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DETERMINATION OF TOTAL AND CONJUGATED

GLUCURONIC ACID IN SERUM AND

URINE EMPLOYING A MODIFIED

NAPHTHORESORCINOL REAGENT

BY

ANGELO MAZZUCHIN

Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy at the
University of Windsor

Windsor, Ontario

1970

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EXTERNAL EXAMINER

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ABSTRACT

A quantitative determination of free, conjugated,
and total glucuronic acid in pure and biological systems
(serum and urine) has been developed employing a modified
naphthoresorcinol procedure. Glucose interference is
eliminated selectively with glucose oxidase which permits
the determination of glucuronic acid in the presence of
high concentrations of glucose. Free glucuronic acid is
decomposed with NaOH to a naphthoresorcinol-negative
product in the determination of O-glucuronides. The
procedure utilizes a modified naphthoresorcinol reagent
and a reduced volume of extracting agent. The method is
six to eight times more sensitive than the previous
naphthoresorcinol procedures. The reagent is stable for
seven days and yields reproducible results. The method
of standard addition is employed for the analysis, which
results in increased accuracy at low concentrations in
the determination of free and conjugated glucuronic acid.
The accuracy of the method is $\pm$ 5% with a precision of
$\pm$ 0.35 µg per 3 ml in the optimum concentration range of
3.5 to 16.6 µg per 3 ml. The total time required for

a complete analysis on either serum or urine is four hours.

A titrimetric determination of glucose, galactose, and xylose has been developed employing N-bromosuccinimide. The reactions are performed at room temperature for 15 minutes and the errors are less than $\pm$ 6% at the milligram level.

ACKNOWLEDGEMENTS

Grateful acknowledgement is made to Professor R. J. Thibert, Ph.D., for his many constructive suggestions regarding the design and direction of this research project. I also wish to gratefully acknowledge Dr. R. J. Walton for his assistance in the execution of this project.

I also wish to acknowledge the financial assistance provided by the Ontario Graduate Fellowships.

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DEDICATION

To Dale, whose love and moral support inspired in me the confidence and patience required throughout graduate school.

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ABBREVIATIONS

GA	glucuronic acid
MNR	modified naphthoresorcinol
NBS	N-bromosuccinimide
NR	naphthoresorcinol
Spike	method of standard addition
P-Gide	phenolphthalein momo-β-glucuronic acid

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CHAPTER I

INTRODUCTION

The uronic acids are generally defined as aldose
derivatives retaining the original carbon skeleton of
the sugar and psuedo-aldehydic group at C-1 and
possessing a terminal carboxylic acid group. The
three common naturally occurring uronic acids are
D-glucuronic acid (I), D-galacturonic acid (II), and
D-mannuronic acid (III), all of which are aldohexuronic
acids. In aqueous solution and probably in the
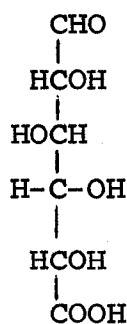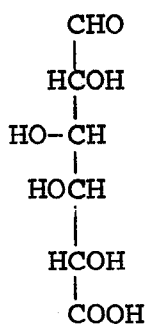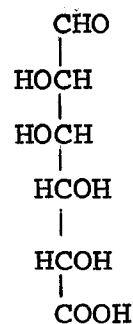
```
      CHO                    CHO                    CHO
       |                      |                      |
     HCOH                   HCOH                   HOCH
       |                      |                      |
     HOCH                  HO-CH                   HOCH
       |                      |                      |
    H-C-OH                  HOCH                   HCOH
       |                      |                      |
     HCOH                   HCOH                   HCOH
       |                      |                      |
     COOH                   COOH                   COOH


       I                     II                    III
```

crystalline state the predominant species of a hexuronic
acid has the cyclic pyranoside structure.

D-glucuronic acid is the most common natural uronic
acid and is found principally in the combined form in
which the linkage is either of the ether or ester types.

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It is found in polysaccharide form in mucopolysaccharides
and mucoproteins of blood and connective tissue (1).

Hyaluronic acid (IV), the predominant mucopoly-
saccharide of connective tissue, contains D-glucuronic
acid, D-glucosamine, and acetic acid. D-glucuronic
acid is also present in other physiologically important
mucopolysaccharides such as; chondroitin sulfates,
dermatan sulfate, and heparin (2). In animals and
humans, it is a structure
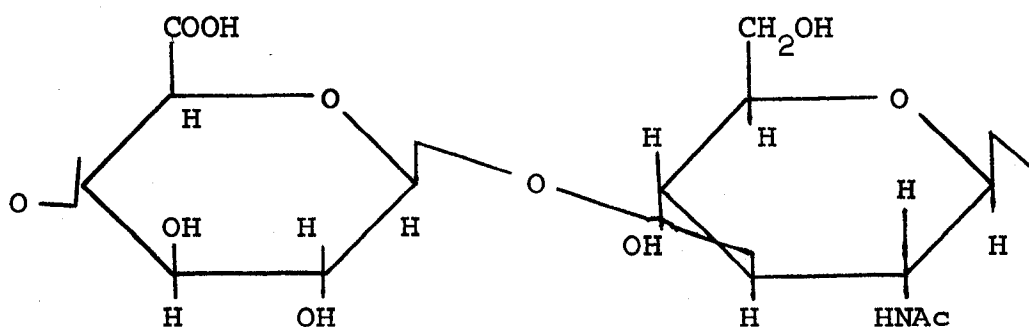
Repeating unit of Hyaluronic Acid (IV)

constituent of essentially all fibrous and connective
tissue. The specific role of glucuronic acid as part of
these polysaccharide is generally unknown.

Considering the above, D-glucuronic acid and
D-glucurone have been utilized in the treatment of
"collagen diseases" by Peterman, Hoakes, Smith, and
Ruggie (3). Collagen diseases such as rheumatic fever,

rheumatoid arthritis, scleroderma, acute lupus
erythematosis and atherosclerosis (4,5) are considered
as diseases of connective tissue where the metabolism of
the mucopolysaccharides have been altered. It would
seem therefore that a method capable of determining
total and combined glucuronic acid in blood serum would
be useful in diagnosis or monitoring of the aforementioned
pathological conditions.

Glucuronic acid is also involved in conjugation
reactions. Conjugations are generally regarded as
detoxification mechanisms, for compounds which undergo
these reactions are converted into products which are
usually less toxic and more rapidly excreted than their
precursors.

The main reactions involving conjugation are those
utilizing glucuronic acid, sulfate, glycine, cysteine,
methyl, and acetyl groups as the conjugating agent. Of
these reactions, glucuronic acid conjugation is probably
the most important, for it occurs extensively in man (6).
The major organs involved in glucuronide biosynthesis
are the liver, kidney, and alimentary tract (7,8). Of
these the liver is considered the major source (9). Thus,
clinical investigators have attempted to correlate the

concentration of glucuronic acid (total and combined) in serum and urine to various liver diseases (10-12). Others have studied the effect of various drugs, hormones, and other diseases on glucuronide biosynthesis in the liver (13-17). However, the interpretations of these results into a general pattern has been hampered by variations in quantitative procedures. The methods for the determination of glucuronic acid seem to have been quite well worked out, but a consideration of the original papers shows that there are as many "normal values" for D-glucuronic acid in biological fluids as there are methods making it quite difficult to interpret which value is really correct.

An accurate, sensitive, and general method for the quantitative determination of glucuronic acid in biological fluids is required. Such a method could be utilized in determining liver damage, monitoring drug therapy, diagnosis of collagen diseases, and provide clinical information leading to the diagnosis of other diseases. However, before outlining our approach to this problem, a brief survey of the available methods seems pertinent at this point.

Glucuronic acid has been quantitatively determined

by many different methods. These methods can be
categorized into two general types; classical wet
methods and instrumental methods of analysis.
Titrimetric (18-21) and manometric methods (22) are
representative of the classical determinations,
whereas gas chromatography (23,24), polarography
(22,25) and spectrophotometry are more modern
methods. The most common type of quantitative
determination employs spectrophotometry. Various
chromogenic reagents have been used, for example:
naphthoresorcinol (26-33); carbazole (34-44);
anthrone (45-47); resorcinol (48,49); sulphuric
acid (50-52); phloroglucinol (53); orcinol (38);
hydroxylamine hydrochloride (45-54); phenol (55).
An excellent review on the experimental methods
for uronic acids have been written by Z. Dische
(56).

The method most commonly employed for the deter-
mination of glucuronic acid in biological fluids is
the naphthoresorcinol reaction. It was introduced
by Tollens (57) as a qualitative test for deter-
mining urinary conjugates of D-glucuronic acid. All
modifications (58-62) of the reaction depart from the

following basic procedures: on boiling an aqueous solution of D-glucuronic acid, naphthoresocinol, and a strong mineral acid, a blue pigment is formed which is extracted into an organic solvent. A colour varying from blue in alcohols to violet-pink in ether results; it is estimated colorimetrically.

The nature of the product of uronic acid and naphthoresorcinol in the Tollens reaction is still in doubt. Guerrero and Williams (63) observed that a blue solid, apparently identical with the pigment, was produced by fusing D-glucuronolactone or D-galacturonic acid with naphthoresorcinol at 100°-120°C. Furfural produced a less intensely coloured product when used in place of uronic acids, while (-)-menthyl-β-D-glucosid-uronic acid gave no colour unless oxalic acid was present suggesting that the reducing group of the sugar residue must be free before pigment formation can occur. These workers suggested for the pigments either a diphenyl-methane structure (V) or that of a xanthene derivative (VI) derived from dehydration of (V), the carboxyl group of the uronic acid not being involved. This, however, does not satisfactorily explain the specific nature of the reaction; why, for example, it is also given by 2-deoxy-D-galactosid-

uronic acids (64), but not D-glucosaminuronic acids

(65).

(V)   (VI)
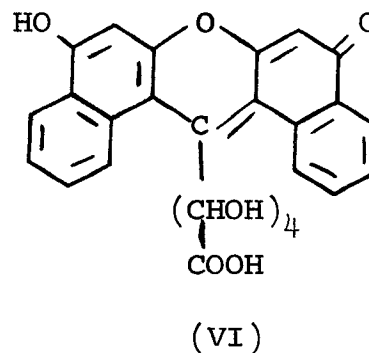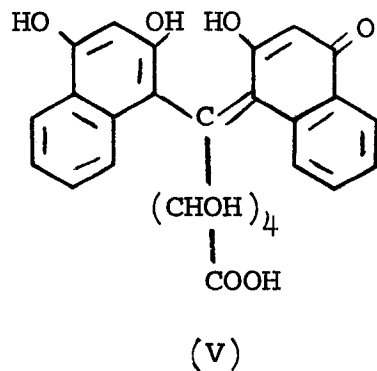
Momose et al. (66,67) methylated with diazomethane

the same fusion product of D-glucuronic acid and

naphthoresorcinol and found a complex mixture of dyes

to be present, containing four chromatographically

separable constituents of differing melting points. The

infrared spectra suggested the presence of lactones,

and the two main derivatives were identified as a pyrone

derivative (VII), and VIII) obtained by partial degrada-

tion of the uronic acids. Both these structures involve

dehydration of the uronic acid molecule. Other structures

have been proposed by Nozaki (68).

CH$_3$ O   O   O   HO   O   O

C

C CH

O

C CH

O C C

OCH$_3$

(VII)

C

C CH

O

C CH$_2$

O C
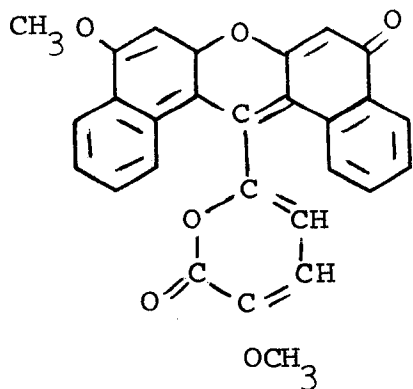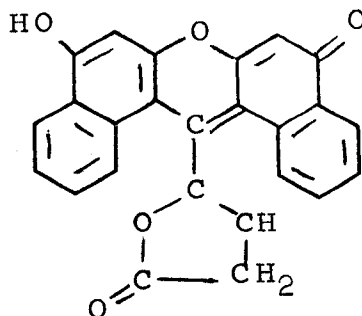
(VIII)

As a quantitative method for the determination of uronic acid, the naphthoresorcinol reaction has been used very widely, and an accuracy of about ± 5% can usually be obtained with pure solutions containing 10-100 μg of uronic acid per milliliter. Its main disadvantages have been the wide variability of the sensitivity, and the background colour varying in intensity with different specimens of naphthoresoricinol or even with different solutions made from the same sample; consequently, repeated controls with standard D-glucuronic acid solutions are essential.

