown in Scheme 2 below:

SCHEME 2: Proposed Coupled Assay for GALT

$$\text{Galactose 1-Phosphate} + \text{UDPG} \xrightarrow{\text{GALT}} \text{Glucose 1-Phosphate} + \text{UDPGal}$$

$$\text{Glucose 1-Phosphate} \xrightarrow[\text{Glucose 1,6-Diphosphate}]{\text{Phosphoglucomutase}} \text{Glucose 6-Phosphate}$$

$$\text{Glucose 6-Phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-Phosphogluconate} + \text{NADPH} + \text{H}^+$$

$$\text{6-Phosphogluconate} + \text{NADP}^+ \xrightarrow{\text{6-PGADH}} \text{Ribulose 5-Phosphate} + \text{NADPH} + \text{H}^+$$

$$2\ \text{Resazurin} + 2\text{NADPH} + 2\text{H}^+ \xrightarrow{\text{Diaphorase}} 2\ \text{Resorufin} + 2\text{NADP}^+ + 2\text{H}_2\text{O}$$

Synthetic galactose 1-phosphate was obtained at a later date from Biosynth Ag., in the hopes of eliminating the problem of the reagent blank. However, it was found that this preparation was also contaminated, and for all future studies it was necessary to include a blank, in which UDPG was omitted from the reaction mixture.

Preliminary studies using hemolysates produced $\Delta F/\Delta t$ signals which were lower than anticipated and the possibility of quenching by the hemolysate was investigated. The fluorescence coefficient of resorufin was found to be lower in the presence of hemolysate. Figure 12 shows the absorption spectrum of hemolysate. There is a double peak at 540 nm and 576 nm, in the region of both the excitation and emission spectra of resorufin. The fact that the peaks correspond to oxyhemoglobin (54) raised the problem of variable quenching from sample to sample, since each sample would have different amounts of hemoglobin.

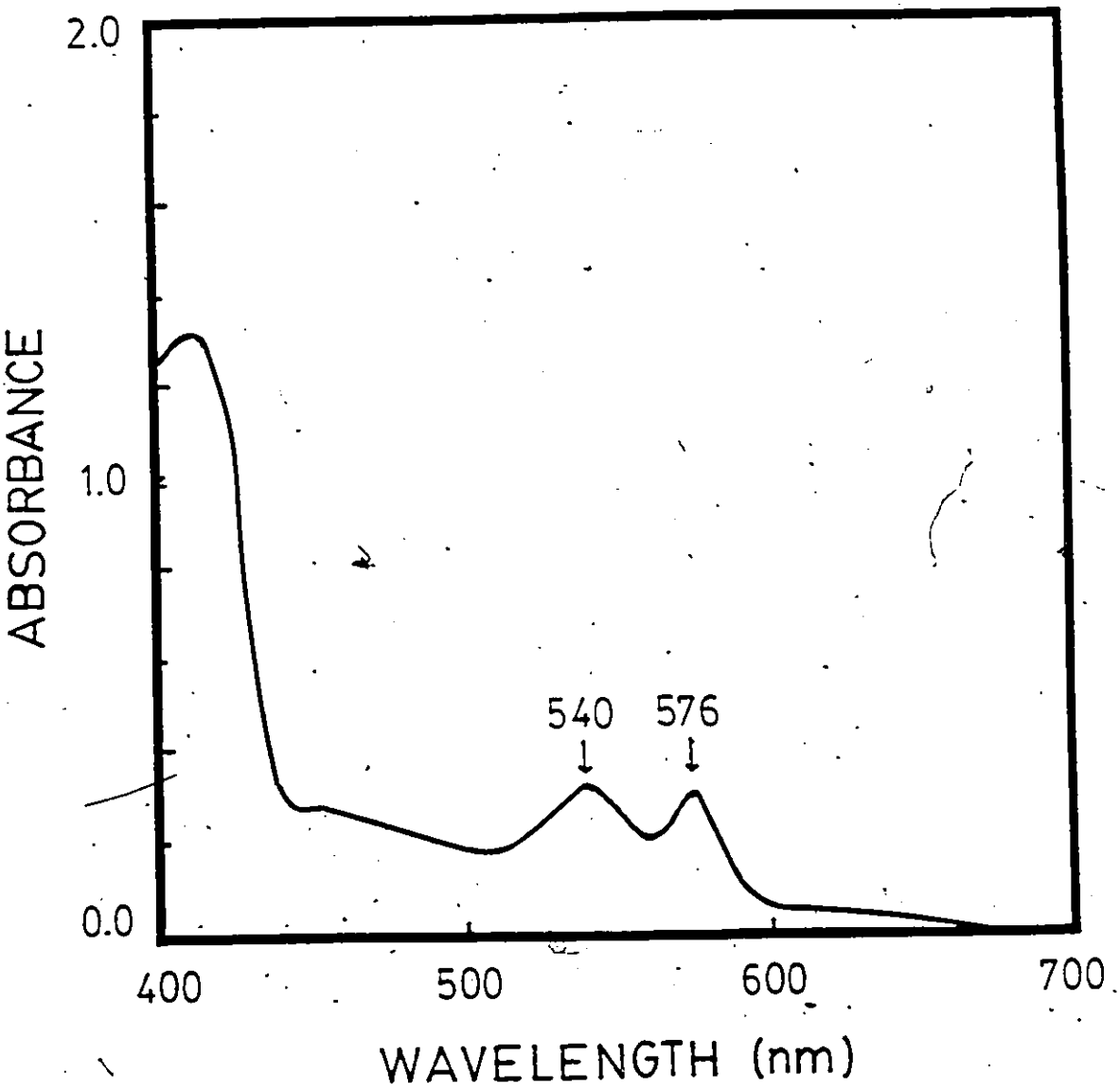
FIGURE 12

ABSORPTION SPECTRUM OF HEMOLYSATE

Legend

A dilute solution was made by adding 10 μL of 1:1 hemolysate (14.9 g Hb/ 100 mL) to 1.0 mL of glycine buffer, pH 8.7. Spectrum was recorded at 25°C.

FIGURE 12



It was, therefore, decided that a calibration curve not be used for the determination of GALT activity. Rather, the fluorescence coefficient of resorufin would be determined for each sample with the use of an internal standard, i.e., a known amount of resorufin is added to reagent mixture containing buffer, resazurin and sample and its fluorescence intensity is measured. The internal standard is subsequently used in the calculation of GALT activity. (See APPENDIX B.)

Figure 13 indicates that the emission spectrum of resorufin is not changed in the presence of hemolysate and resazurin. However, the excitation spectrum is altered, showing two peaks, one at 520 nm and one at 565 nm.

3.3.2 Optimization of GALT Assay

Optimization was carried out on hemolysates prepared from pooled venous blood samples. The "optimum" concentration of each component of the assay was determined by holding all others constant. Each optimum found was then used in the optimization of the next component. A temperature of 37°C was used throughout.

3.3.2.1 Sample Size

Table III lists the change in fluorescence signal observed when various amounts of hemolysate were analyzed. Maximum rate was observed when 10 µL of 1:1 hemolysate (14.2 g/100 mL) was used. This amount constitutes a compromise between greater amounts of GALT and increased quenching, with increasing sample size.

FIGURE 13

FLUORESCENCE SPECTRA OF RESORUFIN IN THE PRESENCE OF HEMOLYSATE AND RESAZURIN

Legend

Fluorescence spectra of resorufin in the presence of 20 µL 1:1 hemolysate/mL and 34 µM resazurin (———). Spectra of hemolysate and resazurin (– – –).

The emission spectra were generated first using exciting light of 520 nm. The excitation spectra were then generated by monitoring the fluorescence at 585 nm.

All spectra were generated at pH 8.7 and 37°C.

FIGURE 13

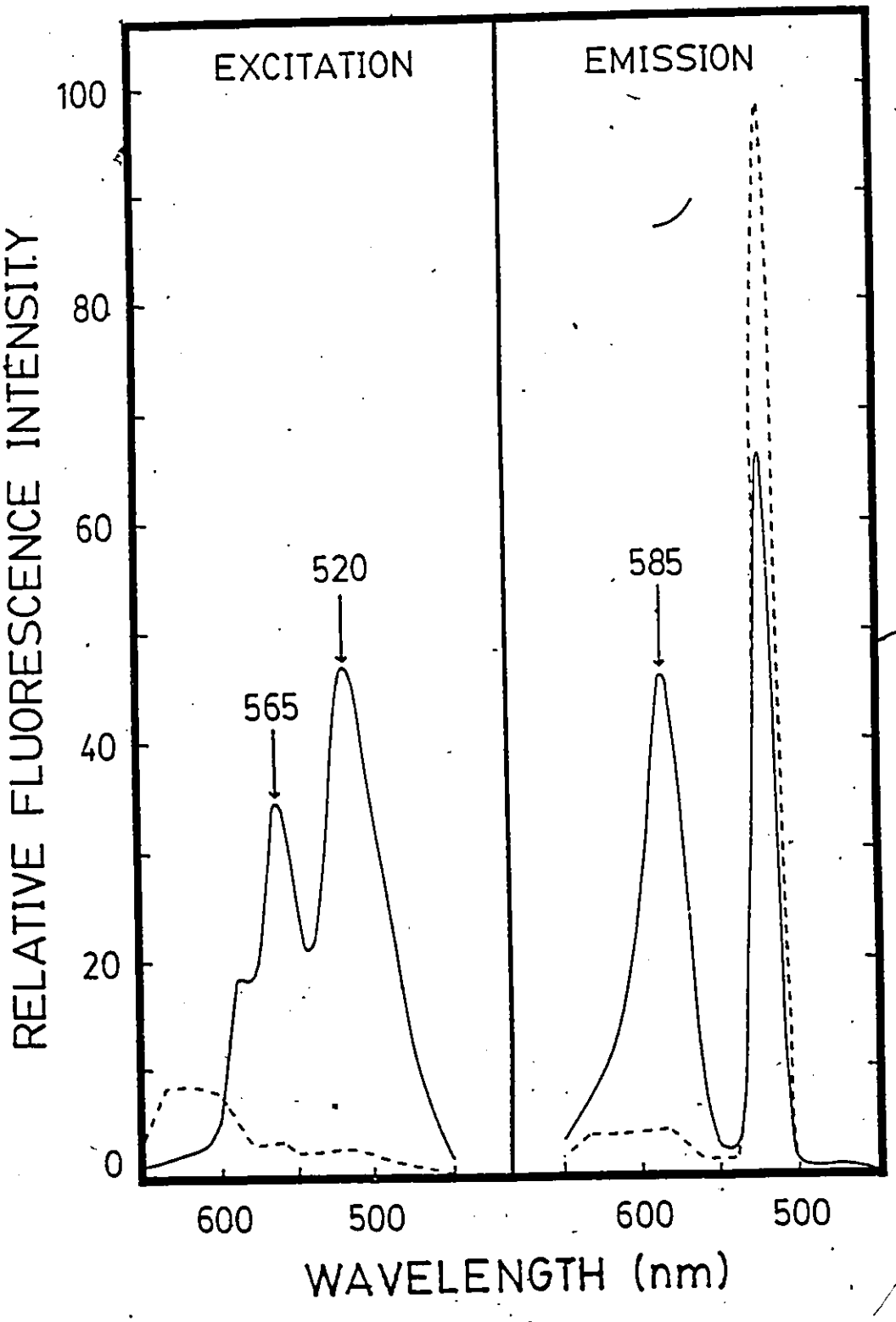

EXCITATION
EMISSION
100
80
60
40
20
0
RELATIVE FLUORESCENCE INTENSITY
520
565
585
600
500
600
500
WAVELENGTH (nm)

TABLE III

EFFECT OF DILUTION OF HEMOLYSATE ON OBSERVED SIGNAL IN GALT ASSAY

Sample[a]	Amount used (μL)[b]	Net ΔF/min[c]
1:1 Hemolysate	20	2.4
1:1 Hemolysate	10	8.2
1:3 Hemolysate	10	8.5
1:7 Hemolysate	10	4.4

(a) Ratio refers to erythrocyte volume: water volume.

