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ANALYSIS OF BILIRUBIN WITH URIDINE-5'-DIPHOSPHATE GLUCURONYLTRANSFERASE.

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ANALYSIS OF BILIRUBIN
WITH
URIDINE-5'-DIPHOSPHATE GLUCURONYLTRANSFERASE

by

ALLY SHIVJI

A DISSERTATION

Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfilment
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1987

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The literature on the determination of bilirubin is voluminous, and there prevails a steady flow of papers describing new or modified techniques. To a large extent this is due to drawbacks and problems with the available methods rather than mere compulsive behaviour by clinical chemists" (A. McDonagh, 1979).

ABSTRACT

ANALYSIS OF BILIRUBIN WITH URIDINE 5'-DIPHOSPHATE

GLUCURONYLTRANSFERASE

by

ALLY SHIVJI

Use of bilirubin uridine 5'-diphosphate glucuronyl-
transferase, for the analysis of bilirubin was studied.
Microsomal suspensions, as well as the solubilized enzyme,
demonstrated capacities for a kinetic assay of albumin:
bilirubin (1:2) solutions and solutions devoid of albumin.
The system was enzymatically coupled for continuous monitor-
ing. Comparison with the Jendrassik-Grof method showed a
very good agreement. Analysis of serum/plasma samples was
met with interference from albumin. Albumin appears to
inhibit the enzyme by sequestering free bilirubin, and
possibly by direct protein-protein interaction with the
enzyme. The low specificity of pyruvate kinase, for a
phosphate acceptor, made it possible to link production of
uridine 5'-diphosphate to H_2O_2 production and bilirubin
oxidation by peroxidase. The activity of bilirubin uridine
5'-diphosphate glucuronyltransferase could be monitored
continously at 460 nm. Production of uridine 5'-diphosphate

is a feature of reactions catalyzed by uridine 5'-diphos-phate glucuronyltransferases. Therefore, the coupling system has the potential to allow assay of a number of UDPGT isoenzymes, as well as their substrates (which may be endogenous metabolites or xenobiotics). The pyruvate kinase can also be used for the analysis of adenosine 5'-diphos-phate, uridine 5'-diphosphate and glucose.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ix

x

LIST OF ABBREVIATIONS

AAP	4-Aminoantipyrine
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BDG	Bilirubin diglucuronide
BILI	Bilirubin
BMG	Bilirubin monoglucuronide
BSA	Bovine serum albumin
ER	Endoplasmic reticulum
FAD	Flavin adenine dinucleotide
HDCBS	3,5-Dichloro-2-hydroxybenzenesulfonate
HSA	Human serum albumin
Kda	Kilo daltons
LD	Lactate dehydrogenase
mM	Millimolar
min	Minute
NAD	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
4-n-p	4-Nitrophenol
PER	Horseradish peroxidase
PEP	Phospho(enol)pyruvate
PK	Pyruvate kinase
Pi	Phosphate
POx	Pyruvate oxidase
PYR	Pyruvate

TPP	Thymine pyrophosphate
UDPGLcNAc	Uridine 5'-diphosphate N-acetylglucosamine
UDP	Uridine 5'-diphosphate
UDPGA	Uridine 5'-diphosphate glucuronic acid
UDPGT	Uridine 5'-diphosphate glucuronyltransferase
UTP	Uridine 5'-triphosphate

CHAPTER I

INTRODUCTION

A. BILIRUBIN

1. General properties

Bilirubin IXα (BILI) is the principle breakdown product of heme metabolism. This compound is yellow at physiological pH and is excreted mainly through bile. Therefore, it is also classified as a bile pigment [1]. The name, bile pigment, is a generic term that now denotes tetrapyrroles with a general structure as the one shown in Fig. 1. It is customary and convenient to use trivial names for bile pigments, because use of systematic names can become overwhelming, especially since bile pigments occur in a number of isomeric forms. Figure 2 shows the structure of bilirubin and its conformation in the solid form and in non-polar solvents. The systematic name for BILI is based on the 21-H-biline (Fig. 1) and is known as 21 H -biline-8,12-dipropanoic acid-2,17--diethenyl-1,10,19,22,23,24-hexahydro-3,7,13,18-tetra-methyl-1,19-dioxo. BILI is also known as a 5,15-biladiene [1].

BILI possesses peculiar physical and chemical characteristics that are believed to arise from its structural conformation. Extensive studies have been conducted to understand its physiochemical properties. It has been

1

FIGURE 1

21 H-BILINE SYSTEM

Legend

The basic nucleus of the bile pigments and the numbering system employed, for naming these compounds is illustrated.

(Borrowed, without permission, from: McDonagh, A. F. (1979) in The Porphyrins. (Dolphin, D. ed.) Vol. VIA, pp. 293-491. Academic Press Inc., New York).

FIGURE 1
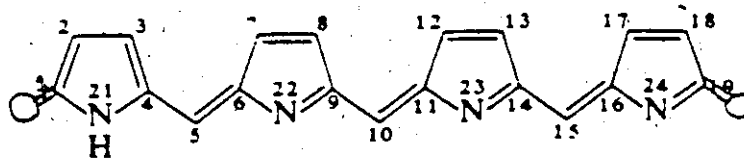
FIGURE 2 .

STRUCTURE AND CONFORMATION OF BILIRUBIN IX α

Legend

The preferred conformation of BILI, with intra-
molecular hydrogen bonds that lock the molecule into a
bent structure, is shown. Shielding of polar groups
gives this conformation a hydrophobic character.

(Borrowed, without permission from: Brodersen, R. (1980)
CRC Crit. Rev. Lab. Sci. 11: 305-359)

FIGURE 2
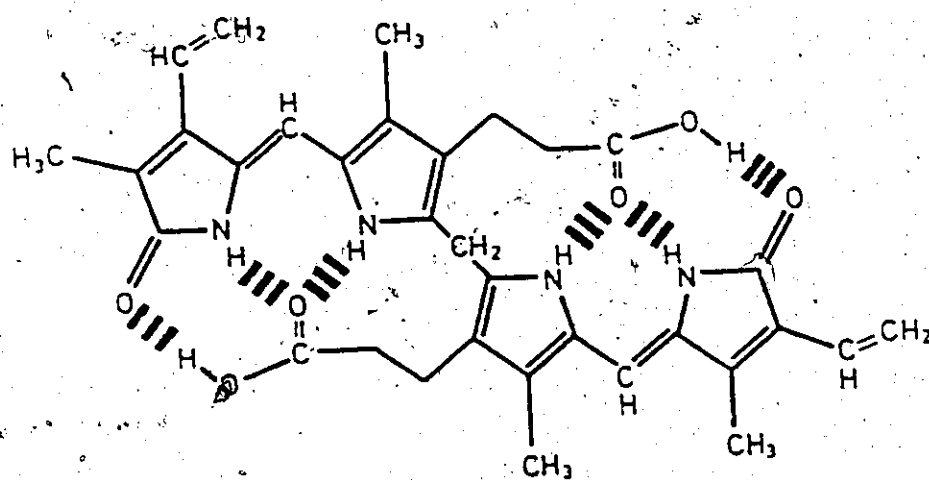
established that this bile pigment exists, predominantly, as a bis-lactam and the unsaturated meso double bonds, at 5 and 15 positions, have a Z, Z configuration [1,2]. In acidic or neutral aqueous solutions, or in non-polar solvents, the conformation is very similar to the one in the solid. The molecule has a folded structure in which the carboxyl groups and N-H groups are able to participate in strong intra-molecular hydrogen bonding (Fig. 2). Each propionic acid side chain from one half of the molecule is hydrogen-bonded with the pyrrole ring of the other half. Consequently, the hydrophilic groups are shielded from the immediate environment, and the molecule takes on rather hydrophobic properties [1,3]. BILI has very limited solubility in non-basic aqueous media, its solubility has been estimated to be about 7 nM at pH 7.4. In fact, it has a tendency to form colloids and surface films [1,2]. Solubility of this bile pigment increases with pH. In slightly basic solutions (pH 7.4-8.5), BILI molecules apparently exist as dimers or aggregates of unknown structures [1]. In more basic solutions, the ionization of carboxyl and N-H groups disrupts hydrogen bonding to bring about solubilization [2].

2. Acid-base properties

5,15-Biladienes are amphoteric molecules; in neutral solutions, these pigments are golden-yellow or orange-brown

(concentrated solutions), and hence, called rubins [1]. In strongly acidic or basic environments, the BILI solutions are deep red. The colour change results from protonation of carboxyl moieties (in acid) or ionization of the N-H group lactam ring (in base). The acid-base properties are poorly understood, and there is a general disagreement regarding the ionization constants. In water, the pK_1 and pK_2 have been estimated to be between 4.3 and 5.3 [1,4].

3. Stability

The central methene group of rubins is highly unstable in visible light and susceptible to attack by electrophiles [1,2]. Visible light oxidizes BILI to biliverdin and other products. This reaction proceeds at a faster rate in the presence of singlet oxygen photosensitizers like riboflavin and porphyrins, and moreover, the oxidation products appear to propagate oxidation of BILI. The mechanism is not clearly understood. There is substantial evidence that suggests blue light (400-500 nm) is responsible for photoisomerization. The Z,Z configuration at meso double bonds is disrupted, and BILI is converted to water soluble isomeric forms collectively known as photobilirubin, [1,2]. The rate of BILI photo-oxidation is dependent on numerous factors which include the physical state of the pigment, concentration, medium, oxygen content of the system, pH, intensity and

wavelength of the light reaching the BILI preparation, etc. BILI in solid state is quite stable under room light. In non-polar organic solvents, the central methylene group is apparently protected by the involuted structure. Therefore, such solutions are also fairly stable. Aqueous BILI solutions are generally unstable, especially at higher pHs or in the presence of transition metals like Ni and Fe. Detergents and albumins, which promote solubilization of BILI also have a protective action.

4. Albumin binding

Aqueous solutions of BILI are commonly prepared with albumin as a carrier molecule. This binding property of albumin makes possible the preparation of fairly concentrated aqueous BILI solutions at physiological pHs, without evidence of colloid or precipitate formation. Albumin binds BILI firmly, yet reversibly [1,2,4]. The maximum binding capacity of human serum albumin (HSA), for BILI, is still a matter of controversy. There appears to be at least one high affinity binding site per molecule of albumin. The number of secondary binding sites has been reported to range from 2-3. The association constants are estimated to be 10^7-10^8 M^{-1} for the high affinity site and about 10^5 M^{-1} for the secondary sites. The binding of BILI is believed to occur to a hydrophobic site through hydrophobic interactions and

hydrogen bonding. Furthermore, the preferrred involuted structure of BILI is supposedly maintained. The binding is characterized by appearance of the absorption maximum at 455 nm (pH 7.4) with optical activity in the visible region. This complex of albumin:BILI is, as mentioned earlier, less susceptible to photooxidation and enzymatic degradation. These observations suggest that the albumin environment protects BILI by limiting access to the central methene group. Bovine serum albumin (BSA) is more commonly employed. It can bind two equivalents of BILI. The association constant for the high affinity sites has been estimated to be around 3-7 x 10^7 M^{-1}. The absorption spectrum maximum occurs at 470 nm.

B. BILIRUBIN METABOLISM

Approximately 250-300 mg of BILI are produced daily [5-7]. The major source (65-75%) of this bile pigment is hemoglobin. The rest is derived from turnover of hemo- proteins present in the liver cells. Contribution from other tissues is relatively insignificant.

BILI is the final product of an oxidative cleavage, of the heme, by an enzymatic system collectively known as the microsomal heme oxygenase system.

The sequence of reactions that form biliverdin, the

immediate precursor of BILI, is schematically shown in Fig. 3. The cleavage of the α-methene bridge carbon is quite specific, however, minute amounts of IXβ, IXγ and IXδ isomers do occur, but they may be products of a non-enzymatic reaction [3]. The major sites of extrahepatic BILI production are the spleen, bone marrow, macrophages, and the proximal tubule cells of the kidney [5]. Biliverdin, a bilatriene, is reduced to bilirubin by a NADPH dependent biliverdin reductase.

Conjugation of BILI is a necessary requirement for excretion. BILI from non-hepatic sources is transported to the liver for conjugation and secretion into the canaliculi. Its lipophilic nature allows BILI to cross membranes readily. In plasma BILI becomes tightly bound to albumin, and is transported to the liver as a complex. Sinusoidal fenestrations, in the liver, provide easy access for the albumin: BILI to the hepatocyte plasma membrane. The mechanism of BILI uptake is not yet fully understood. It appears to be a carrier mediated process. Once inside the cell, BILI supposedly binds to cytosolic proteins - mainly ligandin (glutathione-S-transferase B). The exact role of ligandin is unknown but several roles have been postulated that include storage of the BILI pool, minimizing reflux of cellular bilirubin into plasma, protection of subcellular structures from potentially toxic effects of bilirubin, and transport of

FIGURE 3

OXIDATIVE CLEAVAGE OF HEME

Legend

Shown is a possible mechanism by which heme oxygenase system may break down heme to form biliverdin, the immediate precursor of BILI.

(Borrowed, without permission from: Kikuchi, G. and Yoshida, T. (1980) Trends Biochem. Sci. 5: 323-325).

FIGURE 3
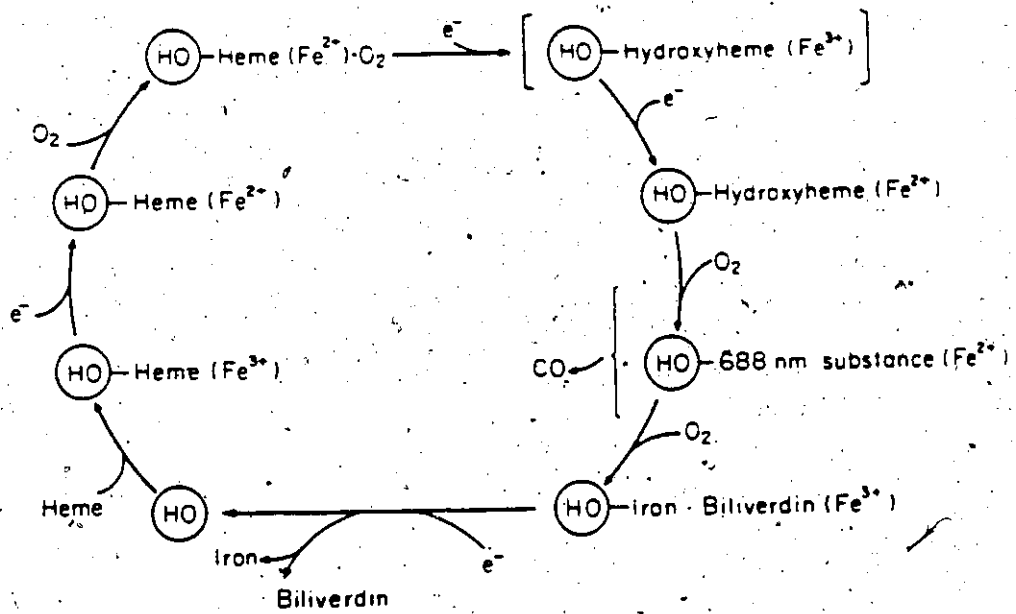
BILI to the endoplasmic reticulum (ER) for glucuronidation. It has been postulated that lipophilic BILI may be transferred to the ER for conjugation via a direct membrane-membrane diffusion process. The intracellular proteins may have a modulating effect on the intracellular movement of BILI, and therefore, on the rate of conjugation [5]. The diffusion process may account for the reflux of internalized bilirubin back into plasma (37%).

Conjugation of BILI is catalyzed by a membrane bound enzyme, BILI uridine 5′-diphosphate glucuronyltransferase (UDPGT) [EC 2.4.1.17]. One or both of the carboxyl substituents may be esterified with a glucuronyl moiety. In humans, the bile contains mainly BILI diglucuronide (BDG), with both monoglucuronide (BMG) isomers in roughly equal proportions. Formation of the diglucuronide apparently takes place in two steps. The monoglucuronide is formed first and uridine 5′-diphosphate glucuronic acid (UDPGA) serves as a cosubstrate.

The conjugated BILI is eliminated by secretion into the biliary tract. In the GI tract, a substantial proportion of the conjugated bilirubin is degraded by bacterial action to colourless tetrapyrroles (mesobilirubinogen, stercobilinogen, urobilinogen) collectively known as urobilinogen. About half of the urobilinogen is reabsorbed and the bulk of it is recycled to the liver, where it is converted back to

BILI [7]. The remaining urobilinogen, in the circulation, is excreted by way of the kidney. Some of the urobilinogen in the GI tract is converted to urobilin (an orange-brown pigment) which gives stool its characteristic pigmentation. Conjugated BILI, under normal conditions, is excreted almost exclusively by the liver. The presence of BILI in the urine may be detected when levels exceed the capacity of the kidney to reabsorb it from the glomerular filtrate [8].

C. EFFECTS OF ELEVATED BILIRUBIN

BILI, because of its lipophilic character, can partition into membrane structures or cross them by diffusion. Elevated levels in the circulation, by mass action, lead to increased levels in the intracellular pools and structural components of cells. When membrane content is sufficient to take on a yellow shade, the tissue is said to be jaundiced or icteric. Chronic or long-term exposure to high levels of BILI can result in irreversible damage to neurological tissue. However, the liver's high capacity to handle increased BILI load offers some protection by keeping levels in the circulation fairly low. In addition, high plasma albumin minimizes the availability of free BILI, the active fraction that can enter cells. Toxic effects of BILI include uncoupling of oxidative phosphorylation, loss of membrane

integrity, and interference with RNA and protein synthesis, carbohydrate metabolism, and a host of enzyme activities. In humans, BILI encephalopathy mostly occurs in rare genetic diseases and in the newborns. At birth, the activity of UDPGT in human liver is quite low, and increases at an exponential rate to reach adult levels within 14 weeks. During this period, the neonates have a limited ability to conjugate and excrete BILI. Consequently, unconjugated BILI can reach very high levels. This condition is termed physiological jaundice, and encephalopathy (kernicterus) may occur if appropriate measures are not taken. The most common therapy to lower levels of circulating BILI is phototherapy, but in certain cases it may be necessary to instigate blood transfusion.

D. DISORDERS OF BILIRUBIN METABOLISM AND HYPERBILIRUBINEMIA

Bilirubin levels in the circulation reflect the balance between its rate of production and clearance. Factors that increase BILI production or interfere with its elimination invariably cause an elevation in circulating levels [5,7]. Hyperbilirubinemia can be due to an elevation in the level of unconjugated BILI, conjugated BILI, or both. Factors that can impinge upon this sytem can be grouped into three types: prehepatic, hepatic or post-hepatic. Some of

the congenital hyperbilirubinemias will be briefly described
to exemplify the BILI profiles.

Prehepatic

Those are mostly hemolytic syndromes, brought about by
hereditary red cell enzyme defects. The disorders involve
the glycolytic pathway, hexose monophosphate shunt, and the
purine-pyrimidine metabolism [9]. Hyperbilirubinemia,
predominantly of the unconjugated type, is a result of
increased red cell fragility [7]. Plasma levels do not
generally exceed levels beyond eight times the normal upper
limit [5].

Hepatic

Defects here can occur before conjugation, at the
conjugation step (UDPGT), or post-conjugation.

The preconjugation defect, such as in Gilbert Syndrome,
involves impaired hepatic uptake of BILI with slightly
decreased levels of UDPGT activity. Mild but persistent
elevation in levels of unconjugated BILI are observed. At
the conjugation level, the impairment can be severe as in
Criggler-Najar Type I disease. This fatal condition is
characterized by total lack of BILI UDPGT activity, and by
very high levels of unconjugated BILI (> 50 times the upper
normal limit). These patients demonstrate pale yellow or

white bile, together with low urinary levels of urobilinogen, a feature of impaired BILI excretion into bile. In Criggler-Najar Type II, the defect is less pronounced, there is some activity of BILI UDPGT. Levels of conjugated BILI do not usually exceed 20 times the upper normal limit. The conjugated fraction consists of mainly BMG.

The defects occurring after the conjugation step are characterized by an accumulation of conjugated BILI and some unconjugated BILI. Defects can be of the functional type (i.e., transport or storage), or obstructive, where the flow of conjugated bili to the GI tract is obstructed as a result of stasis. The Dubin-Johnson Syndrome is characterized by impaired transport of organic ions across the canalicular membrane. As a result, conjugated BILI levels in the circulation become elevated and elimination by way of the kidney takes place.

Posthepatic

The obstructive types, which can involve the liver, or the post hepatic ducts, are marked by stasis which prevents flow of conjugated BILI to the GI tract. Consequently, the bile is pale yellow or white. These individuals excrete elevated amounts of conjugated BILI and low levels of urobilinogen in the urine. Signs of liver damage are also present.

E. ANALYSIS OF BILIRUBIN

Analysis of bilirubin levels in body fluids is a commonly used diagnostic procedure. It can provide important information regarding the functional integrity of the hepatic biliary system. Furthermore, it is necessary to monitor levels of circulating bilirubin in certain disorders and in neonates, to avoid bilirubin-induced encephalopathy. The most common methods of bilirubin analysis in the clinical laboratory, are spectrophotometric ones, either direct or following diazozation with aryl diazonium ions [1].

1. Direct spectrophotometric analysis

The major absorption band of bilirubin occurs between 400-470 nm. The extinction coefficient has been estimated to be 5.0-6.5 x $10^4 M^{-1} cm^{-1}$. The shape and the position of λ max varies with the solvent and the nature of substituents on the pyrrole rings.

In the serum, 40-95% of the yellow colour is due to BILI. The rest is contributed by carotene, xanthophyll ester, bilifuscin and mesobilifuscin [10]. Although direct spectrophotometric analysis is simple and fast, it suffers interference from heme containing pigments (450-460 nm), turbidity, and dietary pigments [1,2,10].

2. Diazotization methods

The most commonly used methods involve the diazo reactions. Of the known bilirubinoids, bilirubin, dehydroxy-bilirubin, mesobilirubin and dehydromesobilirubins react with aryl diazonium ions to produce red-violet dipyrrolic azo derivatives [1,10]. Of these, only BILI is found in the circulation [10].

The electrophilic aryl diazonium ions react at the 9 and, 11 positions to produce two equivalents of diazo-dipyrroles and formaldehyde. With the first attack, the reaction produces one azodipyrrole (vinyl isoneoxanthobilirubic acid (or azopigment)) and one equivalent of hydroxypyrromethane carbinol. The latter in the presence of aromatic amines, reacts with another diazonium ion to form the second azodipyrrole. Since BILI is unsymmetrically substituted, the azopigments thus formed are isomeric forms (Fig. 4).

In 1916, Van den Bergh and Muller [11] showed that two types of diazo reactions occur in serum samples. A direct reaction that occurs in 30 sec and an indirect one which requires addition of alcohol. The direct reacting fraction contained the water soluble conjugated BILI, and the indirect contained unconjugated BILI. Azopigment derived from unconjugated bili is referred to as azopigment A and the one from the esterified form is azopigment B. Some authors

FIGURE 4

REACTION OF BILIRUBIN WITH A DIAZO REAGENT

Legend

Mechanism of the diazo coupling reaction with bilirubin to form azo derivatives that absorb at 530 nm at acidic pHs.

(Borrowed, without permission, from: Hutchinson, D.W., Johnson, B., and Knell, A.J. (1972) Biochem. J. 127: 907-908).

FIGURE 4
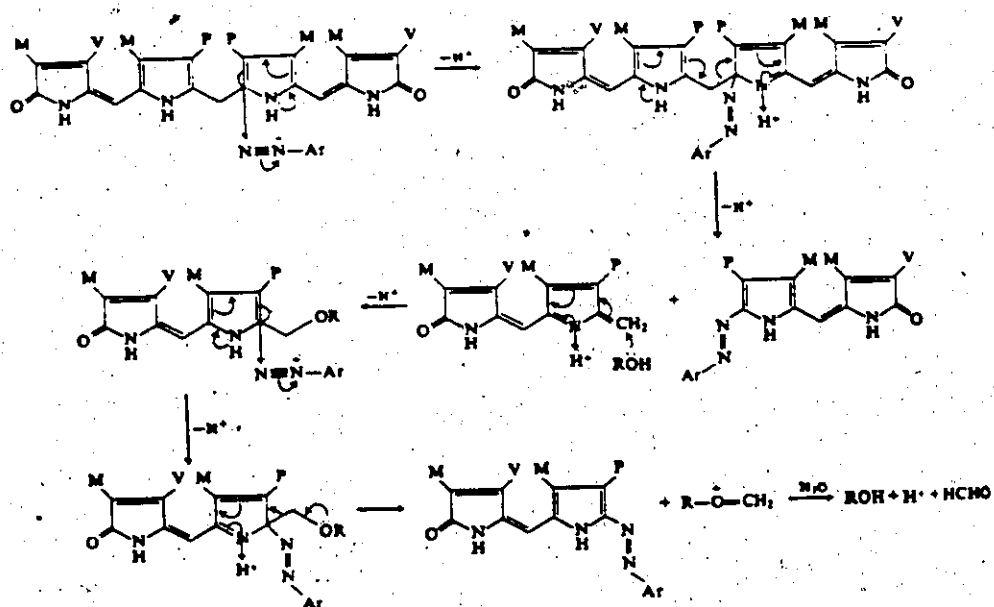
have referred to azocompounds as azobililrubin A or B. This nomenclature is misleading because these names imply that the tetrapyrrolic moiety, after reaction with diazonium ions, remains intact [3].

There are two types of diazo reagents. Those that produce water soluble azocompounds are referred to as type I reagents; type II reagents on the other hand, yield products that are lipophilic or soluble in organic solvents [3]. Consequently, with type II reagents, extraction into organic solvents is necessary before spectrophotometric measurement. Lower blanks and greater sensitivity are the attractive features of such methods [3].

The methods most widely used in the clinical laboratories employ type I reagents for practical purposes. Although many versions have been formulated, the most common ones are those based on ones described by Evelyn and Malloy [12], or Jendrassik and Grof [13]. Both use diazotized sulfanilic acid (p-benzene diazonium sulfonate) as the electrophilic reagent.

Evelyn-Malloy Procedure

These investigators used methanol (at 50% final concentration) to initiate the indirect reaction. The lower concentration of alcohol reduced turbidity from precipitation of serum proteins and loss of BILI (by adsorption to

precipitated protein). Methanol, in neutral or acid solutions apparently promotes the coupling reaction by solubilizing the unconjugated BILI. The disadvantage of this procedure is poor precision due to high sample dilution [10].

Jendrassik-Grof Procedure

In this method caffeine/sodium benzoate mixture is used as an accelerating agent for the indirect reaction. Alkaline tartate is added to shift measurement to 600 nm (from 530 nm) to reduce potential interference from other sample components [10]. The function of the sodium benzoate is to displace BILI from albumin, and caffeine serves to solubilize the BILI and confer stability to the azopigment at the alkaline pH [3]. Precision with this method is much better.

Although used for estimating bilirubin fractions. These methods are not satisfactorily accurate or precise. The diazo reaction is complex and is affected by many variables. Slight modifications yield different results. This obser-vation has been attributed to non-stoichiometry in the reaction [10]. Most of the dissatisfaction lies with the estimation of the direct reacting fraction. Colour development continues past the initial rapid reaction, which poses a problem of selecting an appropriate time to obtain an estimate of the conjugated BILI. Apparently a significant

proportion of the conjugated BILI does not react during the time frames picked for the direct reaction. This is further confounded by contribution of colour development by the unconjugated BILI [10]. For the Evelyn-Malloy, it has been argued that values for the extinction coefficient and λmax differ for aqueous and methanol containing solutions. Therefore, equating values of the total and direct fractions, to obtain levels of unconjugated introduces error.

3. Enzymatic method

Recently, an enzymatic method, employing BILI oxidase, has been developed. This enzyme oxidizes BILI (conjugated as well as unconjugated) to biliverdin and other products. Furthermore, the enzyme has the capacity to act on bound and unbound BILI. Its activity is monitored by observing the decrease in absorbance at 460 nm. The procedure is simple, fast, and quite accurate [14]. However, this procedure can only estimate the total BILI fraction.

F. URIDINE 5'-DIPHOSPHATE GLUCURONYLTRANSFERASES

1. General properties

Uridine-5'-diphosphate glucuronyltransferases (EC 2.4.1.17), also referred to as UDP glucuronosyltrans-ferases, represent activities that catalyze conjugation

of a large number of endogenous metabolites and xenobiotics with glucuronic acid, to facilitate their excretion from the body [15,16]. Glucuronidation is, by far, the most predominant conjugation reaction in the body and an important pathway for detoxication in man [15,16]. The glucuronyl moiety imparts water solubility to hydrophobic compounds, which are then excreted through bile or urine. The mechanism for glucuronidation has not been elucidated. The glucuronic acid functionality is transferred from UDP-α-D-glucuronic acid (UDPGA) to an acceptor (aglycone) to form an β-D-glucuronide. The inversion at the anomeric carbon of glucuronic acid suggests a nucleophilic type of displacement by the aglycone [15,16]. Therefore structures with hydroxyl, carboxyl, sulphydryl, and even carbon can be glucuronidated [15-17].