To explain these variations, it has often been suggested that the chromogenic agent involved is not naphthoresorcinol but an oxidation product derived from it, and artificially aged solutions have been

recommended as being more stable than fresh preparations.
Thus Maughan et al. (58) employed solutions previously
kept at 38°C for 24 hours, then stored at 3°-5°C in the
dark; bubbling oxygen through the naphthoresorcinol
solution before use has also been suggested (69).
Freshly prepared aqueous solutions of naphthoresorcinol
are pale yellow, but darken to a reddish colour within a
few days, especially in the presence of air, and insoluble
material is gradually deposited. Aged solutions give a
pink colour on extraction with most organic solvents and
appreciably higher blank values. The coloured pigment
may be extracted with diethyl ether (58), amyl alcohol
(60), toluene (62), or β,β-dichloroethyl ether (31), the
wavelength at which the colorimetric measurement is
performed being determined by the solvent employed; thus
extraction with amyl alcohol yields a deep blue solution,
and with ether, chloroform, toluene, or ethyl acetate,
violet colours.

All the common hexuronic acids, and some monosub-
stituted uronic acids, give the naphthoresorcinol
reactions, D-glucuronolactone being quantitatively
equivalent, on a molar basis, to D-glucuronic acid.
D-glucosiduronic acids generally react as free D-

glucuronic acid, and the method has been adapted to the determination of total and conjugated D-glucuronic acid in mixtures (62). This particular modification of the naphthoresorcinol reaction described by Fishman has been the preferred method in many biochemical studies (70-74). This method, however, is inaccurate in the presence of high concentrations of glucose since the naphthoresorcinol reaction is actually a group reaction for hydroxy-carbonyl-carboxylic acid, and simple sugars may interfere. The one most serious source of interference in biological fluids is glucose (31,59,75,76). Although many investigators (77-80) have reported success in removing glucose interference, the procedures have been found to be too time consuming and the results were unsatisfactory (31, 60).

The greatest limiting factors in acquiring an accurate method for the determination of total and conjugated glucuronic acid are: the presence of interfering substances (31,59,60); the stability of the naphthoresorcinol reagent (33,58); poor sensitivity at low levels of glucuronic acid; and the inability to selectively eliminate glucose interference and free glucuronic acid. The present study is concerned with the following:

selectively eliminating glucose interference employing glucose oxidose; decomposing free glucuronic acid with NaOH in the determination of glucuronides; studying the effect of varying certain parameters in the naphthoresorcinol procedure with a view to obtaining greater sensitivity, stability, accuracy, and reproducibility; and to utilize this procedure for the determination of total and conjugated glucuronic acid in serum and urine.

CHAPTER II

EXPERIMENTAL

A. Modified Naphthoresorcinol Procedure

Materials and Methods

1) Apparatus

Spectral and absorbance measurements were made with a Beckman DB spectrophotometer employing a Sargent SRL recorder. Stability studies were performed on a Beckman DU monochromator with a Gilford Model 2000 Multiple Sample Recorder and a Haake thermostated circulating water pump ($25 \pm 0.5^\circ$C) with matched silica cells of 1-cm path length. Constant temperature water baths were set at $37 \pm 1^\circ$C and $100^\circ \pm 1^\circ$C. Glass-stoppered test tubes (25 x 200 mm) and centrifuge tubes (16 x 115 mm) were utilized throughout this study. A Vortex-Genie mixer was employed during deproteinization. An International Clinical Centrifuge Model CL was employed for centrifugation and pH measurements were performed on a Fisher Accumet pH Meter Model 19.

2) Reagents

D-glucuronic acid (Grade 1) and phenolphthalein mono-β-glucuronic acid were obtained from Sigma Chemical

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Company, St. Louis, U.S.A. All solutions were made up

in deionized, distilled water. Purified naphthoresorcinol

and anhydrous D-glucose (A.C.S. certified) were obtained

from Fisher Scientific Company, Canada. Glucose Oxidase

Type V (1,000 E.U. per ml) was purchased from Sigma

Chemical Company. One unit of this enzyme will oxidize

1 μmole of glucose to gluconic acid and H_2O_2 per minute

at pH 5.1 at 35°C. The following reagents employed were

of analytical grade: concentrated hydrochloric acid and

sulphuric acid; redistilled ethyl acetate; redistilled

toluene. Analytical grade reagents were utilized in

preparing the following solutions: aqueous 2.0 N

sodium hydroxide; aqueous 10% (w/v) phosphoric acid;

sodium tungstate 5% (w/v); n-octanol.

3) Modified Naphthoresorcinol Reagent (MNR)

A variation of the reagent as utilized by Nir

(33) was prepared. Naphthoresorcinol (NR), 400 mg, was

dissolved in about 80 ml of deionized, distilled water

and 0.5 N sodium hydroxide was added dropwise to adjust

the pH between 8-8.5. After allowing the solution to

stand for 15 min, 10% (w/v) phosphoric acid was added

from a pasteur pipet until a pH 2 - 2.5 was obtained.

The solution should turn light yellow. The solution

was continually stirred by means of a magnetic stirrer.
Sodium bisulfite (200 mg) was added and the volume was
adjusted to 100 ml with deionized water. This solution
was filtered twice, refrigerated (0 - 5°C), and protected
from light until use (100-ml volumetric flask was wrapped
with aluminum foil).

4) Study of Variables in the NR Procedure

The concentration of glucuronic acid was main-
tained at 50 μg per 2.0 ml of test solution throughout
this study. The variables studied were as follows:
hydrochloric acid volume in reaction; concentration of
naphthoresorcinol; sulphuric acid media; toluene as an
extracting agent; decreased volume of ethyl acetate as
the extracting agent. The procedure was basically that
of Nir (33). The sugar solution (2.0 ml) was pipetted
into the glass-stoppered test tubes to which 2.0 ml of
the naphthoresorcinol reagent under study were added.
Three preparations of NR reagent were studied: aqueous
0.2 mg % NR; 0.2 mg % NR (Nir reagent); and 0.4 mg % MNR.
Various acids and volumes of acid are then added to this
mixture. Blanks were prepared by substituting 2.0 ml of
water for the glucuronic acid. The tubes were then
stoppered and the solutions therein mixed (the tubes were

covered with a damp cloth to prevent the stoppers from jumping out during the heat treatment). The samples were then placed in a boiling water bath ($100 \pm 1^\circ C$) for 30 minutes. After the heating step, the samples were immersed in an ice bath for 10 min. Various volumes of extracting agents were then added by burette and the samples were agitated for 30 seconds. The organic layer in each of the tubes were removed by pasteur pipets and transferred directly to a cuvette. The absorbance was read at the appropriate wavelength. The two extracting agents employed were toluene and ethyl acetate. The absorbances were read at 565 nm and 580 nm for toluene and ethyl acetate, respectively.

The effect of time of heating on the reaction mixtures was studied. The reaction mixtures consisted of 2.0 ml of test solution (10 μg/ml glucuronic acid), 2.0 ml MNR and 2.0 ml concentrated hydrochloric acid. Three experiments were performed on these mixtures, as described earlier, except for periods of heating (30, 60, and 90 min periods at $100^\circ C$). After cooling, the samples were extracted with 3.0 ml ethyl acetate. The absorbance in the organic layer was read at 580 nm. All absorbance measurements reported are the average of duplicate

analysis.

5) Spectral and Stability Study of MNR Product

The spectrum and the stability of the reaction product extracted into 3.0 ml redistilled ethyl acetate from a reaction mixture consisting of 2.0 ml glucuronic acid solution (50 μg/2 ml), 2.0 ml MNR reagent, and 2.0 ml of concentrated hydrochloric acid were examined. The basic experimental procedures with regards to handling and cooling times were the same as described in the previous section. The samples were heated for 90 min in a boiling water bath.

6) MNR Procedure for Glucuronic Acid

The following solutions were pipetted into glass-stoppered test tubes: 3.0 ml glucuronic acid solution; 2.0 ml concentrated HCl; 2.0 ml of 0.4 mg %. MNR. Blanks were prepared with 3.0 ml H_2O instead of glucuronic acid solution. The concentration range studied was 0 - 20 μg per 3.0 ml of glucuronic acid. The test tubes were then stoppered and shaken. After having covered the test tubes with a damp cloth, they were then placed in a boiling water bath for 30 min. This study was repeated except that a heating period of 90 min was used. The samples were then immersed in an ice bath for 10 min

and exactly 3.0 ml of redistilled ethyl acetate were added from a burette and the samples shaken for 30 seconds. The ethyl acetate layer was then transferred to a cuvette by means of a pasteur pipet and the absorbance was read at 564 nm.

A comparative study of the sensitivity of the present method with that of Fishman (62,76) and Nir (33) was carried out.

7) Stability of the MNR Reagent

The stability of the MNR reagent was monitored by performing regularily the MNR procedure (90 min heating period) over a two week period utilizing a single preparation of the reagent. The samples, in duplicate, consisted of a blank and 10 μg per 3.0 milliliters of glucuronic acid. The absorbance versus age of reagent was plotted.

8) Glucose Interference Study

Two-milliliter samples containing different concentrations of glucose were incubated with 0.1 ml glucose oxidase (Type V) and 1.9 ml of a 0.1 M acetate buffer pH 5.6 at 37°C for 90 min. The samples were aerated throughout the incubation period. The samples were then deproteinized by the method of Bonsenes and

Taussky (81). Two milliliters of a 5% (w/v) sodium

tungstate solution were pipetted into the samples

contained in the glass-stoppered test tubes. Two

milliliters of 2/3 N H_2SO_4 were then added dropwise from

a burette as the samples were being agitated by a Vortex

mixer. After allowing the samples to stand for 10 min,

they were transferred to centrifuge tubes and centrifuged

in a clinical centrifuge for 5 min. Three milliliters of

the supernatant were then utilized as the samples for the

MNR procedure.

A second study was conducted in which 1.0-ml samples

diluted to 2.0 ml with deionized, distilled water were

incubated for 45 min and 1.5-ml aliquots were taken after

deproteinization. These aliquots were diluted to 3.0 ml

and utilized for the MNR reaction. Blanks in both systems

contained the appropriate volume of water instead of

glucose solution. The concentration range of glucose

studied was 0 - 600 mg %. The absorbance was read at

564 nm.

9) Gluconic Acid Study

The MNR procedure was performed on samples

containing varying amounts of gluconic acid. The samples

were treated in the same manner as described in the glucose

interference study (1.0-ml samples) except, no enzyme
or incubation period was required. The concentration
range of gluconic acid studied was 0 - 600 mg %. The
absorbance was read at 564 nm.

10) Determination of Glucuronic Acid in the Presence
    of Glucose

Glucuronic acid concentration was determined in
the presence of 3 mg per ml glucose following the exact
procedure with respect to incubation time (45 min) and
deproteinization as described in the previous section.
One-milliliter samples were taken and 1.5-ml aliquots
(of the deproteinized samples) were utilized for the MNR
reaction. Blanks contained glucose and all analyses of
samples were carried out in duplicate. The samples were
heated for 30 min in the boiling water bath and the
concentration of glucuronic acid was determined from a
standard curve. Standards run simultaneously with the
blanks and samples contained no glucose.

11) Determination of Conjugated Glucuronic Acid in
    the Presence of Free Glucuronic Acid

One milliliter of 1.0 N NaOH was added to 2.0-
ml samples which contained a mixture of D-glucuronic
acid and phenolphthalein mono-β-glucuronide (P-Gide). A

spectral analysis was performed on these samples. The

glucuronic acid concentration was constant at 10 μg per

ml and the concentration of P-Gide varied from 7.3 μg -

21.9 μg per 2.0 ml. The samples were heated for 20 min

at 100°C and allowed to cool in a cold water bath. The

samples were then subjected to the MNR procedure (90 min

heating) for the determination P-Gide. The concentration

of P-Gide was determined from a standard curve of P-Gide.

12) Determination of Total and Conjugated Glucuronic

Acid in Synthetic Mixtures by the Method of

Standard Addition

The synthetic samples contained D-glucose,

D-glucuronic acid, and P-Gide. The concentration of

glucose was constant in all samples (3 mg per ml) and

the volume of sample taken was 1.0 ml. The concentration

of glucuronic acid and P-Gide in the samples varied from

5 μg to 20 μg and 5.98 μg to 11.96 μg, respectively. The

ratios of glucuronic acid to P-Gide studied were 1:1 and

2:1. Blanks contained 3 mg per ml of glucose.