(b) In a total of 500 μL assay mixture.

(c) Determined in duplicate by $\Delta F_{sample} - \Delta F_{blank}$

3.3.2.2 Activators

Reviewers of the reference method (5) suggested that the hemolysates be pre-incubated with 26 mM dithiothreitol (DTT) to reactivate GALT. When this procedure was tried with the present assay, an increase in the blank was observed. It was consequently discovered that the DTT could non-enzymatically reduce resazurin to produce resorufin. It was decided, therefore, to omit this pre-incubation step.

Williams et al. (12) found that the stability of GALT from erythrocytes was about the same in the absence of DTT as that in its presence up to about eight minutes of incubation at 37°C. Since our assay requires only six to seven minutes, omission of DTT reactivation does not pose a serious problem.

In addition, if the samples are assayed soon enough, GALT retains most of its activity (see Section 3.3.5) and does not require reactivation.

3.3.2.3 Buffer Solution

The buffer solution was comprised of three components: glycine, EDTA and $MgCl_2$. Buffer solutions were prepared by dissolving appropriate amounts of each component in water, adjusting the pH at 25°C with NaOH and making up to volume.

The effect of pH of the buffer was first studied. A broad peak (Figure 14) was observed, with maximum activity between pH 8.3 and 8.7. A pH of 8.7 was chosen due to the fact that this is the one most often quoted for GALT activity in erythrocytes.

Concentrations of up to 200 mM glycine in the buffer solution were used and maximum activity was found at about 100 mM.

FIGURE 14

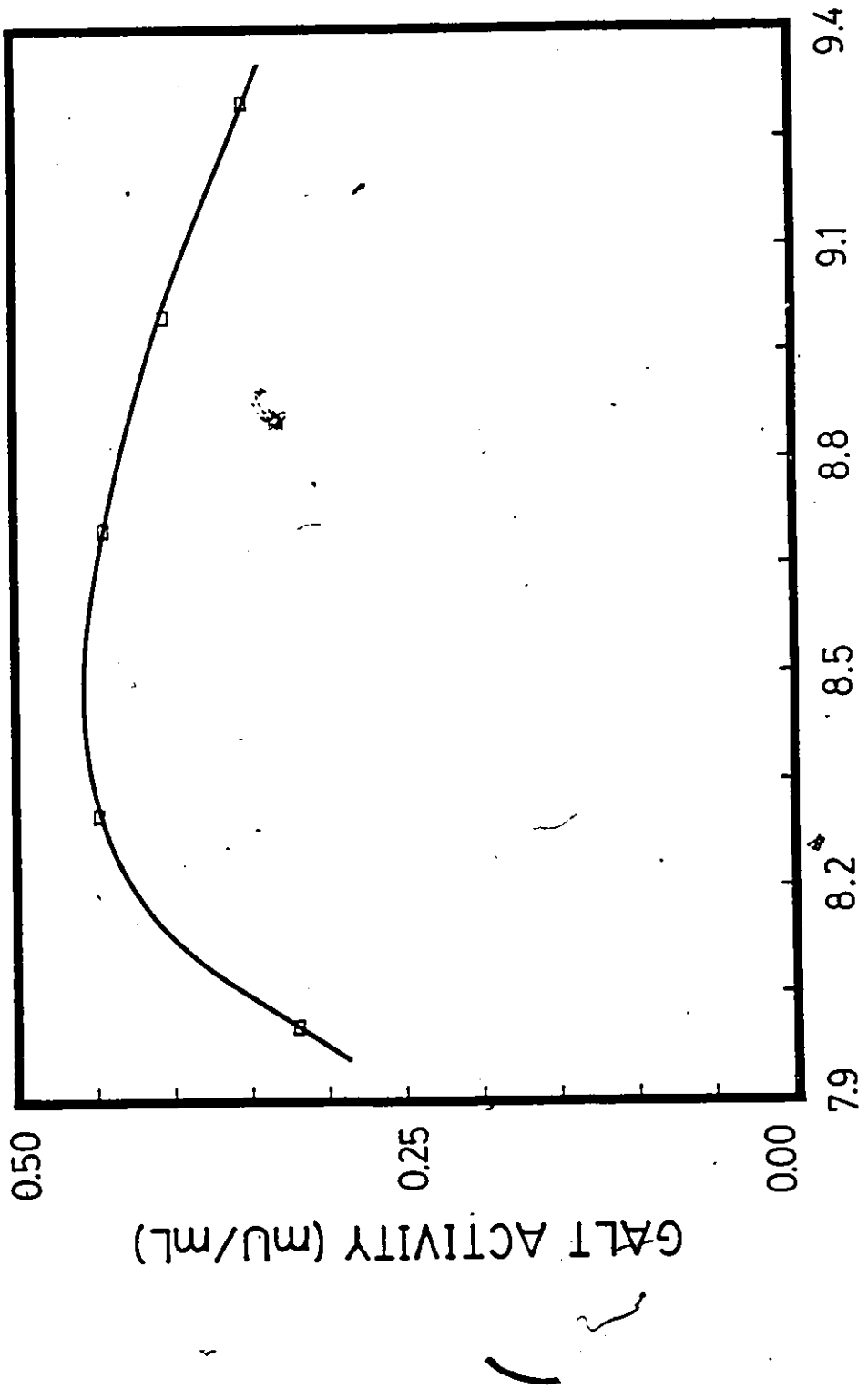
pH STUDY OF GALT ACTIVITY

Legend

All buffer solutions were made to be 100 mM glycine, 3.3 mM $MgCl_2$ and 2.0 mM EDTA, and adjusted to appropriate pH values at 25°C.

GALT activity in a pooled hemolysate sample was determined in duplicate by the present coupled assay. To 410 µL of buffer solution at 37°C were added 10 µL of hemolysate, 20 µL of resazurin (0.85 mM), 20 µL of UDPG (27.5 mM) and 20 µL of enzyme solution (1.0 U/mL PGM, 9.0 U/mL G6PDH, 16 DCPIP U/mL diaphorase). The fluorescence of this solution was set to "zero", after which 10 µL of resorufin (0.20 mM) was added and the RFI recorded. The GALT reaction was initiated by adding 10 µL of substrate solution (0.12 M GAL1P 0.02M $NADP^+$, 1.0 mM G1,6DP), and followed at 37°C by monitoring the rate of increase of fluorescence (λ_{exc} = 565 nm, λ_{emit} = 585 nm). Blank rates were determined by replacing UDPG with water, and were subtracted.

FIGURE 14



EDTA concentration in the buffer solution was varied between 0 and 2.0 mM. With 0.5 mM EDTA in the buffer, GALT activity was maximum.

Figure 15 shows the effect of [$MgCl_2$] on GALT activity. Inhibition of GALT activity was seen at all concentrations of $MgCl_2$, and it was, therefore, omitted from the assay mixture.

3.3.2.4 Cofactors

The effect of glucose 1,6-diphosphate concentrations, up to 52 μM, was studied. Maximum GALT activity was seen at concentrations between 6.5 and 13 μM. At concentrations above 13 μM, activity decreased.

3.3.2.5 Coupling Enzymes

Due to the low specific activity of diaphorase, the reagent blank had to be kept to a minimum. This was done by decreasing the concentrations of phosphoglucomutase and glucose 6-phosphate dehydrogenase. This resulted in decreased blanks but longer lag phases. The compromise between unacceptable lag phases (> 4 min) and intolerable blanks gave rise to the concentrations chosen for these two auxiliary enzymes (PGM and G6PDH).

Pesce et al. (25) discussed the problem of adding extraneous 6-PGADH to their coupled assay for GALT. (This assay scheme is identical to ours except that it lacks the last reaction.) They indicated that 6-PGADH should not be added since it causes an inhibition of GALT activity. However, they go on to argue that in normal erythrocytes, and even in 6-PGADH-deficient samples, the activity of 6-PGADH is sufficient to produce the second mole of NADPH.