The liver is the major site of glucuronidation, however, other tissues also contain some UDPGT activity [15,16]. Inside the cells, the activities are widely distributed, and are present mostly within the membranes of smooth and rough endoplasmic reticulum. Small amounts of UDPGT activities are also associated with the membranes of golgi, nucleus, and mitochondria [15]. Membrane-bound preparations (freshly prepared) exhibit latency. That is, the activities of UDPGT are enhanced after membrane perturbation (e.g., mechanical disruption, aging, freeze/thawing, or exposure to detergents,

proteases, phospholipases or certain chemicals) [15]. Two models have been postulated, based on experimental observations, to explain latency and the associated kinetics. The proponents of the compartmentation model believe the enzyme to be deeply embedded within the membrane lattice with the active site on the luminal side [18-21]. They maintain that latency is an expression of restricted entry of polar cosubstrate (UDPGA) or the egress of the product. So activation removes this barrier or makes it leaky. The supporters of the lipid constraint model, argue that the enzyme's active site is exposed to the cytosol. The membrane environment determines the conformation of the transferase, and thus, modulates its activity [22,23]. Activation is looked upon as a conformational change (i.e., relaxation or readjustment of lipoprotein-complex). These models are a subject of a vigorous debate, and although still speculative, there is ample evidence to support both perspectives [15-17]. It is generally agreed that the enzymes are latent in vivo, and can be activated by endogenous activators like UDP-N-acetyl-glucosamine (UDPGLcNAc). According to the compartmentation model, a transport system (permease) for transferring UDPGA to the enzyme has been postulated. UDPGLcNAc stimulates this transport system, and concurrently, the activity of UDPGT [18]. With the constraint model, the UDP-GLcNAc binds to an allosteric site to activate the enzyme (by

lowering the Km for UDPGA) [24].

The capacity to glucuronidate the wide variety of compounds, has been attributed to existence of more than one form of transferase [15,16,19]. Evidence suggests the existence of a number of isoenzymes with variable degrees of overlapping specificities, which facilitate the conjugation of a wide range of compounds [15,17,25-30]. The current notion is that two distinct populations of UDPGTs exist: one for exogenous compounds and one for endogenous ones [17]. The forms catalyzing the conjugation of the endogenous metabolites have low capacities, high affinities, and show strict substrate specificities. This supposedly ensures effective elimination of these compounds. The forms for the exogenous compounds exhibit low affinities, high capacities (Vmax), diverse specificities, and can be induced to a greater degree. Consequently, drugs can be metabolized and disposed of efficiently [17]. Another major difference between the two populations is presence of activities, associated with exogenous compounds, at birth. The population associated with the endogenous compounds develops after birth [17,30].

2. Bilirubin conjugating activity

A transferase protein with a high specificity for BILI has been isolated in a pure form from rat liver [31-34]. It

has an apparent molecular weight of 53-58 Kda [32]. Intra-
cellularly, this enzyme appears to be widely distributed.
The majority of the activity is concentrated within the
membranes of the ER, with some in the golgi and nuclear
envelope [35]. It has been well established that human and
rat bile contain predominantly BDG. Yet, in vitro studies
with BILI have shown synthesis of mainly BMG with small
quantities of BDG, when BILI is incubated with UDPGA and
UDPGT [34,36-39]. The mechanism of BDG formation has not
been established, and is an issue of an on-going debate. It
is agreed that the synthesis of BDG takes place in two
steps. Formation of BMG occurs first, it is then converted to
BDG. The disagreement lies at the synthesis of BDG from
BMG. One school of thought is, as the long-held classical
presumption, that a second molecule of glucuronic acid is
attached to the second half of BILI by the same enzyme
[5,34,40].

The opponents of the above perspective believe that the
capacity of UDPGT to form BDG in vivo is inadequate, and that
another enzyme is involved. Apparently, this enzyme,
bilirubin monoglucuronide glucuronosyltransferase
(EC 2.4.1.95), is located at the canalicular plasma memb-
ranes. It catalyzes a dismutation reaction (transesterific-
ation) involving two molecules of BMG [38,41-43]:

BMG + BMG ---------------> BDG + BILI
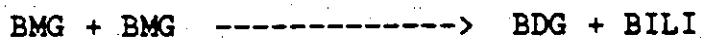
The physiological importance of each mechanism has yet to be
established. A third mechanism has been suggested [44]. These
workers, using radiation inactivation to determine the
molecular weight of UDPGT, obtained an apparent value of
43,000 (compared .to 53-57,000 with conventional biochemical
techniques). They proposed the BILI conjugating system to be
a complex of four subunits, which can be dissociated easily
by detergents. The monomers have the ability to synthesize
BMG at a fast rate but cannot form BDG. Formation of BDG is
only possible when the tetrameric form is intact. This is an
interesting postulate, however, it . does not concur with the
observations that intact microsomes, which presumably have an
intact complex, also synthesize predominantly BMG [45,46].
More recent evidence suggests that the form in which the
substrate is presented may be an important factor. A higher
proportion of BDG, resembling the profile found in rat bile,
was obtained when BILI was presented in a liposomal prepara-
tion, [37]. These results support the notion that BILI is
transferred to UDPGT through membrane-membrane collisions or
diffusion. Furthermore, since composition and structure of
the membrane have a pronounced effect on activity, the
phospholipids may play a role in correctly aligning the BILI
molecule for the enzyme [47]. BILI UDPGT is a thiol

containing enzyme that requires phospholipids and magnesium ions for activity [17,24,31,32,48]. The location of the thiol group is thought to be removed from the active site [49]. Like other UDPGTs, it is latent in freshly prepared microsomes and can be activated by UDPGLcNAc or membrane perturbation.

Kinetic properties of this enzyme have been investigated [23,24,39,50-53]. However, results do not concur. It is thought that differences in isolation procedures may have led to preparations with different phospholipid content [17]. So it is quite possible that forms with kinetically different properties were obtained. Furthermore, modification of experimental conditions for optimization purposes, probably led to altered kinetic behaviour. Therefore, kinetic parameters require a re-evaluation under controlled conditions.

G. OBJECTIVE

The primary objective of this study was to investigate the possibility of employing the membrane bound enzyme, BILI UDPGT, to determine concentrations of BILI in solutions/ fluids. If the initial results were fruitful, then analysis of serum/plasma samples would be studied. The proposed reactions for the analysis of BILI fractions are outlined below:

UNCONJUGATED BILI:

$$\text{SERUM + UDPGA} \xrightarrow{\text{UDPGT}} \text{BMG + UDP}$$

TOTAL BILI:

$$\text{SERUM + B-GLUCURONIDASE} \xrightarrow{\text{UDPGT}} \text{BILI + GLUCURONIC ACID}$$
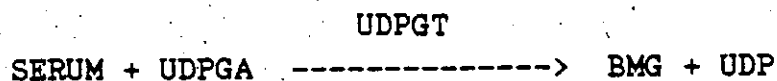
TREATED SERUM + SACCHAROLACTONE + UDPGA ------> TOTAL BILI
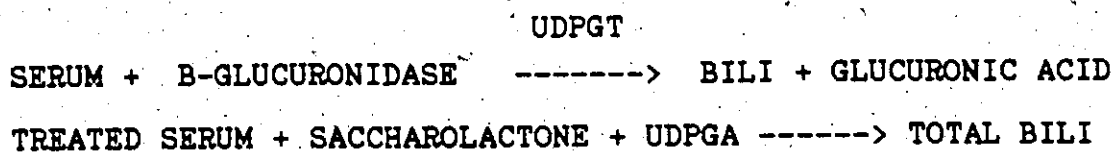
Unconjugated BILI is a substrate for UDPGT, therefore, the first reaction would provide an estimate for the unconjugated fraction. Treatment of sample with B-glucuronidase would convert BILI glucuronides to BILI. 1,4-Saccharolactone, a potent inhibitor of B-glucuronidase, would subsequently permit determination of total BILI. The conjugated fraction would be calculated by subtracting the unconjugated value from the total. An enzyme coupled assay was desired, and the suitability of employing the system described below [25] or a modified version was also investigated:

$$\text{BILI + UDPGA} \xrightarrow{\text{UDPGT}} \text{BMG + UDP}$$

$$\text{UDP + PHOSPHO(ENOL)PYRUVATE} \xrightarrow{\text{PK}} \text{PYRUVATE + UTP}$$

$$\text{PYRUVATE + NADH} \xrightarrow{\text{LD}} \text{LACTATE + NAD}^+$$
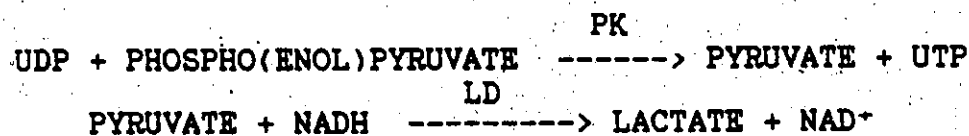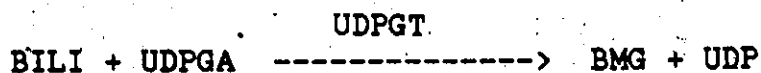
The key enzyme that facilitates an enzyme coupled assay for the UDPGT system is pyruvate kinase (PK). This enzyme has a rather low specificity for a phosphate acceptor. This property was further exploited. Preliminary work to establish suitability of using PK for the analysis of adenosine 5'-diphosphate (ADP), uridine 5'-diphosphate (UDP), and glucose, was also carried out.

CHAPTER II

MATERIALS AND METHODS

A. REAGENTS

4-Aminoantipyrine(AAP), n-butylacetate, 3,5-dichloro-2-hydroxybenzenesulfonic acid (sodium salt)(HDCBS), and 2-pentanone were obtained from Aldrich Chemical Co. (Milwaukee, WIS. USA).

Lactate dehydrogenase (LD) (L-lactate: NAD oxidoreductase, EC 1.1.1.27) was obtained from Amano International Enzyme Co. Inc. (Troy, VA, USA).

Folin and Ciocalteau reagent was obtained from British Drug House (Toronto, ON, Canada).

Pyruvate kinase (PK)(ATP: pyruvate 2-0-phosphotransferase, EC 2.7.1.40, from rabbit muscle) was obtained from Boehringer Mannheim, (Dorval, PQ, Canada).

Octyl-β-D-glucopyranoside and octyl-β-D-thioglucopyranoside were obtained from Behring Diagnostics (Calbiochem^R) (La Jolla, CA, USA).

Bilirubin, caffeine, ethylenediamine tetraacetic acid (disodium salt), and hydrogen peroxide were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA).

Ethyl anthranilate and 2-mercaptoethanol were obtained from Eastman Kodak Co. (Rochester, NY, USA).

33

Glucose calibration serum (ENI) was obtained from Electro-
nucleonics Inc. (Fairfield, NJ, USA).

Pyruvate oxidase (POx) (pyruvate: oxygen oxidoreductase,
phosphorylating, EC 1.2.3.3, from Pediococcus sp.) was a gift
from Finnsugar Biochemicals Inc. (Shaumburg, IL, USA).

Pediatric bilirubin control serum was obtained from Ciba
Corning Diagnostics, Gilford (Irvine, CA, USA)

Lubrol 12A9 was purchased from Imperial Chemical
Industries (Bolton, England).

Emulgen 911 was a gift from KAO Corporation (Tokyo,
Japan).

Hexokinase type V (ATP: D-hexose 6-phosphotransferase,
EC 2.7.1.1, from yeast), horseradish peroxidase type X (PER)
(donor:hydrogen-peroxide oxidoreductase, EC 1.11.1.7),
ascorbic acid, adenosine 5'-diphosphate (ADP) (sodium salt),
adenosine 5'-diphosphate (ATP) (sodium salt), bovine serum
albumin (BSA) (fraction V, fatty acid free), flavin adenine
dinucleotide (disodium salt) (FAD), p-hydroxybenzoic acid
methyl ester (methyl paraben), NADH (grade III), 4-nitro-
phenol (4-n-p), phospho(enol)pyruvate (PEP) (monocyclohexyl-
ammonium salt), phenylbutazone (4-butyl-1,2-diphenyl-3,5-
pyrazolidinedione), D-saccharic acid-1,4-lactone, sulfadi-
methoxine (sodium salt), thiamine pyrophosphate chloride
(TPP) (cocarboxylase, aneurine pyrophosphate),uridine
5'-diphosphate (sodium salt) (UDP), UDP glucuronic acid

(UDPGA) (sodium salt), and UDP-N-acetylglucosamine(UDPGLcNAc)
(sodium salt) were obtained from Sigma Chemical Co.
(St. Louis, MO, USA).

Other reagents, conforming to the standards specified by
the American Chemical Society, were obtained from the
Department Stock Room.

B. PROCEDURES

1. Standard assay conditions

UDPGT activity towards BILI was assayed using slightly
modified conditions published by Burchell [33]. The prod-
uction of BILI glucuronides was assayed with the diazo
procedure described by Van Roy and Heirweigh [45].
Incubations were carried out at 37°C in 5-mL glass screwtop
test tubes. In a total volume of 200 µL, the incubation
mixture contained 90 µM BILI, 120 mM triethanolamine-HCl
buffer pH 7.8, 15 mM $MgCl_2$, 5 mM saccharo-1,4-lactone. 5 mM
UDPGA, 40-50 µL enzyme preparation (0.7-1.3 mg protein) and
2.5 mM UDPGLcNAc (all final concentrations). With the
solubilized fractions, UDPGLcNAc was omitted. Stock BILI
solutions were prepared by dissolving 3.7 mg solid BILI in
0.5 mL of 0.05 M sodium hydroxide. This mixture was mixed
with 9.5 mL of buffered bovine serum albumin (BSA) solution
(0.4 M triethanolamine-HCl, pH 7.8, 20 mg/mL, BSA). A working

stock solution was obtained by diluting one volume of this stock with 2 volumes of 20 mM triethanolamine-HCl buffer, pH 7.8. The incubation cocktail containing everything except UDPGA was preincubated for 5 min. The reactions were initiated by the addition of UDPGA. Cold glycine buffer, pH 2.7 (1 mL), was used to terminate enzyme activity. The resulting solutions were kept on ice until coupling with the diazo reagent could be performed (usually within 10-40 min).

2. Diazo coupling with ethyl anthranilate

Fresh diazo reagent was prepared for each experiment and used immediately. Ethyl anthranilate (0.1 mL), suspended in 10 mL of 0.15 M HCl by vigourous shaking, was mixed with 0.3 mL sodium nitrite (0.1 g/mL) and allowed to stand for 5 min at room temperature. Ammonium sulfamate (0.1 mL, 10 mg/mL) was added to destroy excess sodium nitrite. The solution was allowed to stand for 3 min before use. Samples, treated with 0.5 mL of diazo reagent, were allowed to develop for 30 min. Freshly prepared ascorbic acid (0.5 mL, 0.57 M) was added to consume excess aryl diazonium ions. Azo derivatives were extracted into 1.4 mL of 2-pentanone: n-butyl-acetate (17:3) by vigorous shaking for 1 min. Phase separation was accomplished by centrifugation on a table-top clinical centrifuge (International Equipment Co.) at full speed for 5 min. The absorbance was measured at 530 nm

against appropriate blanks. Blanks contained everything except UDPGA. Measurements were performed with either the Shimadzu Model 240 double beam recording spectrophotometer or the Hewlett Packard Model 8451A diode array spectrophotometer. The entire procedure was carried out under subdued light to avoid photodegradation of BILI. This diazo procedure was used during the evaluation and optimization of the UDPGT assay.

3. Preparation of microsomes

Wistar rat livers were the source of microsomes and solubilized enzyme. Microsomes were prepared using the procedure described by Lewis and Armstrong [54]. Figure 5 outlines the procedure schematically.

Chopped rat liver, suspended in 4-5 volumes of buffer (50 mM Tris-HCl, pH 7.4, 0.15 M KCl, 0.3 mM 2-mercaptoethanol was homogenized in a Potter-Elvehjem glass-teflon homogenizer. Centrifugations were performed on a Beckman L8-55 ultracentrifuge at 4°C. The sequence of centrifugations are outlined in Fig 5. Microsomes were washed with 10 mM EDTA in a buffer system (10 mM Tris-HCl, pH 7.4, and 0.15 M KCl) and stored at -140°C (liquid N_2) as a suspension in an equal volume of 25 mM Tris-HCl buffer (0.3 mM 2-mercaptoethanol, pH 8.0, 15% glycerol).

FIGURE 5

PREPARATION OF MICROSOMES AND SOLUBLE ENZYME

Legend

Flowchart outlines the steps in the preparation of microsomes and the solubilized enzyme.

FIGURE 5

RAT LIVERS

↓

HOMOGENIZATION

↓

CENTRIFUGATION (15,000 xg)

↓

SUPERNATANT   PELLET

↓

CENTRIFUGATION (105,000 xg)

↓

SUPERNATANT   PELLET

↓

EDTA WASH

↓

CENTRIFUGATION (105,000 xg)

↓

MICROSOMES   SUPERNATANT

↓

DETERGENT/SONICATION

↓

STIRRING (30 min)

↓

CENTRIFUGATION

↓

VISCOUS PROTEIN
(SOLUBLIZED ENZYME)
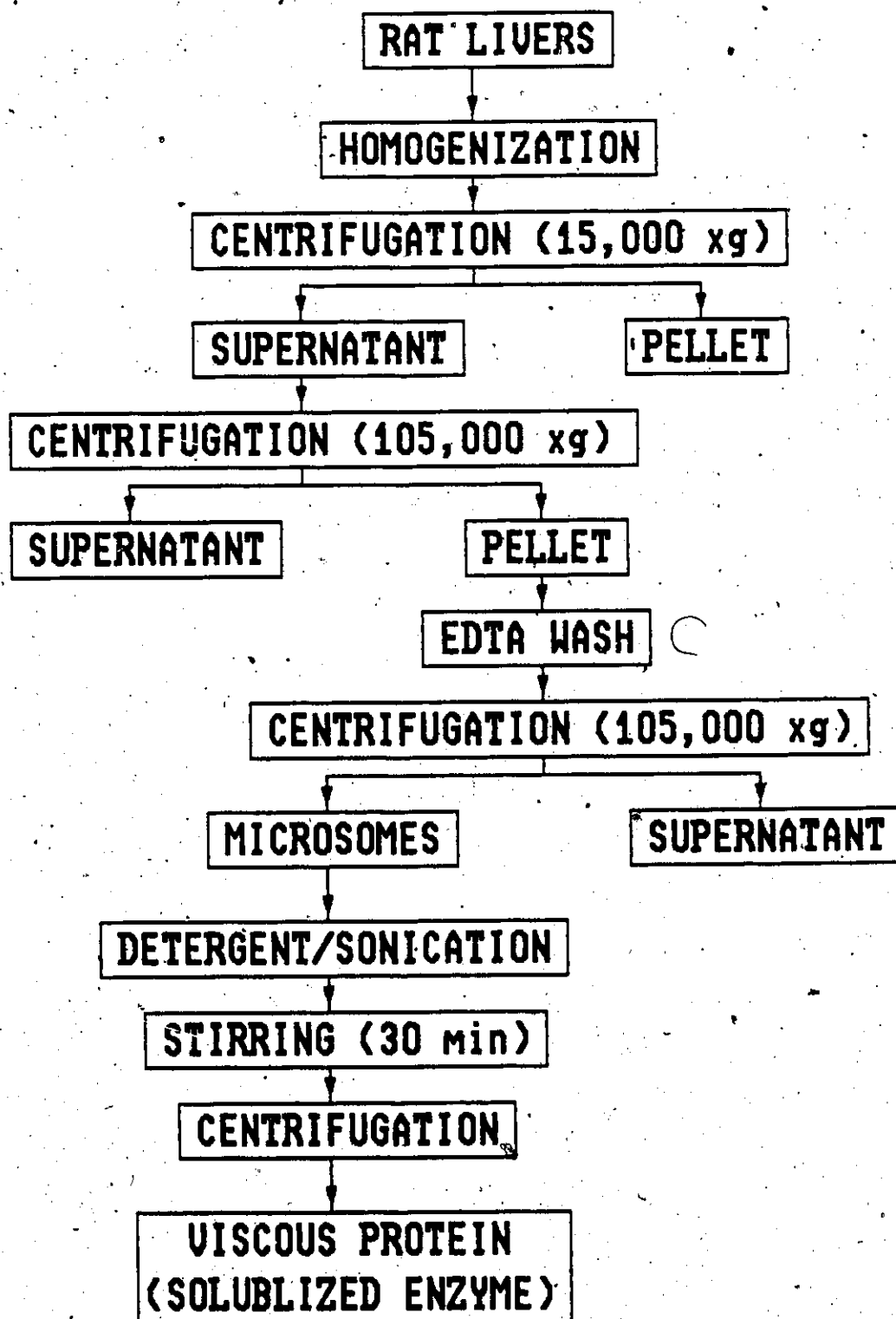
4. Solubilization of UDPGT activity

Microsomes (1 g wet weight ,fresh or frozen) were suspended in adequate amounts of buffer (25 mM Tris-HCl, 0.3 mM 2-mercaptoethanol, pH 8.0, 15% glycerol) to obtain a total volume of 4.5 mL. Detergent solution, 0.5 mL 0.3 M octyl-β-glucopyranoside (34 mM final concentration), was added, and the resulting mixture was mixed with a 10-mL glass-teflon homogenizer by hand for 1 min. Solubilization was faciliated with two 10-sec bursts with a Sonic 300^{Tm} dismembrator (Artek Systems Corp., Farmingdale, NY, USA) (set at 70% of maximum power). The microsomal preparation was immersed in an ice bath at all times to minimize temperature rise. The resulting solution was stirred for 30 min at 4°C before centrifugation at 105,000 x g for 120 min. The thick viscous protein layer above the pellet, which contained more than 90% of the UDPGT activity, was the source of the solubilized enzyme. This protein solution was diluted with an equal volume of the above buffer, and used for study. Protein was estimated by the method of Lowry et. al.[55].

5. Effect of incubation medium components on UDPGT activity

With BILI kept constant at 90 µM, concentrations of the assay components were varied one at a time. Components tested included UDPGA, assay buffer, and MgCl₂.

Concentrations of glycerol, EDTA, and phosphatidylcholine

were also tested, one at a time, for their effect on UDPGT activity. Phosphatidylcholine, obtained as a methanolic solution, was prepared according to the procedure described by Burchell [33]. Methanol was evaporated at 37°C, under nitrogen, and mixed with the assay buffer to obtain a concentration of 10 mg/mL. The solution was used after sonication at 4°C to obtain a suspension of lecithin liposomes.

6. Temperature studies

a. Effect of assay temperature on activity: Buffer solutions for assays at 25°C, 30°C, 37°C, 40°C, and 45°C were prepared at their respective temperatures and used in the assays. Amounts of conjugated BILI produced, at each temperature over a 5-min period were measured.

b. Temperature and stability: The incubation cocktail mixtures, in the absence of UDPGA, were pre-incubated at 37°C, 40°C, and 45°C for varying periods of time. intervals. The pre-incubation intervals were of 5, 10, 20, 25, and 30-min duration. Activities were measured, as described above, at 37°C, immediately following pre-incubation.

7. Studies with albumin-BILI solutions

a. Effect of BILI concentration on UDPGT activity: BILI glucuronide production was measured in the presence of varying amounts of albumin:BILI solution (1:2, molar ratio).

Incubations were of 2, 5, and 10-min duration. Conjugated BILI production was measured, colorimetrically, with the diazo procedure.

b.   Linear range: Various concentrations of BILI were assayed with the UDPGT/diazo method. The concentrations ranged from 30 µM to 350 µM. Measurements were made at 2 and 5-min intervals. Analyses were performed in triplicate.

c.   Standard curve: Stock BILI was diluted 7,6,5,4,3- and 2-fold with 20 mM assay buffer. Aliquots of 60 uL were analyzed. Measurements were made at 2 and 5-min points with the diazo procedure.

8. Comparison with the Jendrassik-Grof method

Stock BILI solution was diluted 7,6,5,4,3- and 2-fold with 20 mM assay buffer. Aliquots of 60 µL of each solution were assayed by the enzymatic method and the Jendrassik-Grof method. In the enzymatic procedure, the amounts of conjugated BILI were assayed with diazotized ethyl anthranilate. With the Jendrassik-Grof method, the amount of total BILI was quantitated with diazotized sulfanilic acid, in the presence of caffeine/sodium benzoate/acetate as the accelerating agent. This procedure was carried out, at Salvation Army Grace Hospital Laboratory, on Flexigem™, a centrifugal analyzer (Electronucleonics Inc. Fairfield, NJ USA).

9. Analysis of bilirubin in serum

a. Serum samples: Patient samples were obtained from S.A. Grace Hospital. Aliquots of patient samples (50 µL) were substituted for BILI solutions and assayed.

b. Recovery study: Varying amounts of BILI solution were assayed in the presence of 0,10, and 30 µL of serum.

c. Effect of varying UDPGA: BILI concentration was fixed at 90 uM. UDPGA was varied from 1 mM to 3.8 mM.

10. Fractionation and electrophoresis of serum

a. Gel filtration: Pooled serum, diluted 1:4 with 0.15 M KCl in 20 mM Tris-HCl (pH 7.8), was subjected to fractionation a column of Sephadex^{Tm} G-200 (1 cm x 120 cm) equilibrated in the same buffer. 5 mL of the diluted serum were applied to the column and 2 mL fractions were collected at a flow rate of 9 mL/hr. Major protein bands (monitored at 280 nm) were pooled and concentrated with Amicon^{Tm} membrane cones (Centriflo^R, 25,000 mwt cut-off). Protein solutions were transferred to the cones and centrifuged at 3000 rpm for 10 min. Centrifugations were performed with a RC2-B Sorvall^R centrifuge with SS-34 rotor. All procedures described above were performed at 4°C. The retained fractions as well as the filtrates were assayed for inhibitory action against UDPGT. This was accomplished by assaying the activity of the preparation in the presence and absence of the various

fractions, 5-min glucuronide production (in the presence of 90 μM BILI) was measured with the ethyl anthranilate diazo procedure.

b. Agarose gel electrophoresis: This procedure was performed at S. A. Grace Hospital, as part of a routine serum electrophoresis run. The procedure employs commercially prepared agarose gels from Ciba Corning Diagnostics (Oberlin, OH USA). Separation (of inhibitory protein band from above) was performed in 50 mM sodium barbital buffer, pH 8.7 for 35 min at fixed setting of 90 volts. Protein bands were visualized by staining with 0.5% Ponceau-S stain in 10% acetic acid (15 min). Destaining was done in 5% acetic acid. Densitometer scans were performed with the Corning 710 densitometer set at 520 nm.

11. Effect of albumin on UDPGT activity

a. Activity studies: Bilirubin and UDPGA concentrations were varied in the presence of 0, 0.2 and 0.4 mg BSA (in addition to that present in substrate solution). Activities were determined with the diazo method, as described before.

b.. Time study: Production of conjugated BILI was monitored over time in the presence of 0, 0.2 and 0.4 mg additional BSA. Measurements were carried out at 2,5,10, and 15-min intervals.

c. Time study with free, BSA- or HSA-bound BILI: Stock solution of free BILI was prepared by dissolving 3.7 mg BILI in 2.5 mL of 0.05 M NaOH. The resulting solution was diluted to 10 mL with water. HSA:BILI solution was prepared as described for BSA solution. Reactions were stopped and assayed at 2,5,10,15, and 20-min intervals.

d. Standard curves for free and bound BILI: BILI (free, BSA-, and HSA-bound) concentrations were varied from 15 µM to 75 uM. Incubations were of 5-min duration.

12. Displacement of bilirubin and UDPGT activity :

Caffeine/benzoate, ethanol, phenylbutazone, p-hydroxy-benzoic acid methyl ester, and sulfadimethoxine were used to displace/solubilize BILI. Concentrations were varied from 0.05 mM to 15 mM in the presence of 0.4 mg additional BSA or 30 uL serum. Activities, obtained in the presence of these agents, were compared with runs without additional BSA/serum or displacing agents (controls). Blanks consisted of control with displacing agent and control with displacing agent but no BILI.

13. Enzyme coupled assays

Measurements for the enzyme coupled assay were carried out at 37°C using the Beckman Model 35 spectrophotometer or the Hewlett-Packard Model HP 8451A spectrophotometer. Both

instruments were equipped with thermostated cuvette holders.

a. Modified Mulder and Van Doorn coupled assay: Conditions described by Colin-Neiger et al. [56] were employed. The assay cocktail (600 uL total volume) contained 150 mM triethanolamine-HCl buffer (pH 7.8), PK (8U), 0.7 mM PEP, LD (0.5U), 0.01 mM NADH, and 50 μL of solubilized enzyme preparation. Reactions were initiated with UDPGA and the decrease in absorbance at 340 nm was monitored.

b. Enzyme coupled assay II: The coupled enzyme coupled assay described by Bozimowski et al. [57] was applied to our system.