A single sample was treated as outlined in Fig. 5.

One-milliliter samples were diluted to 2.0 ml and were

incubated for 45 min with continuous aeration at 37°C.

The incubation mixture consisted of 2.0 ml of sample,

0.1 ml of glucose oxidase and 1.9 ml of 0.1 M acetate buffer, pH 5.6. The samples were then deproteinized and centrifuged as described earlier. After deproteinization the total volume of the reaction mixture is 8.0 ml. Four 1.5-ml aliquots of the supernatant from the sample were pipetted into four glass-stoppered test tubes. One set of duplicates was utilized for the determination of total glucuronic acid and the other set for conjugated glucuronic acid.

In order to determine the endogenous concentration of glucuronic acid of a particular sample, four replicate samples were treated as described above. These four replicates result in sixteen aliquots or eight sets of duplicates after deproteinization. Four sets were utilized for the determination of conjugated glucuronic acid and the remaining four for the determination of total glucuronic acid. Free glucuronic acid was determined by difference between total and conjugated glucuronic acid.

In the determination of endogenous total glucuronic acid three sets were spiked with varying concentrations of P-Gide. The concentration of spike utilized were 5.98 µg, 8.98 µg, and 11.96 µg. (Therefore, the result

of the above treatment was four sets of duplicates of which three were spiked and one unspiked.) All samples were then diluted to 3.0 ml with water and were subjected to the MNR procedure. The absorbance readings of the spiked and unspiked samples were plotted against known concentration of P-Gide spike, and the endogenous total glucuronic acid was determined by extrapolating the straight line back to the X-axis. This straight line was then utilized as the standard curve for the determination of endogenous total glucuronic acid for other synthetic samples - these samples need not be spiked.

In the determination of conjugated glucuronic acid, the remaining four sets were spiked as described for total glucuronic acid and 0.5 ml of 2.0 N NaOH was added and the samples diluted to 3.0 ml with water. The samples were stoppered and covered with a damp towel and placed in a boiling water bath for 20 min. After cooling to room temperature in a cold water bath, the samples were subjected to the MNR procedure and the same calculations were performed as described for total glucuronic acid. To determine the amount of total or conjugated glucuronic acid in the original 1.0-ml sample, the amount determined

from the curve is multiplied by the dilution factor
8/1.5 or 5.33.

13) Glucuronide Study in Serum

To determine any loss of P-Gide during deprotein-
ization 1.0, 0.5, 0.25 ml samples of pooled sera were
taken and spiked before and after deproteinization. The
glucose concentration of the serum was determined by
the alkaline ferricyanide method on the AutoAnalyzer
(83) and was found to vary between 115 - 117 mg%. The
blanks contained 1 mg per ml of glucose. The total
volume of spike and sample was maintained at 2.0 ml
before deproteinization and 3.0 ml after deproteinization.
The samples were treated as described for the synthetic
samples except that to the incubation mixture 0.02 ml
of n-octanol was added to prevent foaming during aeration.
Reproducibility studies were done on sera samples for
both total and conjugated glucuronic acid.

14) Denaturation Studies

In an attempt to increase the recovery of
glucuronic acid in serum protein denaturing agents were
utilized. The denaturing agents employed were urea,
Triton X-100, and sodium deoxycholic acid which were 8M,
0.1% (v/v), and .005M, respectively. Samples were

treated with urea before deproteinization and were made

8M in urea concentration. The MNR procedure described

for the glucuronide study, above, was then carried out.

Serum samples (0.5 ml) were treated as described above

except that 2.0 ml of the other denaturing agent was

added to the protein precipitates and the resulting

solutions were agitated on the Vortex mixer for two

min and allowed to stand at room temperature for 15 min.

The samples were then centrifuged and the supernatants

were transferred by pasteur pipet to the corresponding

supernatants from the initial deproteinizations.

Aliquots (1.5 ml) were utilized and treated as described

in the glucuronide study. The per cent recovery was

calculated by utilizing the curve obtained from spiking

after deproteinization as the standard curve.

15) Analysis in Urine

Three 24-hour urine specimens were collected

from adults and stored under toluene at 0 - 5°C. Five-

milliliter urine samples were heated for 5 min at 100°C.

When precipitates appeared, they were removed by centri-

fugation. One-milliliter of urine was diluted to 2.0 ml

with deionized water and incubated in the same manner as

the synthetic samples. The blanks consisted of 1 mg per

ml glucose and water. The total incubation mixture was then diluted to 100 ml with deionized water in a 100-ml volumetric flask. Aliquots (1.5 ml) were then utilized for the determination of total and conjugated glucuronic acid as described for the synthetic mixtures. Deproteinization was not required. Reproducibility studies were done on all urine samples and the results obtained for all the determinations were compared to one another. Results reported for each sample are the average of five determinations.

B. Titrimetric Determination of Glucose, Galactose, and Xylose with N-Bromosuccinimide

Materials and Methods

1) Reagents

N-Bromosuccinimide (NBS), approximately 0.05N (2.22 grams per 500 ml), was prepared fresh daily. Anhydrous preparation of glucose, galactose, and xylose were utilized. NaOH and KI were of analytical grade. Standard solutions of sodium thiosulfate, approximately 0.01N, were prepared and standardized in the usual manner with 0.01N KIO_3 (primary standard).

All titrations were performed with a pre-calibrated

50-ml burette and 250-ml conical flasks with stoppers were employed as the reaction vessels. pH measurements were performed on a Fisher Accumet pH Meter, Model 19.

2) pH Studies

The reaction procedures were performed as described in section 3B for all three sugars with respect to time and volumes of reagents, except 100-ml beakers were utilized and various normalities of NaOH were added in order to study the pH range from 4 to 12. The concentration of sugars was maintained constant throughout this study (5 mg per ml). pH measurements were made before and after the 15 min reaction time. The per cent recovery was calculated as in section 3B, except for xylose whose equivalent weight is 75.07.

3) Titrimetric Determination of Glucose

A 1.0-ml aliquot of the test solution was transfered to 250-ml conical flasks to which 10.0 ml of 0.05N NBS was added. In the case of low concentrations (1 mg per ml), 5.0-ml aliquots of the test solution were employed. Blanks contained the appropriate volume of water instead of sugar solution. The solutions were made alkaline with 2.0 ml of 0.2N NaOH. The flasks were stoppered, shaken, and allowed to stand for 15 min at

room temperature (in the dark). The solutions were then
acidified with 10 ml of 1N HCl and approximately 2 grams
of potassium iodide were added. The flasks were again
stoppered and shaken. The liberated iodine was titrated
with the standard sodium thiosulfate solution using
starch as an indicator. The amount of glucose was
calculated applying the following relationship:

$$\text{mg glucose} = \text{Eq. Wt.} \times N_{Na_2S_2O_3} (V_{T_{Blank}} - V_{T_{Sample}})$$

where;   Eq. Wt. = equivalent weight of the particular
                       sugar

$N_{Na_2S_2O_3}$ = Normality of sodium thiosulfate

$V_{T_{Blank}}$ = Volume of sodium thiosulfate required
                to titrate blank

$V_{T_{Sample}}$ = Volume of sodium thiosulfate required
                to titrate sample.

The recoveries reported are the average of five deter-
minations.

4) <u>Titrimetric Determination of Galactose and Xylose</u>

The procedure for the determination of galactose
and xylose is different with regards to volume and normality
of NaOH added. For galactose 3.0 ml of 0.2N NaOH and for
xylose 2.0 ml of 0.5N NaOH were utilized. The reaction

time for both sugars was maintained at 15 min.

CHAPTER III

RESULTS AND DISCUSSION

A. Modified Naphthoresorcinol Procedure

The results of the study of the various para-

meters affecting the naphthoresorcinol (NR) procedure

are reported in Table 1. Experiments 1 - 3 illustrate

the effect of increasing the concentration of the NR

reagent. The results indicate an increase in sensitivity

with increasing concentrations of NR. This agrees with

the results of Mosher (31). These experiments also

indicate that the modified NR reagent is more sensitive

than Nir's reagent (33) by a factor of about 1.3. The

results of experiments 3 - 5 illustrate that when the

volume of concentrated hydrochloric acid is increased,

the sensitivity reaches a maximum at 4.0 ml and then

decreases. This decrease in sensitivity most probably

results from acid hydrolysis of some of the ethyl acetate

during the extraction, thereby preventing the complete

extraction of the NR pigment formed. The substitution

of concentrated sulfuric acid in the NR procedure

resulted in a turbid ethyl acetate layer and therefore

was not utilized as the acid media. Toluene was found

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to be inferior to ethyl acetate as an extracting agent due to the turbidity of the organic phase.

Table 1 (experiments 8 - 11) demonstrates the effect of increasing the volume of the extracting agent. As one would expect, an increase in volume results in a decrease in absorbance or sensitivity. The volume of ethyl acetate which resulted in the greatest sensitivity was 3.0 ml. An attempt to increase the sensitivity, even further, by increasing the volume of hydrochloric acid resulted in a complete loss of the ethyl acetate layer.

Increasing the time of heating in the boiling water both from 30 to 90 min also causes an increased absorbance which is in agreement with previously reported data (30, 31,62). Therefore, the conditions for maximum absorbance or sensitivity based on the above observations are: 2.0 ml of 0.4 mg % of MNR; 2.0 ml concentrated hydrochloric acid; 90 min heating period; 3.0 ml redistilled ethyl acetate as the extracting agent.

TABLE I

Study of variables in NR procedure

Expt. No.	Concentration NR mg %	Volume of acid (ml)$_d$	Volume of extracting agent (ml)$_e$	Absorbance$_f$
1	0.2[a]	2.0	10.0	0.175
2	0.2[b]	2.0	10.0	0.235
3	0.4[c]	2.0	10.0	0.307
4	0.4	4.0	10.0	0.371
5	0.4	6.0	10.0	unstable
6	0.4	2.0 (H_2SO_4)	10.0	turbid
7	0.2	2.0	10.0 (toluene)	turbid
8	0.4	2.0	3.0	1.159
9	0.4	2.0	4.0	0.814
10	0.4	2.0	5.0	0.623
11	0.4	2.0	6.0	0.505

a aqueous NR

b NR reagent made up according to Nir (33)

c MNR reagent

d The acid utilized in this study was concentrated hydrochloric acid where mentioned in parenthesis.

e Ethyl acetate was employed as the extracting agent except where mentioned in parenthesis.

f The results reported are the average of duplicate analysis.

The above conditions were studied with respect
to absorption maxima and stability of the NR pigment
finally extracted with 3.0 ml redistilled ethyl acetate.
The absorption maximum for glucuronic acid under the
above conditions is at 564 nm (Fig. 1). The normal
blank versus ethyl acetate has an absorption maximum
at 510 nm. The stability of both sample and blank
was studied by monitoring the change of absorbance
with time. The sample and blank were both stable for
the length of study (1 hour).

The MNR procedure obeys Beer's law in pure
solutions as illustrated by the linear relationship
in Fig. 2 (curve 1). This linear relationship is
also true for the MNR procedure when a heating period
of 30 min is employed. Figure 2 (curve 3, 4) illustrate
the Beer's law relationships for the methods of Fishman
(76) and Nir (33). Curve 5 represents the data reported
by Nir (33). The results obtained in this laboratory
employing Nir's method are lower than those reported
by Nir. The molar absorptivity, therefore, was
calculated from the data reported by Nir. We could
make no such comparison with Fishman's method (61,62,
76), due to the lack of data required to calculate

the molar absorptivity. The molar absorptivity

was calculated from the data (curve 3) obtained

using the method of Fishman in this laboratory.

The molar absorptivity (liter mole^{-1} cm^{-1}) of the

MNR procedure for 90 min and 30 min heating periods

are 30.1 x 10^3 and 16.5 x 10^3, respectively. The

molar absorptivities for Nir's and Fishman's methods

are 3.8 x 10^3 and 5.0 x 10^3, respectively. This

demonstrates that the present method is six times

more sensitive than Fishman's method and eight times

more sensitive than that of Nir. The optimum

concentration ranges as calculated by the Ringbom

method (83) for the MNR procedure at 90 min and

30 min heating are 3.5 to 16 μg per 3.0 ml and 5 to

25 μg per 3.0 ml, respectively (the concentration

range for the MNR at 30 min heating was calculated

from curve 2 (Fig. 2) extrapolated to 30 μg). The

results of a typical calibration curve indicate

standard errors of estimate to be ± 0.015 O.D.

units and ± 0.5 μg per 3.0 ml. The MNR procedure

involves errors in the optimum concentration range

that are less than ± 5%.