FIGURE 15

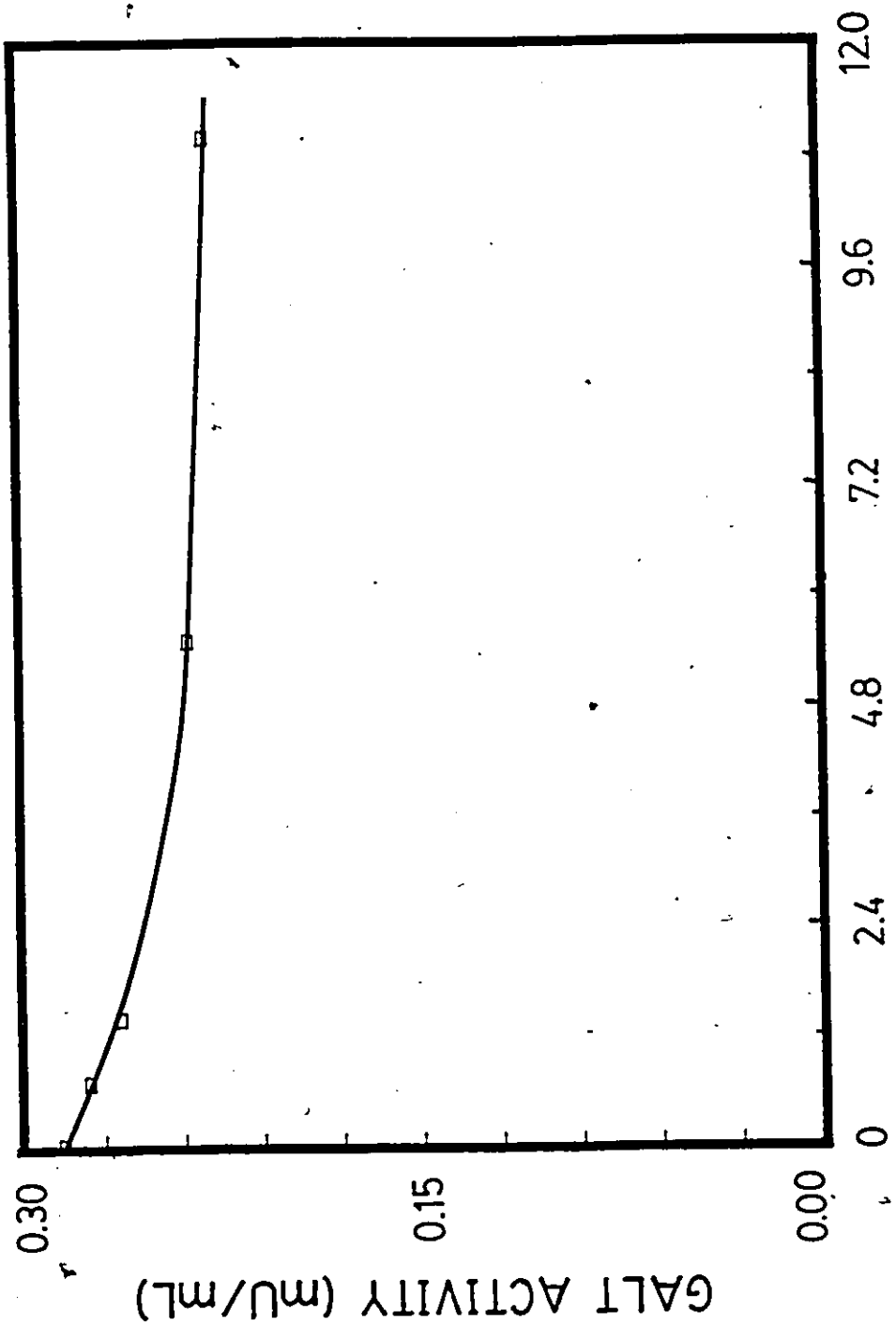
EFFECT OF $MgCl_2$ ON GALT ACTIVITY

Legend

Buffer solutions were made to be 100 mM glycine, 0.5 mM EDTA and contained varying amounts of $MgCl_2$. Concentrations shown are those in the final assay mixture.

GALT activity in a pooled hemolysate sample was determined in duplicate by the present coupled assay. To 410 µL of buffer solution at 37°C were added 10 µL of hemolysate, 20 µL of resazurin (0.85 mM) 20 µL of UDPG (27.5 mM), and 20 µL of enzyme solution (1.0 U/mL PGM, 9.0 U/mL G6PDH, 16 DCPIP U/mL diaphorase). The fluorescence of this solution was set to "zero", after which 10 µL of resorufin (0.21 mM) was added and the RFI recorded. The GALT reaction was initiated by adding 10 µL of substrate solution (0.12 M GAL1P, 0.02 M $NADP^+$, 1.0 mM G1,6DP) and followed at 37°C by monitoring the rate of increase of fluorescence (λ_{exc} = 565 nm, λ_{emit} = 585 nm). Blank rates were determined by replacing UDPG with water, and were subtracted.

FIGURE 15



This was tested in our system by adding an amount of 6-PGADH (600 U/L of hemolysate) which would cause minimum inhibition of GALT activity (25). GALT activity was about 8% lower than when 6-PGADH was omitted from the sample. This indicates that the endogenous 6-PGADH is sufficient to produce the second mole of NADPH; otherwise, the added 6-PGADH would have increased the GALT activity.

Figure 16 shows the effect of adding different amounts of diaphorase to the assay mixture. Maximum GALT activity was seen at diaphorase concentrations of 0.32 U/mL for both normal and deficient samples.

3.3.2.6 Substrates

$NADP^+$ was varied from 0.03 mM to 0.94 mM, and no significant difference in GALT activity was observed in that range.

Table IV shows the effect of resazurin concentration on GALT activity. Maximum activity was seen at concentrations of 34 μM and greater. At lower concentrations, the reduced GALT activity is probably due to the decrease in diaphorase activity which becomes rate-limiting.

The Michaelis constants for UDPG and GAL1P were determined using two different pooled hemolysates. By Hanes-Woolf analysis (Figure 17), the K_m for GAL1P was found to be 0.57 mM (± 0.02 mM) and 0.64 (± 0.19 mM). These values are higher, but agree well with values obtained by other workers (25). A concentration of 2.3 mM GAL1P (about 4 x K_m) was chosen for the GALT assay.

FIGURE 16

EFFECT OF DIAPHORASE ON GALT ACTIVITY

Legend

GALT activity in a normal sample (□) and deficient sample (Δ) was determined in duplicate using the present coupled assay.

To 410 µL of buffer solution (100 mM glycine, 0.5 mM EDTA, pH 8.7) at 37°C, were added 10 µL of sample, 20 µL of resazurin (0.85 mM), 20 µL of UDPG (27.5 mM) and 10 µL of enzyme solution (1.5 U/mL PGM, 20 U/mL G6PDH) and 10 µL of diaphorase (varying concentrations). The fluorescence of this solution was set to "zero", after which 10 µL of resorufin (0.23 mM) was initiated by adding 10 µL of substrate solution (0.12 M GAL1P, 0.024 M $NADP^+$, 0.64 mM G1,6DP), and followed at 37°C by monitoring the rate of increase of fluorescence (λ_{exc} = 520 nm, λ_{emit} = 585 nm). Blank rates were determined by replacing UDPG with water, and were subtracted.

FIGURE 16

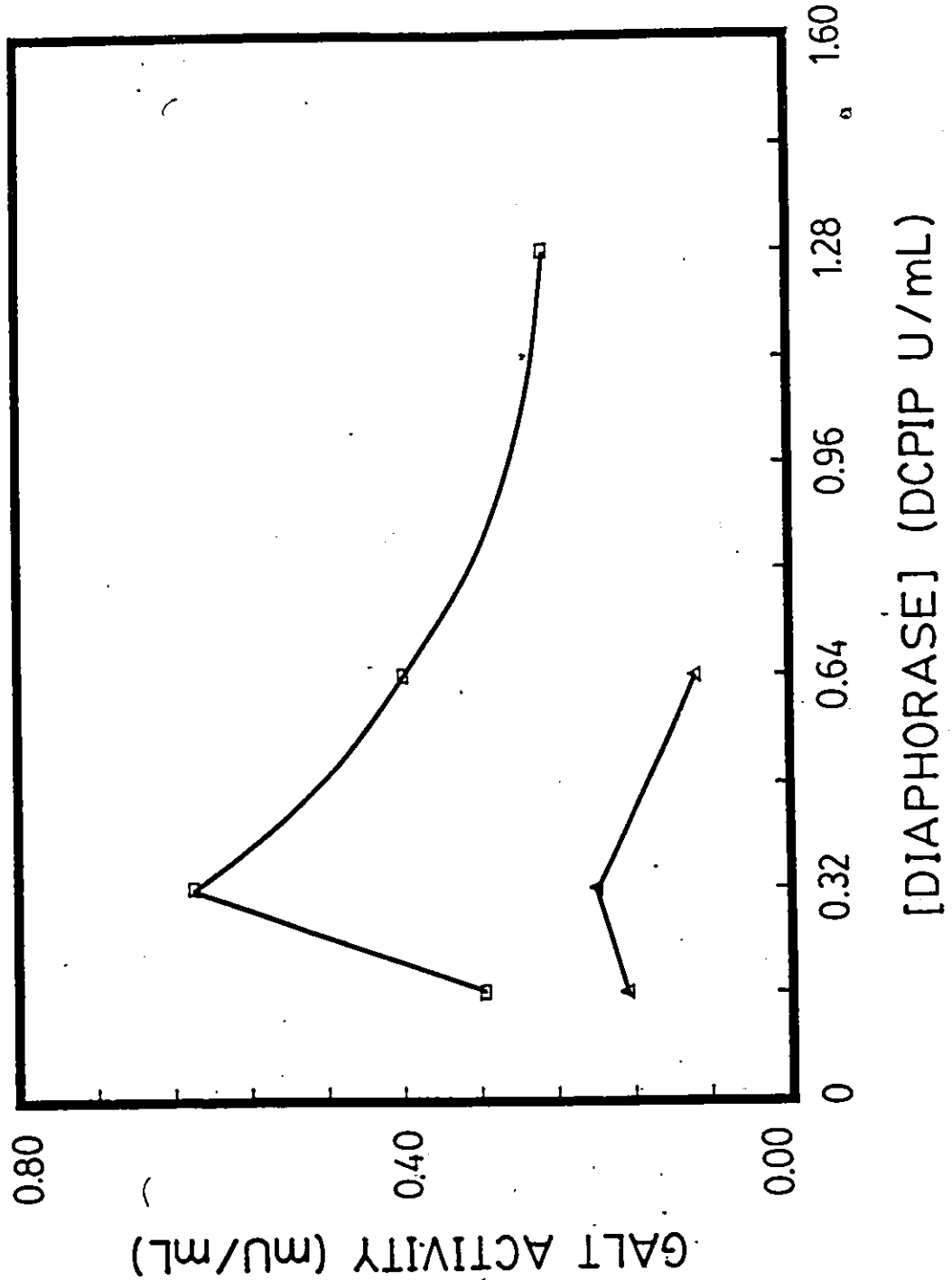

GALT ACTIVITY (mU/mL)
0.80
0.40
0.00
0
0.32
0.64
0.96
1.28
1.60
[DIAPHORASE] (DCPIP U/mL)

TABLE IV

EFFECT OF RESAZURIN ON GALT ACTIVITY

Resazurin (μM)	GALT activity[(a)] (U/mL, x 10^4)
68	5.04
34	4.78
17	3.80
8.5	2.54

(a) Activity was determined in duplicate using the present coupled assay. To 410 μL of buffer solution (100 mM glycine, 0.5 mM EDTA, pH 8.7) at 37°C, were added 10 μL of hemolysate, 20 μL of resazurin (varying concentrations), 20 μL of UDPG (27.5 mM) and 20 μL of enzyme solution (0.75 U/mL PGM, 10 U/mL G6PDH, 8.0 DCPIP U/mL diaphorase). The fluorescence of this solution was set to "zero", after which 10 μL of resorufin (0.20 mM) was added and the RFI recorded. The GALT reaction was initiated by adding 10 μL of substrate solution (0.12 M GAL1P, 0.024 M $NADP^+$, 0.64 mM G1,6DP), and followed at 37°C by monitoring the rate of increase of fluorescence (λ_{exc} = 520 nm, λ_{emit} = 585 nm). Blank rates were determined by replacing UDPG with water, and were subtracted.