BILI conjugating activity: PK (8U), 0.7 mM PEP, POx (1U), peroxidase (2U), 10 uM FAD, and 2 mM TPP, were added to the standard assay mixture. Total reaction volume was 600 μL. The reactions, initiated with UDPGA, were monitored continuously by following the decrease in absorbance at 460 nm. For convenience, a working cocktail with all the components, except UDPGT, UDPGA and BILI was prepared in advance. This solution was placed in an ice bath in the dark and used within 2 hr.

c. 4-n-p conjugating activity: Standard assay components with PK (8U); 0.7 mM PEP, POx (1U), 10 uM FAD, 2mM TPP, peroxidase (2U), 0.5 mM HDCBS, and 0.5 mM AAP were used. BILI solutions were substituted with that of 4-n-p. Reactions were initiated with UDPGA, and monitored

continuously at 510 nm. Working cocktail solutions were also used here. Total reaction volume was 600 µL.

14. Enzyme coupled assay for ADP and UDP

The cocktail used for the assay of 4-n-p activity was used. However, 4-n-p, UDPGT, and UDPGA were omitted. Aliquots of ADP and UDP stock solutions were pre-incubated with the assay cocktail solution, at 37°C for 5 min. Reactions, were initiated by adding PEP. Analysis of ADP was carried out in the presence of 5 mM KCl.

15. Analysis of glucose

In addition to the components for the assay of ADP, the cocktail also contained hexokinase (1U). All samples were pre-incubated at 37°C (5 min) before measurement. Reactions were initiated with ATP (0.16 mM, final concentration). Aqueous glucose solutions or glucose serum standard (from Electronucleonics Inc.) were used for calibration purposes. Serum samples were diluted 20-fold and 60-µL aliquots were analyzed.

16. Effect of albumin on oxidation of BILI by peroxidase/H_2O_2

Assay components were peroxidase (1U), 10 µM BILI, 120 mM triethanolamine-HCl buffer, pH 7.8,. Oxidation was initiated with 60 nmol of H_2O_2 (100 µM final concentration,

600 µL total volume).   The rate of decrease of absorbance was monitored, at 460 nm, in the presence of 0, 0.5,   1.5, 2 and 2.5 mg of extraneous BSA.

CHAPTER III

RESULTS

1. Ethyl anthranilate diazo method

Before using the ethyl anthranilate assay for our enzyme studies, some time was spent becoming familiar with the methodology. For this purpose, Gilford pediatric control serum was used as a source of our conjugated BILI. Figure 6 shows the spectra of the solvent system, diazo ethyl anthranilate, and the azopigment (in the presence of BILI). Figure 7 shows the spectra, before and after derivatization, with various concentrations of BILI. It was observed that presence of BILI contributed to the absorbance at 530 nm. Incubation of unconjugated BILI with the diazo reagent, up to a period of 20 min, did not produce detectable amounts of azopigments at all concentrations studied (up to 160 μM). Therefore, under the experimental conditions, unconjugated BILI did not react (significantly) to produce detectable amounts of diazo derivatives. Some colour development was observed when microsomal proteins, in the absence of BILI, were incubated with the colour reagent. The colour development is thought to occur as a result of the reaction of the diazo reagent with amino acid residues of proteins [10]. These results indicated the inclusion of BILI,

49

FIGURE 6

SPECTRAL PROPERTIES OF THE DIAZO SYSTEM

Legend

I. Absorption spectrum of the solvent (2-pentanone:
    butylacetate, 17:3)

II. Absorption spectrum of 1% ethyl anthranilate in
    the solvent

III. Absorption spectrum of diazotized ethyl
    anthranilate (1%) in solvent

IV,V. Absorption spectra of azopigments in the presence
    of unconjugated BILI. Aliquots of pediatric
    control serum (Gilford) were reacted with diazo-
    tized ethyl anthranilate. The concentrations of
    conjugated BILI in curves IV and V correspond to
    0.75 µM and 1.5 µM, respectively.

FIGURE 6

ABSORBANCE

WAVELENGTH(nm)
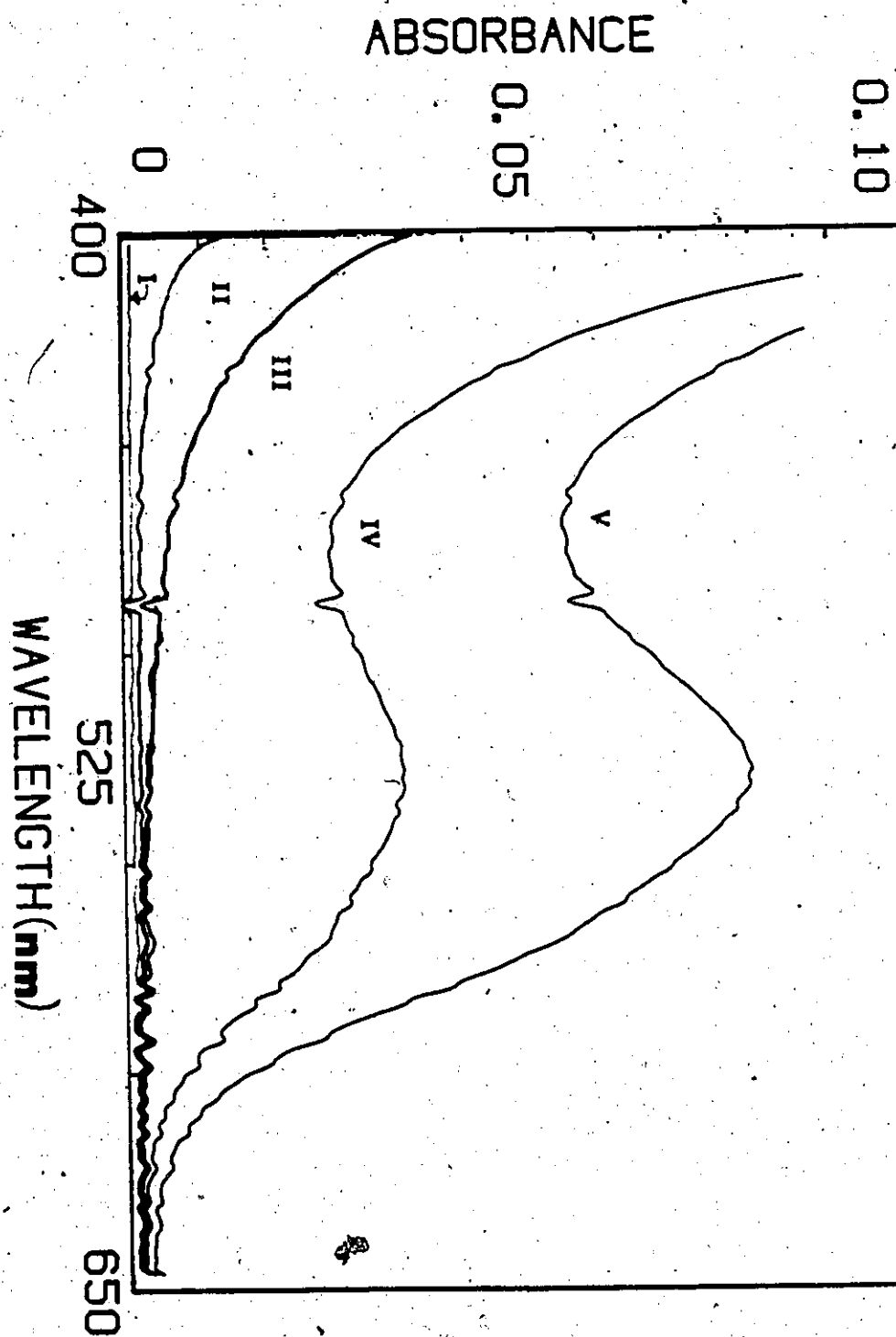
FIGURE 7

SPECTRAL PROFILES OF BILIRUBIN AND AZOPIGMENTS

Legend

Curves A, B, C, and D represent the spectra of pure BILI in pentanone:butylacetate (17:3). The final concentrations of BILI are A. 7.0 uM; B. 4.5 uM; C. 3.0 uM; D. 1.5 uM. Curves A', B', C', 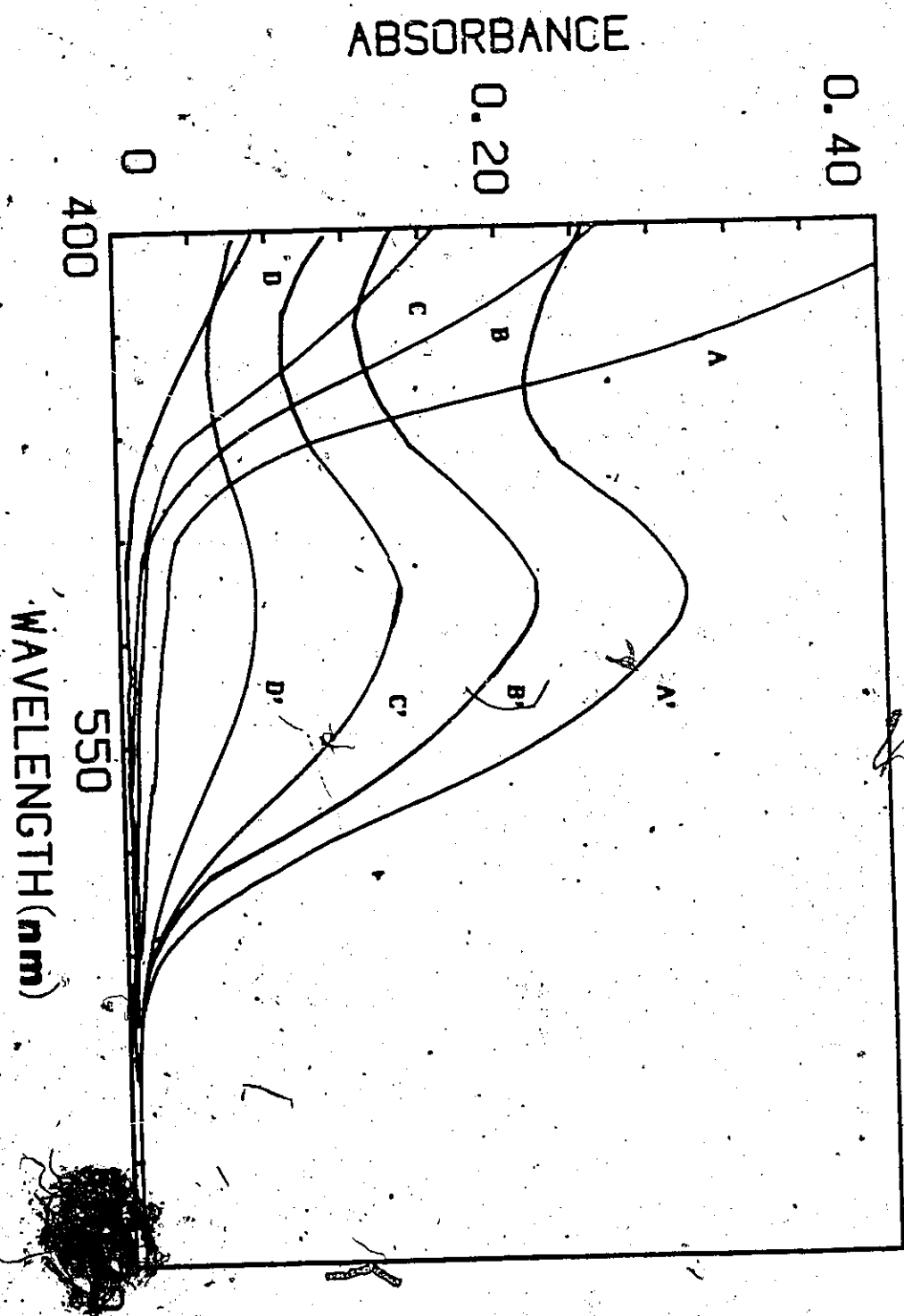and D' are the azopigment spectra obtained with 1.5 uM, 2.2 uM, 3.0 uM, and 3.6 uM conjugated BILI (pediatric control serum), respectively.

FIGURE 7

diazo reagent, and the enzyme protein for the blanks. There-
fore, blank tubes contained all components except UDPGA.
Blanks with UDPGA (added after the glycine buffer, or
when glycine was added immediately following the introduction
of UDPGA) were practically identical.

2. Enzyme preparations

Purification of the UDPGT, using the procedures outlined
by Burchell [32], was attempted. Owing to limited experience
with membrane-bound enzymes, low yields, and instability of
the resulting preparations, preliminary work was carried out
with microsomal suspensions. Work with the solubilized form
and perhaps the semipurified enzyme was to follow, if results
with the microsomes were favourable.

The isolated microsomes, stored in buffered glycerol
solution in liquid nitrogen, possessed activity for at least
3 months. For BILI studies, both fresh and frozen prepar-
ations were employed. Microsomes, suspended in two volumes of
storage buffer, were used in the assays. In the presence of
5mM UDPGLcNAc, the activities of the microsomal preparations
ranged from 0.35-1.1 nmol glucuronide/min/mg microsomal
protein, which compared quite favourably with activities
reported in the literature (0.3-1 nmol/min/mg protein)
[20,33,34,51].

For studies with the solubilized enzyme, UDPGLcNAc

was omitted, because of its inhibitory effect on the enzyme. This inhibition has been reported before and has been attributed to altered kinetic behavior. The inhibition does not appear to be competitive because it is additive in the presence of other competitive substrates. Inhiition by UDPGLcNAc is not observed in the absence of Mg^{+2}, and therefore, it has been surmised that UDPGLcNAc binds to the allosteric site to bring about the inhibition [58].

Solubilization of the enzyme with Triton X-100, deoxycholic acid, Lubrol 12A9, Emulgen 911, octyl-B-D-thioglucopyranoside, and octyl-B-D-glucopyranoside, was carried out. In our experience, the most favourable preparations (with the highest activities) were obtained with octyl-B-D-glucopyranoside. Effect of the detergent concentration on enzyme activity, was studied. Table I shows the progressive inhibition by increasing concentrations of this detergent. Dialysis of the preparation against the storage buffer(~50 volumes), for 2 hr did not produce a more active preparation. The concentration of the detergent in the assay medium was estimated (through volume dilution) to be approximately 0.05%. Solubilization of UDPGT with detergents has been known to delipidate the enzyme and cause inactivation [18,20,31]. Addition of phosphatidylcholine liposomes restores most of the activity. Effect of adding the lecithin preparation was studied. Table II shows the liposomes to have

TABLE I

INHIBITION OF UDPGT ACTIVITY BY DETERGENT

Detergent(%)[1]	UDPGT activity[2] (nmol/min/mg protein)
0.05	1.80
0.10	1.50
0.15	1.28
0.20	1.10
0.25	0.84
0.30	0.68

1. Detergent (octyl- -D-glucopyranoside) was introduced into the assay mixture before the pre-incubation period.
2. Activities were assayed by measuring the 5-min BILI glucuronide formation with the diazo reagent. The assays were performed in triplicate.

TABLE II

EFFECT OF PHOSPHATIDYLCHOLINE ON UDPGT ACTIVITY

Phospholipid[1] μg/assay	UDPGT activity[2] (nmol/min/mg protein)
0	1.85
40	1.52
80	1.54
130	1.77
200	1.78
2000	1.76

1. Phospholipid liposomes were added at the time of pre-incubation.
2. Activities were measured with the ethyl anthranilate diazo procedure as for table I. All analyses were performed in triplicate.

no apparent effect on our preparation.

It has been shown that as the protein/detergent ratio is increased (for solubilization of membrane-bound proteins), the size of the resulting micellar protein aggregate also increases [59]. A larger protein/detergent ratio (compared to commonly used ratios [59]), at the detergent concentration just above its critical micellar concentration (25 mM), was used in hopes of obtaining large protein aggregates. Implicit in this reasoning was that the enzyme delipidation would be minimized. The results of adding extraneous phospholipid supports this notion. Freezing and lyophilization destroyed most of the activity. The preparation was relatively stable at 4°C; showing less than 40% loss in activity after two weeks.

3. Assay conditions

The assay protocol of Burchell [33], slightly modified for optimum activity, was employed. Several different buffers were tested. These included sodium phosphate; Tris-HCl; Tris-maleate; and triethanolamine-HCl (all at pH 7.8). Of these, activity was the highest in triethanolamine-HCl. Concentration of the buffer system was varied, and the optimum activity was obtained at concentrations greater than 0.1 M (Fig. 8). It is possible that increased BILI solubility, brought about by increased ionic strength,

. FIGURE 8

EFFECT OF BUFFER CONCENTRATION ON UDPGT ACTIVITY

<u>Legend</u>

The final concentration of buffer in the assay was varied from 0.05M - 0.20M. Shown are the Activities of the solubilized preparation (0.8 mg protein/assay), measured as the amount of BILI glucuronide formed within a 5-min incubation period. Assays were performed in triplicates, at 37°C, in the presence of 90 uM BILI (final concentration). Measurements were done at 530 nm with the ethyl anthranilate diazo procedure.

FIGURE 8

ABS 530 nm

BUFFER CONCENTRATION(M)
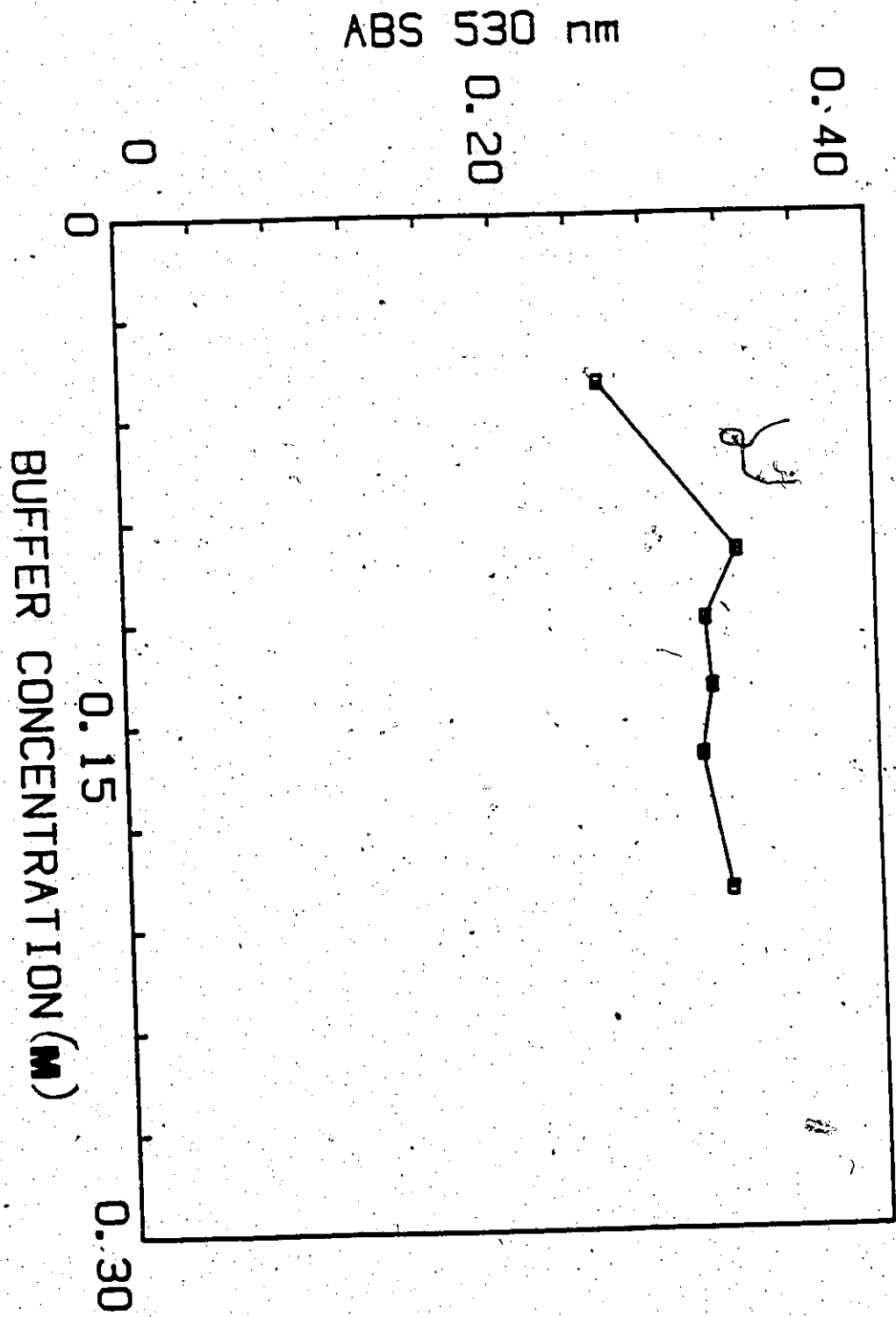
could have been the basis for increased enzyme activity [60]. Alternatively, the buffering capacity, at concentrations below 0.1M, may not have been optimal.

Magnesium ions facilitate the conjugation reaction by enhancing the solubility of BILI [60] and promoting the binding of the allosteric effector in microsomes [58]. The effect of Mg^{+2} has been reported to be biphasic, that is, stimulation followed by progressive inhibition [45,46,52,61]. The maximal stimulation has been reported to occur between 5 and 10 mM. Figure 9 shows the results obtained with our system which shows Mg^{+2} to have only an activating effect up to a concentration of approximately 18 mM. These results agree with that of Gregory and Strickland [52], who did not observe inhibition below 20mM.

The effect of temperature on the activity of UDPGT was studied. The activity was observed to be linearly proportional to the temperature, up to at least 45°C (Fig. 10). The stability study of the enzyme over time at 37°C, 40°C, and 45°C, showed the enzyme to be quite stable at 37°C and 40°C. This was true for both, microsomal and soluble forms. Gradual loss of activity was observed at 45°C with both preparations (Fig. 11). The soluble preparation was slightly more labile, it lost activity faster.

Glycerol was present in assays because it imparted stability to preparations during storage. Increasing
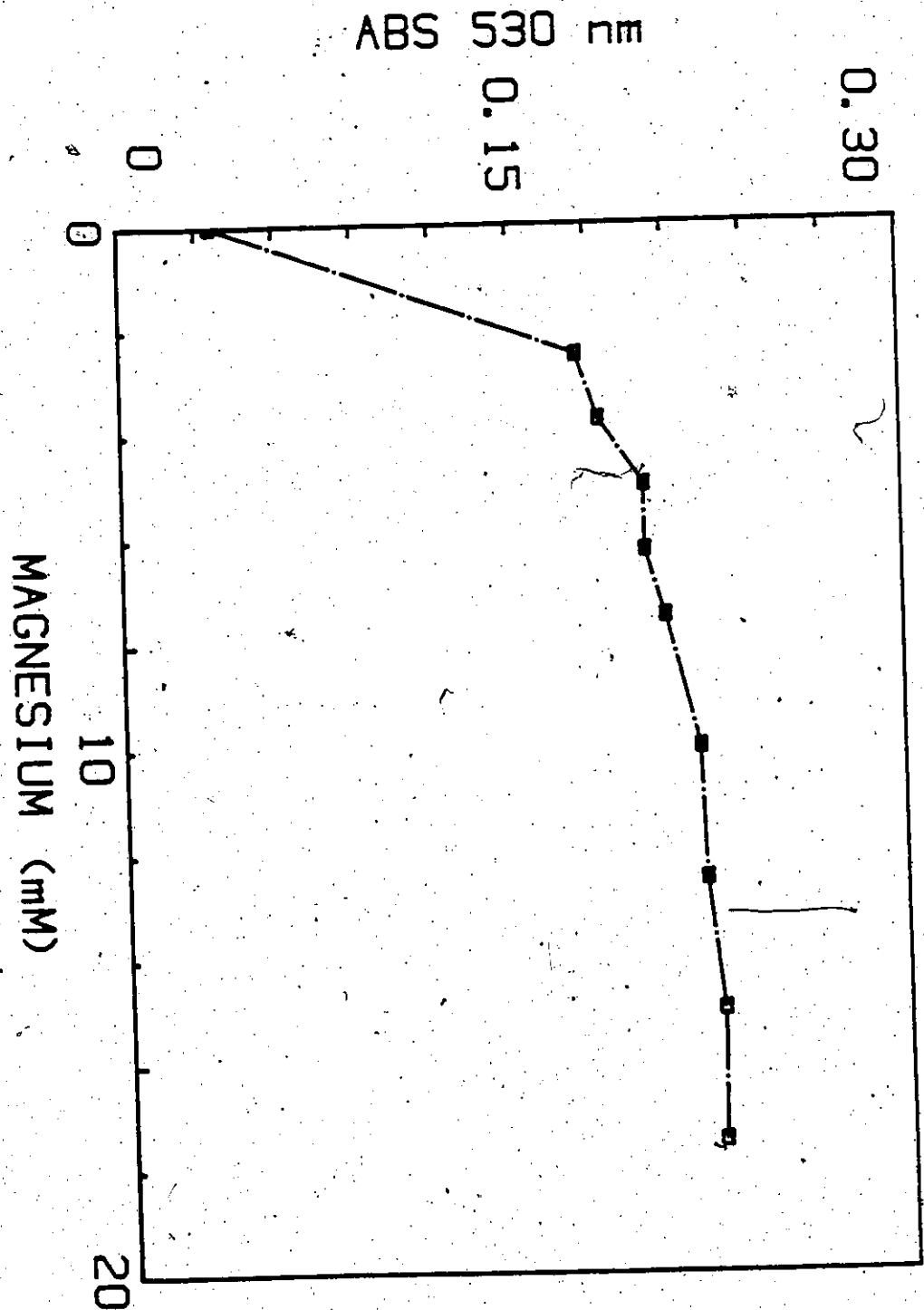
FIGURE 9

EFFECT OF MAGNESIUM ION CONCENTRATION ON UDPGT ACTIVITY

Legend

The concentration of $MgCl_2$, in the final assay mixture
was varied from 0 to 18 mM. Activity of UDPGT was measured
as described for Fig. 8, p. 59. Shown are the results with
the soluble preparation. Each point represents an average of
3 measurements.

FIGURE 9

FIGURE 10

EFFECT OF TEMPERATURE ON UDPGT ACTIVITY

Legend

UDPGT activities were measured at 25°C, 30°C, 37°C, 40°C
and 45°C. The assays were performed as described for
Fig. 8, p. 59, at the respective temperatures. Assay
buffers were prepared at the appropriate temperatures
to maintain the pH. Both, microsomal and solubilized
activities varied linearly with temperature. Shown are
the results with the solubilized preparation, performed
in triplicate.