Legend to Figure 1

Fig. 1   Absorption spectra of various NR products:

Curve 1, 14.6 μg per ml of P-Gide reacted with

NaOH read against blank containing NaOH; Curve 2,

14.0 μg per ml glucuronic acid read against normal

blank; Curve 3, Blank containing NaOH read against

redistilled ethyl acetate; Curve 4, normal NR

blank read against redistilled ethyl acetate.

Figure 1   35
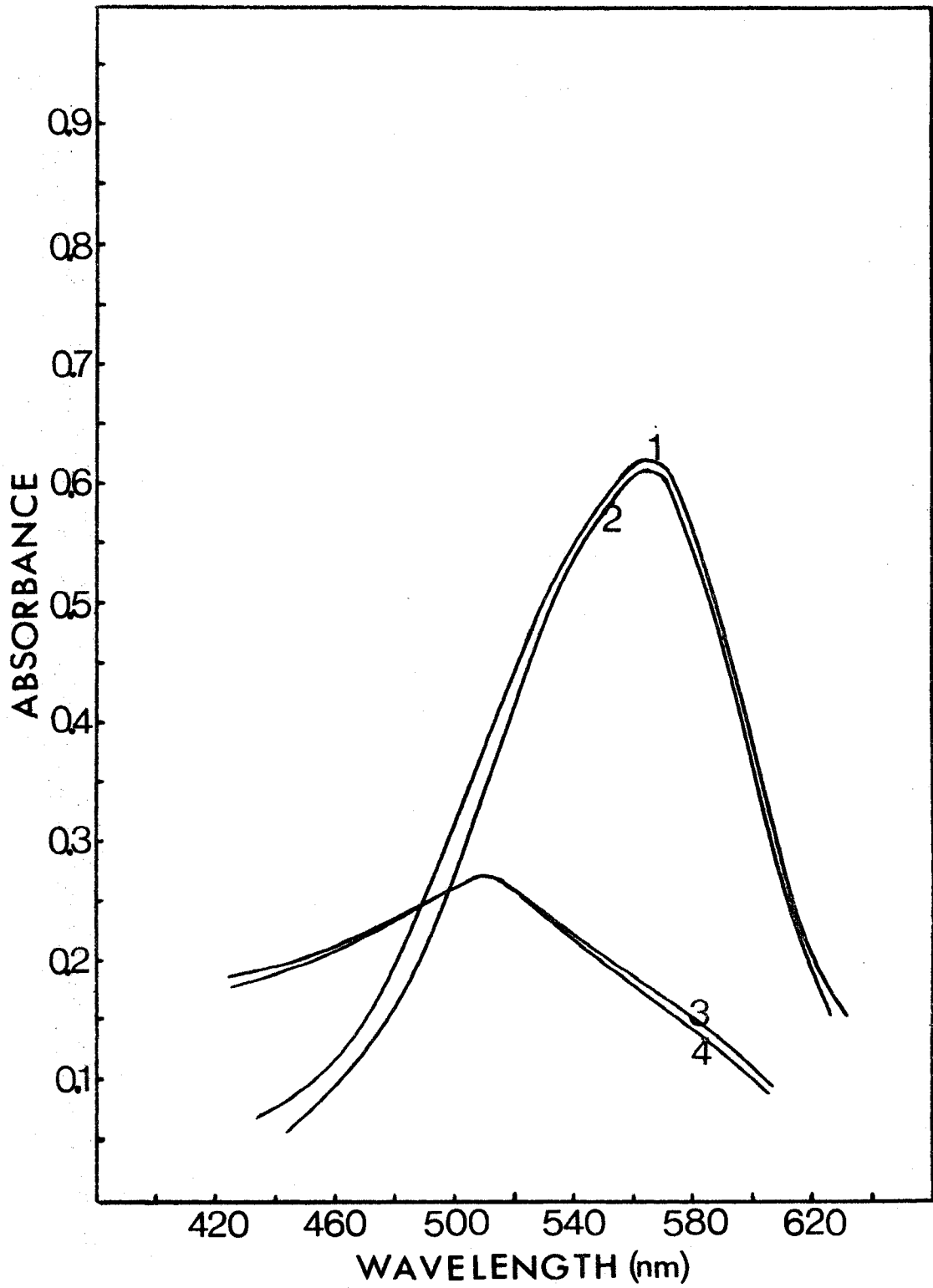
Legend of Figure 2

Fig. 2 Beer's Law study of various NR procedures. Each

curve represents the following: Curve 1, MNR

procedure, 90 min heating, μg per 3.0 ml, 564

nm; Curve 2, MNR procedure, 30 min heating, μg

per 3.0 ml, 564 nm; Curve 3, Fishman method

(44); Curve 4, Nir method (33), μg per 2.0 ml, 580

nm; Curve 5, published data of Nir (33).

Figure 2   37
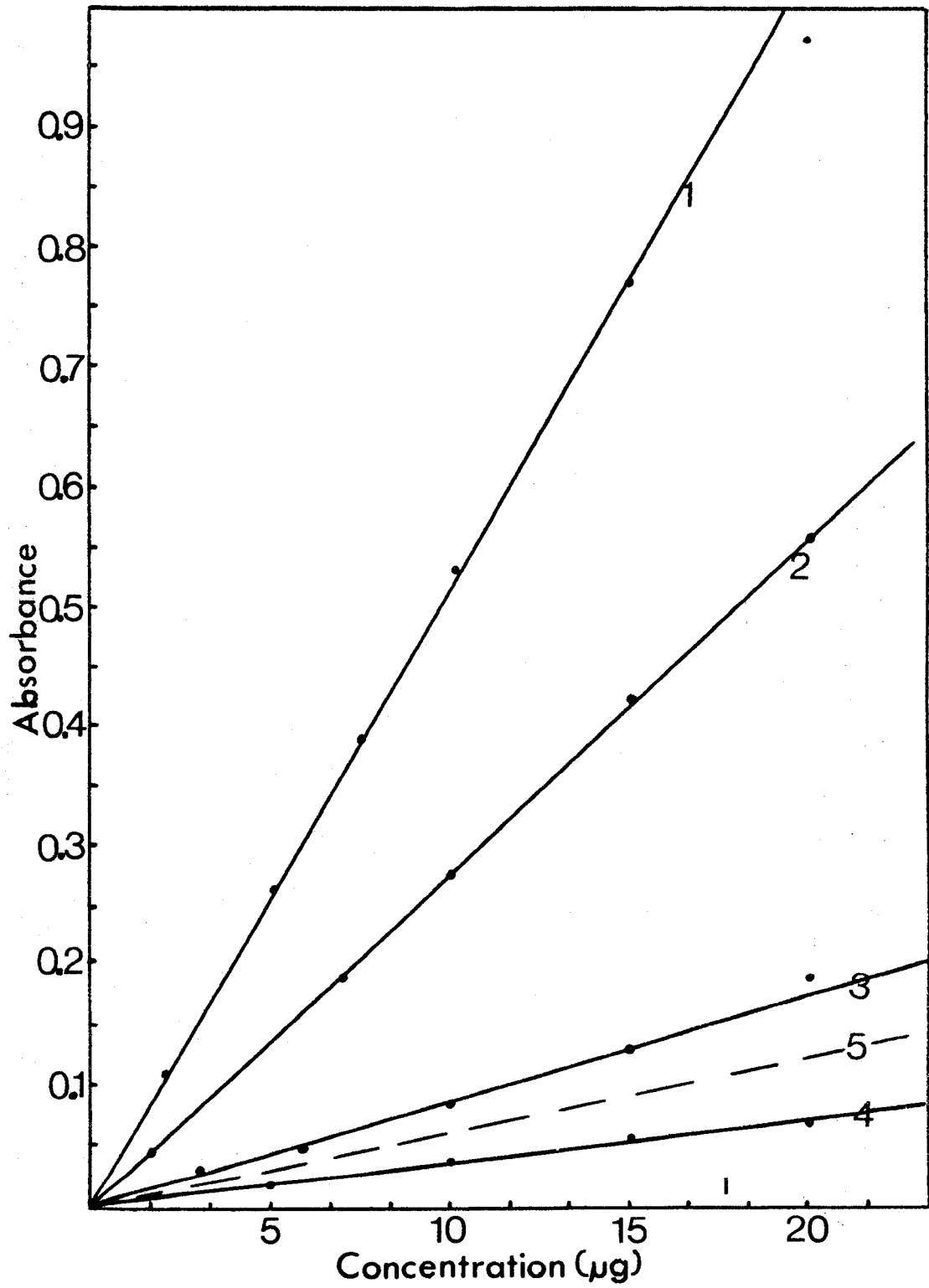
The results of the stability study are illustrated in Figure 3. Curve 1 indicates that the colour produced by naphthoresorcinol with 10 µg per 3.0 ml of glucuronic acid is reproducible over a period of seven days. The colour of the blank (curve 2) reaches a plateau which also is maintained for seven days after which the absorbance increases quite sharply. The results, therefore, illustrate that the reagent can be used up to a period of seven days but it is this author's suggestion that fresh MNR preparations be utilized. The stability of the MNR reagent is not as good compared to Nir's reagent which is proposed to be stable for a period of 3 months. However, Nir's reagent has approximately one-eighth the sensitivity of the MNR reagent.

The present method, therefore, provides the advantage of sensitivity which is a requirement for the determination of low concentrations of glucuronic and glucuronides in serum. The reagent is fairly stable and reproducible. The method proves to be more accurate at these low concentrations when compared to other methods. The method is flexible with respect to heating time and sample size. If glucuronic acid concentrations above 16.6 µg per 3.0 ml are to be determined, then a 30 min heating period and

Legend to Figure 3

Fig. 3   Stability study of the MNR reagent.   The MNR

procedure was performed regularly over a period

of two weeks: Curve 1, sample containing 10 μg

per 3.0 ml of glucuronic acid, 90 min heating;

Curve 2, blank containing 3.0 ml of H_2O, 90

min heating.

Figure 3   40
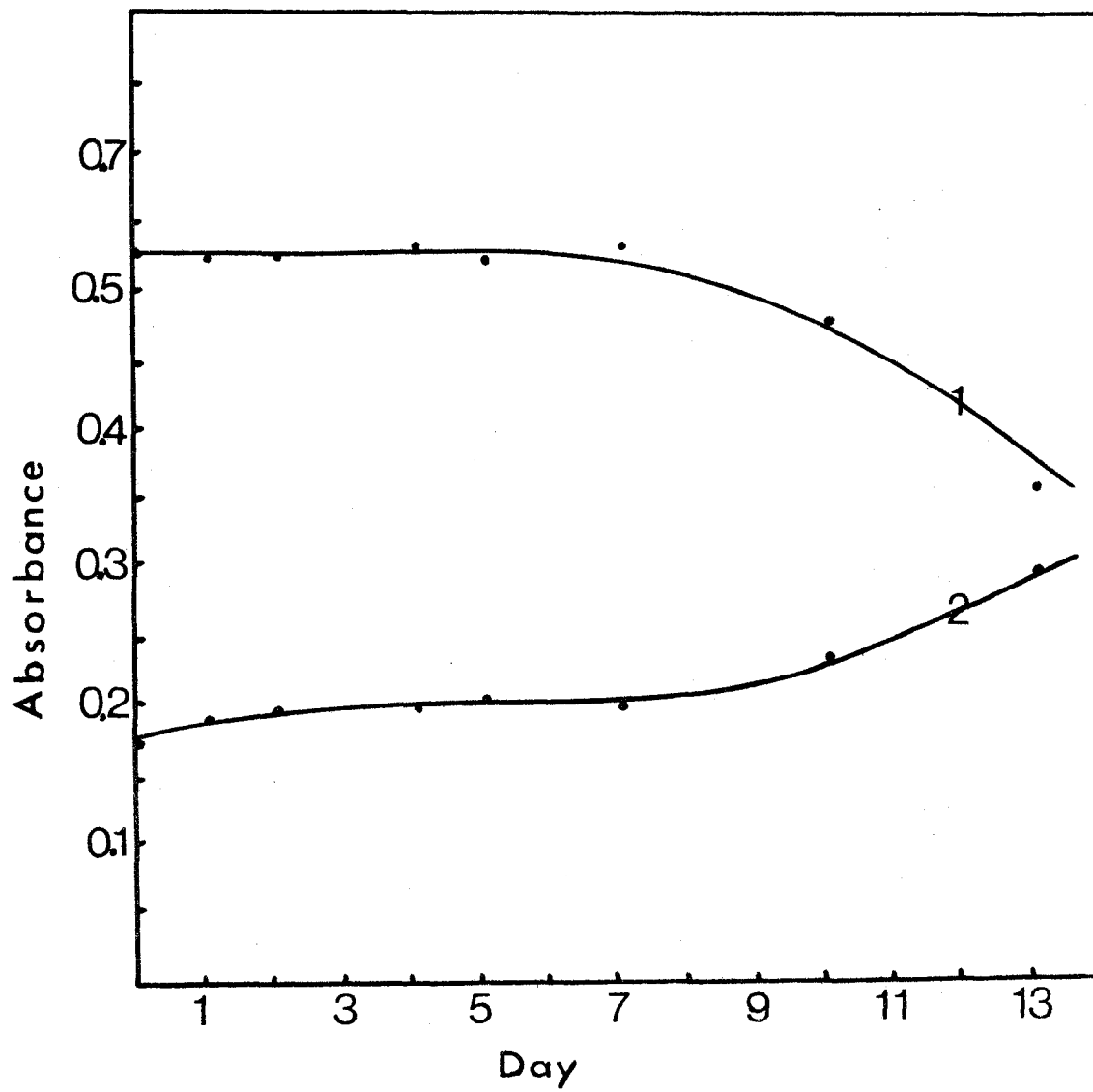
dilution of sample could be utilized to obtain results quickly and accurately. The time required to carry out a complete analysis in pure systems is one to two hours depending on heating time.