FIGURE 17

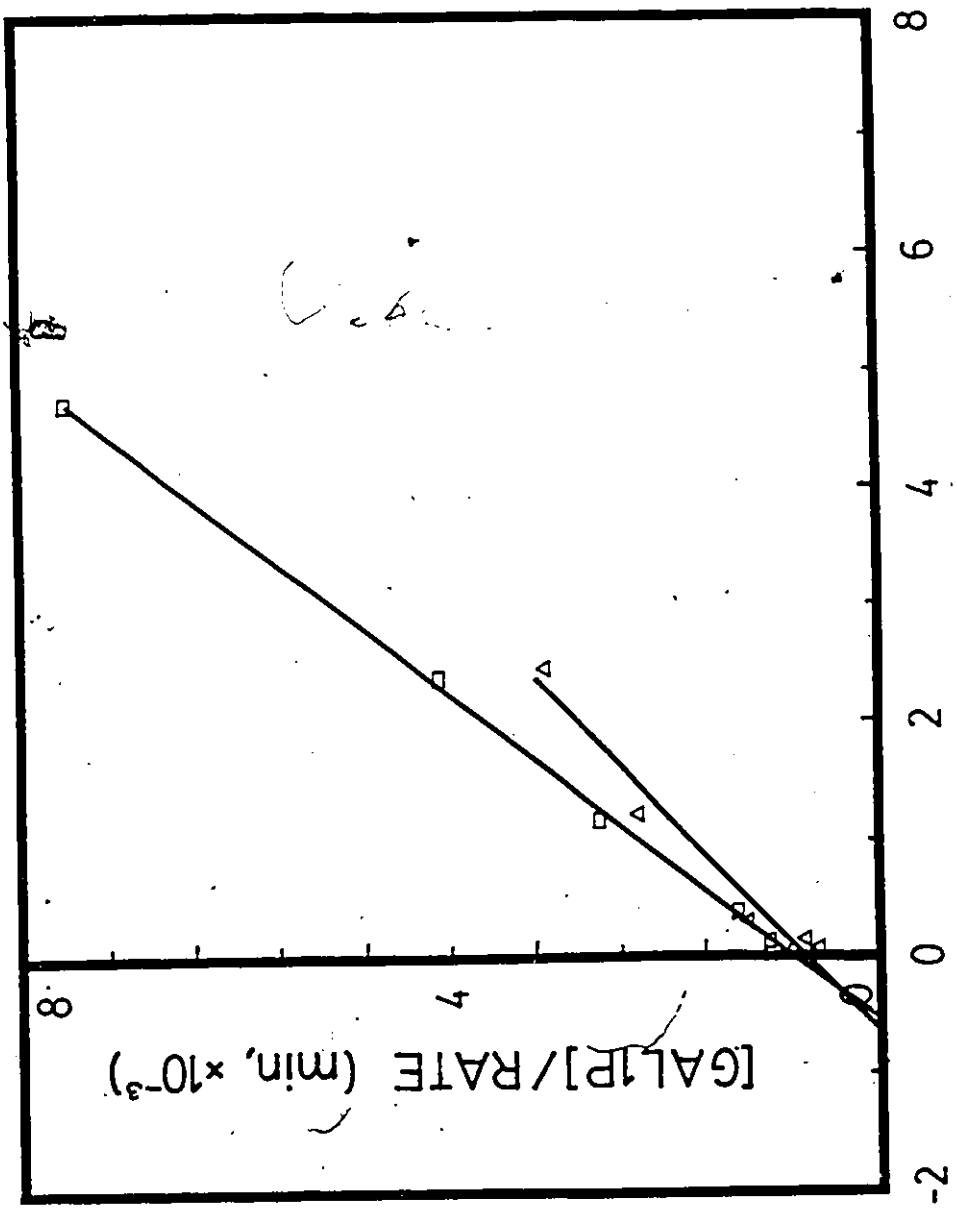
HANES-WOOLF PLOT OF GALT ACTIVITY

WITH RESPECT TO GALACTOSE 1-PHOSPHATE

Legend

GALT activity in two normal pooled samples was determined using the present coupled assay in the presence of 1.30 mM UDPG and varying amounts of GAL1P. Blanks were determined at each GAL1P concentration. Procedure followed was that described in Chapter 2, Section 2.2.3.2 c., p. 23.

FIGURE 17



By Hanes-Woolf analysis (Figure 18), the K_m for UDPG was found to be 0.081 mM (± 0.002 mM) and 0.083 mM (± 0.017 mM). This value is lower but agrees well with the K_m quoted by Tedesco and Mellman (2), but is significantly lower (2-fold less) than that quoted by others (25). A UDPG concentration of 1.10 mM (about 14 x K_m) was chosen for our assay. At higher concentrations, GALT activities were lower.

Table V provides a summary of the optimum conditions of the present coupled assay for GALT.

3.3.2.7 The Internal Standard

As discussed previously (see Preliminary Studies, Section 3.3.1, p. 53), the necessity of using an internal standard arose as a result of the variable quenching of the resorufin fluorescence caused by the variation of hemoglobin from sample to sample.

In the initial stages of the optimization, the internal standard was made by adding the standard amount of resorufin to the assay mixture itself in order to make the procedure easier. The procedure followed was as described in CHAPTER 2, Section 2.3.2.2 c., p. 23 with the following modifications: 1) everything except the "substrate" solution was added to a cuvette and allowed to incubate for several minutes at 37°C; 2) cuvette was placed in fluorometer and the instrument was "zeroed"; 3) the resorufin was added and the RFI measured; 4) the "substrate" solution was then added to the cuvette; 5) the mixture was allowed to incubate at 37°C for 3 minutes and then the rate of increase in fluorescence measured; 6) steps 1-5 were repeated for the blank by substituting water for UDPG.

FIGURE 18

HANES-WOOLF PLOT OF GALT ACTIVITY WITH RESPECT TO UDPG

Legend

GALT activity in two normal pooled samples was determined using the present coupled assay in the presence of 2.3 mM GAL1P and varying amounts of UDPG. Procedure followed was that described in CHAPTER 2, Section 2.2.3.2 c., p. 23.

FIGURE 18

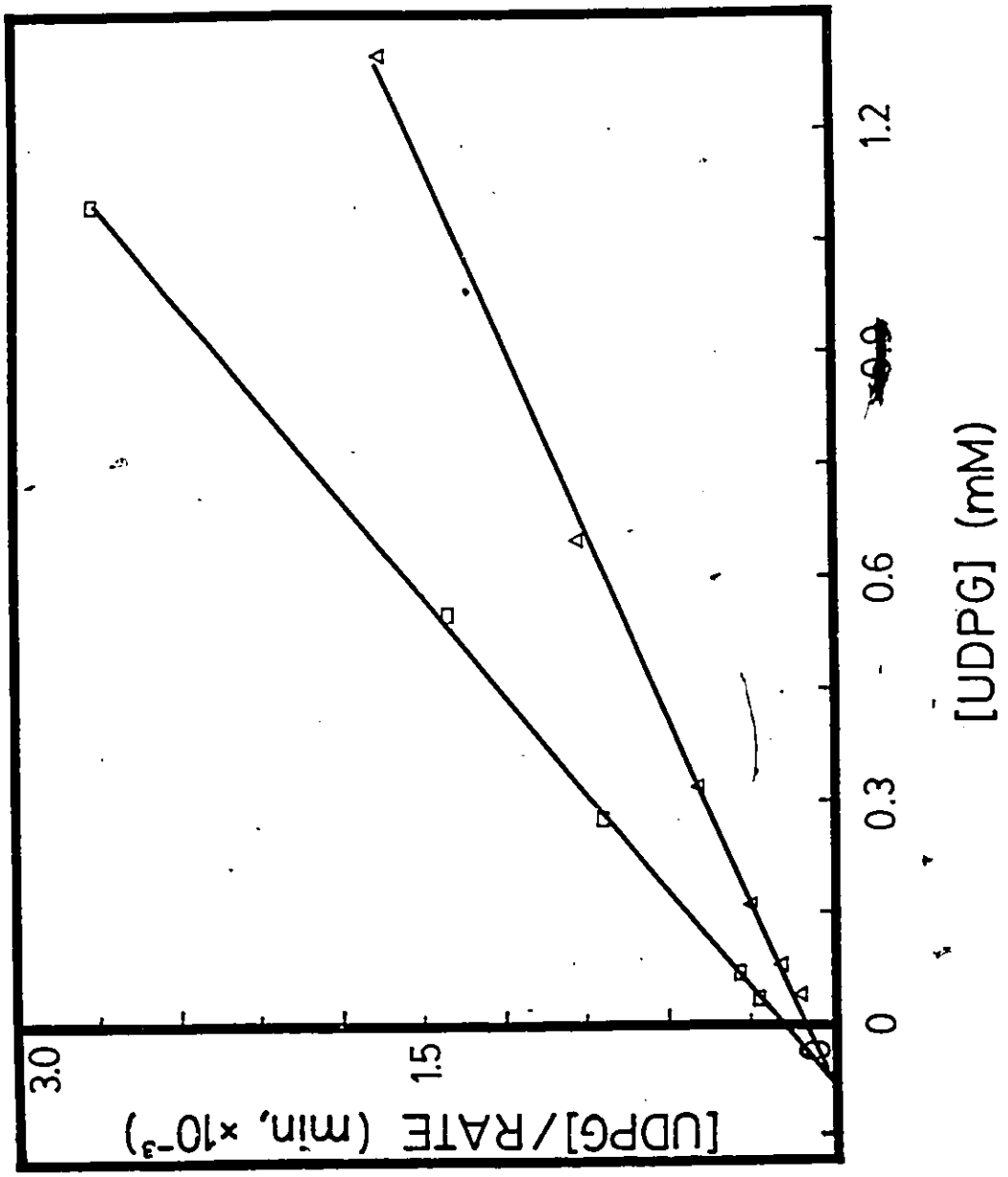


TABLE V

FINAL CONDITIONS FOR COUPLED ASSAY OF GALT

Component	Condition in assay
pH	8.7 (at 25°C)
Temperature	37°C
Glycine buffer	81 mM
EDTA	0.4 mM
Glucose 1,6-diphosphate	13 μM
$NADP^+$	0.47 mM
Resazurin	34 μM
UDPG	1.1 mM
Galactose 1-phosphate	2.3 mM
Phosphoglucomutase	0.03 U/mL
Glucose 6-phosphate dehydrogenase	0.40 U/mL
Diaphorase	0.32 DCPIP U/mL

As the optimization proceeded, it became clear that lower GALT activities than expected were being recovered. The effect of adding different amounts of resorufin was, therefore, tested and the results are shown in Figure 19. As can be seen, adding resorufin to the assay mixture directly caused a decrease in GALT activity, proportional to the concentration of resorufin.

It was believed that the phenomenon was due to the effect of the resorufin on diaphorase activity and the hypothesis was tested. Inhibition of diaphorase activity was observed (Figure 20) which was proportional to the amount of resorufin added.