FIGURE 10
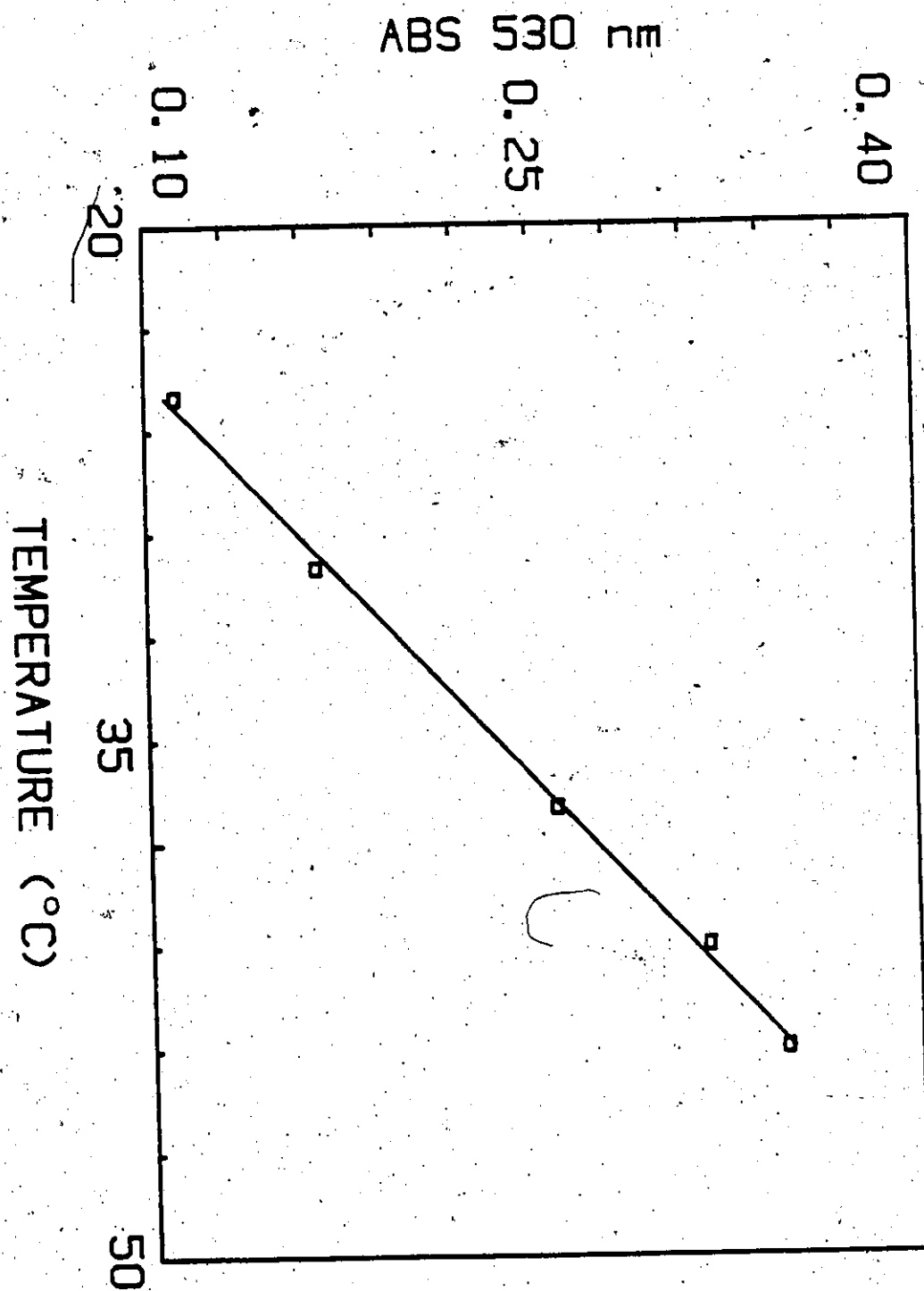
FIGURE 11

THERMAL STABILITY OF ENZYME PREPARATIONS

Legend

Activities in the enzyme preparations, pre-incubated
at the various temperatures for appropriate lengths of
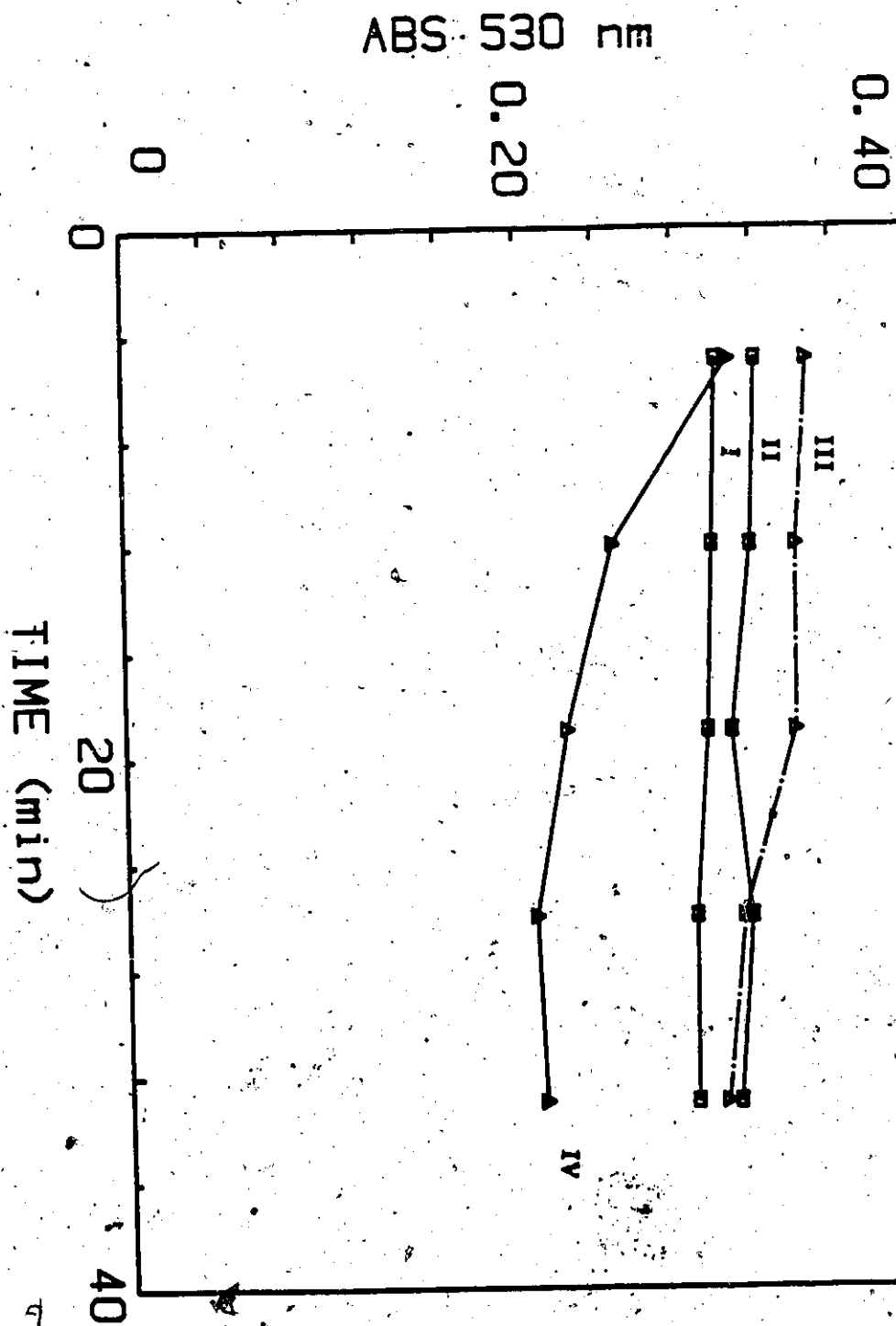time, were measured at 37°C. The 5-min glucuronide
synthesis was measured as described for Fig. 8, p. 59.
All measurements were performed in triplicate.
(I: microsomes, 37°C; II: microsomes, 40°C; III: micro-
somes, 45°C; IV: solubilized enzyme, 45°C).

FIGURE 11

ABS 530 nm

concentrations of glycerol were inhibitory to the intact microsomal enzyme, but not to the solubilized form (Fig. 12). This inhibitory effect may possibly be a result of increased viscosity of the reaction medium. Ethylenediamine tetra-acetic acid (EDTA) has been reported to have an activating effect on the enzyme at low concentration and inhibitory effects at higher concentrations [45]. Our results contra-dicted these observations; EDTA did not have any effect on our preparations (Fig. 13). The activation observed may have been due to removal of inhibitory metals by EDTA[46]. However, in our case, such metals would have been removed by the EDTA wash (in the course of microsomal preparation). The inhibition may have been a result of Mg^{+2} chelation by EDTA to levels below that required for optimal activity [50].

4. Analysis of albumin:BILI solutions

The initial studies were aimed at establishing an appropriate type of assay (endpoint or kinetic). Time-based studies clearly showed glucuronide production to vary with BILI concentrations (Fig. 14). The data was transformed and replotted as a kinetic type of assay. The absorbance at the 2-min point was subtracted from the 5 and 10-min readings, and plotted against their respective concentrations (Fig. 15). This linear relationship prompted an investigation for a kinetic type of assay. A wide range of BILI concentra-

FIGURE 12

EFFECT OF GLYCEROL ON UDPGT ACTIVITY.

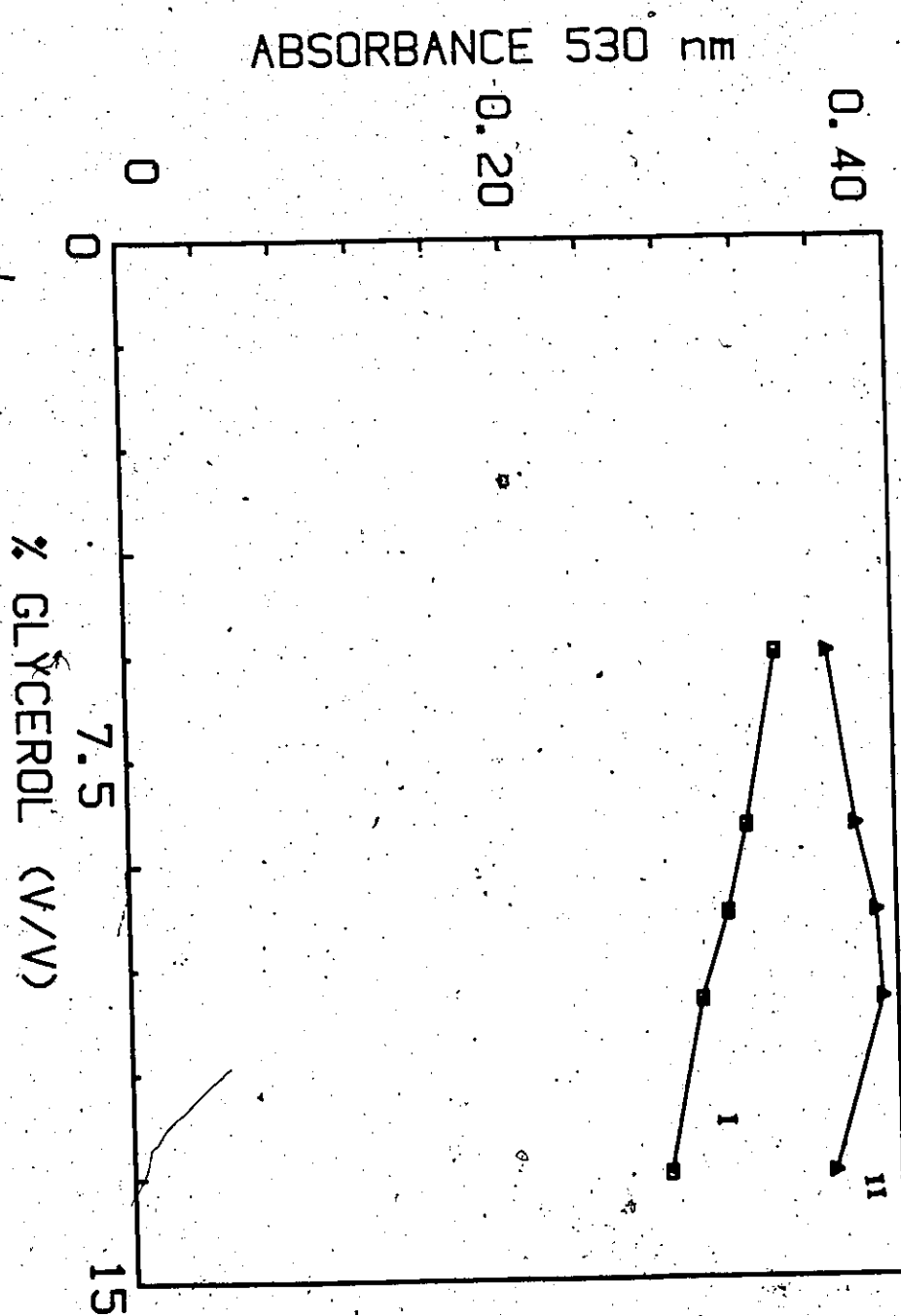### Legend

Assay of BILI conjugating activity, in microsomes and the solubilized preparation was performed in the presence of varying amounts of glycerol. The final glycerol concentrations ranged from 6 - 13.5% (v/v). Activity measurements were made as described for Fig. 8, p. 59. Average of triplicate measurements are shown. (I: microsomes; II: solubilized enzyme).

FIGURE 12

ABSORBANCE 530 nm

FIGURE 13

EFFECT OF ETHYLENEDIAMINE TETRAACETIC ACID
ON UDPGT ACTIVITY

Legend

Shown is the effect of varying amounts of EDTA, on
the activity of UDPGT (solubilized preparation).
Response with the microsomal suspension was similar.
Enzyme activity (UDPGT) at each EDTA concentration was
measured as described for Fig. 8, p. 59, in triplicate.

FIGURE 13
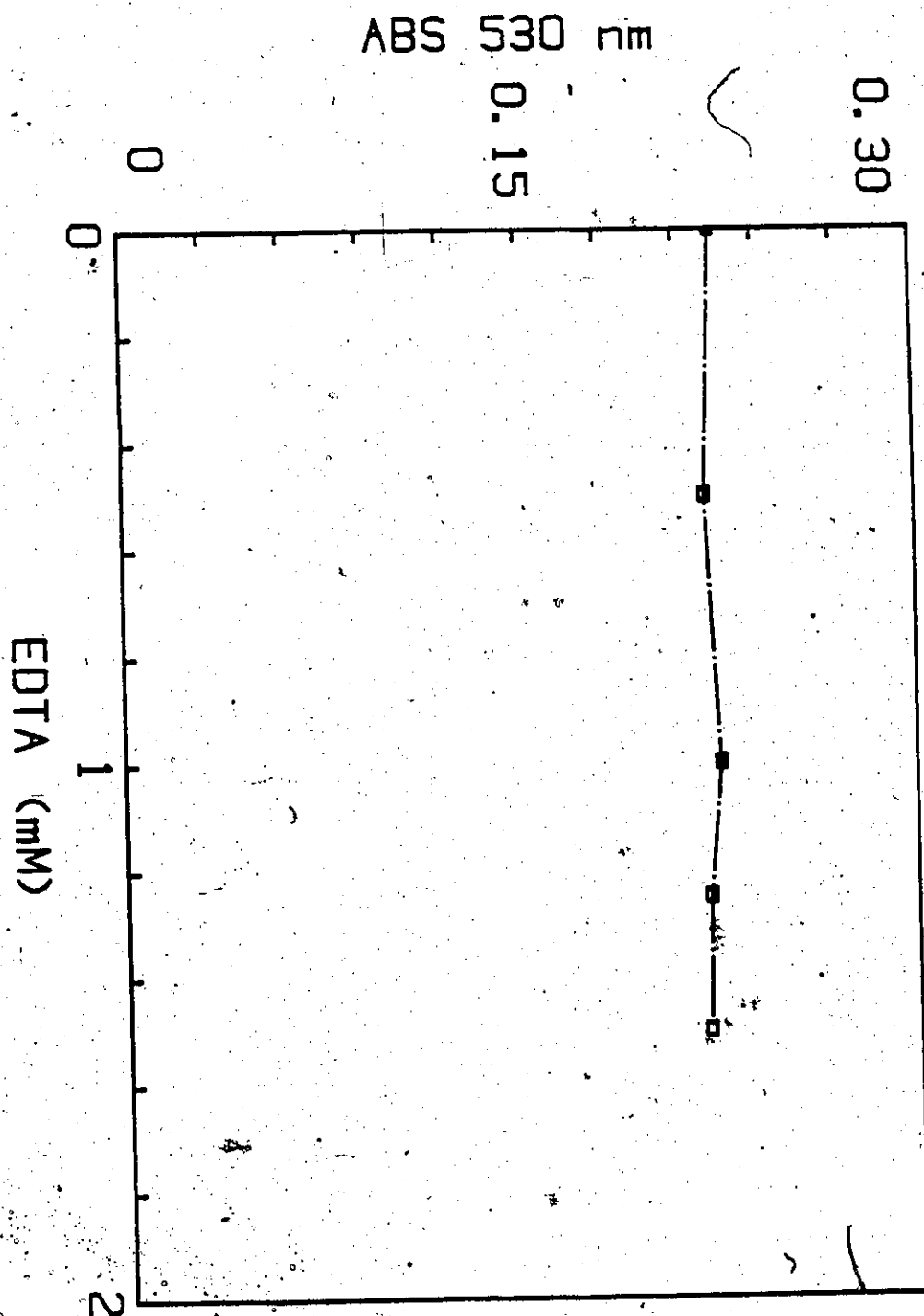
FIGURE 14

BILIRUBIN GLUCURONIDE SYNTHESIS WITH TIME

Legend

BILI glucuronide production, by microsomal susp-
ension and solubilized enzyme, was studied as a function
of time and.BILI concentration. Measurements were made
at 2, 5 and 10-min intervals, with the ethyl
anthranilate diazo colorimetric assay. Shown. are the
average of triplicate measurements obtained with the
solubilized preparation. (Concentrations of BILI: I: 16
uM; II: 24 uM; III: 32 uM; IV: 48 uM).

FIGURE 14

ABSORBANCE 530 nm
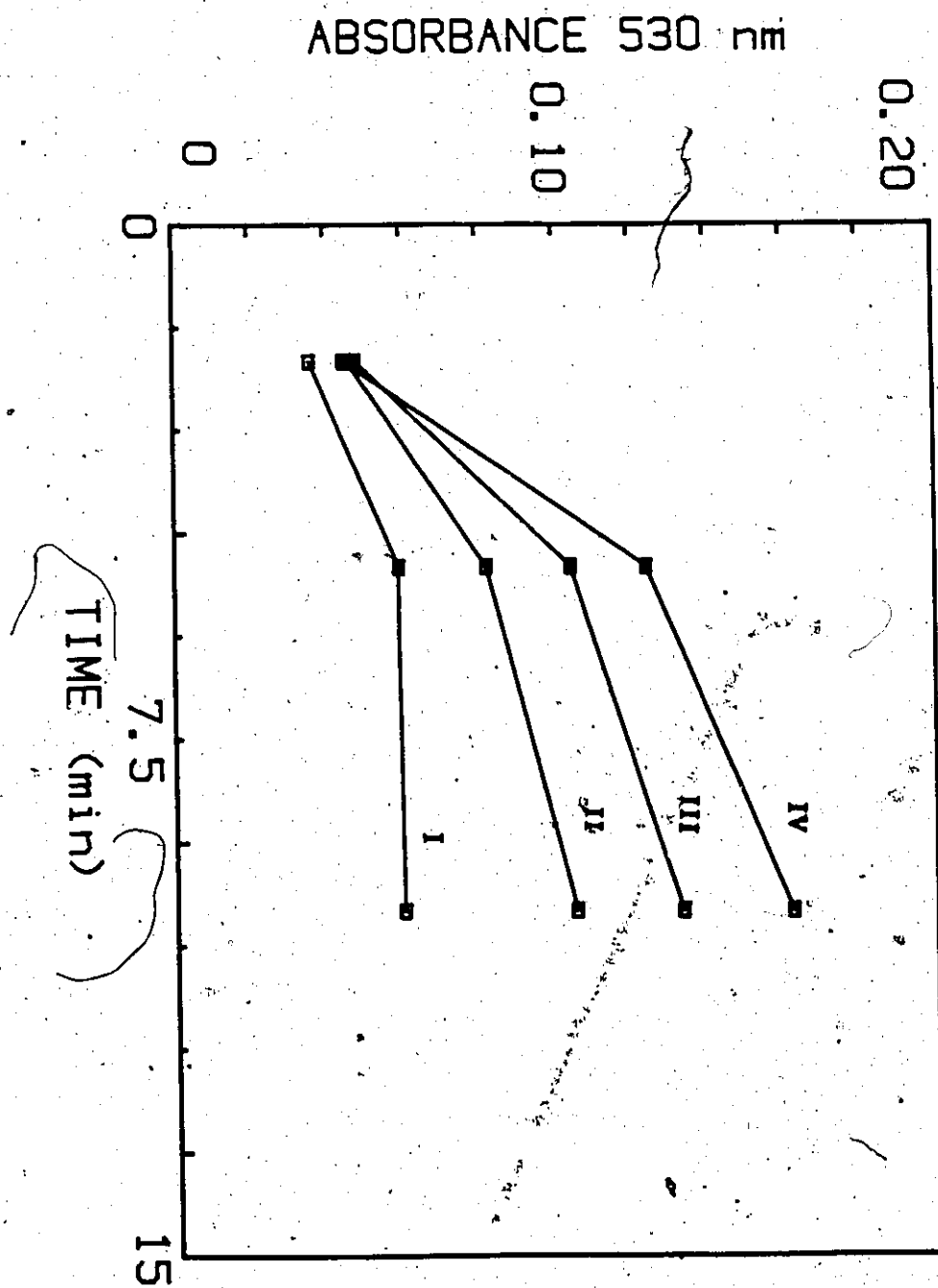
FIGURE 15

DEPENDENCE OF UDPGT ACTIVITY ON BILIRUBIN CONCENTRATION

Legend

The data from Fig. 14 was replotted by subtracting
values at the 2-min point, from the respective 5 and
10-min measurements. The rate of change of absorbance
was plotted against the respective concentration.
(I: 5-min points; II: 10-min points).

FIGURE 15

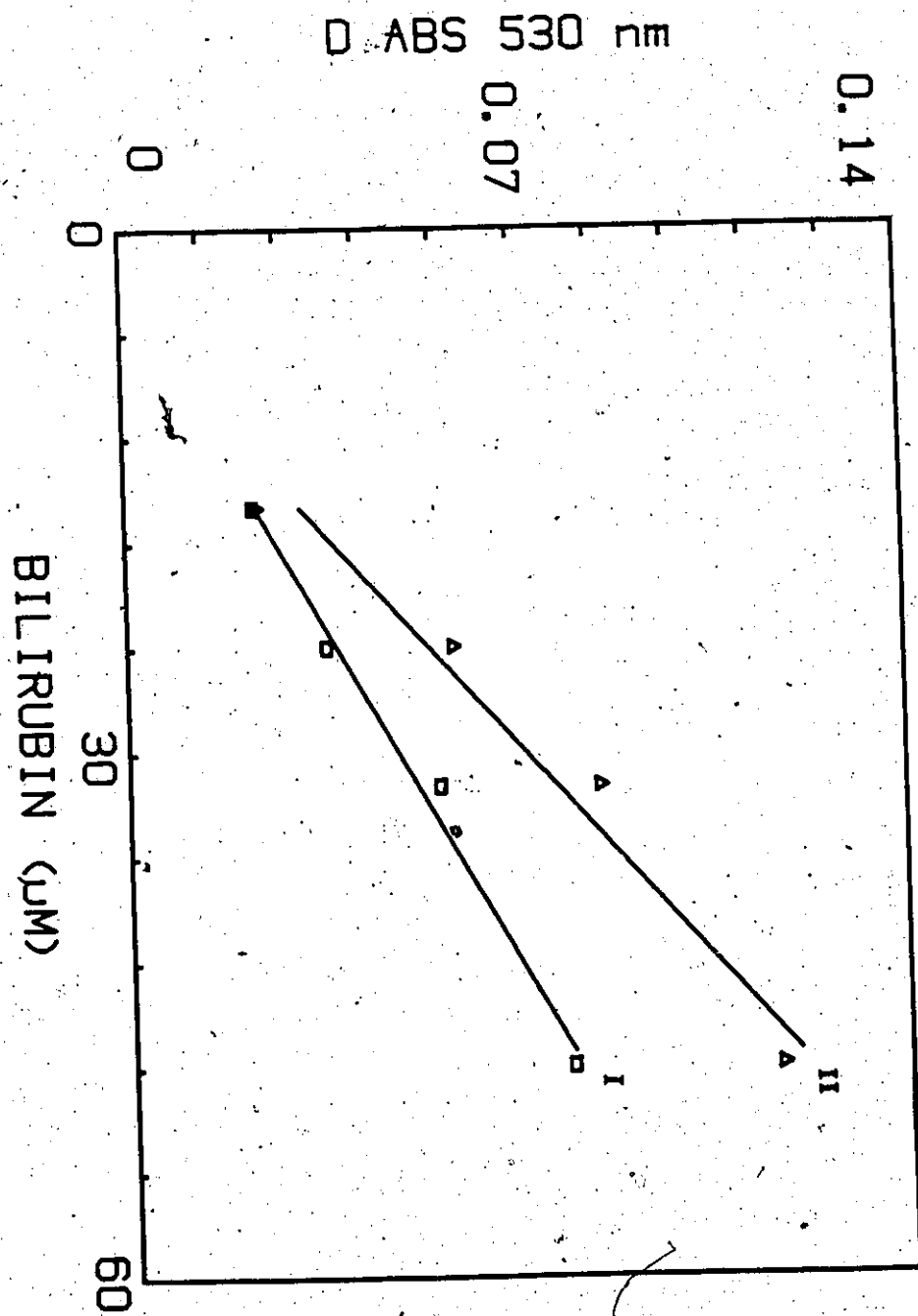tions were analyzed to establish the limit of linearity, which appeared to be below 60 μM (Fig. 16). Figure 17 shows the analysis of different concentrations of BILI within this range. All data points (triplicates) were plotted to graphically exemplify the precision. A close examination of the data points showed the relationship to be non linear. The linear range was observed to extend up to a final assay concentration of 20 μM. Comparison of the data, as absorbance change/3 min (D ABS 530 nm) and as absorbance after a 5-min incubation (data not shown) were very comparable. The standard curves with both types of data yielded similar values of x for given values of y. Therefore, both types of data were acceptable. The linear ranges for microsomes and the solubilized preparations were very similar. Figure 18 shows the results of analyses performed with the solubilized system, and as the total fraction, with the Jendrassik-Grof method. Within the linear range for the UDPGT assay (20 μM, see inset), the correlation of determination was very good (r^2=0.998) with the equation of the regression line being y = 1.12 X - 13. Table III shows the results of the precision study performed with albumin:BILI solutions.

5. Analysis of serum samples

Work with serum/plasma samples was not successful. Very low UDPGT activities were observed with spiked samples

FIGURE 16

RANGE OF LINEAR RESPONSE

Legend

Shown are the relative activities of the solu-bilized UDPGT preparation at various concentrations of BILI, and the location of the linear response. Measure-ments at the 2-min point was subtracted from the respective 5-min measurement to obtain "D ABS 530 nm". BILI glucuronides were assayed with the ethyl anthranilate diazo method. The assays were all done in triplicate.

FIGURE 16

D ABS 530 nm
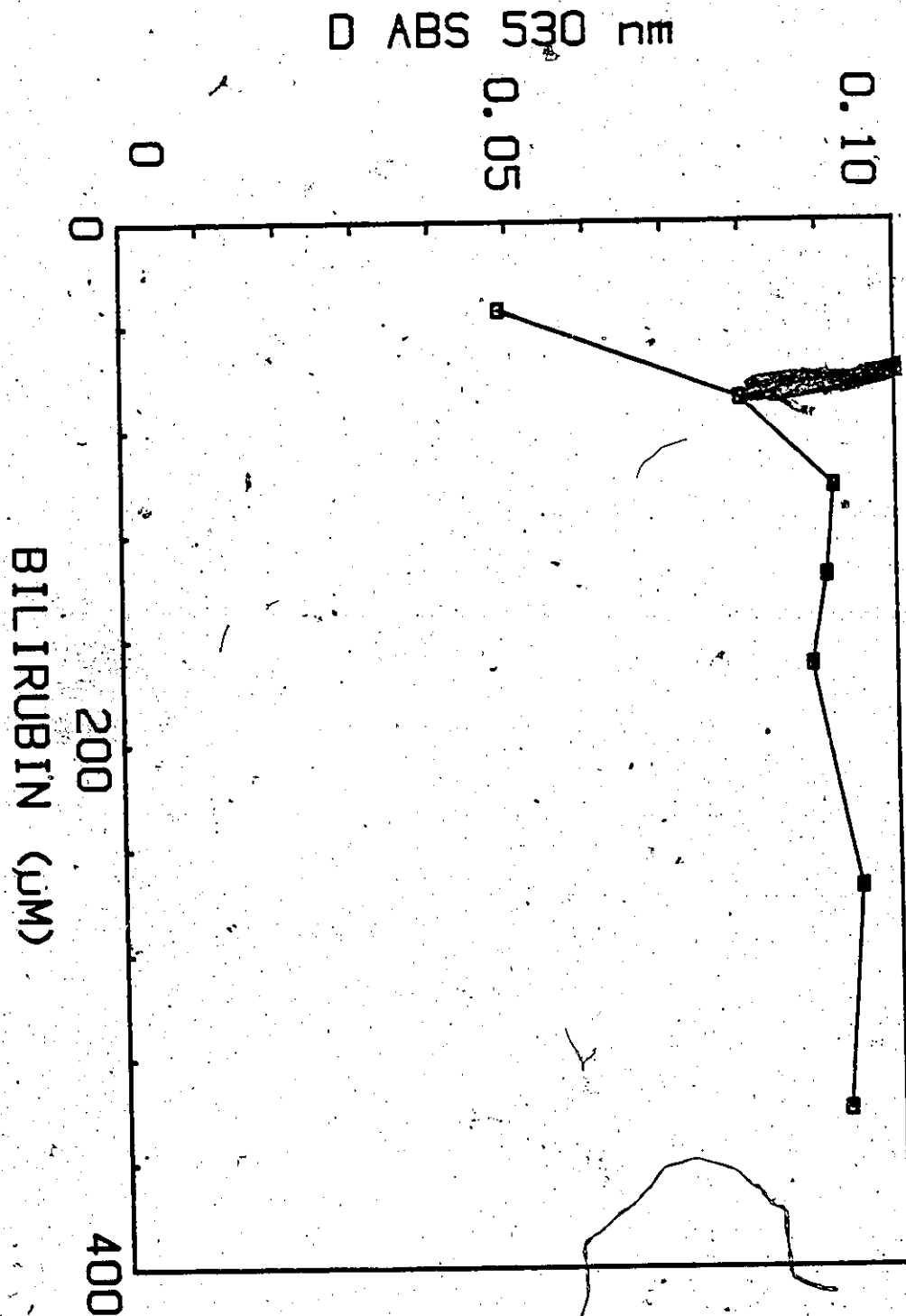
BILIRUBIN (μM)

FIGURE 17

STANDARD CURVE OF UDPGT ACTIVITY AND BILIRUBIN

CONCENTRATION

Legend

Analysis of albumin:BILI solutions (1:2) was done with the solubilized enzyme preparation. Glucuronide formation over a 5-min period was measured with the ethyl anthranilate diazo procedure. The absorbances obtained were plotted as a function of BILI concentration. The individual results, of the analyses done in triplicates, are plotted to graphically illustrate the precision. The line of best fit within the linear range was calculated to be y = 0.0009X - 0.004 (r^2 = 0.976).