In the determination of both glucuronic acid and glucuronides in serum or urine, many investigators have reported that glucose, which is at much higher concentrations with respect to glucuronic acid (particularly in serum) interferes with the colour reaction (31,59,60,62, 76). Several authors have reported success in removing glucose from solutions with yeast (77-79). This has been found to be too time consuming and the results were unsatisfactory (31,60). An alternative was to compensate by adding known amounts of glucose to the standards (31, 80). This procedure was satisfactory when the glucose concentration was within normal limits (80 - 120 mg per 100 ml). The obvious disadvantages in this procedure are the necessity to determine exactly the concentration of glucose in the samples and an increase in the complexity of preparing the standard solutions.

Fishman (62) was able to determine conjugated glucuronic acid in the presence of free glucuronic acid by oxidation of free glucuronic acid with hypoiodite to

saccharic acid which is insensitive to the NR reaction.
The hypoiodite reaction also oxidizes glucose to gluconic
acid which is also insensitive to the NR reaction (84).
Therefore, in this determination of conjugated glucuronic
acid or glucuronide, glucose interference is eliminated.
However, total glucuronic acid (free glucuronic acid
plus glucuronide) cannot be determined accurately by
this procedure since the hypoiodite reaction is not allowed
to occur. (Glucose is not oxidized, therefore, interfer-
ence does occur.) Free glucuronic acid cannot be deter-
mined accurately since it is calculated as the difference
in the values for glucuronic acid before and after the
oxidation procedure. The values for both total and free
glucuronic acid would be too high. This method, as was
mentioned previously, has been widely utilized and was
reported to be inaccurate in the presence of high concen-
trations of glucose (75,76). In a later communication
Fishman et al. (84) employed glucose oxidase to eliminate
glucose interference. However, the procedure utilized a
crude preparation of the enzyme which resulted in high
blank readings, loss in sensitivity, an extended incubation
period (3 hours), and tedious pH adjustments for the
incubation procedure.

In the present study glucose oxidase was also
utilized. It was observed that crude preparations of
the enzyme contained high concentrations of NR positive
impurities, and consequently could not be employed.
Glucose oxidase Type V from Sigma Chemical Company (1000
E.U. per ml) was found to be most satisfactory in eliminat-
ing glucose interference. The incubation is performed
before deproteinization in order to eliminate pH adjust-
ments and to minimize as much as possible any contributions
to the MNR colour reaction due to the enzyme preparation.
Figure 4 illustrates the interferences one can expect from
glucose when the glucose oxidase system is used to convert
glucose to gluconic acid. Figure 4 (curve 1) exhibits a
plateau region of absorbance between 150 to 270 mg % of
glucose and then the absorbance increases with increasing
glucose concentration. The interference at this plateau
is equivalent to approximately 1.3 μg of glucuronic acid.
Curve 2 illustrates that the absorbance reaches a plateau
between 100 to 450 mg % of glucose. The interference at
this plateau is equivalent to approximately 0.45 μg of
glucuronic acid. The results, therefore, indicate that
the interference in this plateau region is constant. Since
glucose is converted to gluconic acid by the enzyme,

Legend to Figure 4

Fig. 4   Glucose interference study with glucose oxidase:

Curve 1, 2.0 ml samples, 90 min incubation, 3.0 ml

aliquot, 564 nm; Curve 2, 1.0 ml samples, 45 min

incubation, 1.5-ml aliquot, 564 nm; Curve 3, 1.0

ml samples of gluconic acid, no enzyme, 1.5-ml

aliquot, 564 nm.

Figure 4
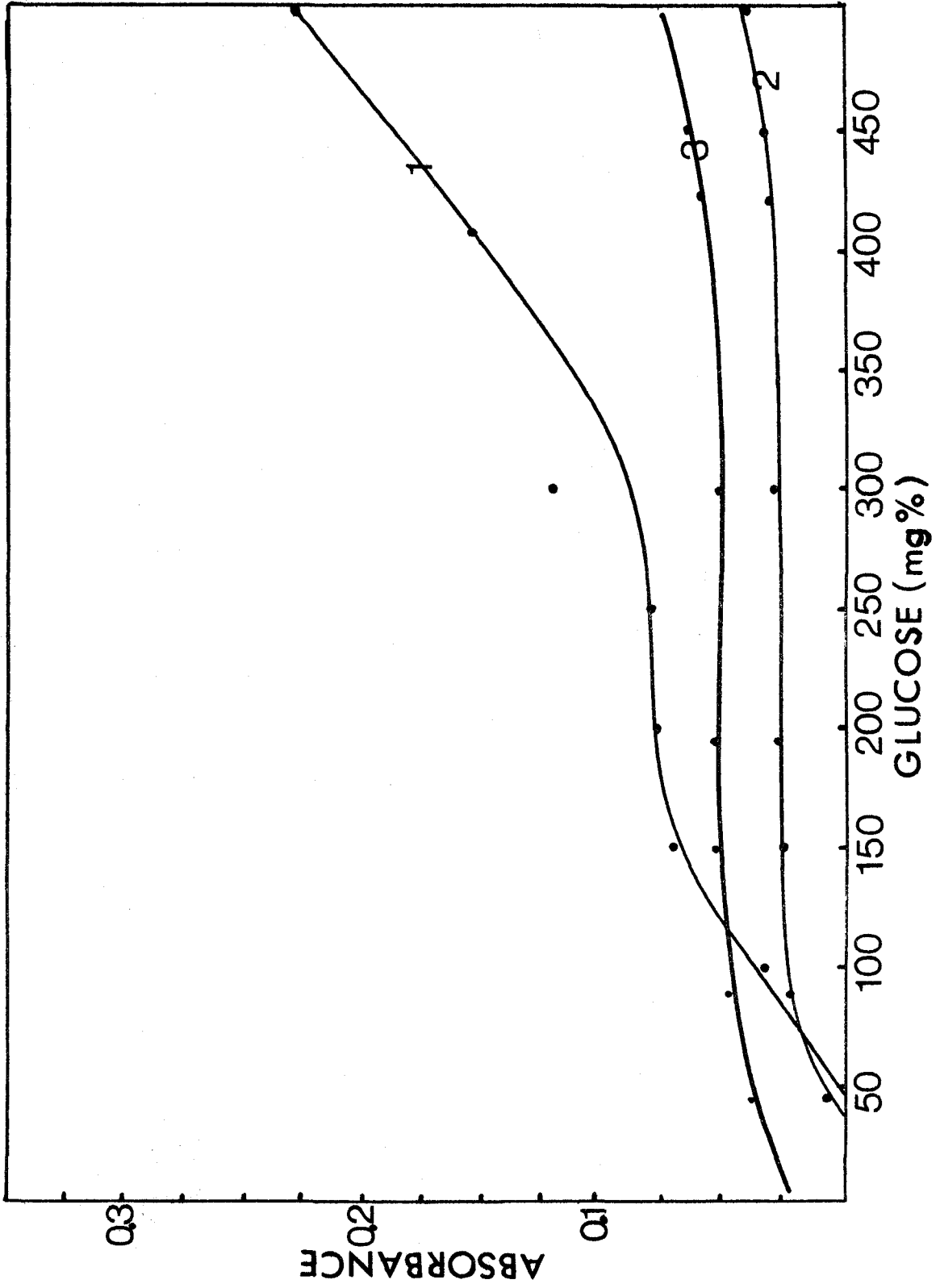
glucose oxidase, the behaviour illustrated in Fig. 4 (curve 2) results from gluconic acid interference since it was observed in this laboratory that gluconic acid is in fact NR positive at high concentrations (curve 3). The interference of pure gluconic acid samples (curve 3) at concentrations equivalent to the glucose samples displays a similar curve to that obtained with glucose samples treated with glucose oxidase. This interference is constant due to the fact that the MNR reagent simply does not quantitatively react with gluconic acid. The interference of 0.45 µg of glucuronic acid equivalent can be eliminated completely by the addition of glucose and glucose oxidase to the blanks. The quantity of glucose added to the blank is not critical since the interference is constant in the range of 100 to 450 mg % of glucose. Furthermore, the necessity for pre-determining the glucose level in the samples is obviated. The blank would also eliminate any interferences resulting from the enzyme preparations. The total time of incubation is 45 min instead of 3 hours and no pH adjustments are necessary except for preparing the acetate buffer.

The results of the quantitative determination of gluconic acid in the presence of 3 mg % of glucose are

reported in Table II (samples 1 - 5). The errors involved
are in the range of ± 4%. Therefore, we can accurately
determine low concentration of glucuronic acid in the
presence of high concentrations of glucose.

In the determination of conjugated glucuronic acid
free glucuronic acid must be eliminated. Fishman, as
mentioned previously, utilized sodium hypoiodite to
oxidize the free glucuronic acid to saccharic acid (62).
Halogen reactions, in general, are susceptible to many
sources of error (85). The reactions are light and pH
sensitive. The procedure requires additional reagents
and pH adjustments which are time consuming and tedious.
To eliminate these sources of error and pH adjustments,
NaOH is utilized to decompose free glucuronic acid to a
product insensitive to the MNR reagent (86,87). Table II
(samples 6 - 8) demonstrates that phenolphthalein mono-β-
glucuronide (P-Gide) can be quantitatively determined in
the presence of glucuronic acid with an error of - 2.6%.
The absorption maximum for the product formed in this
reaction occurs at 564 nm (Fig. 1, curve 1) and at 510
nm for the blank (curve 3). Therefore, NaOH does not
alter the absorption maximum of the NR pigment. The
reaction requires 20 min heating in a boiling water bath.

Legend to TABLE II

NOTE: The concentrations expressed above are the amounts expected in the aliquot (3.0 ml). To obtain the amount in the original sample multiply amount recovered by 8/1.5. Samples 1 - 3 were heated for 30 min in the MNR procedure and samples 4 - 8 were heated for 90 min. The results reported are the average of duplicate analysis.

TABLE II

Determination of D-glucuronic acid (GA) and P-Gide in various mixtures

Sample No.	Glucose concentration (mg/ml)	Amount GA expected (µg)	Amount P-Gide expected (µg)	Amount GA recovered (µg)	Amount P-Gide recovered (µg)	% error
1	3.0	7.5	–	7.3	–	-2.6
2	3.0	7.5	–	7.2	–	-4.0
3	3.0	7.5	–	7.3	–	-2.6
4	3.0	11.25	–	11.40	–	+1.34
5	3.0	3.75	–	3.90	–	+4.0
6	–	10.0	7.3	–	7.1	-2.7
7	–	10.0	14.6	–	14.3	-2.1
8	–	10.0	21.9	–	21.2	-3.2

The above procedure is accurate and has the following advantages: no pH adjustments are required; the only additional reagent required is NaOH; the method requires less time and is not as sensitive to reaction conditions as the hypoiodite method.