It was obvious then that the internal standard had to be prepared separately rather than by adding the resorufin to the assay mixture itself. It was known that the resazurin and the sample itself quenched the resorufin signal and had to be present in the internal standard. To see whether any other component of the assay mixture had to be included, the fluorescence coefficient of resorufin in the presence of each of the UDPG, enzyme and substrate solutions was compared to that when only resazurin and sample were present. The results shown in Table VI, indicate that there is no further quenching of resorufin by any other component of the GALT assay mixture. In studies, thereafter, only the sample and resazurin were present in the internal standard, as described in CHAPTER 2, Section 2.3.2.2. c., p. 23. The amount of resorufin used in the internal standard was chosen so that a reasonable RFI was obtained at the same fluorometer settings used to monitor the GALT activity.

FIGURE 19

EFFECT OF ADDING RESORUFIN TO ASSAY MIXTURE ON GALT ACTIVITY

Legend

GALT activity in hemolysate was determined using the present coupled assay, as described in CHAPTER 3, Section 3.3.2.7, p. 69. To 410 µL of buffer solution (100 mM glycine, 0.5 mM EDTA, pH 8.7) at 37°C, were added 10 µL of hemolysate, 20 µL of resazurin (0.85 mM), 20 µL of UDPG (27.5 mM), and 20 µL of enzyme solution (0.75 U/mL PGM, to U/mL G6PDH, 8.0 DCPIP U/mL diaphorase). The fluorescence of this solution was set to "zero", after which 10 µL of resorufin (varying concentrations) was added and the RFI recorded. The GALT reaction was initiated by adding 10 µL of substrate solution (0.12 M GAL1P, 0.024 M $NADP^+$, 0.64 mM G1,6DP), and followed at 37°C by monitoring the rate of increase of fluorescence (λ_{exc} = 520 nm, λ_{emit} = 585 nm). Blank rates were determined by replacing UDPG with water, and were subtracted.

FIGURE 19

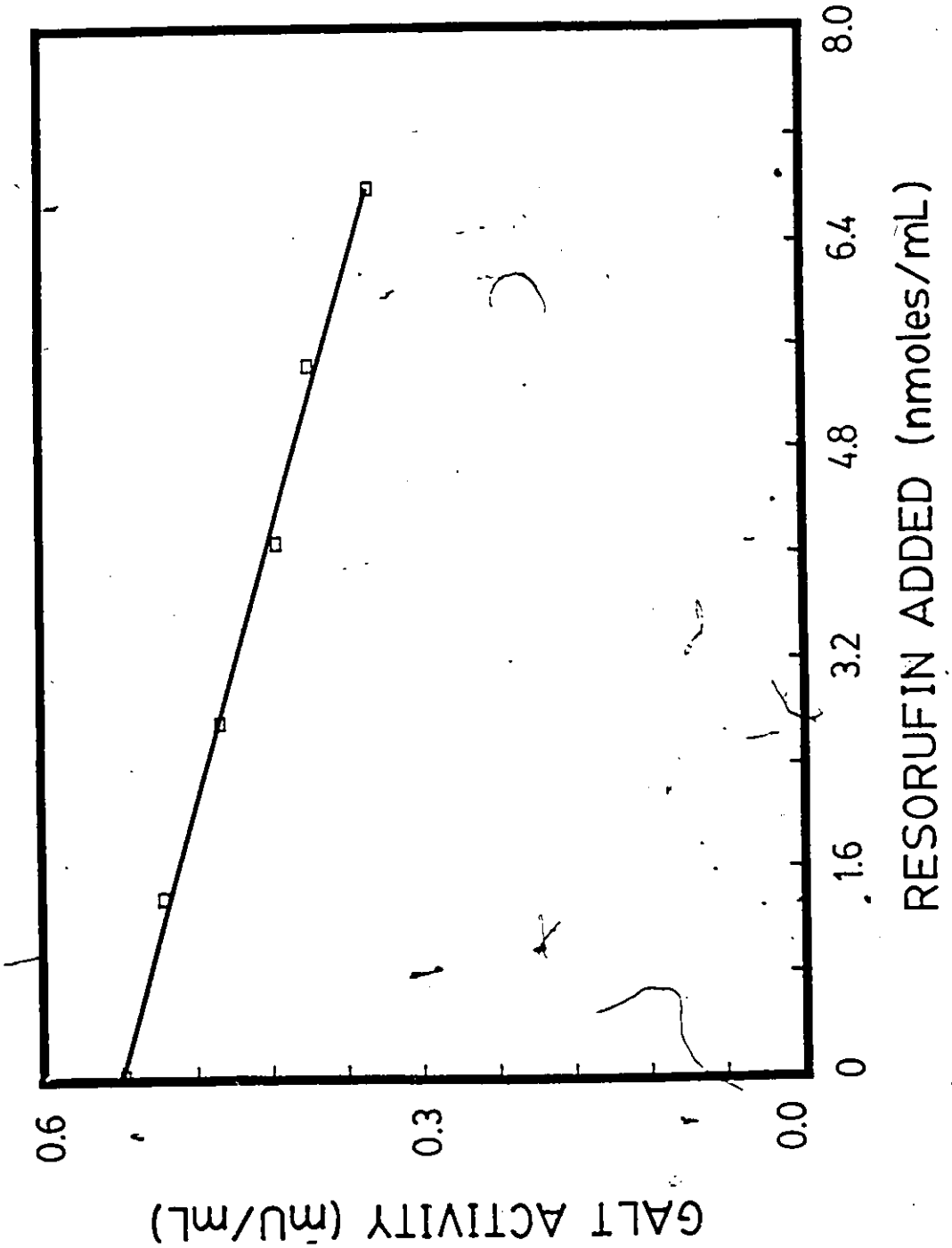

GALT ACTIVITY (mU/mL)
0.6
0.3
0.0
0
1.6
3.2
4.8
6.4
8.0
RESORUFIN ADDED (nmoles/mL)

FIGURE 20

EFFECT OF ADDING RESORUFIN TO ASSAY MIXTURE ON DIAPHORASE ACTIVITY

Legend

Diaphorase activity was determined at pH 8.7, 37°C, by monitoring the rate of increase in fluorescence (λ_{exc} = 568, λ_{emit} = 582 nm).

To a solution containing 450 µL buffer (100 mM glycine, 0.5 mM EDTA), 20 µL resazurin (0.85 mM) and 10 µL NADPH (1.0 mM), were added 10 µL of resorufin solution (varying concentrations). The reaction was initiated by adding 20 µL of diaphorase (8.0 DCPIP units/mL).

Rates of reaction are expressed as a percentage of that obtained when no resorufin was added.

FIGURE 20

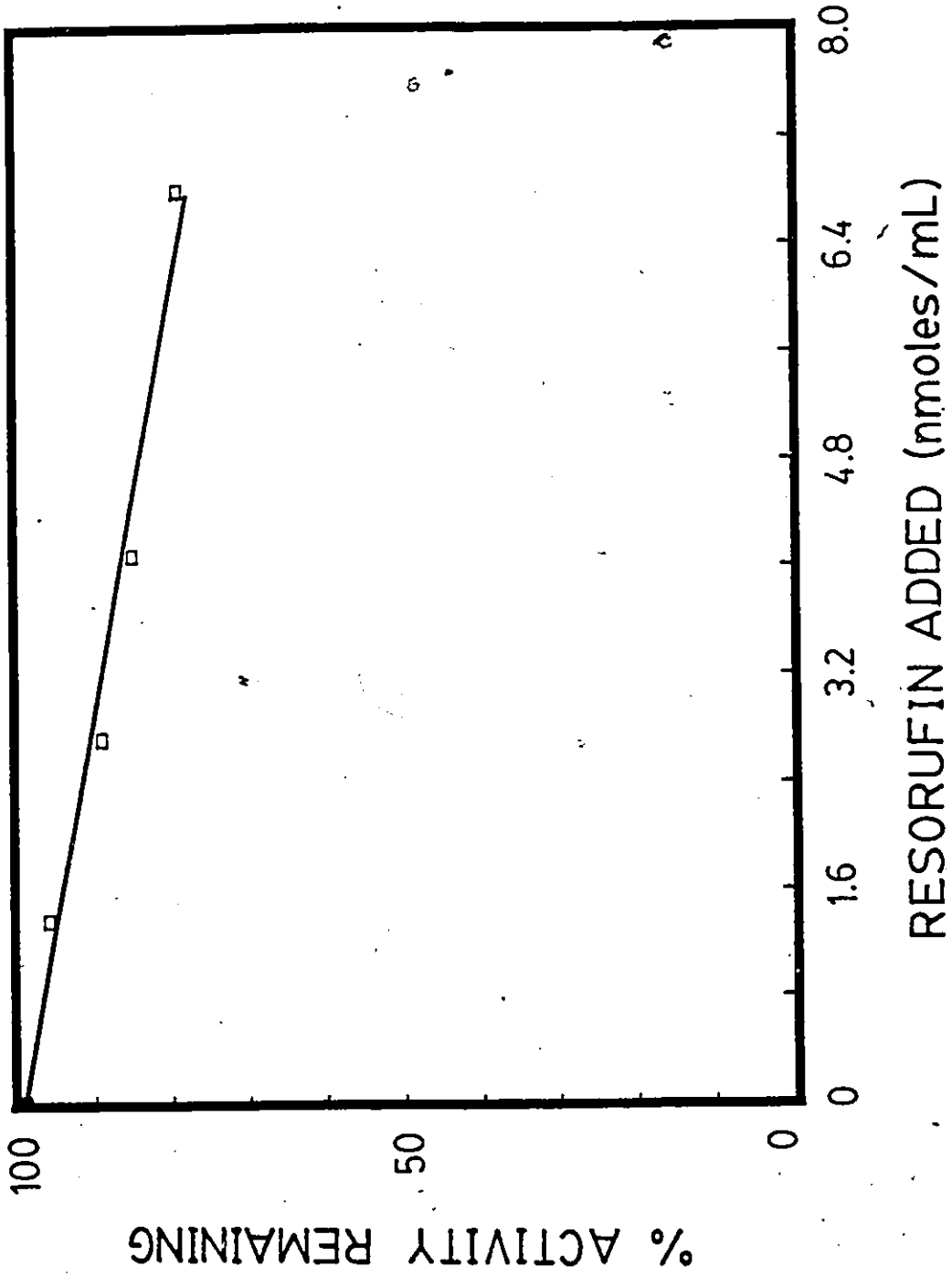

% ACTIVITY REMAINING
100
50
0
RESORUFIN ADDED (nmoles/mL)
0
1.6
3.2
4.8
6.4
8.0

TABLE VI

FLUORESCENCE COEFFICIENT OF RESORUFIN IN THE PRESENCE OF EACH COMPONENT OF GALT ASSAY MIXTURE

Component present[a]	Fluoresence coefficient[b]
Sample, resazurin	2.72×10^7
Sample, resazurin, UDPG	2.72×10^7
Sample, resazurin, enzymes	2.69×10^7
Sample, resazurin, substrates[c]	2.72×10^7

(a) Each component was present in the concentrations indicated in Table V.

(b) Fluorescence intensity was measured in duplicate at (5, x 10) by first zeroing the instrument with mixture containing appropriate amounts of each component indicated, and then adding 10 µL of 2.0×10^{-4} M resorufin.