FIGURE 17
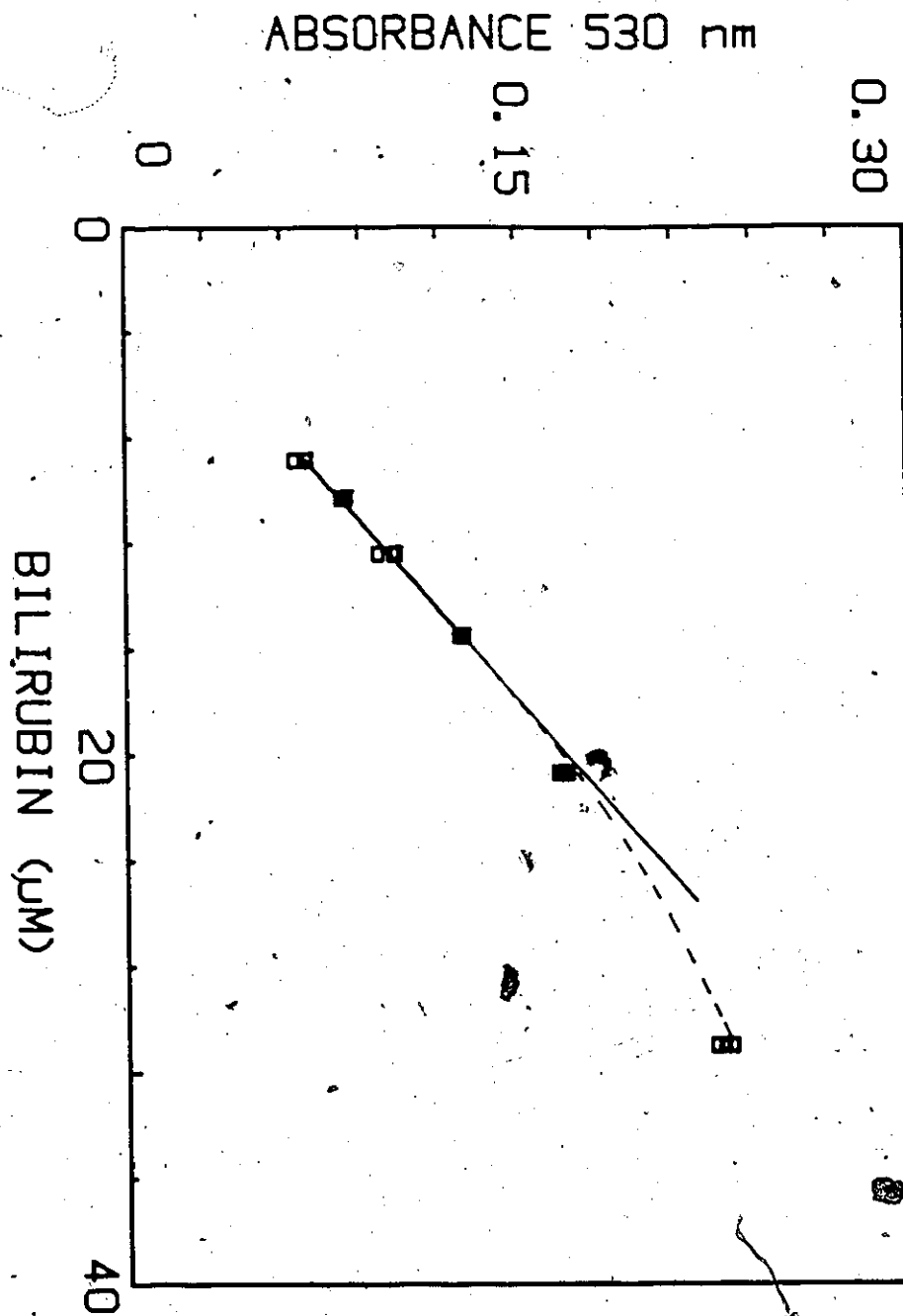
FIGURE 18

COMPARISON OF TEST METHOD WITH A DIAZO PROCEDURE

Legend

Analysis of total·BILI with UDPGT/ethyl anthranilate method (UDPGT/E.A) was compared against the commonly used Jendrassik-Grof procedure. The latter was performed on a centrifugal analyzer, Flexigem™. Values were calculated to represent concentration (uM) in sample volumes of 60 uL instead of 200 uL as in Fig. 17. UDPGT/E.A. analyses were performed in triplicate, and the analysis by Jendrassik-Grof procedure was done in duplicates. The inset graph shows the graph when values from the linear range were compared. The equation for line of best fit (inset graph) was determined to be y = 1.12X -13 (r^2 = 0.998).

FIGURE 18

UDPGT/E. A. METHOD (μM)

JENDRASSIK-GROF   METHOD (μM)
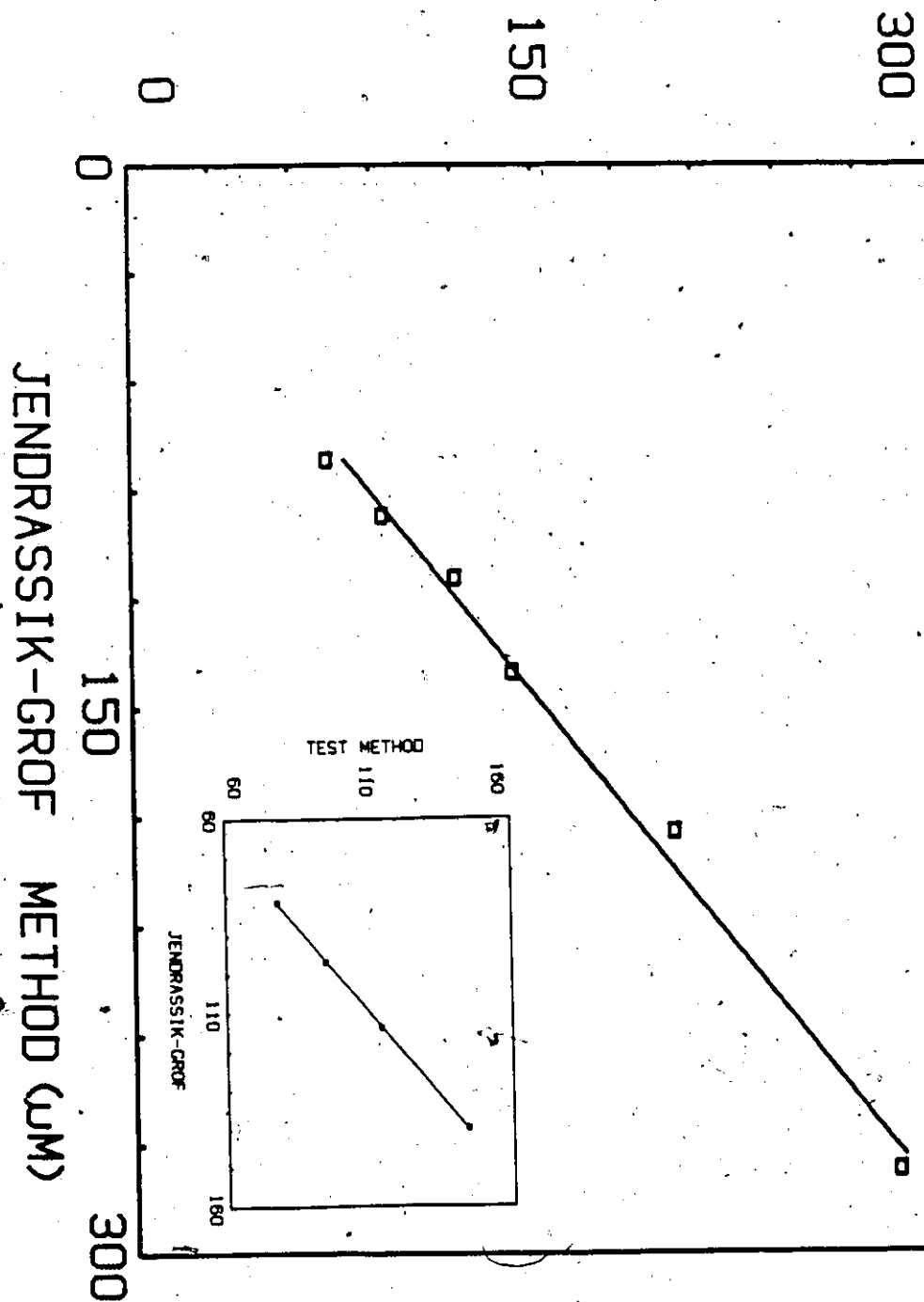
TABLE III

PRECISION STUDY WITH THE UDPGT/ETHYL ANTHRANILATE SYSTEM

	BILI concentration	
	30 μM	60 μM
Within-run CV[1]	2.93%	1.25%
Between-run CV[1]	3.57%	1.4%

Analyses of albumin:BILI solutions (1:2, molar ratio), with the solubilized enzyme preparation, were performed at the concentrations indicated. 5-min glucuronide synthesis was measured with the diazo procedure.

1. Samples/run : 10

(Figs. 19,20). Serum samples and blood treated with anti-coagulants like heparin, citrate buffer, and EDTA were analyzed. Similar results were obtained with all these samples. Citrated plasma, however, showed less inhibition. Time progress study showed serum/plasma to prolong lag phase and suppress activity (Fig. 21). Preliminary fractionation of the serum by gel filtration column chromatography was performed with Sephadex™ G-200. The inhibitory protein in the serum was eluted in the protein fraction immediately following the void fraction. Results of agarose gel electro-phoresis showed the inhibitory fraction to be mainly composed of a protein that had the same electrophoretic mobility as albumin. Small amounts of proteins (<18% of total) with the mobilities that coincided with α_1, α_2, B and γ fractions were also present (Fig. 22). Since albumin is known to inhibit UDPGT [39,51], it became a strong candidate for further investigation.

6. Activity of UDPGT with free and albumin-bound bilirubin

When albumin solutions were substituted in place of serum, results were quite similar to those obtained with serum samples (see Figs. 19,20). The data obtained were replotted as UDPGT activity versus albumin:BILI ratio. This was done for all the BILI concentrations studied (Fig. 23). It is quite evident that a non-linear inverse relationship

FIGURE 19

EFFECT OF SERUM ON UDPGT ACTIVITY

Legend

Activity of solublilized UDPGT was measured in the presence and absence of serum. 5-min glucuronide production, as measured by the ethyl anthranilate diazo procedure was plotted against BILI concentration. All assays were done in triplicate. (I: no serum; II: 10 µL serum; III: 30 µL serum).
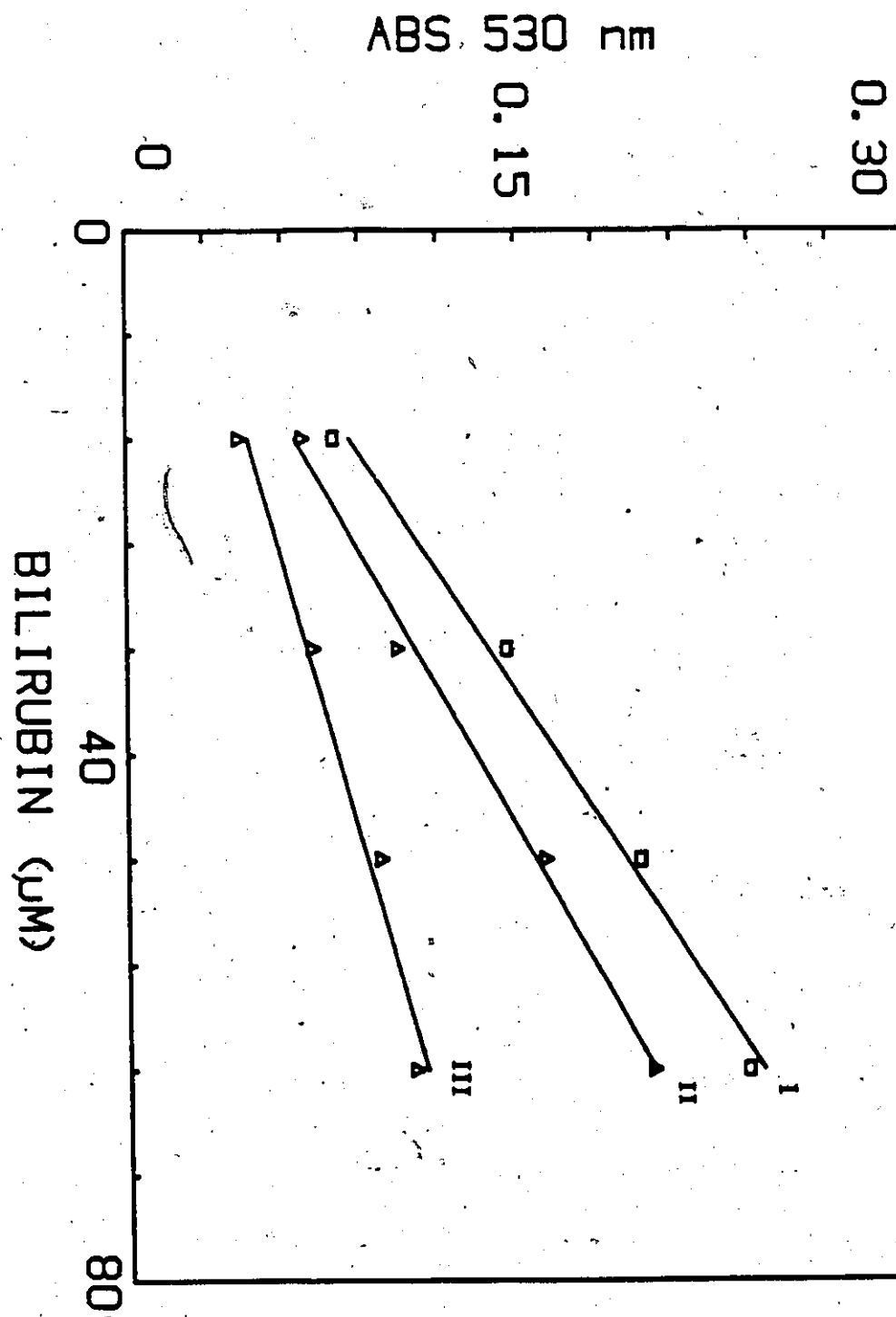
FIGURE 19

FIGURE 20

EFFECT OF SERUM AT VARYING UDPGA CONCENTRATIONS

Legend

UDPGT activity (solubilized form) was assayed in the presence and absence of serum at several different concentrations of UDPGA. BILI concentration was fixed at 90 μM. BILI glucuronide formed within the 5-min incubation period was assayed with ethyl anthranilate diazo procedure. Points represent average values of 3 individual measurements (I: no serum; II: 10 μL serum; III: 30 μL serum).
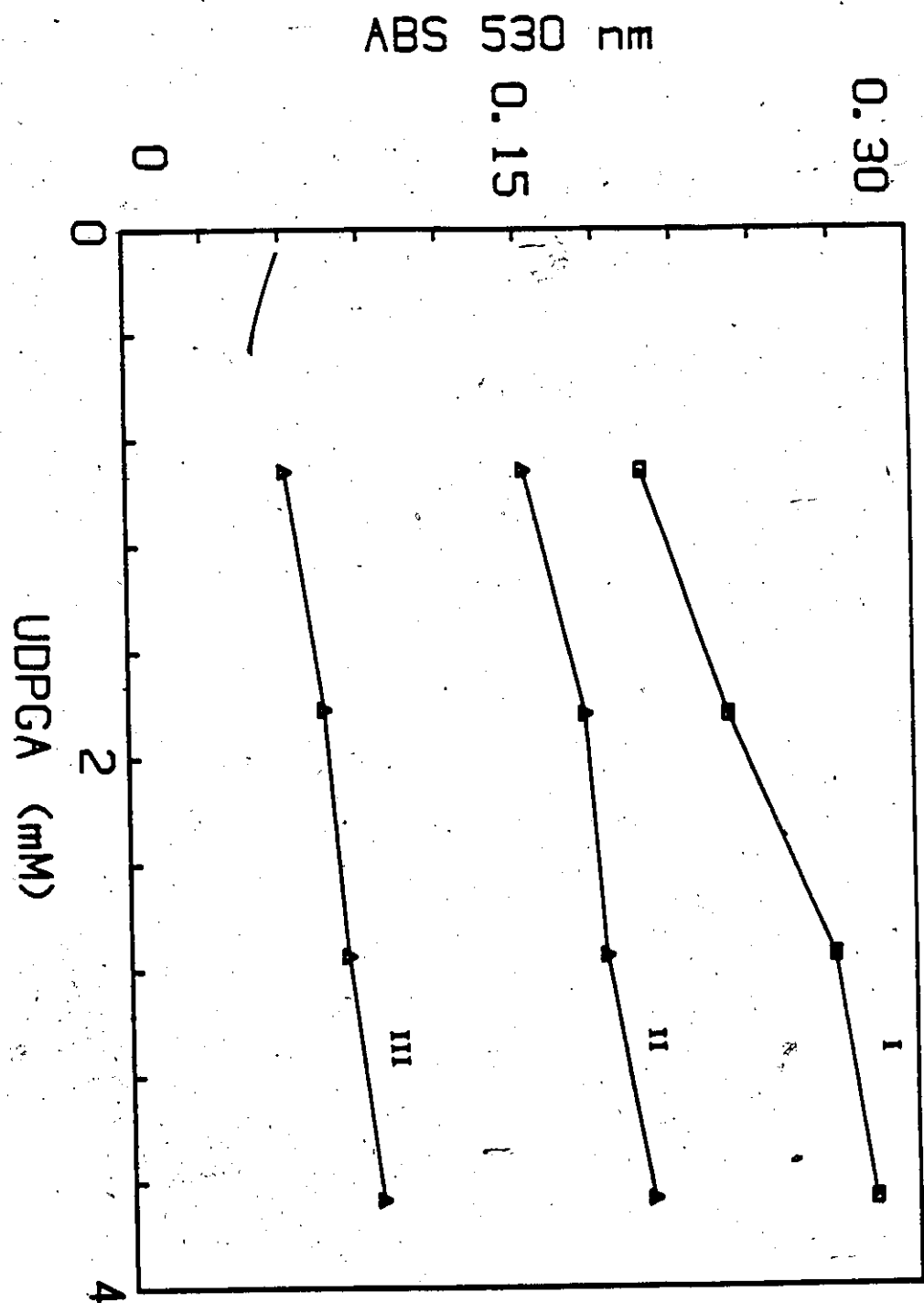
FIGURE 20

FIGURE 21

TIME PROGRESS CURVES IN THE PRESENCE AND ABSENCE OF SERUM

Legend

BILI glucuronide synthesis by solubilized UDPGT, was measured as a function of time. Assays (triplicate) were run in the presence and absence of serum. Reactions were stopped at 2, 5, 10 and 15-min intervals, and the glucuronides were assayed with the ethyl anthranilate diazo procedure. (I: no serum; II: 10 µL serum; III: 30 µL serum).

FIGURE 21

ABSORBANCE 530 nm
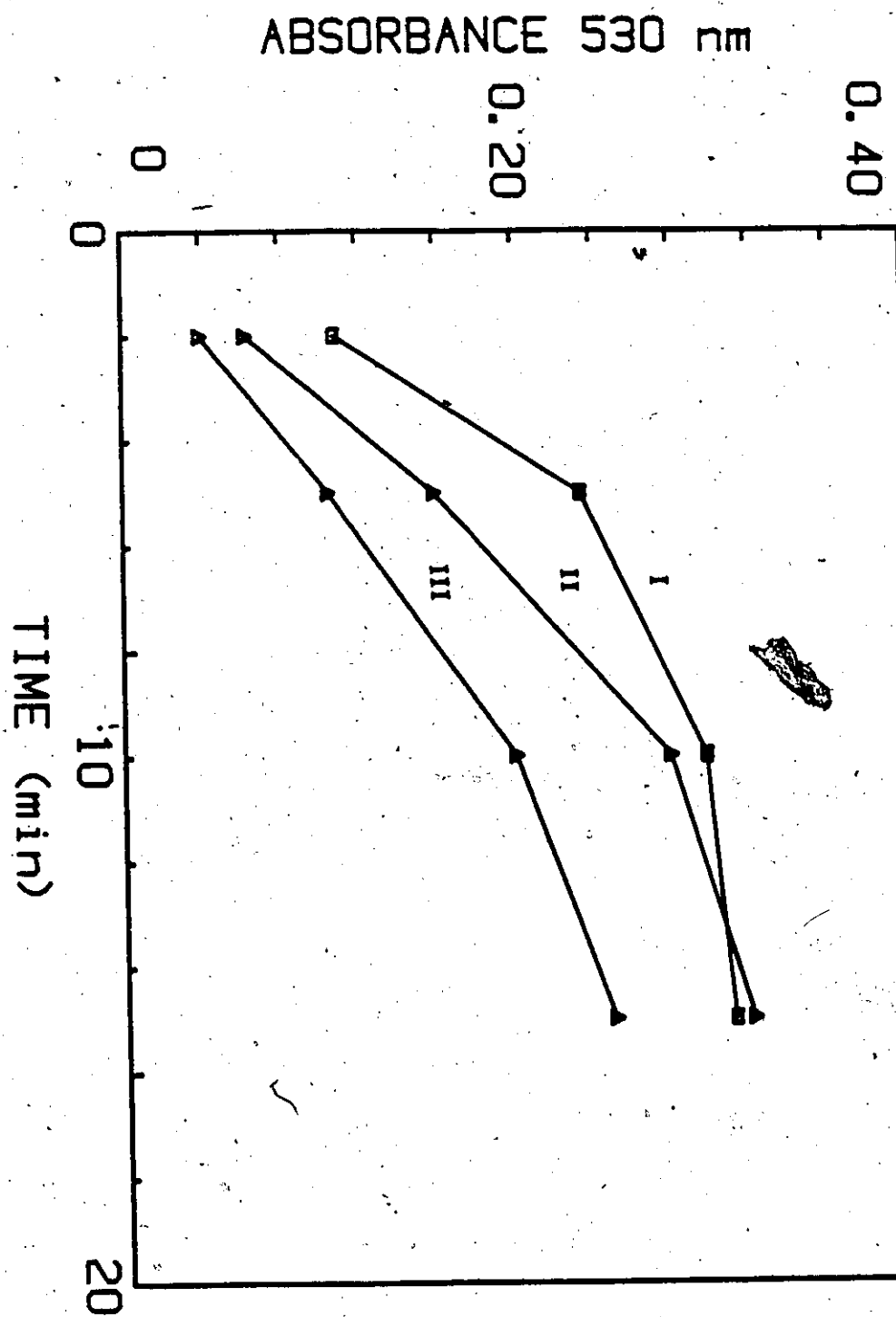
FIGURE 22

GEL ELECTROPHORESIS PROFILE OF THE INHIBITORY FRACTION

Legend

The inhibitory protein band from Sephadex™ G-200 gel column, was subjected to electrophoretic separation. Electrophoresis was performed, on commercially prepared agarose gels(Universal gels, Corning Diagnostics) at pH 8.7 in sodium barbital buffer. Bands were visualized with Ponceau-S stain. The labels on the horizontal axis indicate the positions of the standard serum protein bands.

FIGURE 22

Peak no. 1 (fastest migrator) at right of profile.

CORNING 722 output card

Patient
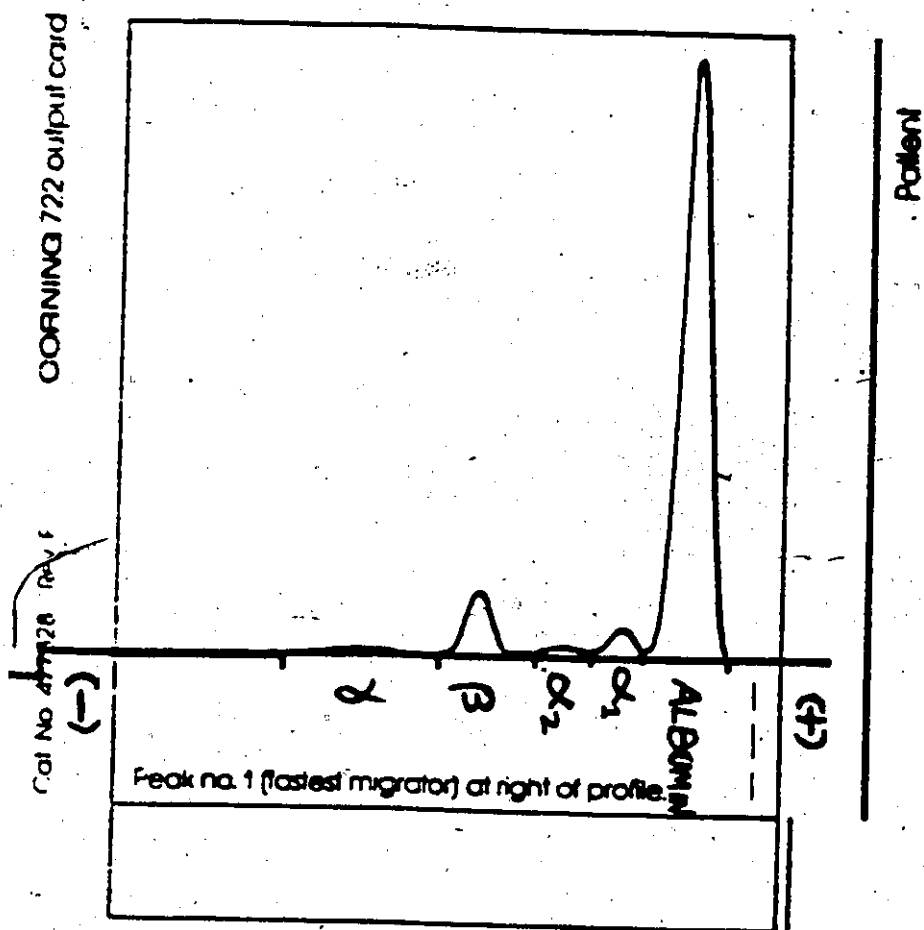
FIGURE 23

EFFECT OF ALBUMIN:BILIRUBIN RATIOS ON UDPGT ACTIVITY

Legend

The rates of BILI glucuronidation (solubilized preparation) were measured in the presence of varying amounts of albumin, different concentrations of BILI. Absorbances (average of triplicates) obtained after treatment with the ethyl anthranilate diazo reagent, were plotted against the respective albumin:BILI ratios. for each concentration of BILI. Incubations were of 5-min duration. (I: 16 µM BILI; II: 24 µM BILI; III: 40 µM BILI).

FIGURE 23

ABS 530 nm
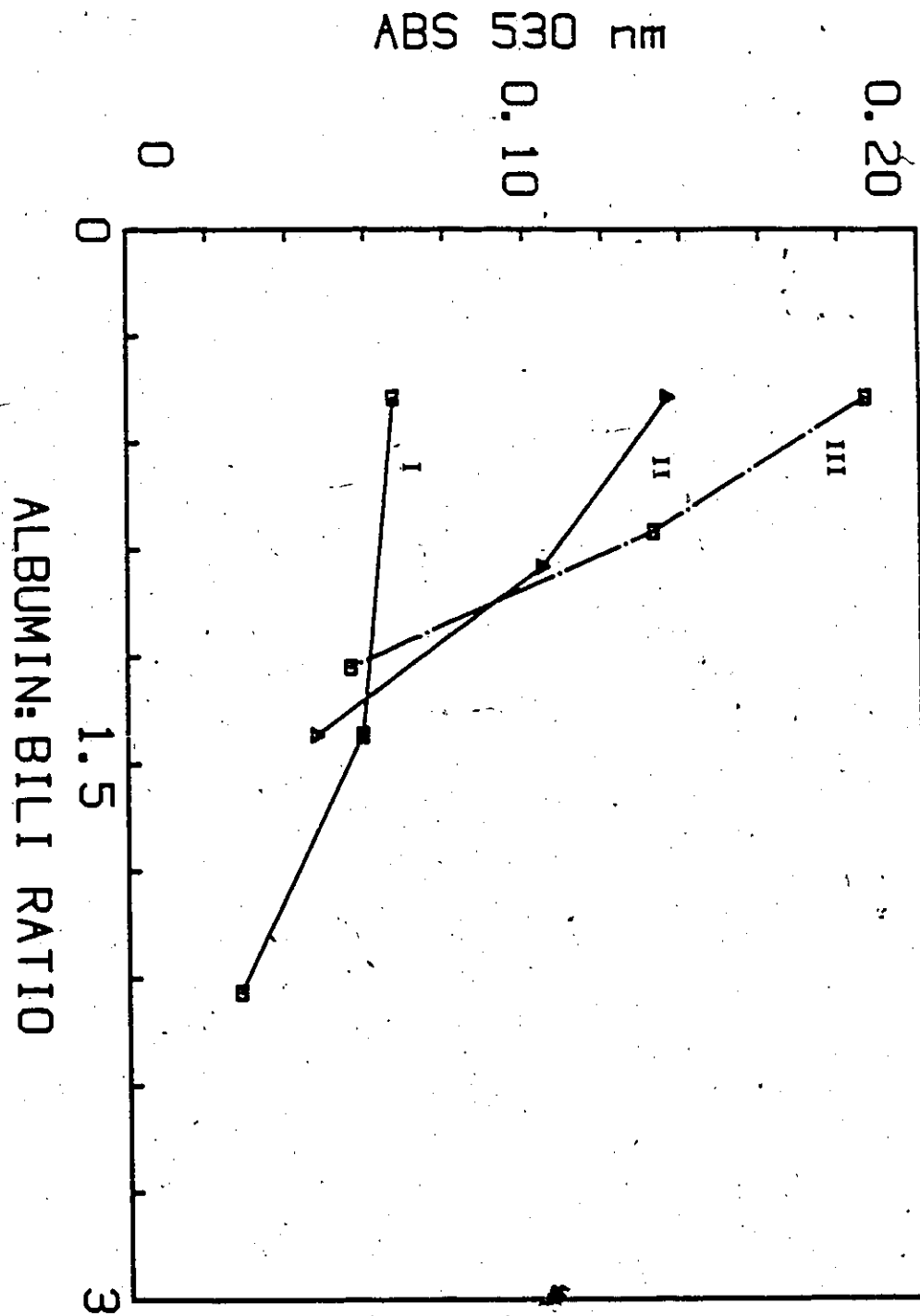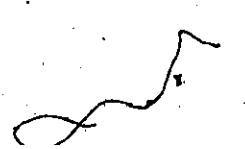
ALBUMIN:BILI RATIO

exists between UDPGT activity and albumin:BILI ratio.
Furthermore, this relationship became more pronounced as
substrate concentration was increased (that is, in the
presence of more BILI and albumin). Time-progress curves
showed longer lag phases and slower rates of
BILI conjugation in the presence of albumin (when more was
added to the assay mixture) (Fig. 24). Again, the profiles
were very similar to those obtained with serum.