In order to establish the reliability of the procedures, quantitative determinations were performed on synthetic mixtures. Glucuronic acid and P-Gide (total and conjugated) were determined in the presence of 3 mg per ml glucose. The procedure is outlined in the flow diagram (Fig. 5) and the results are reported in Table III. The error ranges from - 0.5 to - 5.4 % in the optimum concentration range of the MNR procedure. The error is at a maximum at low concentrations (-9.2%) but the deviation at these low concentrations is less than ± 0.3 μg per 3.0 ml.

Fishman (62) and others determine the amount of glucuronic acid and glucuronides from standard curves of glucuronic acid. This requires that the standards be treated in the same fashion as the samples and that they are analyzed simultaneously with the samples. This must be done since the NR reaction varies with changes in reaction conditions and the reagent, itself, undergoes

Legend to Figure 5

Fig. 5 Flow diagram outlining procedural steps for a

single sample. Volume (ml) is marked in

parentheses and effect of dilution is recorded

by arrow above volume. $\Longrightarrow$ Urine; $\longrightarrow$

Synthetic sample or serum. "Total" refers to

total Glucuronic acid and glucuronide and

"Conjugate" refers to glucuronide.

Figure 5
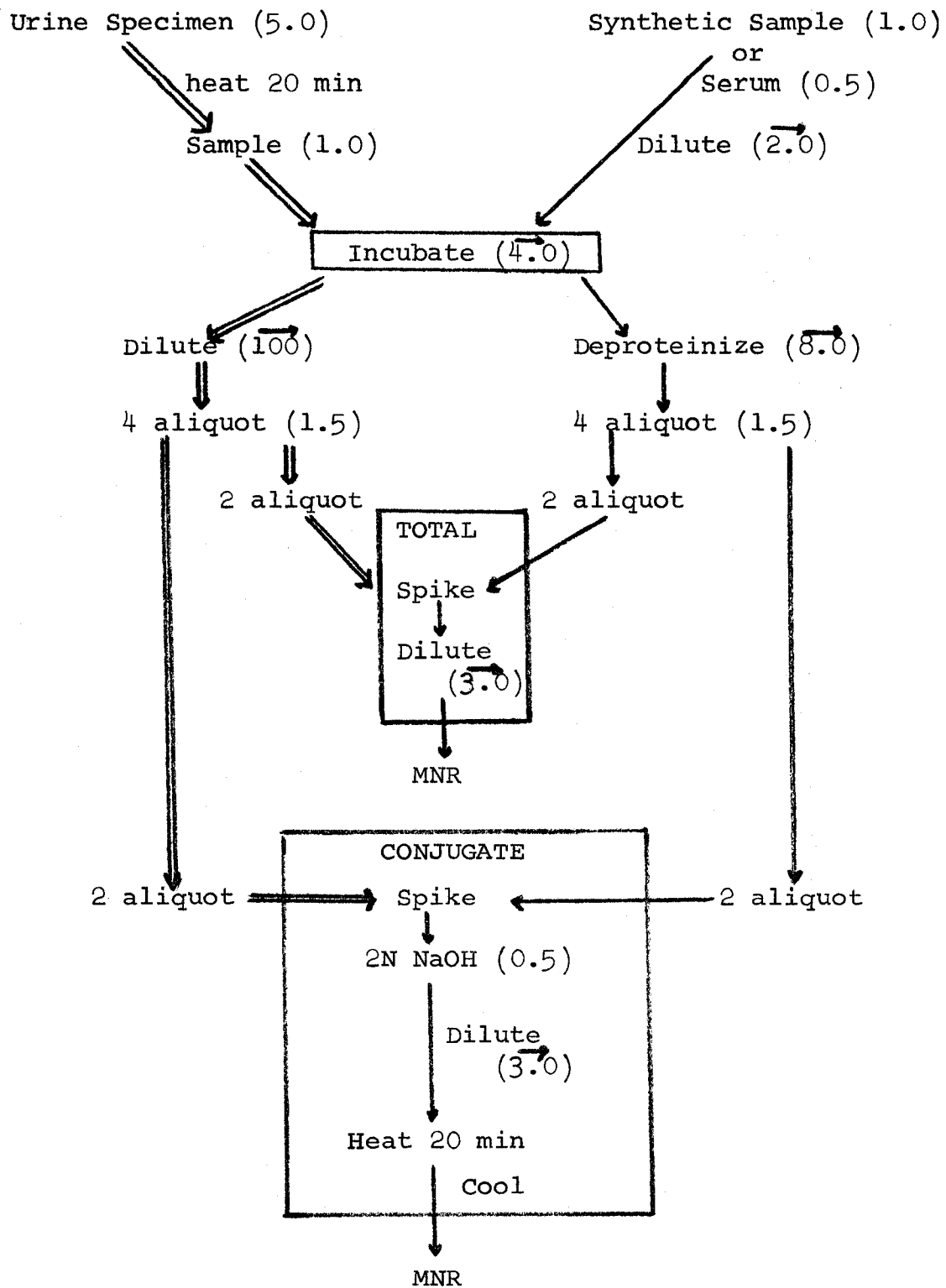
TABLE III

Determination of glucuronides and glucuronic acid

in synthetic mixtures

Sample No.	TOTAL			CONJUGATED		
	Expected (μg/3ml)	Recovered (μg/3ml)	% error	Expected (μg/3ml)	Recovered (μg/3ml)	% error
1	2.06	1.9	-9.2	1.12	0.95	-6.2
2	5.05	4.8	-5.0	4.11	3.95	-3.9
3	11.04	10.8	-2.1	10.10	10.05	-0.5
4	14.03	13.8	-1.6	11.30	11.15	-1.3
5	8.97	8.6	-4.1	13.09	12.94	-1.2
6	7.11	7.24	+2.0	5.23	4.95	+5.4

NOTE: The amounts expressed above denote the

concentrations expected in the 1.5-ml aliquots

diluted to 3.0 ml which were utilized in the

MNR reaction. To obtain amount in original

sample multiply the concentrations by 8/1.5.

The results reported are the average of

duplicate analysis except in sample one

where the recovery was determined from

extrapolation of the curve to the X-axis.

changes with time (31,62,88). The calibration curve, however, cannot account for any endogenous reactions occurring in the system as a result of added reagents. It does account for the effects of reagents on the particular species being analyzed. The determination is also restricted to the error limits imposed by the calibration curve. The method of standard addition has the following advantages: increased accuracy at low endogenous concentrations of glucuronic acid; takes into consideration any endogenous reactions; does not require the preparation of a standard curve; the curve obtained from one sample utilizing this technique can then be employed as the standard curve for unknown samples; elimination of tedious preparations of standards to account for glucose interference and other endogenous interferences. All that is required is the preparation of the appropriate blank for the particular system. The blank system consists of glucose and glucose oxidase. The spike solution is prepared from a P-Gide stock solution. The variation in concentration from our standard curve in the pure system was $\pm$ 0.5 μg per 3 ml, yet, employing the method of standard addition the variation at concentration range was - 0.3 μg and - 0.16 μg per 3.0 ml at the maximum

and minimum, respectively. An example of the type of extrapolation required is illustrated in Fig. 6. The curves for total and conjugated glucuronic acid are linear and parallel which demonstrates obedience to Beer's law. The curve should be parallel since both curves originate from the same sample, except under one set of conditions the endogenous free glucuronic acid is decomposed with NaOH.

The method as outlined in Fig. 6 was studied for precision or reproducibility in both serum and urine. Before this study was attempted in serum it was essential to investigate whether the P-Gide could be completely recovered from serum. This was considered necessary since it has been established that free glucuronic acid is in fact adsorbed or coprecipitated with proteins (70, 76). Therefore, samples of pooled sera were spiked before and after deproteinization. The samples were spiked in such a manner that the concentration of P-Gide in the final aliquots would be the same if no adsorption occurred. Figure 7 illustrates that adsorption of the P-Gide does in fact occur. If adsorption had not occurred curves 1, 2 would be identical as would curves 3, 4. The amount recovered was determined by employing

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Legend to Figure 6

Fig. 6 Determination of total and conjugated glucuronic

acid in synthetic mixtures by method of standard

addition. The curves illustrate the method of

extrapolation; Curve 1 total glucuronic acid;

Curve 2, conjugated glucuronic acid.

Figure 6
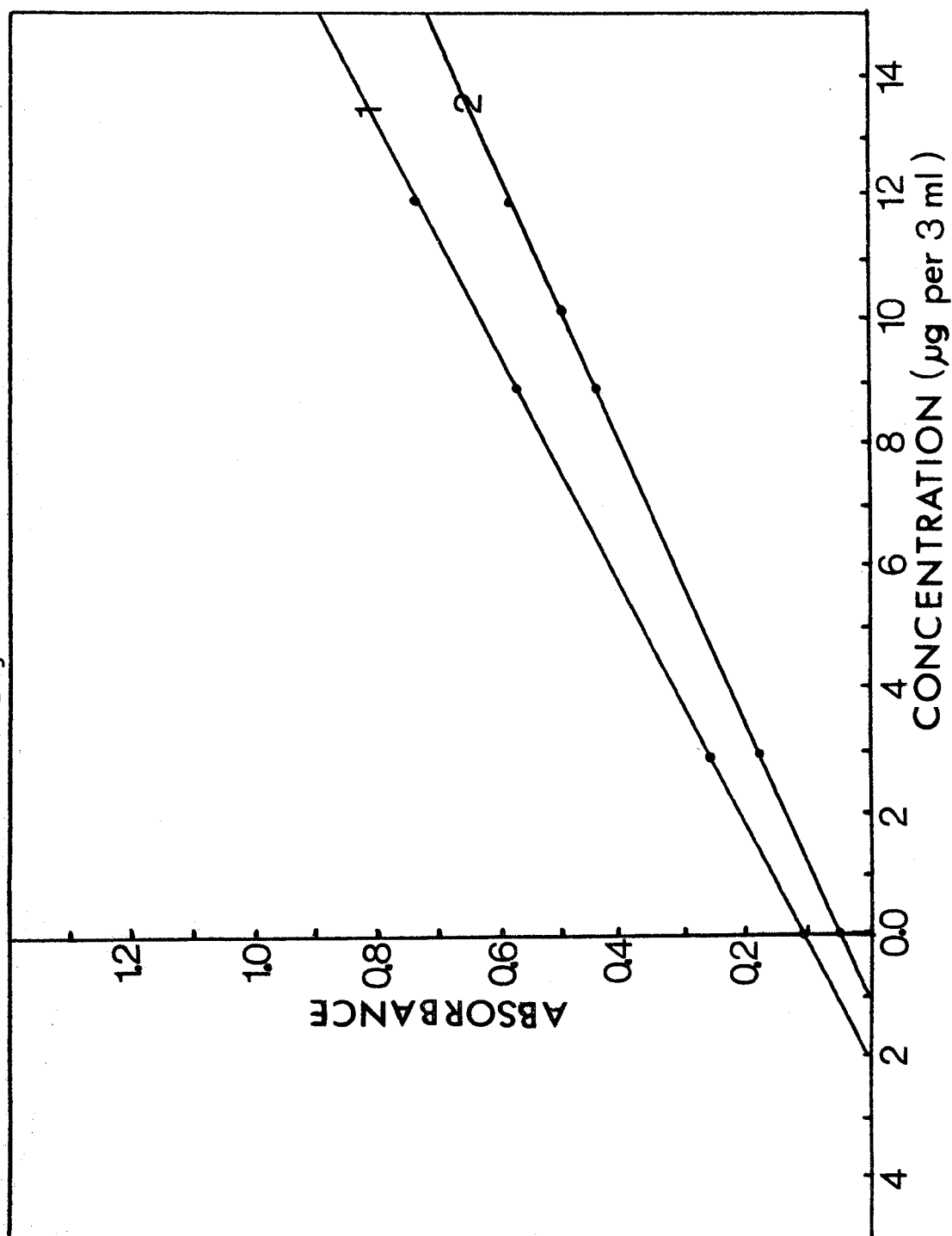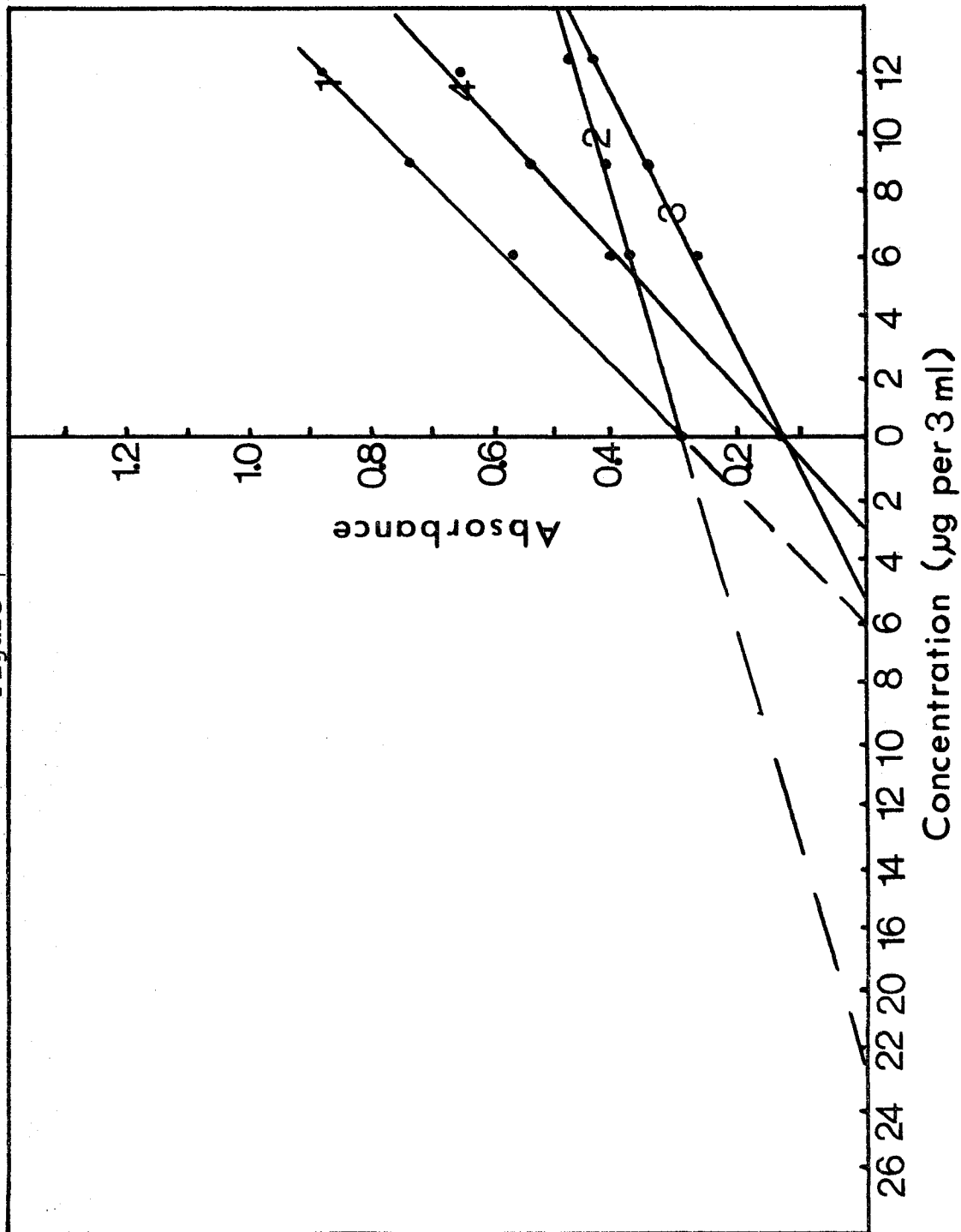
Legend to Figure 7