(c) Substrates comprised of GAL1P, G1,6DP and $NADP^+$.

3.3.3 Linearity of GALT Assay

The linearity of the GALT assay was determined using a commercial preparation of GALT. Figure 21 shows that the assay is linear up to about 1.4×10^{-3} units/mL and can detect less than 1×10^{-4} units of GALT. It should be noted that in this study, 6-PGADH was not present in the assay mixture.

3.3.4 Precison

The within-run CV of the UDPG-consumption method (5) was 5.4% at 318 U/kg Hb. The within-run precision for the present GALT assay at three different levels of GALT activity is shown in Table VII. The higher CV seen for our assay is primarily due to the manner in which the internal standard was prepared, i.e., the small volume of resorufin used led to large variability of fluorescence coefficient from run-to-run.

The significantly higher CV in the GALT Deficient Control can be attributed to the fact that the commercial preparation contained a substance or substances capable of reducing resazurin. Due to the dynamic blank, the determination of the fluorescence coefficient of the internal standard was difficult.

3.3.5 Stability of GALT

The stability of GALT in hemolysates stored at $4^{o}C$ and $-20^{o}C$ is shown in Table VIII. The results indicate that GALT is more stable at $4^{o}C$, and should be assayed within one day after the preparation of the hemolysate.

FIGURE 21

LINEARITY OF GALT COUPLED ASSAY

Legend

Dilutions of a stock solution, containing a commercial preparation of GALT, were made with 1% BSA and assayed.

GALT activity was determined by the present coupled assay as described in CHARTER 2, Section 2.2.3.2 c., p. 23. It should be noted that 6-PGADH was not present in the assay mixture for this study.

FIGURE 21

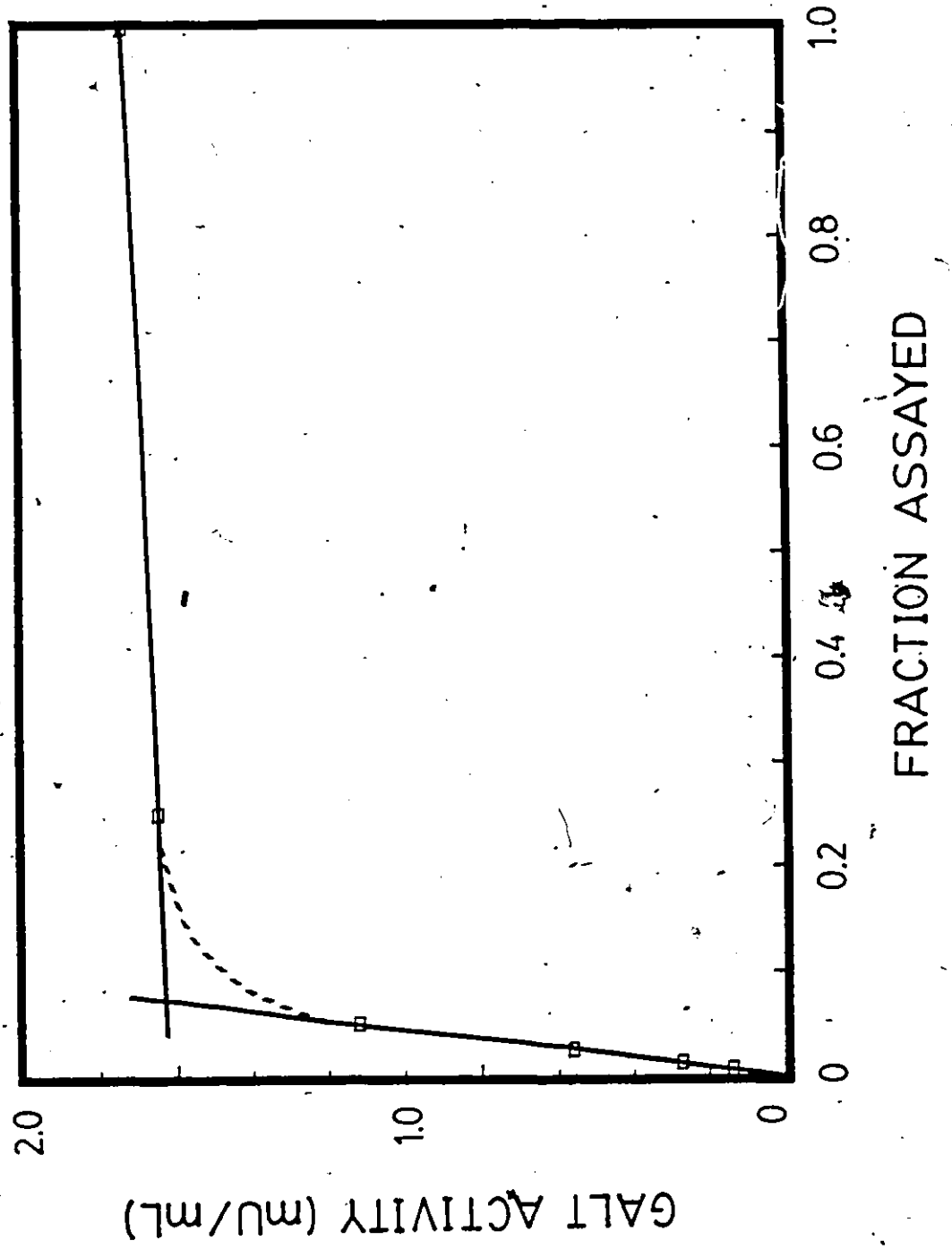


TABLE VII

WITHIN-RUN PRECISION OF PRESENT COUPLED ASSAY FOR GALT[a]

No. of replicates	Mean (U/kg Hb)	S.D. (U/kg Hb)	C.V.[b] (%)
10	466	36	7.7%
10	365	31	8.5%
8	127	19	15.0%

(a) GALT activity was determined in 1:1 hemolysates, as described in CHAPTER 2, Section 2.2.3.2 c., p. 23 .

(b) C.V. = S.D./Mean x 100

TABLE VIII

STABILITY OF GALT IN HEMOLYSATES[a]

Days of storage	GALT activity (U/kg Hb)[b]	
	4°C	-20°C
0	332	332
1	351	338
2	308	286
3	248	208
5	206	150

(a) Hemolysates were 1:1 packed cell volume: water volume.

(b) GALT activity was determined in duplicate as described in CHAPTER 2, Section 2.2.3.2 c., p. 23.

For samples which must be kept longer before analysis, it has been suggested by other workers that the sample be stored as whole blood or packed cells at 4°C (14,25).

3.3.6 Comparison of Methods

The present assay was compared with the reference UDPG-consumption method of Ibbott (5). There is good agreement (r = 0.977) between the two methods (Figure 22). The equation of the line of best fit (y = 5.4 + 0.82x) does not pass through the origin, but this is not clinically significant. It should be noted that one point was discarded as a statistical outlier (55). If this point is included, the statistical parameters are: r = 0.958, y = 26.5 + 0.77x.

Analysis of the data using the t-test revealed that a statistically real bias exists between the two methods. Indeed, the slope of the regression line (< 1.0) suggests that a proportional error exists in our method (56).

The suggestion of a proportional error raised the possibility that there was an incomplete transfer of electrons from NADPH to resazurin. This idea was tested by converting known amounts of NADPH to resorufin using diaphorase and determining the percent recovered. Table IX shows that, indeed there is not an equivalence between the amount of NADPH added and the amount of resorufin produced. In fact, there is only about a 40% efficiency in the presence of hemolysate. For this reason, GALT activity in our assay has been quoted as μmoles resorufin produced per minute.

If one considers our coupled assay, in which it is assumed that 2 moles of NADPH are produced per mole of UDPG consumed, one would then expect about 40% x 2 or 0.80 moles of resorufin are produced per mole of UDPG consumed. This value agrees fairly well with what was seen in the Comparison of Methods study, i.e., a slope of 0.82 and explains the discrepancy between the two methods.

FIGURE 22

COMPARISON OF PRESENT COUPLED ASSAY WITH UDPG-CONSUMPTION METHOD

Legend

Normal samples, the GALT Deficient Control and some samples, created by the combination of the two, were analyzed in duplicate by the present coupled assay (as described in CHAPTER 2, Section 2.2.3.2 c., p. 23) and the UDPG-consumption method of Ibbott (5).

GALT activity for the present assay is expressed as μmoles resorufin produced per minute/kg Hb at pH 8.7, 37°C. GALT activity for the UDPG-consumption method is expressed as μmoles UDPG consumed per minute/kg Hb at pH 8.7, 37°C.