The effects of BILI as free, BSA-bound and HSA-bound
are shown in Fig. 25. The surprising observation was the
similarity in activities between free and bound, at the
various concentrations. These observations suggest equivalent
availability of the free BILI in both preparations. Time-
progress curves with free BILI, standard BILI solution, and
in the presence of additional BSA (0.4 mg) are shown in Fig.
26. The almost equivalent profiles of the free BILI and the
standard BILI solution (albumin:BILI, 1:2), lends further
support to the notion that the availability of free BILI in
these preparations are similar.

7. Displacing agents and UDPGT activity

Several different agents were experimented with in an
effort to displace BILI from albumin. Benzoate/caffeine,
sulfadimethoxine, ethanol, phenylbutazone, and p-hydroxy-
benzoic acid methyl ester were used. Figure 27 shows the

FIGURE 24

EFFECT OF ALBUMIN CONCENTRATION ON THE RATE OF

GLUCURONIDE SYNTHESIS

Legend

Bovine serum albumin made up in assay buffer, was added to assay mixtures containing solubilized preparation of UDPGT. Reactions were stopped at 2, 5, 10 and 15-min intervals and product formation was assayed with the ethyl anthranilate diazo procedure. Triplicate measurements were made in all cases. (I: albumin:BILI (1:2); II: albumin:BILI + 0.2 mg BSA; III: albumin:BILI +0.4 mg BSA).

FIGURE 24

ABS 530 nm

TIME (min)
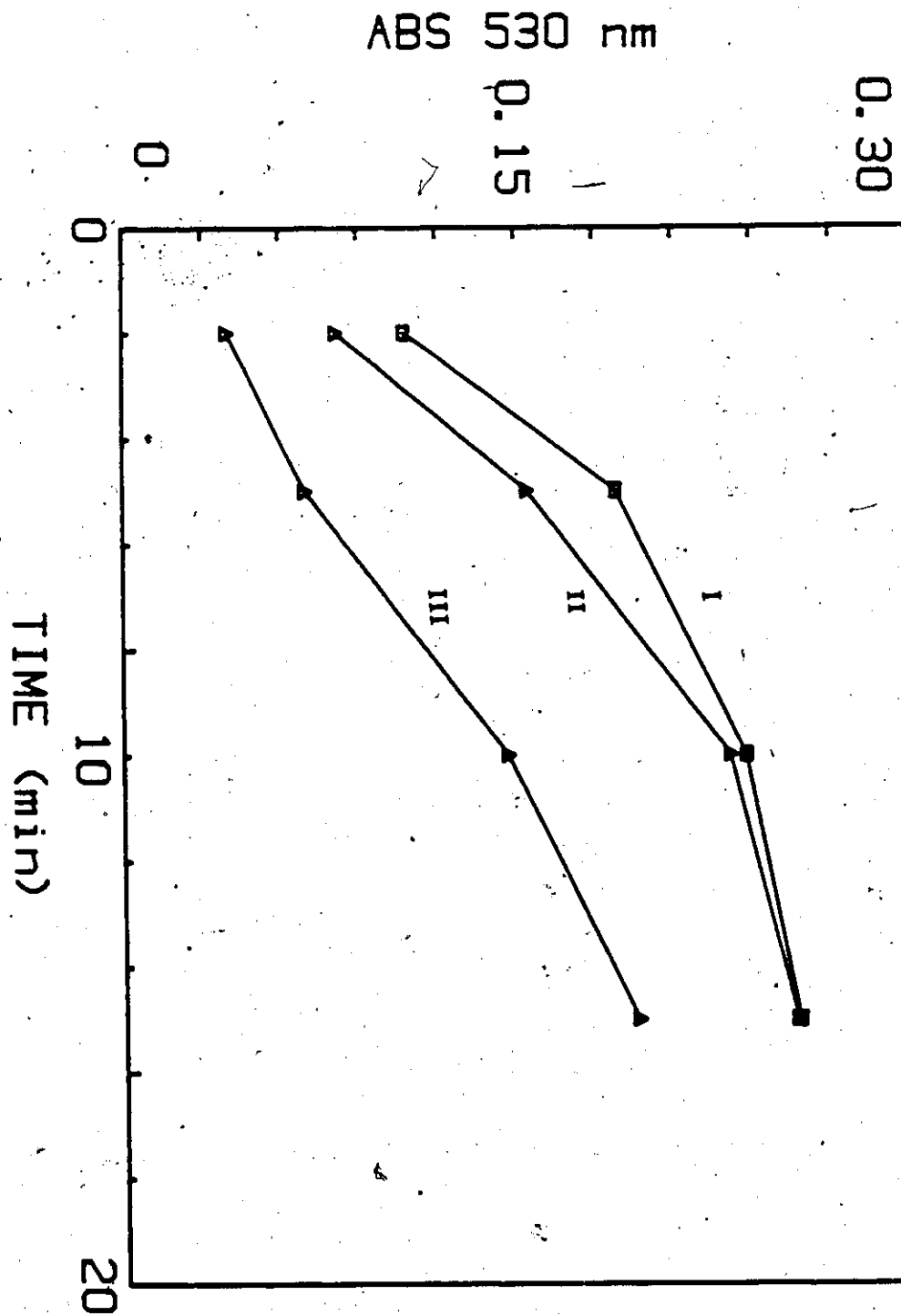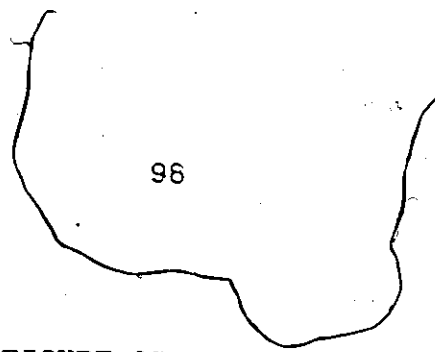
FIGURE 25

COMPARISON OF FREE AND ALBUMIN-BOUND BILIRUBIN

AS SUBSTRATES

Legend

Activity of UDPGT with free, BSA-bound BILI (1:2) and HSA-bound BILI (1:2) was measured at various concentrations of BILI. Solubilized preparations were employed, and the 5-min glucuronide synthesis (run in triplicate) were measured with the ethyl anthranilate procedure (I: HSA-bound BILI; II: BSA-bound BILI; III: free BILI).

FIGURE 25
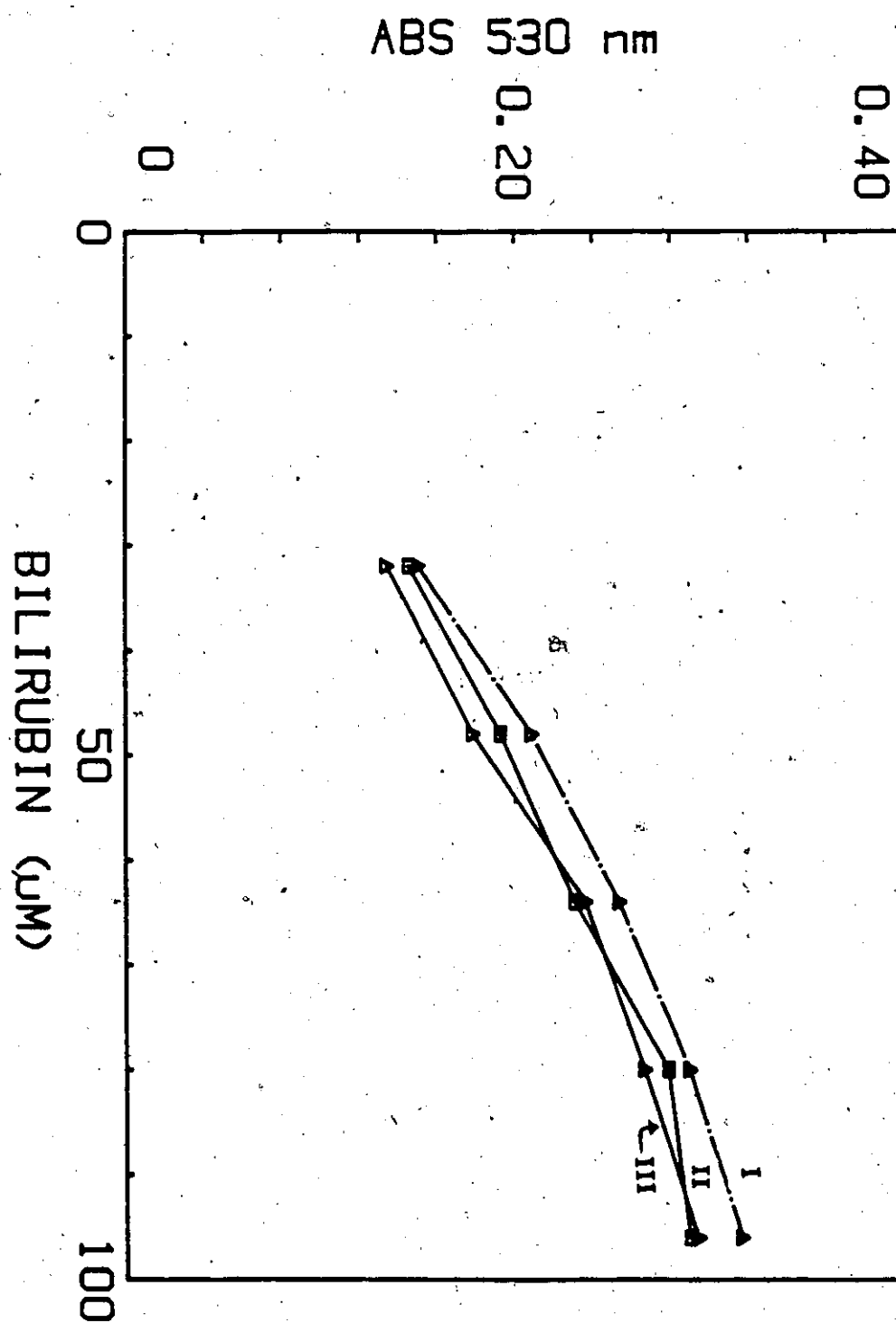
FIGURE 26

TIME-PROGRESS CURVES FOR FREE AND BOUND BILIRUBIN

Legend

BILI glucuronide synthesis was studied as a function of time with BILI in free form, bound to BSA and in the presence of extraneous albumin. Shown are the average of triplicate measurements, obtained with the solubilized enzyme preparation. The concentration of BILI was 90 uM (final). Reactions were stopped at the various intervals and BILI conjugates were measured with the diazo reagent. (free BILI (I); standard substrate (II); standard substrate + 0.4 mg BSA (III).

FIGURE 26

ABSORBANCE 530 nm
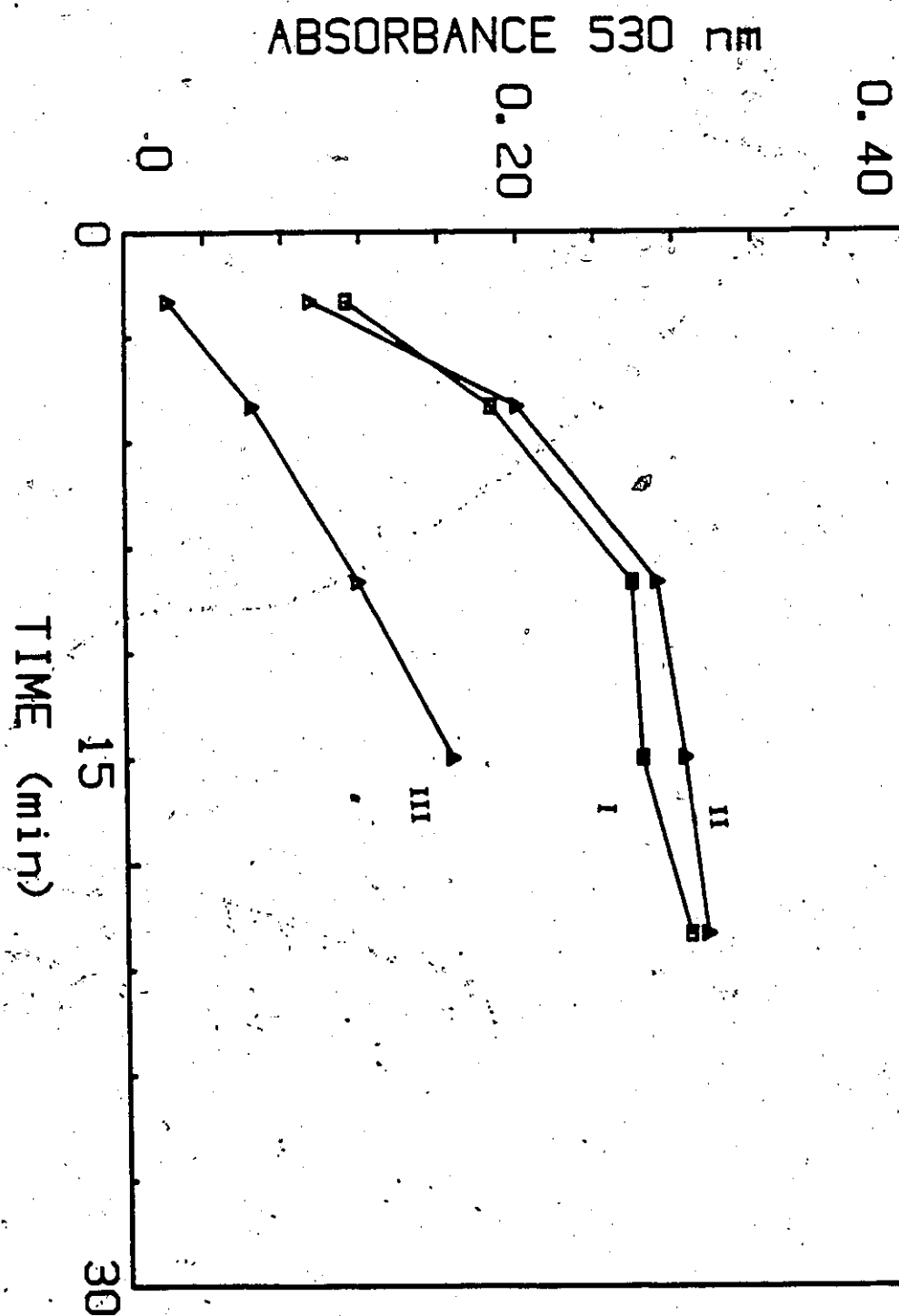
FIGURE 27

UDPGT ACTIVITY IN THE PRESENCE OF DISPLACING AGENT

Legend

Varying amounts of benzoate/caffeine (molar equivalence) were added to assay mixtures containing 30 µL of serum and 90 µM BILI. Shown are the results of the 5-min glucuronide production obtained with the solubilized enzyme preparation. Activities, as the amount of colour development after diazo reaction, were compared against a control (which had no serum or displacing agent). As with other studies, all assays were done in triplicate.

FIGURE 27

ACTIVITY (% OF CONTROL)
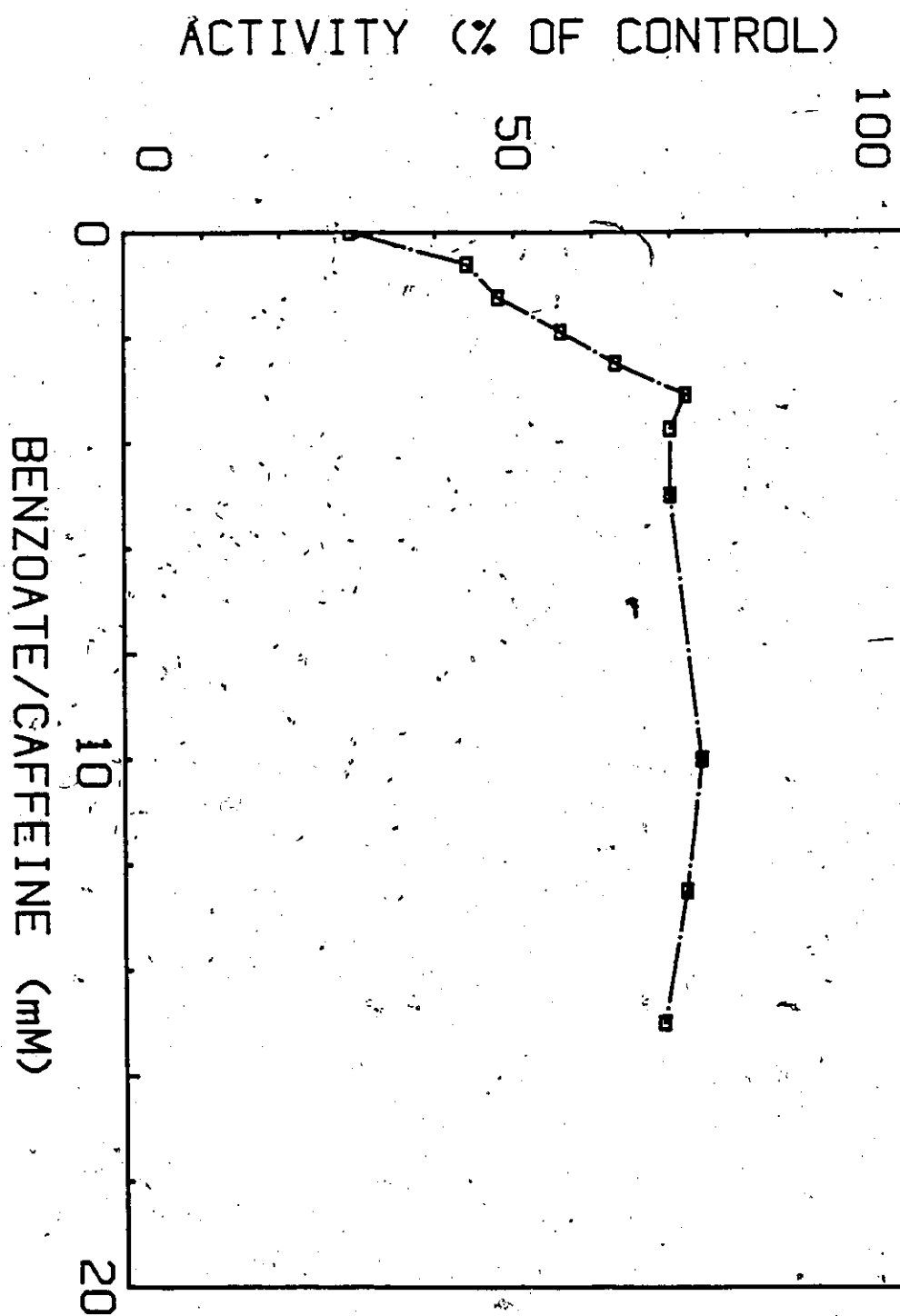
activity of UDPGT relative to a control that contained no added albumin or serum (and no displacing agent). The increase in activity was presumed to be an effective displacement of BILI from its binding site by benzoate. Of the compounds studied, sulfadimethoxine and benzoate/caffeine were the most effective, with a recovery of activity at 75% of the control. Other agents were not as effective, and they also inhibited UDPGT activity (observed in the controls containing displacing agent but no serum).

8. Enzyme-coupled assay of bilirubin

Initial work was performed with the system described by Mulder and Van Doorn [25]. The production of UDP was coupled to NADH consumption. The reaction was monitored by following the decrease in absorbance at 340 nm. The method was found to have limited sensitivity, and because of the high absorbance (due to presence of BILI, protein and NADH), it was prone to effects of light scattering. The system was modified for measurement in the visible region. The system described by Bozimowski et al. [57] was modified slightly for use with our system (see DISCUSSION, CHAPTER, IV p. 134 for equations). Analysis of BILI was accomplished by monitoring the decrease in absorbance at 460 nm (Fig. 28). This figure shows the various spectra of the system (before and after BILI oxidation). The absorbance at 460 nm decreased

FIGURE 28

SPECTRAL CHANGES IN THE PRESENCE OF H_2O_2/PEROXIDASE

Legend

Shown are the spectral profiles of the UDPGT enzyme coupled system in the visible region.

I. Visible region spectrum of the coupled system with all components, except BILI and UDPGA.

II. Spectrum in the presence of BILI (35 µM).

III. Spectrum after initiation of reaction with UDPGA which led to oxidation of BILI by H_2O_2/peroxidase.

FIGURE 28

ABSORBANCE
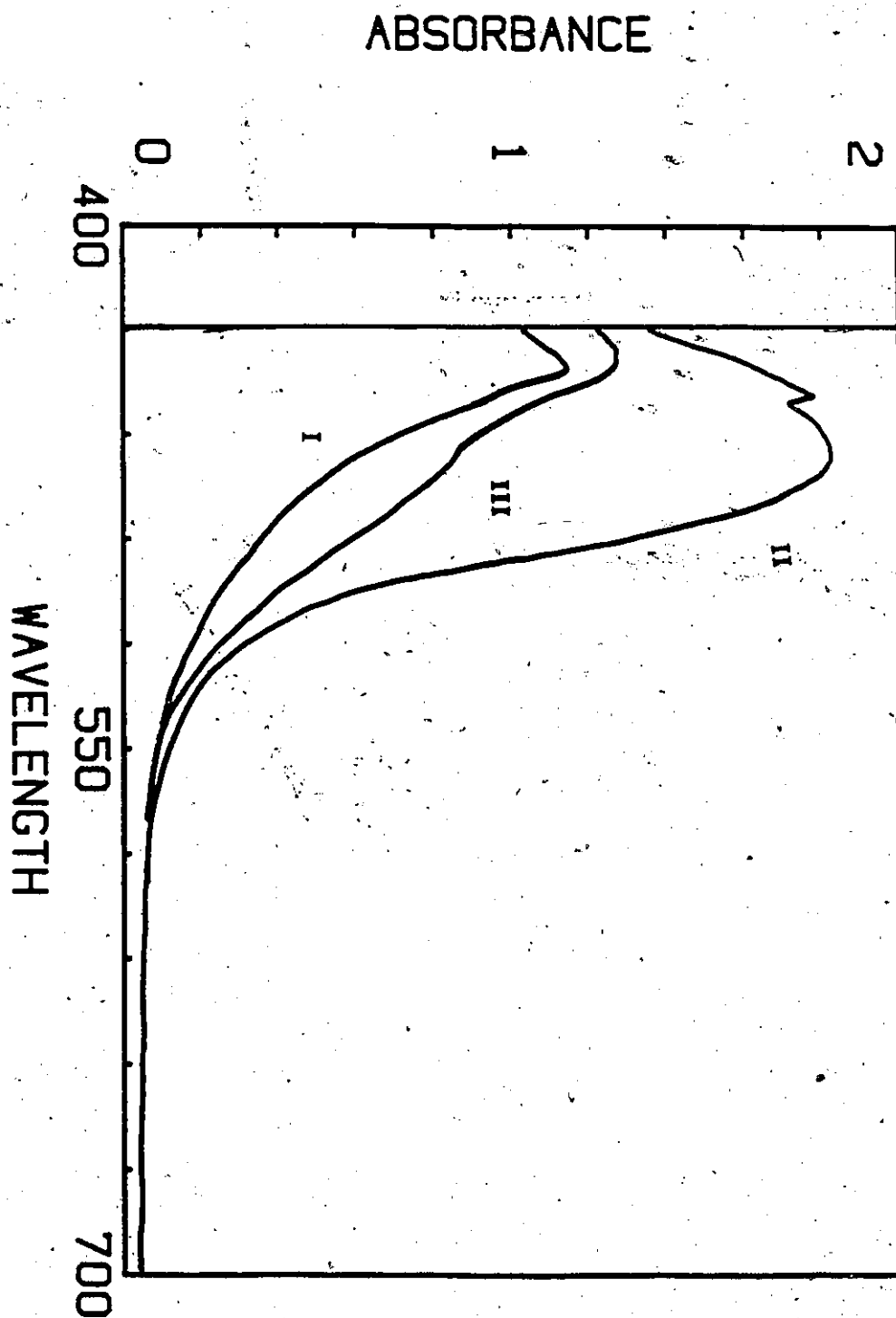
when UDPGA was added (except that due to photodegradation). Various concentrations of the albumin:BILI solutions were analyzed. Results with this coupled assay system are shown on Fig. 29, and compared with the ethyl anthranilate method in Fig. 30. A point of concern in using this coupled system, is that analysis of dilute samples (< 10 μM in cuvette) was hampered by photodegradation from the light beam in the spectrophotometer (preincubations had to be carried out in the dark). Correction for photodegradation was accomplished with the rate observed in the blank. At the other extreme, high BILI concentrations (> 60 μM) resulted in high absorbances. Consequently, substantial light scattering was observed. Enzyme coupling permitted continuous monitoring of the UDPGT reaction with time. Figure 31 shows tracings of the reactions at several different concentrations of BILI (with the same albumin:BILI ratio). It is quite apparent that the lag phase becomes longer with increasing concentrations of BILI. Whether this effect is due to BILI or albumin is not known. All UDPGTs use a common cosubstrate, UDPGA. To show that this system could be used to follow other UDPGT activities, 4-nitrophenol conjugating activity, in the solubilized preparation, was assayed. Results are graphically shown in Fig. 32.

FIGURE 29

BILIRUBIN ANALYSIS WITH THE ENZYME COUPLED ASSAY

Legend

The enzyme coupled system was applied to the analysis of albumin:BILI solutions (1:2). Activity of the solubilized UDPGT preparation was monitored continuously at 460 nm. The activities were calculated from the rate of decrease in absorbance, expressed as nmol/min, and plotted against the respective BILI concentrations. The data point represents an average of duplicate measurements.