Fig. 7   Curves illustrate the loss of P-Gide on

deproteinization in the determination of

conjugated glucuronic acid: Curve 1, 1.0 ml

samples, spiked after deproteinization;

Curve 2, 1.0 ml samples, spiked before

deproteinization; Curve 3, 0.5 ml samples,

spiked before deproteinization; Curve 4,

0.5 ml samples, spiked after deproteinization.

Figure 7

the curve obtained from spiking after deproteinization
as the standard curve. The results are reported in
Table IV. The amounts recovered from 1.0-ml samples of
serum varied from 53.5 to 73.2% with a mean recovery of
62.7%.

To determine more conclusively that per cent recovery
is related to protein concentration, 0.5-ml and 0.25-ml
samples of serum were utilized instead of 1.0 ml. If
adsorption is a factor of protein concentration, the
recoveries should increase and the slopes of the curves
obtained from spiking before and after deproteinization
should approach each other. This would be exemplified
by an increase in the slope of the curves utilizing
0.5-ml and 0.25-ml samples which were spiked before
deproteinization. Curve 3 (Fig. 7) illustrates an
increase in slope in comparison to curve 2 (0.5 ml used
as example). If recoveries did not increase, all the
respective curves obtained from the data before and
after deproteinization would be parallel. This is true
for curves 1 and 4 which were obtained from samples spiked
after deproteinization but not so for curves 2 and 3 which
were obtained from samples spiked before deproteinization.
The recoveries in the system (Table IV, samples 5 - 8)

Legend to TABLE IV

Recovery Studies in Serum

a   Samples 1 - 4 employed 1.0 ml serum

b   Samples 5 - 8 employed 0.5 ml serum

c   Samples 9 - 12 employed 0.25 ml serum

d   Samples 13 - 16 employed 0.5 ml serum and sodium

    deoxycholic acid

NOTE: The amounts recovered in samples 1, 5, 9,

        and 13 were determined by the method of

        standard addition.   Recoveries in other

        samples were determined from curve

        obtained after deproteinization and are

        the result of duplicate analysis.

TABLE IV

Recovery studies in Serum

Sample	TOTAL			CONJUGATED		
	Amount Expected (μg/3ml)	Amount Recovered (μg/3ml)	% recovery	Amount Expected (μg/3ml)	Amount Recovered (μg/3ml)	% recovery
1[a]	-	9.9	-	-	6.00	-
2	15.84	11.6	73.2	11.94	7.95	66.5
3	18.87	12.5	66.2	14.97	8.45	56.4
4	22.24	13.5	60.7	18.34	9.83	53.5
5[b]	-	4.90	-	-	3.00	-
6	9.84	8.20	83.3	8.94	5.80	64.9
7	13.87	9.65	69.5	11.97	7.80	65.2
8	17.24	11.3	65.5	15.34	10.6	69.1
9[c]	-	2.80	-	-	0.80	-
10	6.78	5.80	85.5	4.78	3.8	79.5
11	8.80	7.50	85.2	6.80	5.40	79.4
12	11.05	9.40	85.1	9.05	7.4	81.8
13[d]	-	3.00	-	-	1.7	-
14	6.18	5.50	88.9	4.88	4.0	81.9
15	7.80	6.60	84.6	6.50	5.20	80.0
16	9.80	8.10	82.6	8.30	6.90	83.1

varied from 64.9 to 83.3% with a mean recovery of 70.2%.

Therefore, there was in increase in recoveries in utilizing

0.5-ml samples when compared to 1.0-ml samples. Recoveries

were increased further when 0.25-ml samples were utilized

(Table IV, samples 9 - 12). The recoveries ranged from

79.4 to 85.5% with a mean value of 82.8%. The difference

in slopes reported in Table V, however, illustrate this

increase much more conclusively. The results indicate

very little change in the slopes of curves obtained from

samples spiked after deproteinization but the slopes

approximately increased by factors of two and three for

0.5 and 0.25-ml samples, respectively. In order to obtain

a more accurate value of the amount of free glucuronic

acid and bound glucuronic acid in serum the recommended

procedure for deproteinization is dialysis (70). However,

this method experiences recoveries between 90 - 95% (70)

but requires a minimum of three hours.

Protein denaturing agents (Triton X-100, urea, and

sodium deoxycholic acid) were utilized in an attempt to

free the glucuronic acid (free and combined) from the

protein and therefore, increase our recoveries. The

results obtained in preliminary studies with Triton X-100

indicated that this reagent affected the MNR colour

TABLE V

Effect of protein concentration on slopes

| Volume sample (ml) | SLOPE | | | |
| | TOTAL | | CONJUGATED | |
	After deprotein-ization	Before deprotein-ization	After deprotein-ization	Before deprotein-ization
1.0	0.045	0.013	0.050	0.014
0.5	0.047	0.025	0.046	0.024
0.25	0.050	0.039	0.049	0.037
0.5[a]	0.059	0.045	0.061	0.043

a Sample treated with sodium deoxycholic acid

development and therefore was not employed. Recoveries

with urea utilizing 0.25-ml samples of serum did not

improve compared to normal samples (Table IV, samples

9 - 12). Sodium deoxycholic acid, however, did cause an

increase in recovery as reported in Table IV, samples 13 -

16. The recoveries ranged from 80.0 to 88.9% with a mean

value of 83.5%. These results compared with recoveries

obtained with similar samples which were not treated with

sodium deoxycholic acid (Table IV, samples 5 - 8) indicate

an increase of 13% recovery in the mean values. Therefore,

recoveries can be increased employing sodium deoxycholic

acid. However, this increase is not substantial compared

to recoveries obtained utilizing 0.25-ml samples (Table

IV, samples 9 - 12) and therefore, the time and effort

required with sodium deoxycholic acid is not warranted.

Therefore, I suggest that 0.5 or 0.25-ml samples of serum

be utilized depending on the final concentration of

glucuronide being determined. This should not be any

lower than 1 μg per 3.0 milliliters.

Reproducibility data employing the present method in

the determination of total and conjugated glucuronic

acid in serum are reported in Table VI. The reproduc-

ibility is ± 0.2 μg per 3 ml of aliquot and the results

TABLE VI

Determination of glucuronic acid and glucuronide

in serum

Sample	TOTAL		CONJUGATED		FREE
	found (μg/3ml)	mg %	found (μg/3ml)	mg %	mg %
1[a]	9.9 ±0.2	5.3 ±0.1	6.0 ±0.15	3.2 ±0.1	2.1
2[b]	4.9 ±0.2	5.2 ±0.2	3.0 ±0.2	3.2 ±0.2	2.0
3	4.1 ±0.2	4.4 ±0.2	3.2 ±0.1	2.4 ±0.1	1.9

a   Sample volume was 1.0 ml

b   Sample 2 and 3 utilized 0.5 ml serum samples

NOTE: Pooled sera was employed in this study.   To

obtain amount in original sample multiply

amount found by 8/1.5 x reciprocal of the

volume taken.   The results reported are

the average of five determinations.

are in fair agreement with reported ranges for total and conjugated glucuronic acid in serum (61). However, due to the problem of adsorption, ranges could vary with the deproteinization procedure employed. It is also important to note that pooled sera was utilized since the main concern in this study was to establish precision.

Urine does not require deproteinization and the reproducibility reported in Table VII is approximately ± 0.2 μg per 3.0 ml. Due to the high dilution requirement, this factor results in a deviation of ± 16 mg per 24-hour sample of urine.

To determine the variation, if any, in reproducibility from day to day with fresh and aged reagent, all the curves obtained in the urine study were compared to each other. Figure 8 (curve 1) illustrates that these curves coincided very well, which demonstrates that the system is reproducible, or that little or no change is observed when fresh and aged reagent (not more than 3 days old) is utilized. To determine the reproducibility throughout the entire study, the curves from the pure and biological systems were compared to each other. Figure 8 (curve 3) corresponds to the system in pure solutions. The results obtained from the serum samples coincided with the urine

TABLE VII

Determination of Glucuronide and glucuronic acid

in urine

| Sample | TOTAL | | CONJUGATED | | FREE |
	found μg/3.0 ml	mg/ 24 hour	found μg/3.0 ml	mg/ 24 hour	mg/ 24 hour
1[a]	9.5 ±0.04	633 ± 3	4.7 ±0.35	313 ± 23	320
2[b]	6.8 ±0.3	544 ± 24	5.0 ±0.2	400 ± 16	144
3[b]	4.6 ±0.13	644 ± 18	3.5 ±0.23	490 ± 32	154

a 1.5-ml aliquots utilized for MNR

b 1.0-ml aliquots utilized for MNR

NOTE: The concentrations reported are the average of

five determinations. To determine amount in

original sample multiply amount found by

(1/1.5 or 1) times 100. 1.5- or 1.0-ml

aliquots are taken but are diluted to 3.0

ml in the MNR procedure.

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Legend to Figure 8

Fig. 8 Comparison of the Beer's law curves obtained

throughout this study. Curve 1 was the result

of all the data obtained in the biological

systems. Curve 2 is the composite curve

between the pure and applied systems. Curve

3 was obtained from the Beer's law study in the

pure system (90 min heating).