FIGURE 22

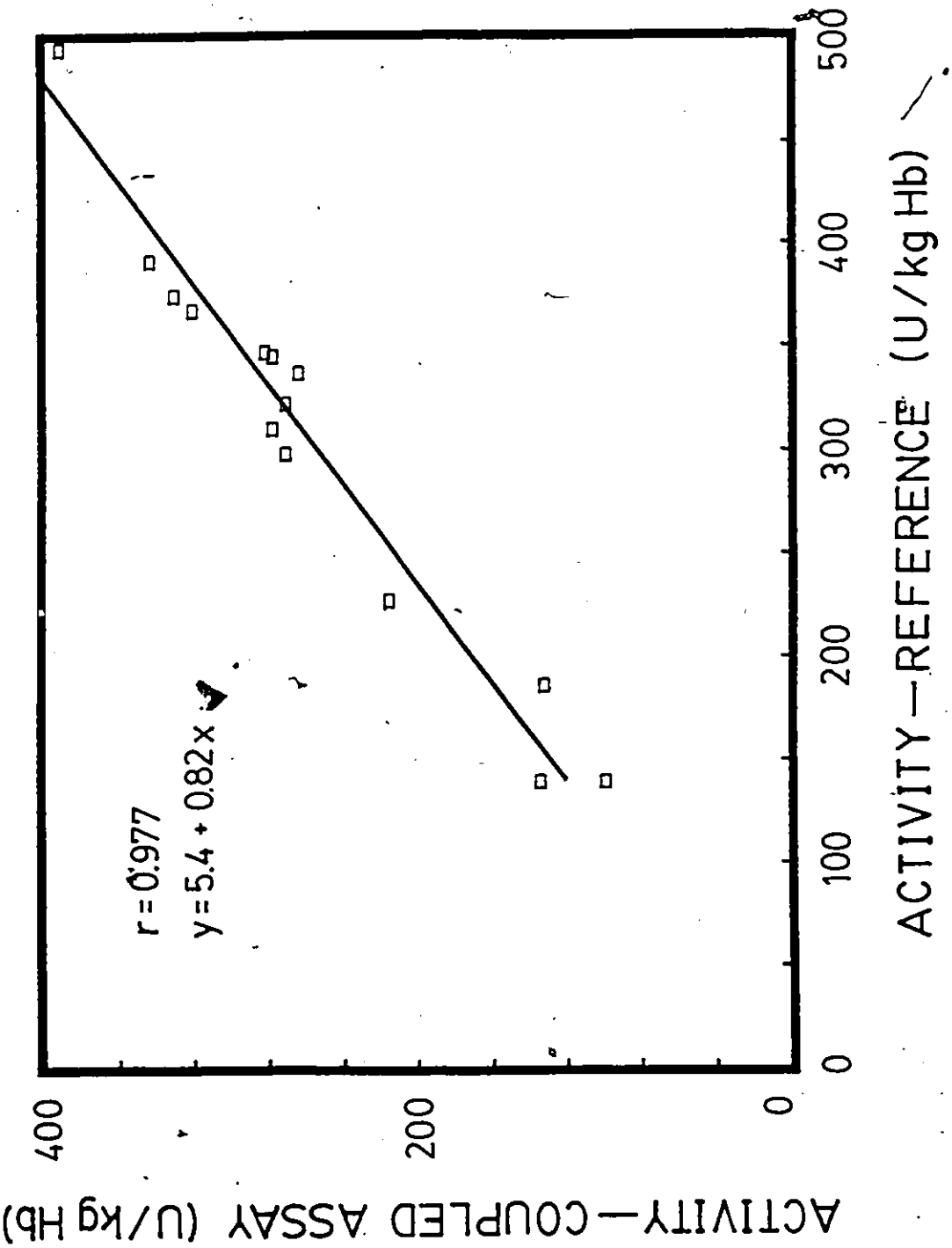

ACTIVITY—COUPLED ASSAY (U/kg Hb)
ACTIVITY—REFERENCE (U/kg Hb)
0
200
400
0
100
200
300
400
500
r = 0.977
y = 5.4 + 0.82x

TABLE IX

PERCENT OF NADPH REDUCING EQUIVALENTS TRANSFERRED TO RESAZURIN[a]

Hemolysate	NADPH added (nmoles)	Resorufin produced[b] (nmoles)	% transferred
Not added	3.66	1.46	40
	2.74	1.02	37
	1.83	0.71	39
	0.92	0.35	38
Added (10 μL)	2.74	1.07	39
	1.37	0.56	41

(a) Determined, in duplicate, at pH 8.7 37°C, using 0.32 U/mL diaphorase, 34 μM resazurin.

(b) Determined from a calibration curve, generated by measuring the RFI of known amounts of resorufin in the presence of diaphorase and resazurin (NADPH was omitted). A second calibration curve was generated for RFI of resorufin in the presence of 10 μL of hemolysate.

It is difficult to say what the exact cause of the inefficiency of the resazurin/NADPH/diaphorase system is, since it has not been discussed by previous workers using the system. Diaphorases, by their very nature, can transfer electrons to a variety of compounds.

It has been shown by other workers (57,58) that resorufin can undergo further reduction to a compound (dihydroresorufin), which is likely non-fluorescent. This fact, along with the observation that the addition of resorufin caused a decrease in the diaphorase activity, raised the question of whether resorufin, as it is produced, competes with resazurin for the electrons from NADPH. This was tested by trying to reduce resorufin using 0.32 DCPIP U/mL diaphorase and 20 μM NADPH at pH 8.7, 37°C. When 1.3 nmoles/mL of resorufin (on the order of what is produced in the GALT assay) was used, a slow rate of decrease in fluorescence was observed. However, this corresponded to a rate on the order of 10^{-6} μmoles resorufin consumed per minute, significantly lower than the rates encountered in the GALT assay. This would suggest that resorufin does not compete with resazurin for the electrons.

Worsfold et al. (59), using phenazine methosulfate as a catalyst for hydrogen transfer from NADPH to electron acceptors, noted that oxygen competes with the reaction to a significant extent. This raised the question of whether oxygen may act in the same way when using diaphorase as a catalyst. Kaplan et al. (60) performed the early studies on diaphorase from *Clostridium kluyveri*. They found that the rate of reaction using oxygen as an electron acceptor is only about 1×10^{-3} times that when using DCPIP. In our assay, 0.32 DCPIP U/mL of diaphorase are used, which is the equivalent of about 0.3 mU/mL in terms of oxygen. This is on the same order of magnitude of GALT activity being measured and suggests that oxygen may be a source of interference. This, however,

would have to be proven experimentally under the conditions of our assay, namely pH 8.7 and 37°C.

3.3.7 Recovery Study

The results from the recovery study are shown in Table X. With the exception of one run, greater than 100% recoveries were obtained for the entire range of GALT activity tested. It should be noted that the "added" GALT was assayed in a mixture not containing 6-PGADH. It was assumed that when added to hemolysates, containing 6-PGADH, twice as much activity would be observed.

The work of Massa and Farias (61) showed that the reduction of a tetrazolium salt (thiazolyl blue tetrazolium bromide) by NADH, in the presence of phenazine methosulphate (PMS) was enhanced by the presence of erythrocyte membrane. This led us to wonder whether the erythrocyte membranes present in our hemolysate preparations were acting similarly, to enhance the reduction of resazurin. A recovery study of NADPH, in the absence of hemolysate was, therefore, performed (Table IX). An average recovery of 38% was seen, as compared to 40% in the presence of hemolysate. The difference does not seem significant enough to explain the greater than 100% recoveries of GALT seen in hemolysates.

In the determination of the activity of "added" GALT, no activator was present in the assay mixture. The presence of reduced glutathione in erythrocytes (13) may have led to the activation of GALT when added to the hemolysates and produced greater than 100% recoveries. This hypothesis, however, must be tested experimentally.

TABLE X

RECOVERY OF GALT IN HEMOLYSATES

GALT activity[a] (units, x 10^{-4})				
Sample	Added[b]	Measured	Recovered	% recovery[c]
3.88	2.72	7.36	3.48	128
	2.04	6.66	2.78	136
	1.80	6.22	2.34	130
	1.38	5.40	1.52	110
	0.90	4.88	1.00	111
	0.70	4.70	0.82	117
0.32[d]	4.32	5.20	4.88	113
	2.16	3.32	3.00	138
	1.08	1.64	1.32	122
	0.54	0.80	0.48	89

(a) Determined as described in CHAPTER 2, Section 2.2.3.2 c., p. 23.

(b) Commercially prepared GALT was used.

(c) % Recovery = Recovered/Added x 100

(d) Sample was GALT Deficient Control.

3.3.8 Reference Range of GALT in Erythrocytes

Figure 23 shows the time course of the present GALT assay for normal and deficient samples. The assay clearly distinguishes between normal and deficient samples.

A small clinical study (23 samples) was carried out. The distribution of GALT activity in patients is shown in Figure 24. Values of GALT in normal samples ranged from 240-456 U/kg Hb, with a mean value of 322 U/kg Hb and standard deviation of 52 U/kg Hb.

FIGURE 23

TIME COURSE OF COUPLED ASSAY OF GALT

Legend

The increase in fluorescence (λ_{exc} = 520, λ_{emit} = 585 nm) using the present coupled assay (as described in CHAPTER 2, Section 2.2.3.2 c., p. 23) is shown for hemolysates containing normal and deficient levels of GALT. UDPG was included in the SAMPLE and omitted from the BLANK. Reactions were initiated with the "substrate" solution, i.e., GAL1P, $NADP^+$ and G1,6DP.

FIGURE 23

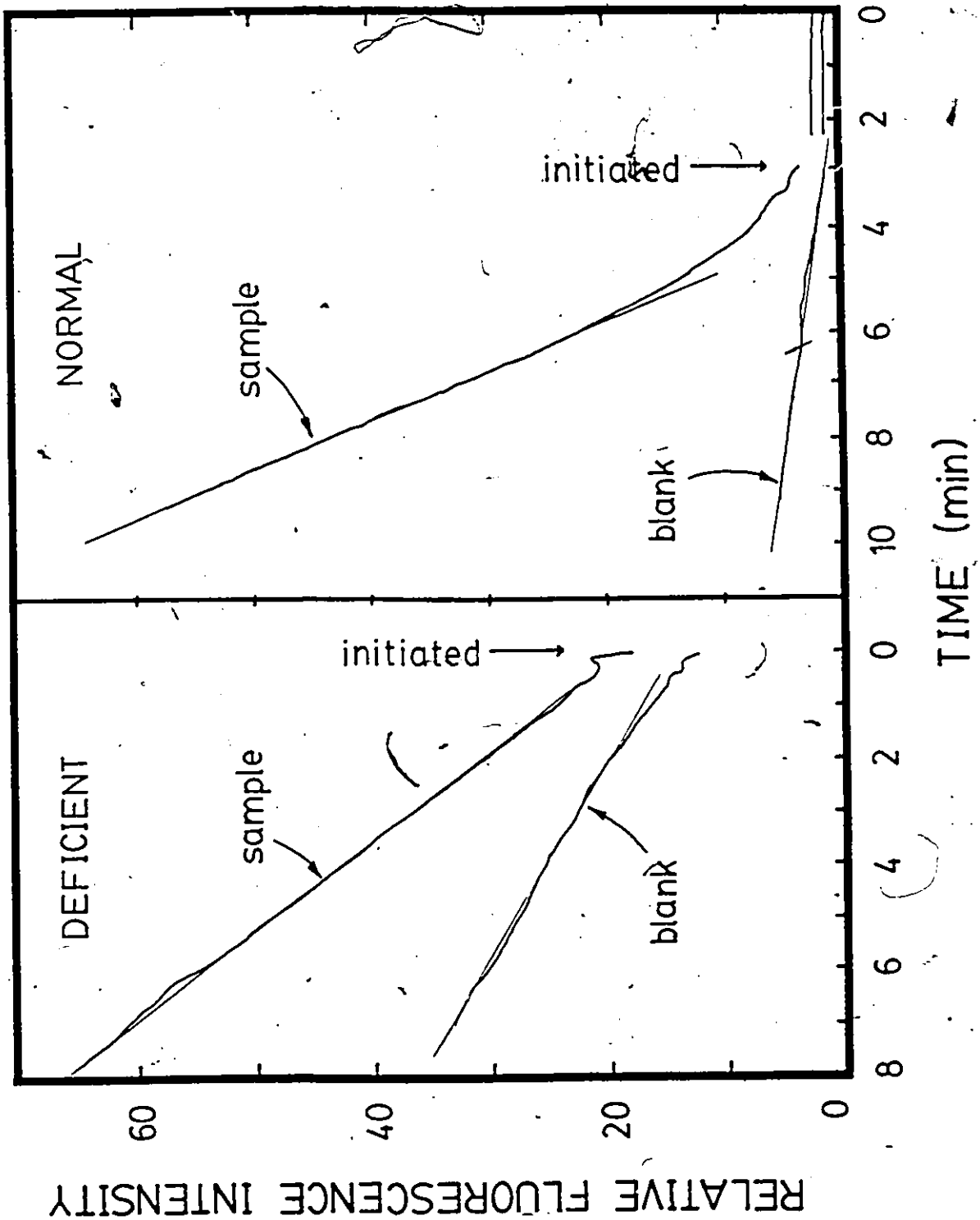

NORMAL
initiated
sample
blank
DEFICIENT
initiated
sample
blank
0
2
4
6
8
10
TIME (min)
RELATIVE FLUORESCENCE INTENSITY
60
40
20
0