FIGURE 29

VELOCITY (nmoles/min)
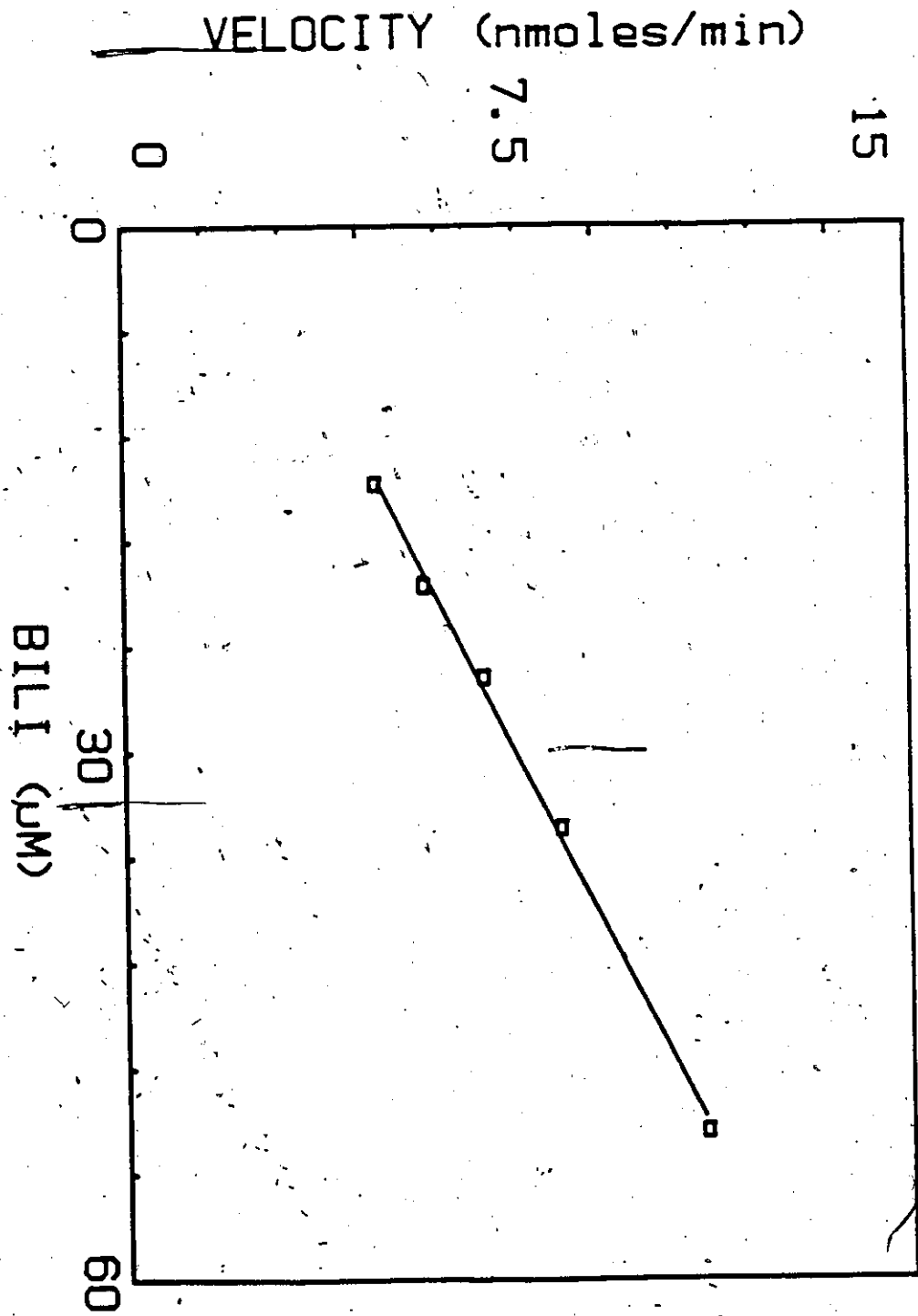
FIGURE 30

COMPARISON OF DIAZO AND ENZYME COUPLED SYSTEMS

Legend

Albumin:BILI solutions (1:2), were analyzed by UDPGT/ethyl anthranilate diazo procedure (UDPGT/E.A.) and by the enzyme coupled procedure. Soluble enzyme preparations were employed in both types of analyses. The diazo based analysis was performed in triplicate, and the enzyme coupled assays were done in duplicate. Concentrations of BILI (final concentrations), as determined by both procedures, were plotted against each other. (r^2 = .990, equation for line of best fit: y = 0.992X + 0.2).

FIGURE 30

UDPGT/PEROXIDASE (µM)
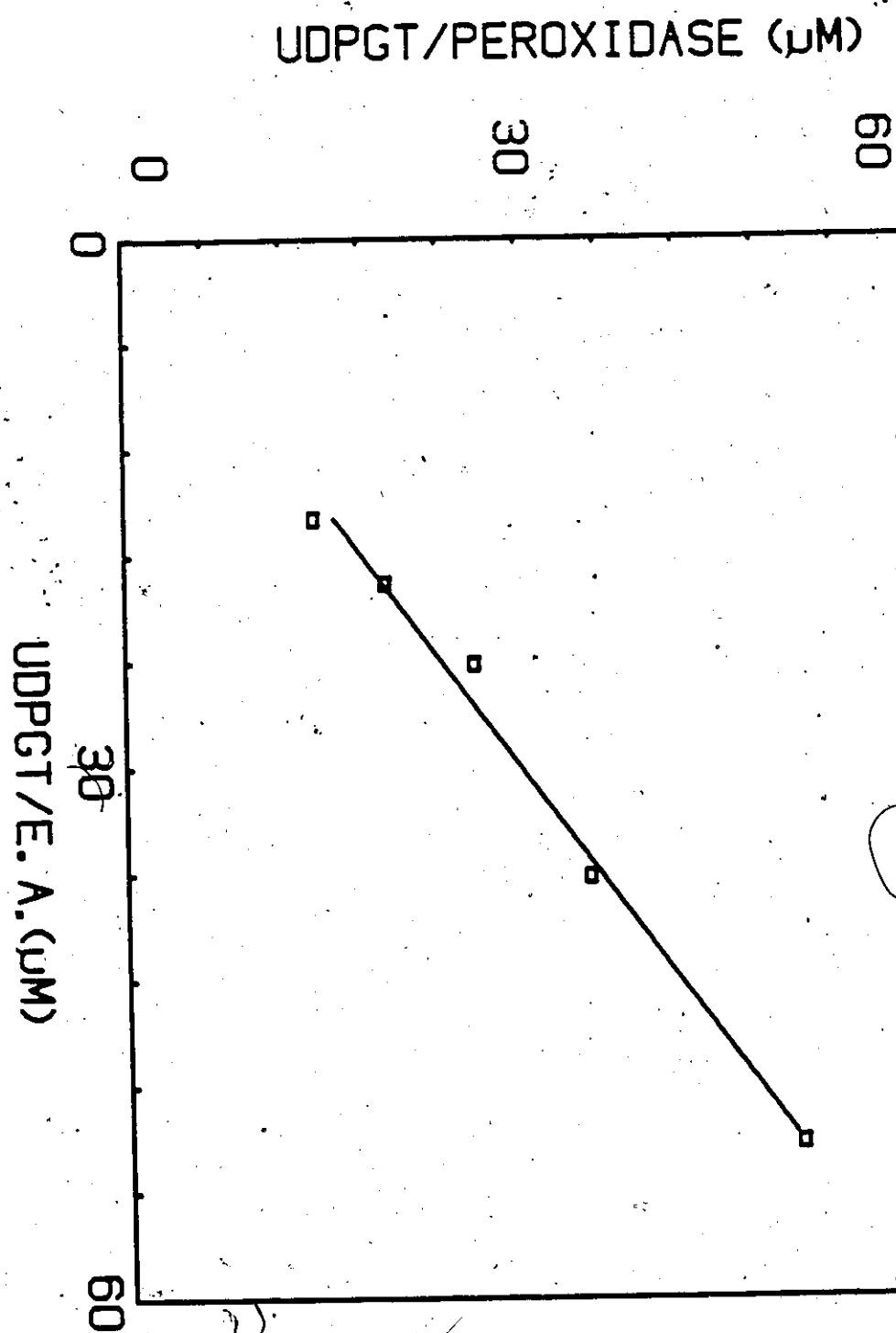
FIGURE 31

CONTINUOUS ASSAY OF UDPGT ACTIVITY WITH BILIRUBIN

Legend

The activity of the solubilized UDPGT was monitored continuously with the enzyme coupled system. Shown are the time-progress curves obtained with different concentrations of BILI. The reactions were initiated, after a 5-min pre-incubation at 37°C in the dark, by adding UDPGA. Reactions were followed by observing the decrease in absorbance at 460 nm. (A: 15 µM; B: 24 µM; C: 36 µM; D: 51 µM).

FIGURE 31

ABS 460 nm

TIME (sec)
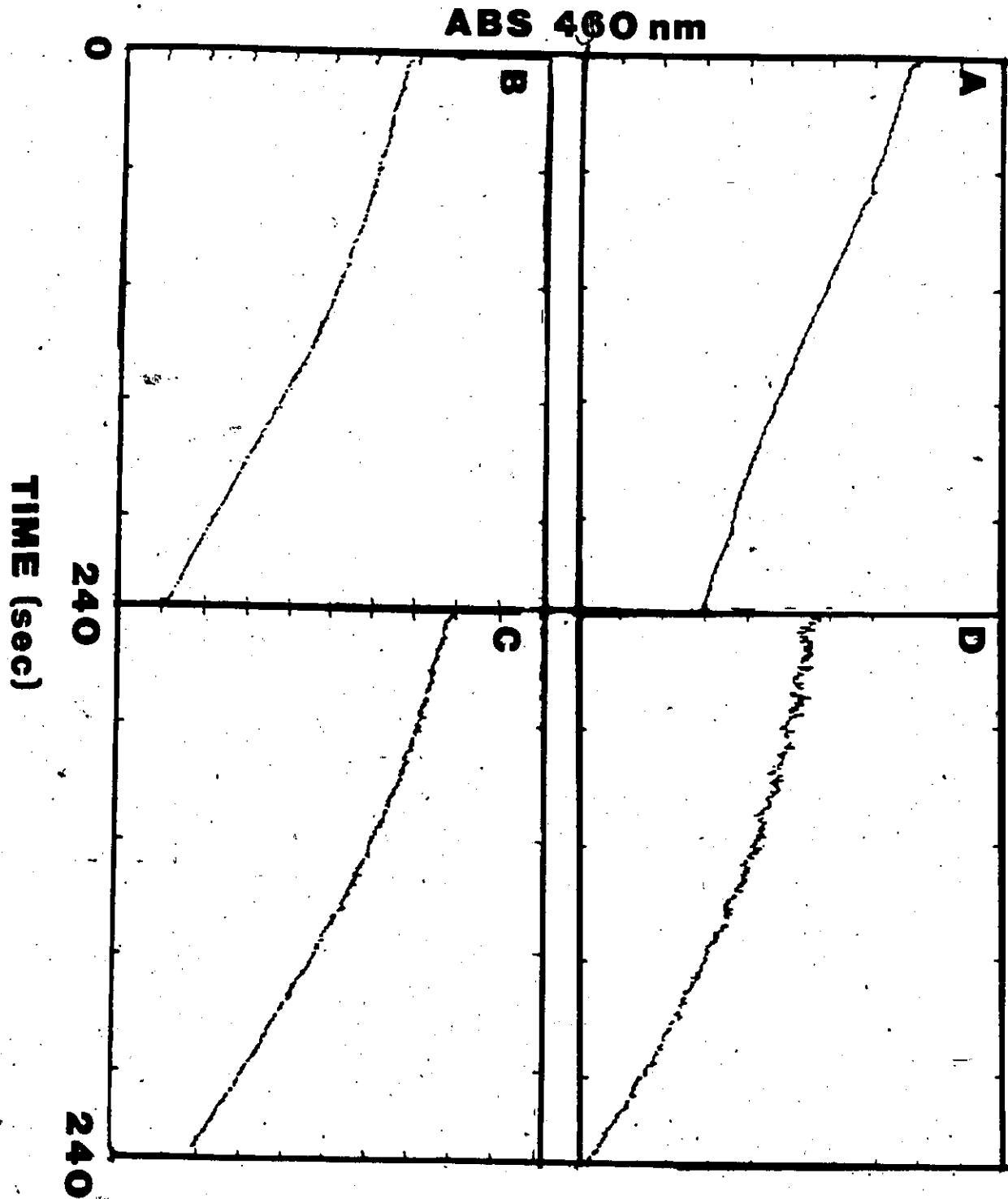
FIGURE 32

ENZYME COUPLED ASSAY FOR 4-n-p CONJUGATING ACTIVITY

Legend

The 4-n-p conjugating activity in the solubilized preparation was assayed at different concentrations of 4-n-p. The production of UDP was linked to formation of a quinoneimine dye. As a result, 4-n-p conjugating activity could be monitored continuously at 510 nm. Average of duplicate measurements (absorbance change/ min) were plotted against 4-n-p concentrations.

FIGURE 32

ABSORBANCE/MIN
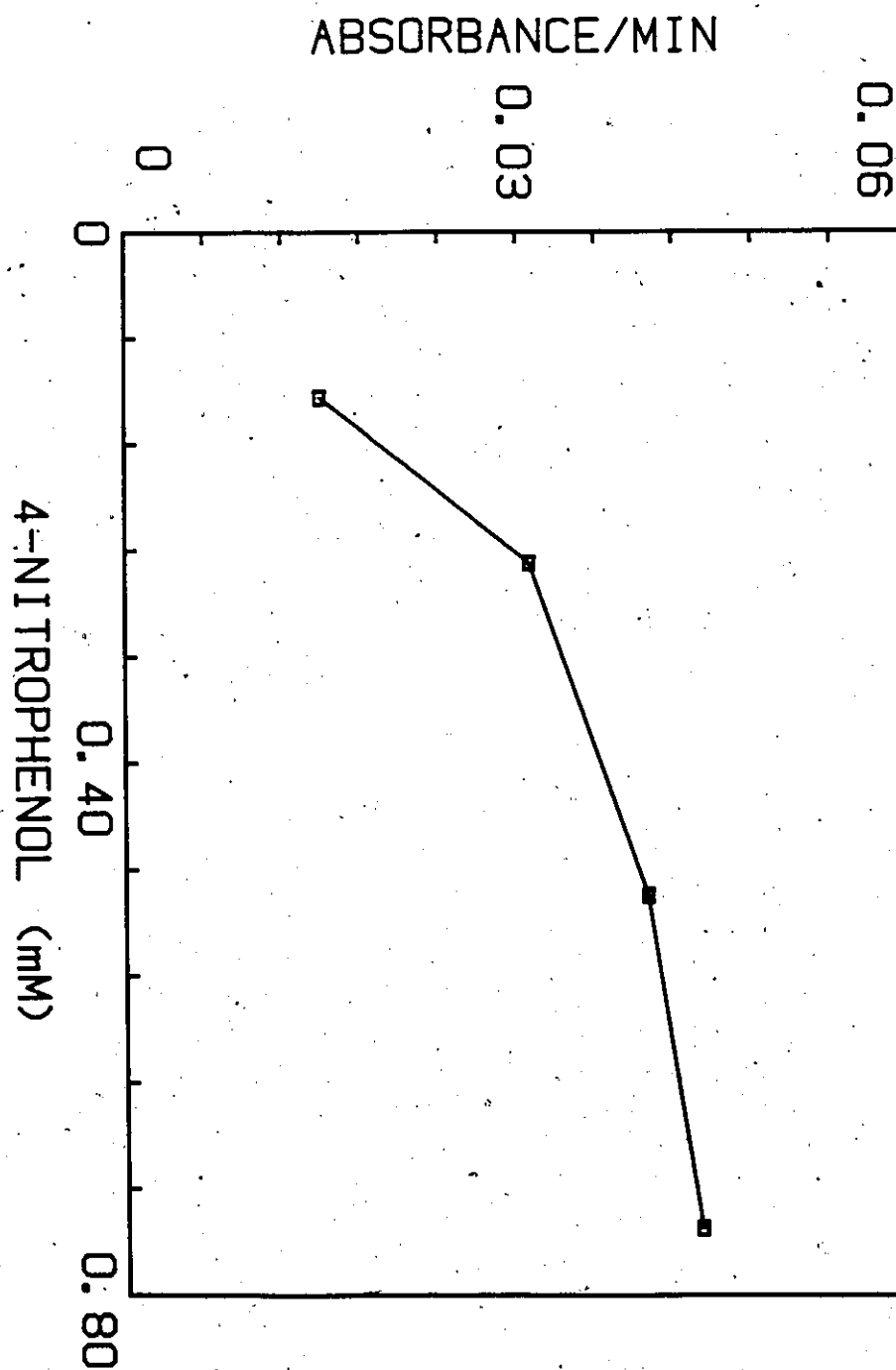
9. Analysis of UDP, ADP, and glucose

The analysis of UDP and ADP was carried out with the coupling reactions used for UDPGT. The results are presented in Figs. 33 and 34, respectively. The limit of detection for both nucleotides with this system was estimated to be about 10 µM (~ 5 nmol). The linear range for both nucleotides, extended to at least 180 µM. Some preliminary work, regards to monitoring reactions that employ or produce these co-factors was also carried out. Analysis of glucose, in the presence of hexokinase and ATP, was studied (Fig. 35). Progress of the reaction, with time, is shown on Fig. 36. Serum samples were analyzed using this system. Quantitative recoveries were obtained with spiked samples. Values obtained with this were compared with the method used on the Perspective^R (American Monitor Corporation). The method "Neochrome II" (American Monitor Corporation), uses glucose-oxidase coupled with a modified Trinder coupling system. The method employs 2,4,6-tribromophenol (instead of phenol) to form a brominated quinoneimine dye. The reaction is monitored at 500 nm. For comparison, the results obtained with the two methods are shown in figure 37.

10. **Effect of albumin on oxidation of BILI by peroxidase**

Figure 38 shows the effect of increasing albumin:BILI ratios on the oxidation of BILI by horseradish peroxidase, in

the presence of excess H_2O_2. Activities were compared against that obtained with albumin:BILI ratio of 1:2. Peroxidase oxidizes free BILI to biliverdin and other products. Increasing amounts of albumin has a profound effect on the activity of peroxidase. This observation is presumably due to decreased availability of free BILI in the reaction medium.

FIGURE 33

ENZYME COUPLED ASSAY FOR UDP

Legend

Various concentrations of UDP were assayed with pyruvate kinase, coupled to a modified Trinder reaction. Reaction was monitored at 510 nm, and all samples were assayed in duplicate. Activities (as absorbance change/min) were plotted against the respective concentrations. Equation for the line of best fit: $y = 0.0011X - 0.013$ ($r^2 = 0.996$).

FIGURE 33
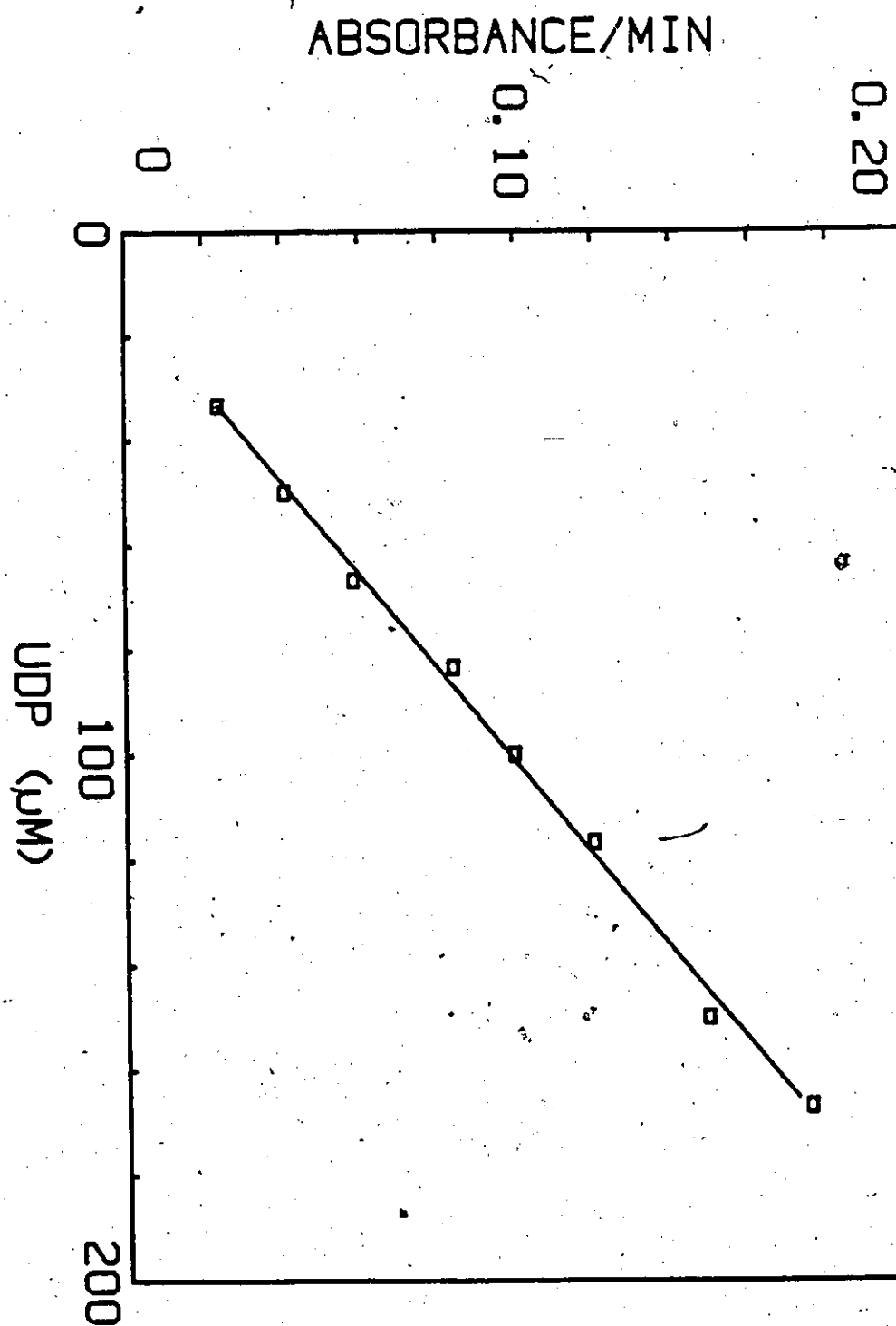
FIGURE 34

ENZYME COUPLED ASSAY FOR ADP

Legend

Analysis of ADP was performed with the enzyme coupled system used for UDP (Fig. 33, p.118). However, KCl (5mM) was required for optimal activity of pyruvate kinase with this cosubstrate. The points represent an average of duplicate runs. The equation for the line of best fit: $y = 0.0013X - 0.013$ ($r^2 = 0.996$).

FIGURE 34

ABSORBANCE/MIN
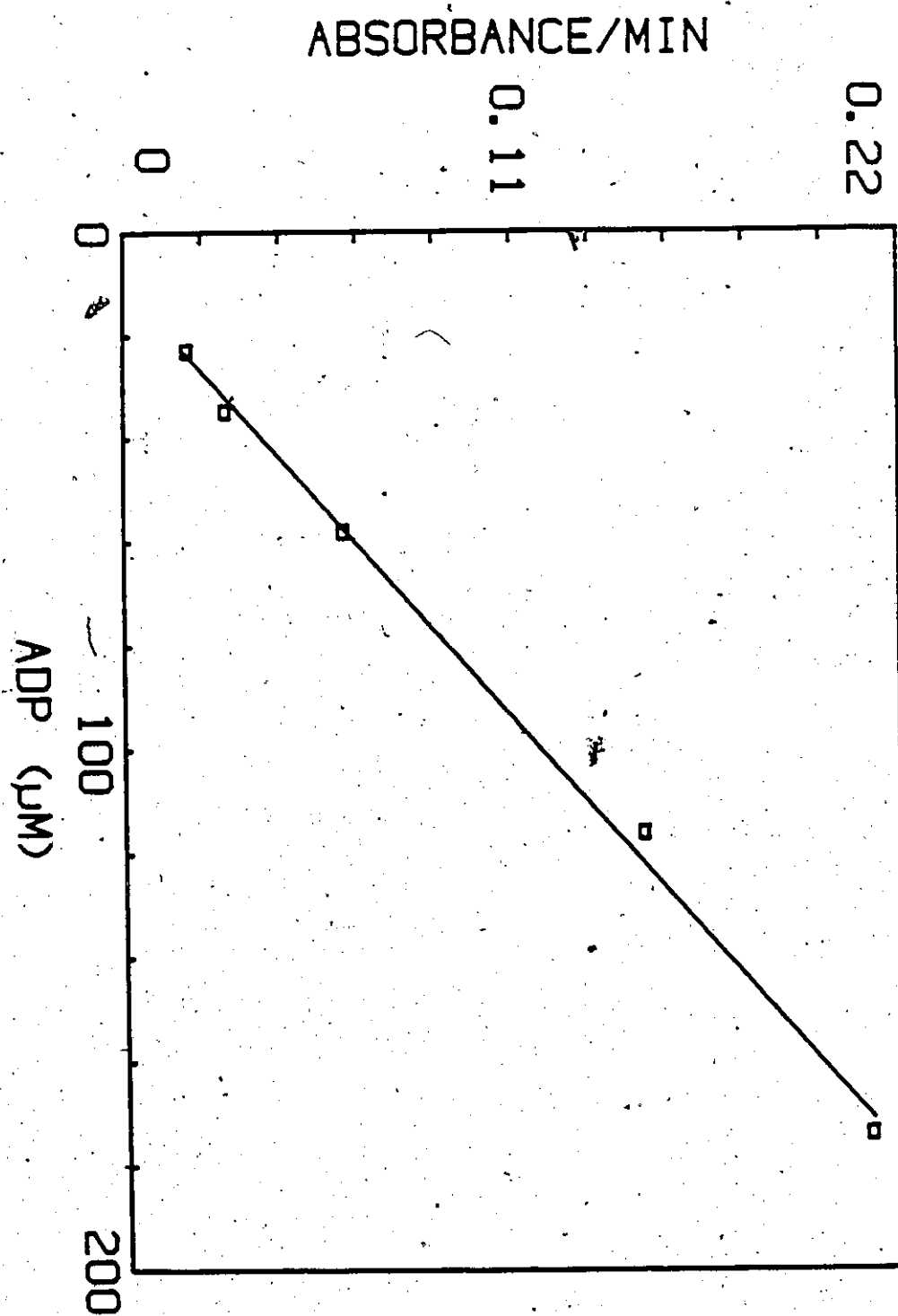
FIGURE 35

AN ENZYME COUPLED ASSAY FOR GLUCOSE

Legend

Various concentrations of aqueous glucose solutions were assayed with hexokinase. The activity of this enzyme was coupled with the system described for ADP (Fig. 34, p.120). All analyses were performed in duplicate. Equation for the line of best fit: $y = 0.002X - 0.02$ ($r^2 = 0.992$)

FIGURE 35
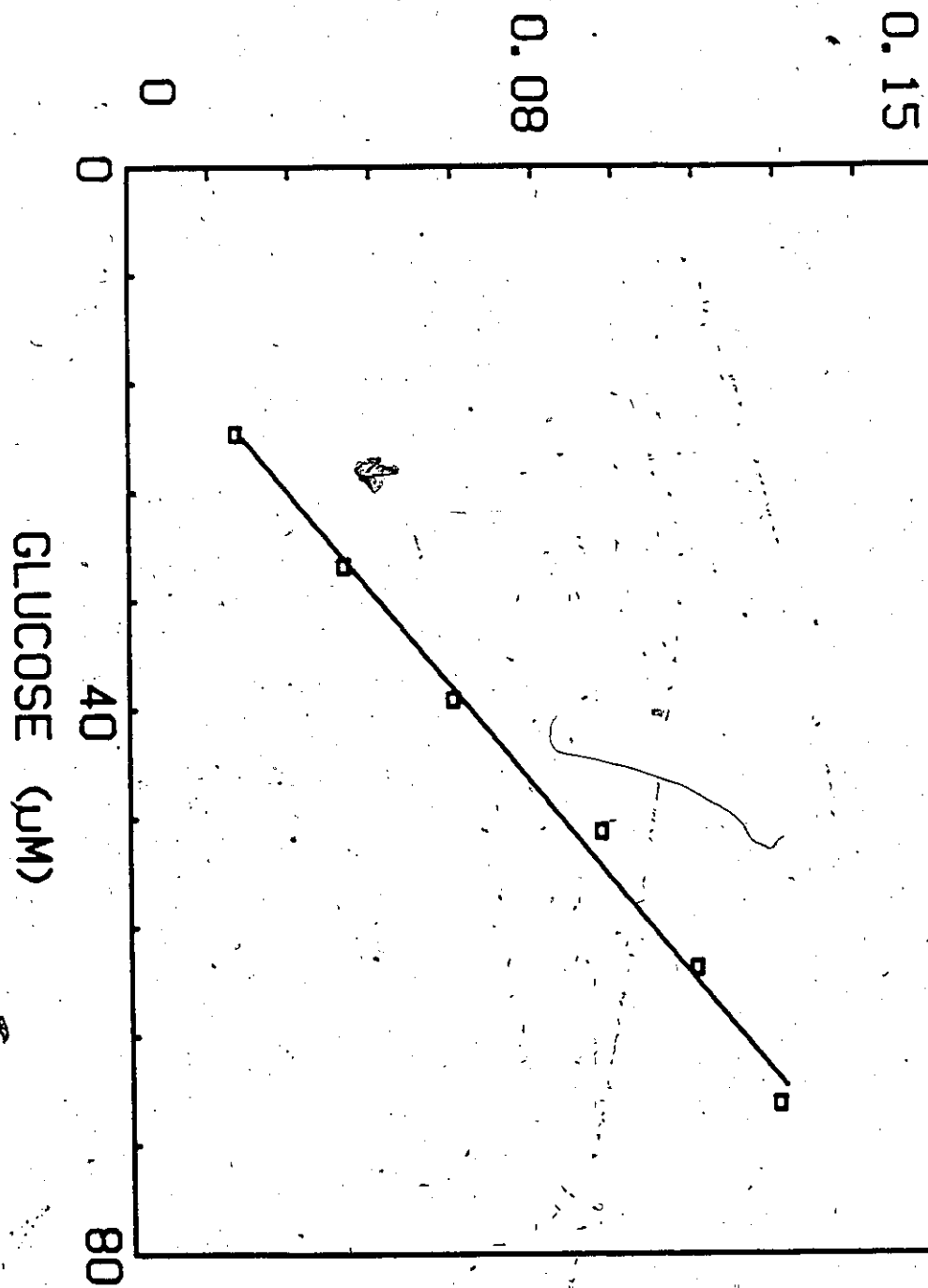
FIGURE 36

TIME PROGRESSION CURVES FOR GLUCOSE ANALYSIS

Legend

Shown are reaction profiles obtained with two different serum samples. The serum samples were first diluted 20-fold, and then 60 uL of the resulting solutions were assayed for glucose. This corresponded to presence of 3 uL of serum sample in a reaction volume of 600 µL. The analysis was carried out with the enzyme coupled system described for Fig. 35, p. 122.