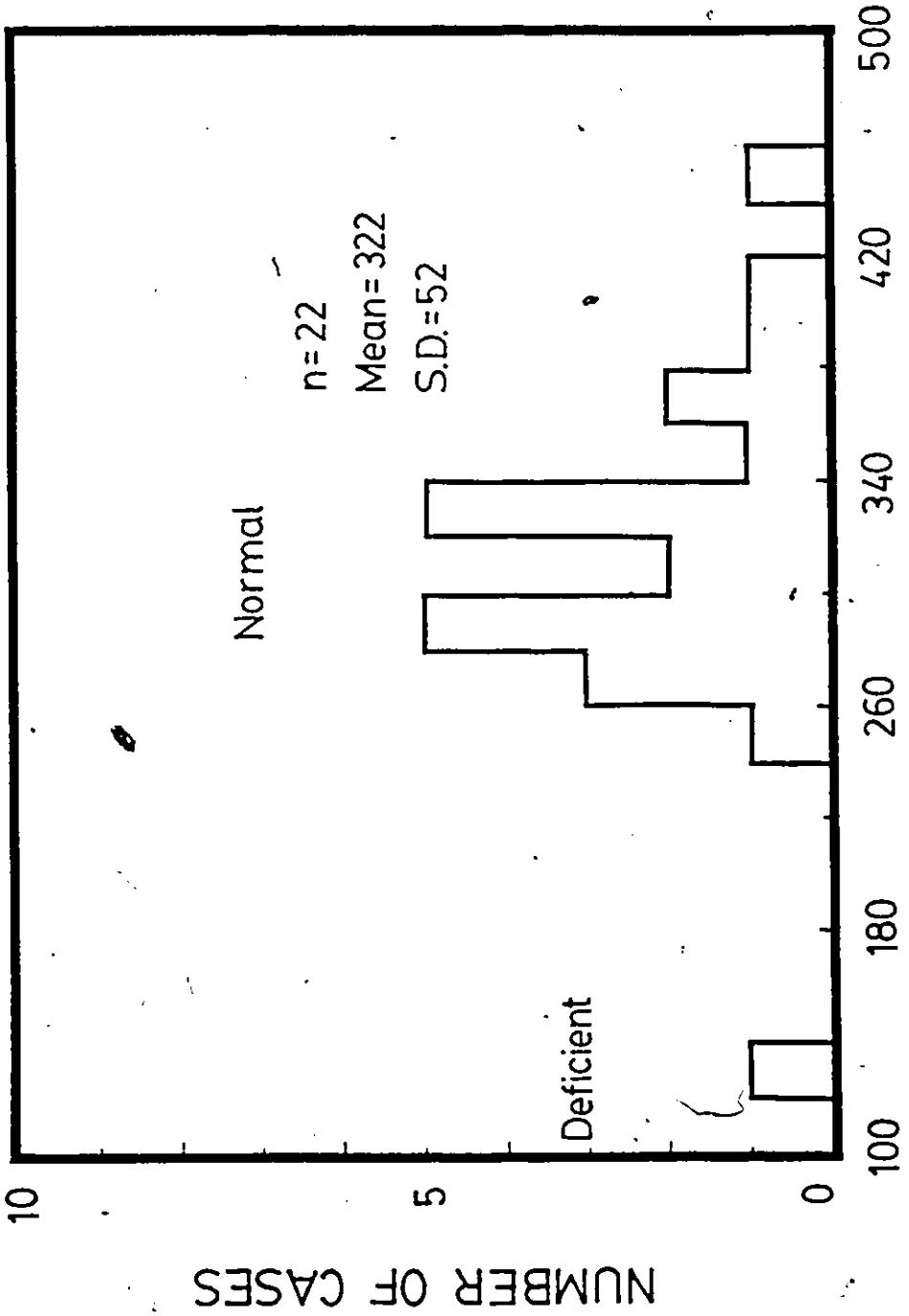
FIGURE 24

DISTRIBUTION OF GALT ACTIVITY IN PATIENTS

Legend

GALT activity was determined in hemolysates in duplicate using the present coupled assay as described in CHAPTER 2, Section 2.2.3.2 c., p. 23. Deficient sample was the GALT Deficient Control.

FIGURE 24


NUMBER OF CASES
10
5
0
100
180
260
340
420
500
GALT ACTIVITY (U/kg Hb)
Normal
n=22
Mean=322
S.D.=52
Deficient

CHAPTER 4

SUMMARY AND CONCLUSIONS

4.1 The Resazurin/Diaphorase System

1. We have found that the specific activity of diaphorase when using resazurin (6.7 μM) as a substrate is only about 2×10^{-3} that when using DCPIP as a substrate. This differs from the findings of Guilbault (53) who found the factor to be only about 5×10^{-2}.

2. Specific activity of diaphorase can be increased by using higher concentrations of resazurin. The concentration chosen is influenced by the size of fluorometric cuvette used. In a 3.0-mL cuvette, concentrations above 13.4 μM result in lower signals due to the quenching of the fluorescence by resazurin. In a 1.0-mL cuvette, the quenching effect is less severe and a higher concentration (34μM) of resazurin can be used.

3. Diaphorase activity is first order in the concentration range up to 34 μM of resazurin, and the K_m could not be calculated from the range tested.

4. The K_m of diaphorase with respect to NADPH is dependent on the concentration of resazurin used. It is 0.78 (± 0.04) μM and 1.31 (± 0.04) μM at resazurin concentrations of 6.7 and 34 μM, respectively.

5. We have improved on the kinetic assay of Guilbault et al. (29,31) for determination of dehydrogenase activity by increasing both the amount of resazurin and diaphorase used. This has increased the sensitivity and produced calibration curves which have an extended linear range and better separation of points.

6. Cellosolve should be replaced with water as a solvent for resazurin solutions. This has two advantages: a) more concentrated solutions of resazurin can be prepared; b) it eliminates the use of a teratogen.

4.2 Present Coupled Assay for GALT

4.2.1 Advantages

a. Due to the high fluorescence of resorufin, the assay is more sensitive than others, being able to detect less than 1×10^{-4} units of GALT activity in hemolysates.

b. The present assay has a much shorter lag phase (about 3 minutes) than other methods, which require anywhere from 15-20 minutes pre-incubation to generate enough product to be measured.

c. The procedure does not require the tedious separation steps required by UDPG-consumption and radioisotopic methods.

4.2.2 Limitations

a. The method is primarily limited by the reagent blank, which is a result of the contamination of galactose 1-phosphate. Due to the low specific activity of diaphorase only very low blanks can be tolerated and this, in turn limits the amount of PGM and G6PDH that can be added to the assay mixture. This gives rise to the lag phase present in our assay. However, if the purity of GAL1P could be improved, then the lag phase would be further reduced and possibly eliminated altogether.

b. The variable quenching of resorufin, from sample to sample, makes it necessary to include an internal standard for the assay of GALT. This is complicated by the fact that the internal standard cannot be added

to the assay mixture itself, due to the inhibitory effect on diaphorase, but must be made up separately.

However, it may be possible to determine hemoglobin concentration in samples from calibration curves based on the quenching of resorufin. The internal standard therefore could also be used for this purpose and eliminate the necessity of determining hemoglobin by a separate procedure.

c. The conversion of NADPH reducing equivalents to resorufin using diaphorase is only about 40% in hemolysates. It is our belief that oxygen is the cause of this inefficiency, but further studies should be done in order to determine the exact cause and develop ways to counter its effect. In addition, although the inefficiency of the diaphorase/resazurin effects the sensitivity of the assay, the method correlates well with the UDPG-consumption method and is still clinically useful for the determination of GALT activity in erythrocytes and ultimately the diagnosis of galactosemic patients. The assay clearly distinguishes between normal and deficient samples.

APPENDIX A

CORRECTING OBSERVED FLUORESCENCE INTENSITIES ON PERKIN-ELMER FLUORESCENCE SPECTROPHOTOMETER

The Perkin-Elmer 204 fluorescence spectrophotometer has 2 knobs which allow amplification of the observed fluorescence intensity.

The "sensitivity control" ranges from 1 to 12. Increasing by one division approximately doubles the RFI. The "selector control" can be set at x 0.1, x 1, or x 10, and this knob causes the RFI to be multiplied exactly by the corresponding factor.

When an RFI is reported with a number in brackets beside it, this refers to the settings at which the above controls were at the time of recording. (The first number in the brackets refers to the sensitivity control setting, and the second refers to the selector control setting.) To compare RFI taken at different settings, one must take the differing amplification into consideration.

Therefore, to correct the RFI obtained at (a, x b) to (1, x 1) the following equation was used:

$$RFI\ (1, x\ 1) = RFI\ (a, x\ b) / 2^{(a-1)} \times b$$

For example, a RFI of 80 at (4, x 10) would have a RFI (1, x 1) of:

$$RFI\ (1, x\ 1) = 80 / 2^{(4-1)} \times 10 = 1$$

APPENDIX B

CALCULATION OF GALT ACTIVITY USING AN INTERNAL STANDARD (IS)

Activity (U/mL)

= μmoles resorufin produced/min/mL

$$= [(\Delta F/min)_{sample} - (\Delta F/min)_{blank})] \times \frac{\mu moles\ resorufin\ added\ (IS)/mL}{RFI\ (IS)}$$

$$= \frac{Net\ \Delta F/min}{RFI\ (IS)} \times \frac{mg\ reso. \times 10^{-3}\ g/mg \times 1\ mole/235\ g}{100\ mL} \times \frac{0.01\ mL}{0.5\ mL} \times \frac{1 \times 10^{6}\ \mu mole}{mole}$$

$$= \frac{Net\ \Delta F/min}{RFI\ (IS)} \times mg\ resorufin \times 0.852 \times 10^{-3}\ \frac{\mu mole}{mg \times mL}$$

where, mg resorufin is the amount of resorufin weighed for stock solution;

100 mL is total volume of stock solution;

0.01 mL is volume of resorufin solution used for IS;

0.5 mL is total assay mixture volume.

To determine GALT activity per kg of hemoglobin (Hb) in hemolysates, the following equation was used:

$$Activity\ (U/kg\ Hb) = \frac{Activity\ (U/mL) \times 0.5\ mL}{[Hb](g/100\ mL) \times (1 \times 10^{-2}) \times (1\ kg/10^{3}g) \times (1 \times 10^{-2}\ mL)}$$

$$= \frac{Activity\ (U/mL)}{[Hb](g/100\ mL) \times 2 \times 10^{-7}\ (kg\ Hb)}$$

where, 0.5 mL is volume of assay mixture;

1×10^{-2} is factor to convert g/100 mL to g/mL;

1×10^{-2} mL is volume of hemolysate used for assay.

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