FIGURE 36

ABSORBANCE 510 nm

TIME (SECONDS)
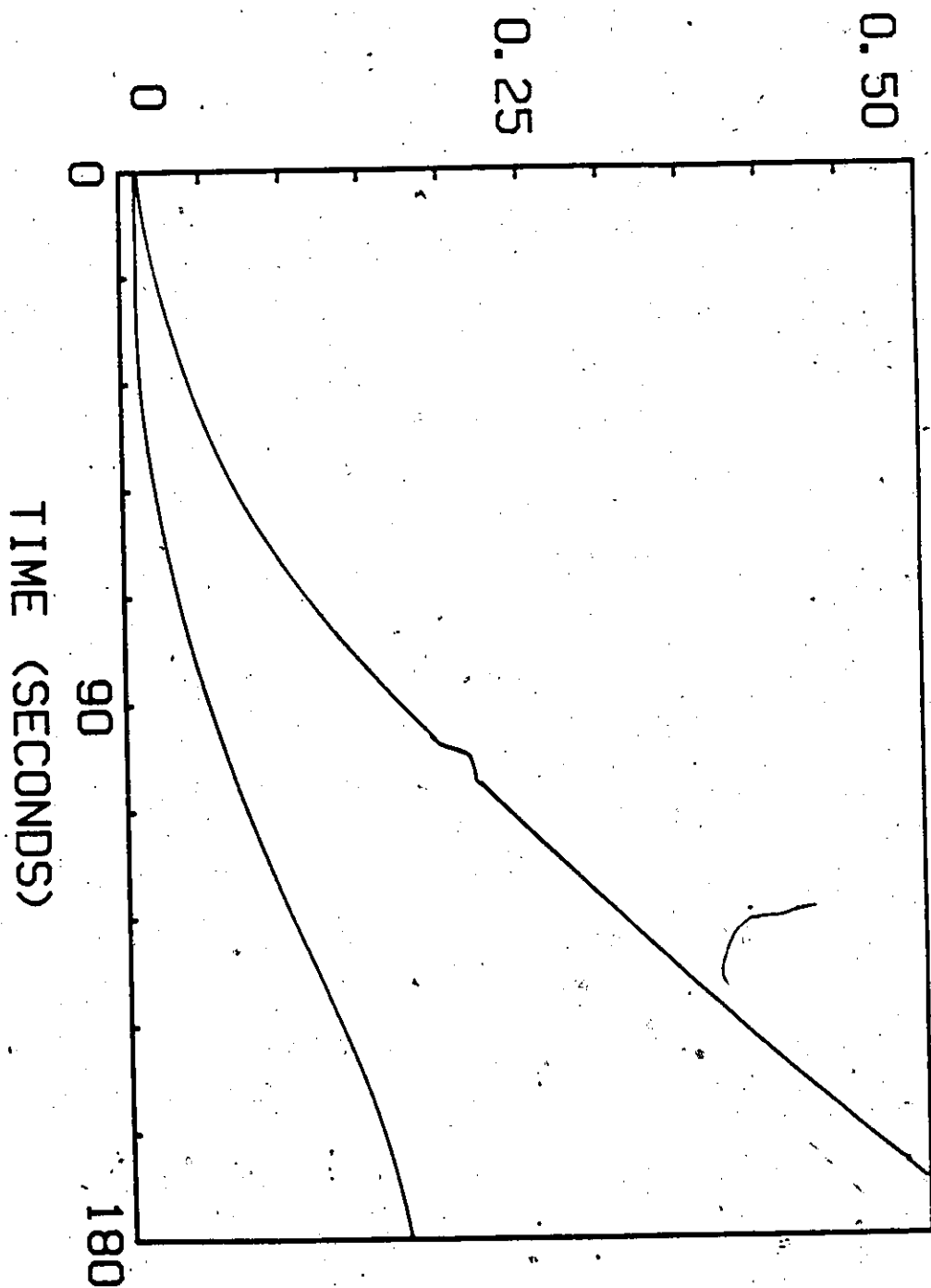
FIGURE: 37

COMPARISON OF TEST METHOD WITH GLUCOSE OXIDASE METHOD

Legend

Glucose levels in patient serum samples were assayed with the enzyme coupled assay developed in this project (See Fig. 35, p. 122 for description). Results were compared with a commercially available procedure. The method employs glucose oxidase coupled with a modified Trinder reaction. The latter was performed as part of a routine run on an automated instrument "Perspective™" (American Monitor Corporation). Data presented are individual determinations for each patient sample. Values reflect the original concentrations of glucose in patient samples. The equation for the line of best fit: $y = 0.86X - 0.3$ ($r^2 = 0.990$).

FIGURE 37
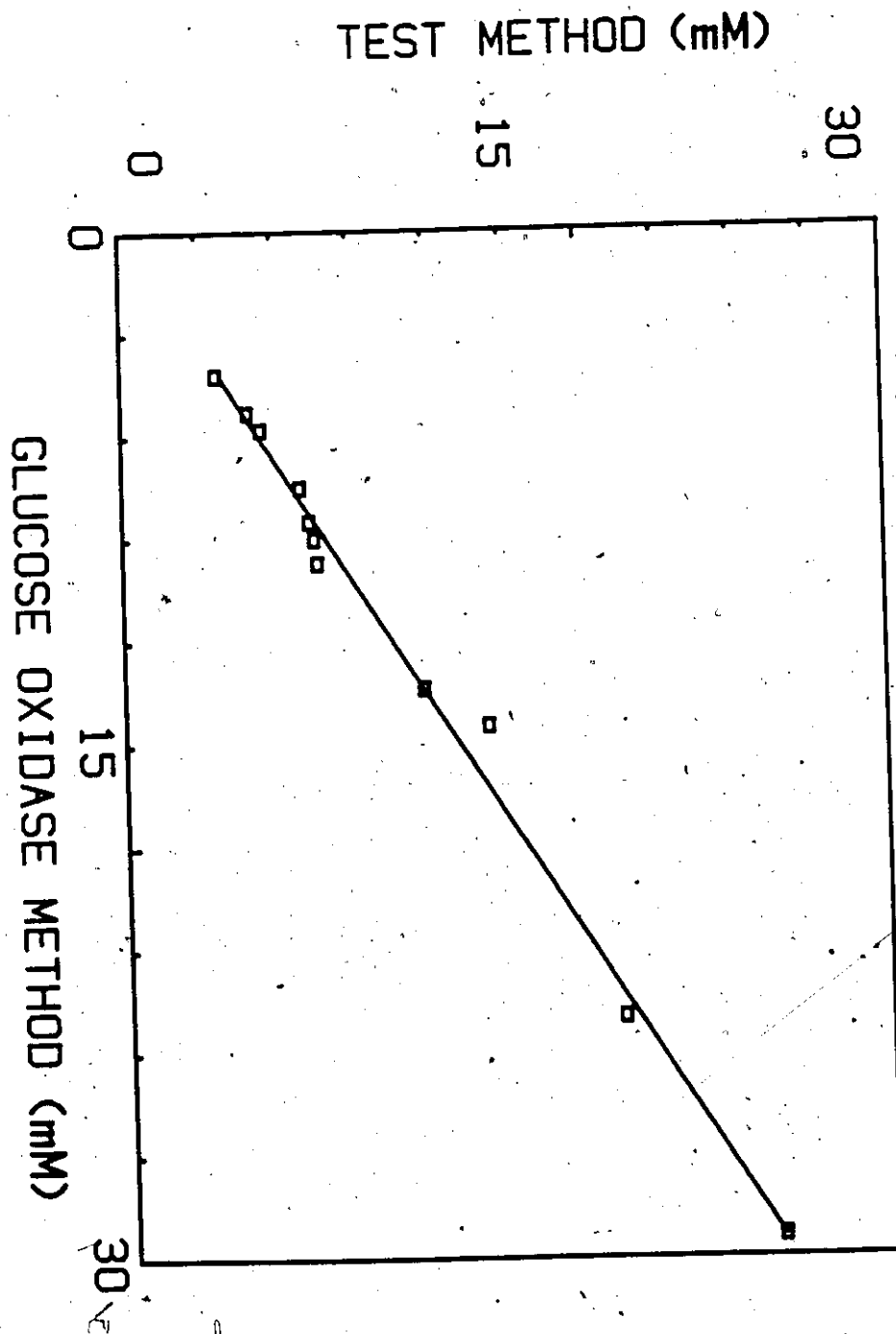
FIGURE 38

EFFECT OF ALBUMIN ON PEROXIDASE ACTIVITY

Legend

The peroxidase coupled oxidation of BILI was measured in the presence of different albumin:BILI ratios. Reactions were initiated by adding excess H_2O_2 to assay medium containing BILI and peroxidase (1U). Measurement of peroxidase activity (as rate of decrease in absorbance) was performed at 460 nm. The results obtained were compared against those obtained with standard BILI solution (albumin:BILI, 1:2). All assays were run in duplicate.

FIGURE 38

% ACTIVITY

ALBUMIN: BILI RATIO
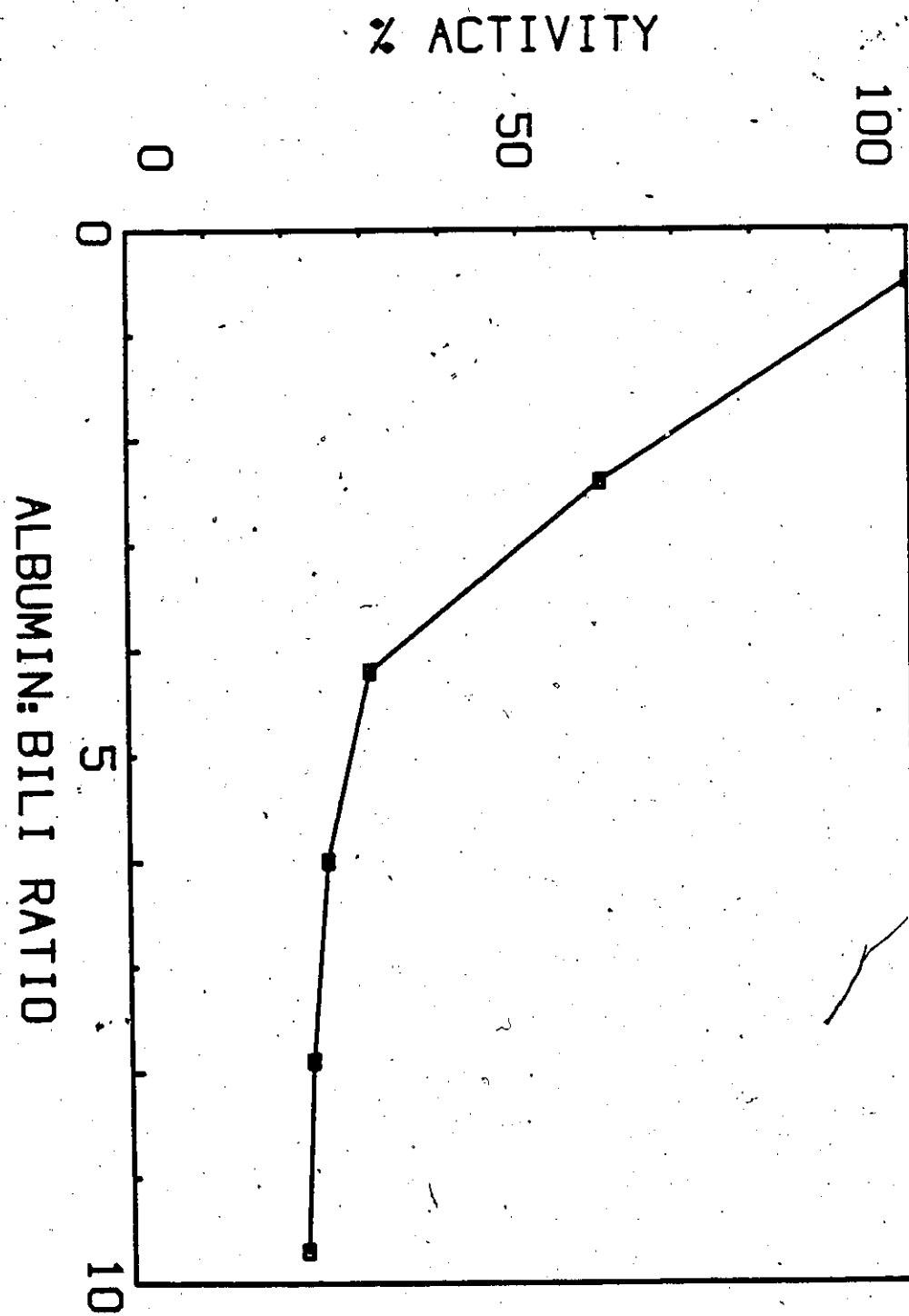
CHAPTER IV

DISCUSSION

Incubation of BILI with UDPGA and UDPGT is known
to result in the synthesis of mainly BMG. In the evaluation
phase, the assay was monitored with a sensitive diazo system
that was specific for the conjugated fraction. At pH 2.7,
the diazotized ethyl anthranilate is unable to react with
unconjugated BILI. The involuted structure hinders the
approach of the larger ortho-substituted ion to the central
methene bridge [45].

Results, of the work with albumin:BILI solutions, showed
some promise for a kinetic type of assay with UDPGT. The
linearity of the method (5-min incubation, followed by diazo
derivatization) fell between 5 and 20 μM (Fig. 17). The
performance, in terms of precision and correlation with a
commonly used method, was quite respectable. The within-run
and between-run CV's were less than 5%, and the coefficient
of determination, when compared to the Jendrassik-Grof
procedure, was 0.998.

Application to serum samples was seriously interfered
with by presence of an "inhibitor". Preliminary results,
obtained from gel filtration fractionation of serum and
agarose gel electrophoresis of the inhibitory fraction,

suggested involvement of albumin. Albumin was a strong candidate, even though small amounts of other proteins were present in the inhibitory fraction, isolated by gel-filtration chromatography with Sephadex™ G-200. The reasons for suspecting albumin were 2-fold: 1. albumin is present in serum/plasma in a large quantity (35 g/L); 2. the inhibitory effect of albumin has also been reported by others [24,39, 50,51].

Use of albumin, to facilitate solubilization of BILI in aqueous buffers, is a common practice. Addition of albumin to poorly water soluble substrate increases the availability of the active form. Without solubilizing agents colloid formation and precipitation can reduce the availability of the substrate to the enzyme [63]. However, the resultant albumin-BILI complex is not a substrate for the enzyme. Free BILI is believed to be the active substrate form for UDPGT [24,46]. Albumin:BILI ratios have a profound effect on UDPGT activity [24,39,50,51]. Activity of UDPGT has been observed to be inversely related to the concentration of albumin (relative to that of BILI). Albumin binds BILI quite strongly, yet reversibly. Increased levels of albumin appear to lower the availability of free BILI through binding [24,51], and hence, bring about decrease in UDPGT activity. The results of our study, with the effects of albumin, correlated quite well with the published ones [24,39,50,51].

An increase in the ratio of albumin:BILI was associated with decreased UDPGT activity and a longer lag phase (Fig. 24). Such an observation is consistent with lower substrate concentration. The activities obtained with BILI as free, HSA- and BSA-bound, were similar. Since the unbound fraction is thought to be the substrate, it can be inferred that the concentration of free BILI is similar in the two preparations. Therefore, it appears that solubility of BILI, under the reaction conditions, are similar. In addition, it was demonstrated that under the reaction conditions used, the solubility of BILI is adequate to support the activity of UDPGT, and does not require albumin. These results are in agreement with the findings of Wong [46], but not completely with those obtained by Cuypers et al. [39]. This group found a 1:1 ratio to be optimal. Our results suggested that the concentrations of free BILI, at an albumin:BILI ratio of 1:2 is similar to when albumin is omitted. An increase in this ratio was associated with a decrease in UDPG activity. It was presumed that larger albumin: BILI ratios (> 1:2) reduced the concentration of free BILI, and therefore, the availability of the substrate for the enzyme. Our studies also showed that this relationship of albumin:BILI ratio with UDPGT activity was more pronounced in the presence of higher substrate concentration (Fig. 23). Our results agree with those of Heirwegh et al. [63], who observed a hyperbolic

relationship of UDPGT activity with albumin:BILI ratio. The steeper slopes at higher concentrations of BILI (Fig. 23) may be an outcome if albumin-BILI binding kinetics, or perhaps due to interaction of albumin with the enzyme protein (UDPGT). Analysis of albumin:BILI solutions with the enzyme coupled system demonstrated longer lag phases with higher substrate concentrations (Fig. 31). This was an interesting observation, because the albumin:BILI ratio was fixed at 1:2 for all the solutions analyzed. It is known that albumin can inhibit oxidation of BILI by peroxidase [64]. Analysis of BILI solutions with various concentrations of albumin:BILI solutions did not have such an effect. Although increasing ratios of albumin:BILI solutions did progressively inhibit the peroxidase reaction (Fig. 38). So it is likely that the effect on the lag phase is not an artifact of albumin effect on peroxidase reaction. These results appear to support the notion that albumin may also alter UDPGT activity by direct protein-protein interaction. It is quite possible that the more pronounced inhibition, observed with higher substrate concentration (Fig. 23) was also due to greater albumin-enzyme interaction. In light of these observations, it is not surprising that the activity of UDPGT was only barely detectable with serum samples (albumin:BILI ratios in serum exceed 30:1).

Use of agents, that have substantial capacity to

displace BILI from albumin [65], was met with limited
success. Increased activities (upto 75%) were obtained in the
presence of serum or albumin, when sulfadimethoxine or
caffeine/ sodium benzoate was added. These compounds
apparently compete for the high affinity site [64]. Inhib-
ition of enzyme activity was observed with phenylbutazone,
ethanol, and p-hydroxybenzoic methyl ester at all concen-
trations . Sulfadimethoxine and caffeine/benzoate inhibited
at concentrations greater than 20 mM. It is quite apparent
that a quantitative displacement of BILI would be necessary,
due to dependence of UDPGT activity on albumin:BILI
ratio. BILI is bound rather avidly, and whether a quanti-
tative displacement of BILI can be achieved, without inter-
ference with UDPGT activity, is a matter of speculation.
However, such a task would entail an exhaustive study with
various agents or their combinations. On a philosophical
note, use of such an ideal displacer would certainly simplify
matters greatly, because the system would be self-blanking.
In the absence of the displacer, the sample could provide a
blank rate, which could be subtracted from the rate obtained
after adding displacer.

The coupled system as described by Mulder and Van Doorn
[25] was found unsuitable. Modification for measurement in
the visible region was accomplished by combining reactions of
Mulder and Van Doorn with that of Bozimowski et al. [57].

The reactions are shown below:

$$\text{AGLYCONE(BILI)} + \text{UDPGA} \xrightarrow{\text{UDPGT}} \text{GLUCURONYL ADDUCT} + \text{UDP}$$

$$\text{UDP} + \text{PEP} \xrightarrow{\text{PK}} \text{PYR} + \text{UTP}$$

$$\text{PYR} + \text{TPP} + \text{FAD} + P_i \xrightarrow{\text{POx}} \text{ACETYLPHOSPHATE} + H_2O_2 + CO_2$$

$$\text{BILI} + H_2O_2 \xrightarrow{\text{PER}} \text{Oxidized "BILI"}$$
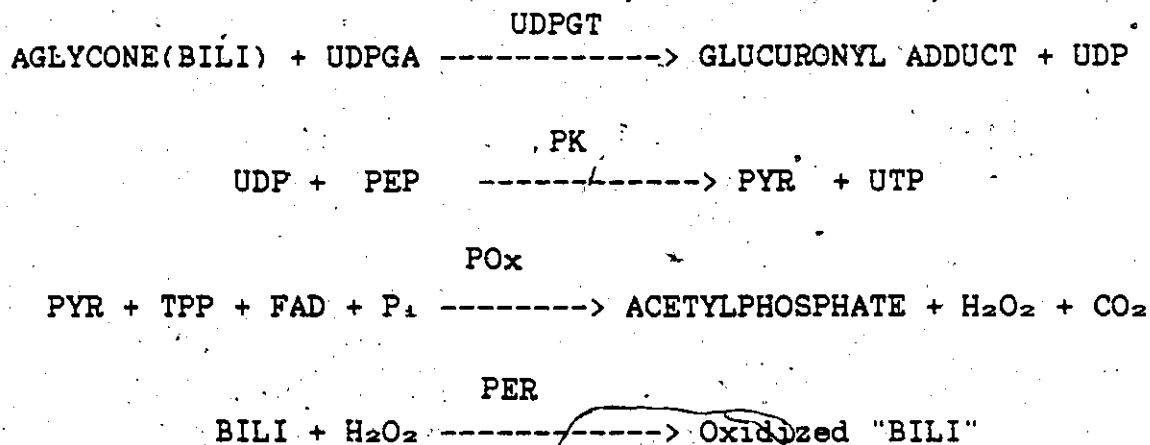
(TTP: thiamine pyrophosphate; FAD: flavin adenine dinucleo-
tide; P_i: phosphate; "BILI": oxidation products of BILI,
i.e., colourless, polar dipyrroles + ?; PK: pyruvate kinase;
POx: pyruvate oxidase; PER: horseradish peroxidase)

In this coupled sytem, the production of hydrogen peroxide is
linked with the production of UDP, and is dependent on UDPGT
activity. Peroxidase, in the presence of H_2O_2, oxidizes BILI
[64] and brings about a concomitant drop in absorbance at 460
nm (Fig. 28). It was necessary to use an enzyme preparation
that was devoid of 2-mercaptoethanol. Thiol groups are
efficient scavengers of peroxide, and therefore, would
interfere with the coupling system. The activity of UDPGT
varied linearly with BILI concentration. The range of linear

response was observed to be larger, than, when the diazo procedure was used (upper limit: 50 µM versus 20 µM for diazo coupled procedure). A possible reason for the extended linear range may be the determination of more representative activities, with the enzyme coupled system. With the diazo coupled procedure, the 5-min incubation provided an averaged velocity. This precluded correction for increase in the lag phase. Furthermore, since the diazo procedure involves sample manipulation prior, to spectrophotometric measurement, it would not have been practical to correct for the dynamic (with respect to BILI concentration) lag phase. Tavoloni et al. [51] observed a biphasic effect of BILI concentration on UDPGT activity; an initial stimulation in activity was followed by gradual inhibition. It is quite likely that, at "inhibiting" concentrations, the lag phase was increased substantially to reduce the value of the overall "average" rate of reaction. The use of this continuous assay system has an advantage in permitting selection of the actual rate of reaction, corresponding to a particular concentration. The 10-min or 30-min incubations, that are commonly used, represent average velocity, and the kinetic parameters determined on such bases may not be representative.

The coupled enzyme system has the versatility to monitor activities of other UDPGTs. This is possible because all

UDPGTs use a common cosubstrate, UDPGA. To demonstrate this capacity, the system was modified slightly for monitoring 4-nitrophenol conjugating activity:

$$\text{4-n-p} + \text{UDPGA} \xrightarrow{\text{UDPGT}} \text{4-n-p GLUCURONIDE} + \text{UDP}$$

$$\text{UDP} + \text{PEP} \xrightarrow{\text{PK}} \text{PYR} + \text{UTP}$$

$$\text{PYR} + \text{TPP} + \text{FAD} + P_i \xrightarrow{\text{POx}} \text{ACETYLPHOSPHATE} + H_2O_2 + CO_2$$

$$\text{HDCBS} + \text{AAP} + H_2O_2 \xrightarrow{\text{PER}} \text{QUINONEIMINE DYE}$$
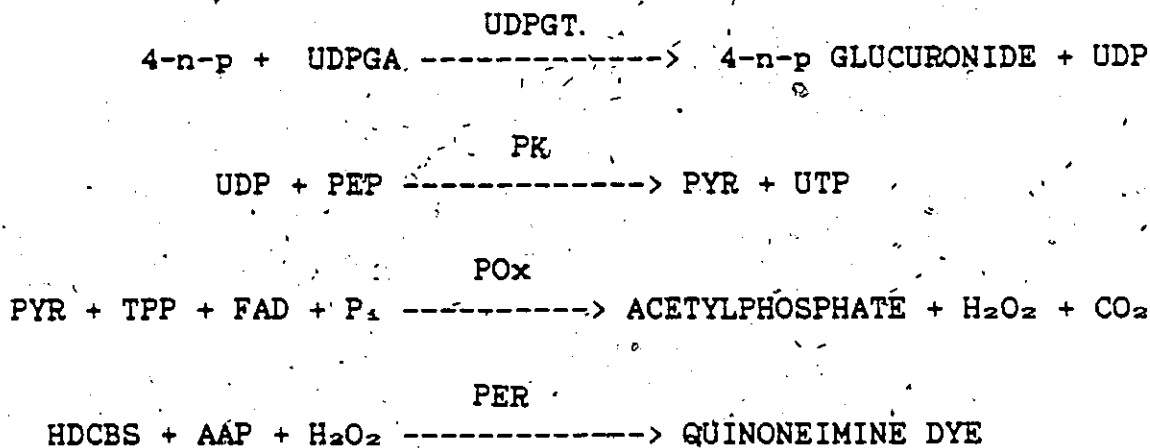
(HDCBS: 3,5-dichloro-2-hydroxybenzene sulfonate; AAP: 4-aminoantipyrine).

The last reaction is a modified Trinder-type reaction [62], that produces a chromogen with maximal absorbance at 510 nm and an extinction coefficient of about 32500 $M^{-1}cm^{-1}$. The advantage of this version is the feature of monitoring an increase in absorbance with time. Like the BILI system, it was necessary to use a preparation free of 2-mercaptoethanol.

The feasibility of applying this system to the analysis of the 4-n-p conjugating activity was demonstrated (Fig. 32). This coupling system may serve as a tool for monitoring activities of a number of UDPGTs. The method is sensitive

(can detect nmole quantities of UDP), simple, fast, continuous, and precludes sample processing (which is not only time consuming, but can introduce error). In contrast, other procedures for assaying the various forms of UDPGTs are uniquely designed for each UDPGT activity, and almost all require a substantial amount of manipulation prior to analysis. The methods, so far, have been colorimetric, fluorometric, radiometric, or chromatographic [27,36,40, 66]. Many of these also require costly equipment.

The enzyme coupled assay was possible because of the low specificity of pyruvate kinase for the phosphate acceptor. In addition to ADP, it can utilize GDP, IDP, UDP and CDP [67]. The results demonstrated the useful capacity of this system for analysis of purine and pyrimidine nucleotides and/or associated reactions.

CHAPTER V

CONCLUSION

Important information regarding the functional integrity of the hepato-biliary system can be obtained from levels of BILI fractions in the serum/plasma. Current methods used in clinical laboratories, especially for determination of conjugated BILI, are not satisfactorily specific. Although such methods do exist, they are not practical for the clinical laboratory.

This project involved a feasibility study of using BILI UDPGT for analysis of BILI. Activity was shown to vary linearly with BILI concentration, well beyond normal physiological concentrations. Comparison with the Jendrassik-Grof method showed a very good correlation. An enzymatically coupled assay that permitted continuous monitoring was established. Activity of UDPGT could be monitored, at 460 nm, by following the decrease in absorbance. Correlation with the diazo method was also quite good. Albumin appeared to be the probable cause of interference that was observed with serum samples. High albumin concentrations inhibited UDPGT activity, as well as that of peroxidase, presumably by lowering the availability of free

BILI, the substrate form for these enzymes. UDPGT activity was sensitive to the albumin:BILI ratio; ratios greater than 1:2 progressively inhibited UDPGT. Under the experimental conditions, albumin was not required to facilitate solubilization of BILI. Therefore, it was demonstrated that analysis of BILI, with UDPGT, is possible and can be enzymatically coupled for an effective continuous assay. However, quantitative displacement of BILI from albumin would be required, if serum samples are to be assayed. The free BILI, so produced, would be a suitable substrate for the enzyme.

The pyruvate kinase reaction, and its low specificity for the phosphate acceptor, proved to be very valuable. This step permitted formulation of an enzyme coupled assay for BILI conjugating UDPGT. Furthermore, since all UDPGTs produce UDP, the coupling system, linked with the modified Trinder reaction, has the potential to facilitate assay of other UDPGT activities. Such procedures would be simple, rapid, and sensitive coupled assays. Besides, allowing coupling of the UDPGT system, the PK reaction permits enzyme coupled analysis of ADP, UDP, and compounds/reactions associated with above nucleotides.

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