Figure 8

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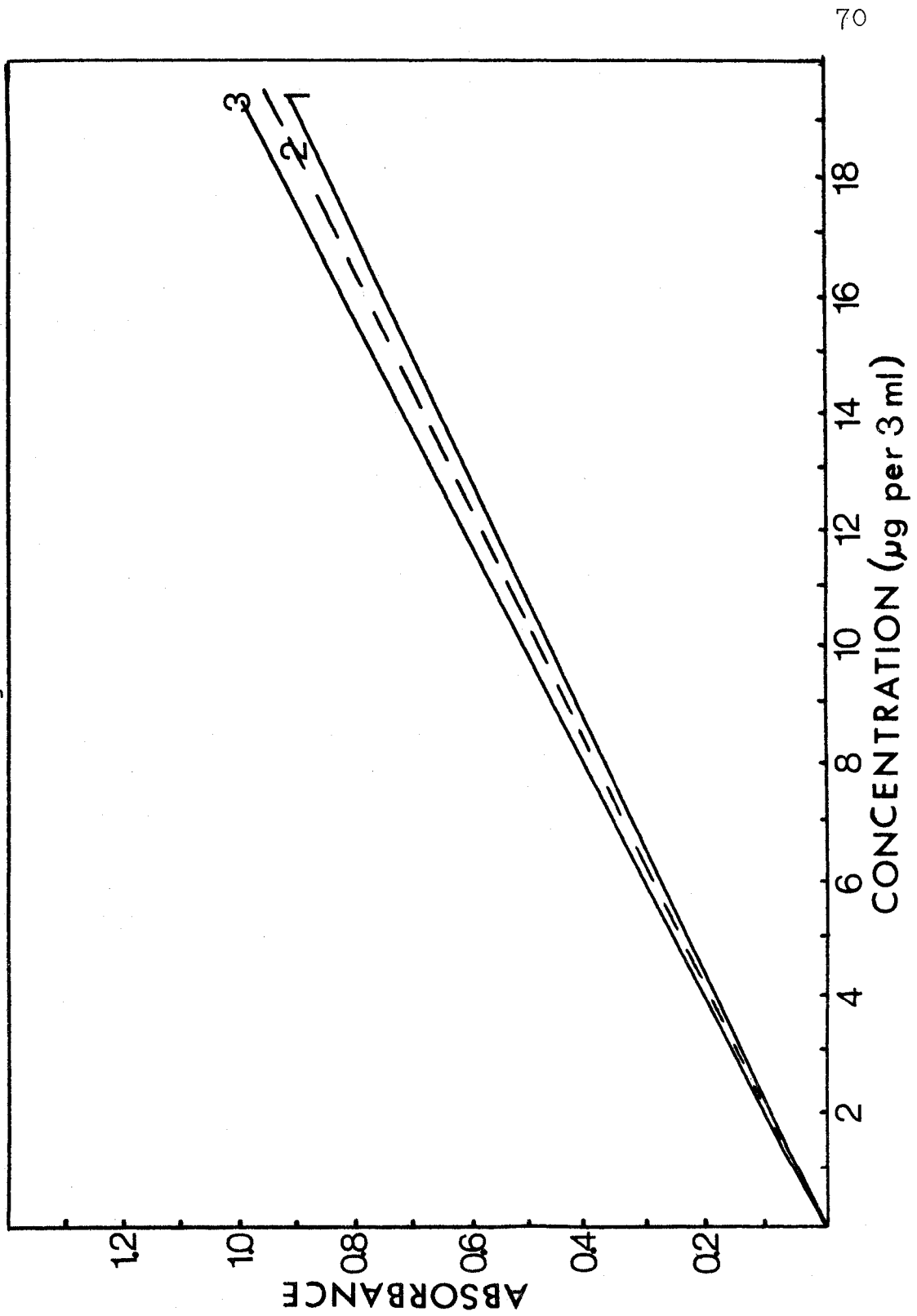
curve (curve 1). Therefore, the two curves (2 and 3) have established or defined the limits of variance over the entire study employing the MNR procedure. Curve 2 is a composite curve which was drawn and could be employed as the calibration curve for all systems with a maximum error limit of not more than $\pm$ 5%. This demonstrates that the procedure is reproducible over a period of time, and that little variation occurs due to different reagent preparations and matrices of the systems under investigation. This provides an obvious advantage over other systems, since in the past, the NR reagent sensitivity increased with age, and since no two reagent preparations are exactly alike, different results were obtained with fresh reagents (88).

B. Titrimetric Determination of Glucose, Galactose,
and Xylose with N-Bromosuccinimide

N-Bromosuccinimide (NBS) has been applied as an agent for allylic brominations. This type of halogenation is presently known as the Wohl-Ziegler reaction (89). An excellent review on this subject has been published by Djerassi (90). Under various conditions, N-halo compounds also react with olefins to add bromine to the double bond,

or they act as a source of hypohalous acid in aqueous

solution. Compounds belonging to this group include

N-chlorosuccinimide, N-iodosuccinimide, and N-bromo-

phthalimide. Aside from the advantages of these compounds

as halogenating agents, several of them have been found

to be useful for oxidations and dehydrogenations. The

reactions and uses of N-bromosuccinimide have been

reviewed in 1951 by Waugh (91) and an excellent survey of

the chemistry of this compound has been published by

Horner (92). Therefore, we investigated the possibility

of utilizing NBS as the oxidizing agent to eliminate
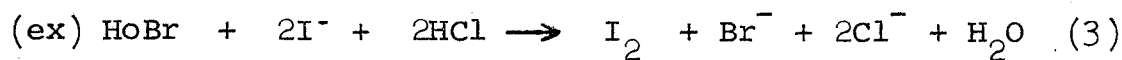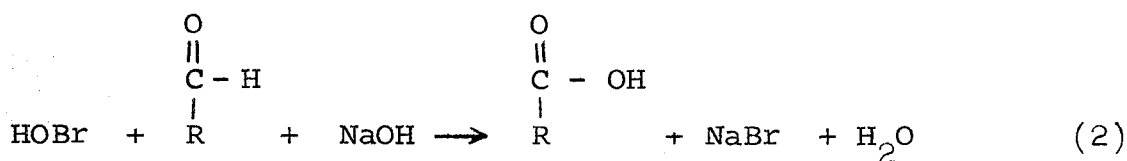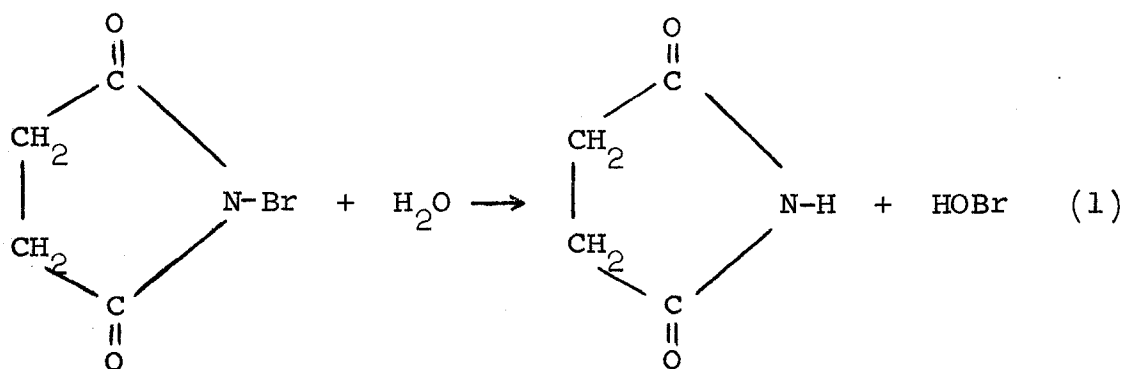
glucose interference in the MNR reactions. Our approach

was first to establish a procedure employing NBS to

quantitatively determine glucose in pure systems and then

apply the procedure to the MNR reaction in biological

fluids. However, after having quantitized the procedure

for glucose, it was discovered that tne excess NBS

interfered with the colour development of the MNR reaction

and attempts to eliminate this excess with cysteine,

thiourea, and thioacetamide were to no avail. We then

became interested in applying NBS to determination of other

carbohydrates such as galactose, ribose, xylose, and

mannose. We were able to quantitate the method for

glucose, galactose, and xylose but were unable to do so for ribose and mannose which probably results from stereochemical considerations.

The products developed are essentially the same for all three sugars except that the optimum pH or basicity varied with each sugar. The procedure is thought to proceed as outlined in the following reaction:

$$HOBr + \underset{\underset{R}{|}}{\overset{\overset{O}{\|}}{C}-H} + NaOH \longrightarrow \underset{\underset{R}{|}}{\overset{\overset{O}{\|}}{C}-OH} + NaBr + H_2O \quad (2)$$

$$(ex)\ HOBr + 2I^- + 2HCl \longrightarrow I_2 + Br^- + 2Cl^- + H_2O \quad (3)$$

$$I_2 + 2NaOH(ex) \longrightarrow NaOI + NaI + H_2O \quad (4)$$

$$OI^- + I^- + 2HCl \longrightarrow I_2 + 2Cl^- + H_2O \quad (5)$$

$$I_2 + 2S_2O_3^{-2} \longrightarrow S_4O_6^{-2} + 2I^- \quad (6)$$
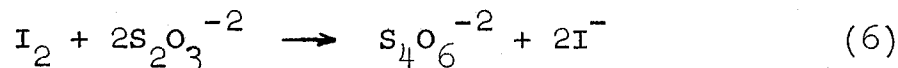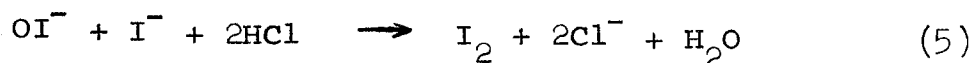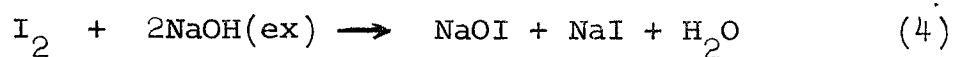
NBS hydrolyzes in water (equation 1) to form succinimide

and hypobromous acid (93). Hypobromous acid in the presence

of base oxidizes the aldehydic group to a carboxylic group

(equation 2). The excess hypobromous acid oxidizes iodide

to iodine which is then titrated in the usual manner with

standard sodium thiosulfate using starch as indicator.

The calculations involved have been described in section

B (Chapter II).

The reaction proceeds at room temperature and the pH

is very critical. The pH optimum range for each sugar

is reported in Table VIII. The recovery study for glucose

illustrated that little or no recovery was obtained above

or below the pH range described above (8.4 - 9.5). However,

with both galactose and xylose, recoveries increased with

an increase in pH. The maximum recoveries for galactose

and xylose occurred at a pH of 11.5. The recoveries

reported in Table VIII are only qualitative since: no

precautions were taken to avoid iodine evaporation

(operations performed in beakers to facilitate pH

measurements); loss of sample on electrodes; and samples

were only carried out in duplicate. However, the conditions

required to obtain maximum recoveries were repeated with

appropriate precautions such as, utilizing stoppered

TABLE VIII

pH Range for maximum recovery

Sugar	Recovery %	pH range
Glucose	90	8.4 to 9.5
Galactose	92	11 to 12
Xylose	98	12.1

flasks and the solutions were kept in the dark during the reaction time and after the addition of potassium iodide. The recovery studies were performed at three different levels for each sugar and the results reported in Table IX are the average of five determinations.

The results in Table IX indicate that the deviation in titrations was always less than $\pm$ .20 milliliters. Glucose was determined with an accuracy of $\pm$ 3%. At low levels of galactose (1 mg per ml) the error reached a maximum of -9%. This could have resulted by a change in basicity due to the fact that 5.0-ml aliquots of sample were utilized. The maximum amount of error one could expect, otherwise, for all sugars is $\pm$ 6%. However, in most cases, the per cent error is much less as illustrated in Table IX. The only solution that requires standardization is the sodium th. sulfate and a complete determination considering pipetting, reaction time, and titrating can be performed in approximately 45 minutes.

TABLE IX

Recovery studies

Sugar	Amount added (mg)	Volume sodium thiosulfate (ml)				Amount recovered mg	% Recovery
		Blank	Deviation ±	Sample	Deviation ±		
Glucose	*1.0	47.80	0.11	42.89	0.16	1.03	+3.00
	4.0	47.69	0.19	43.37	0.13	3.87	-3.25
	7.0	47.56	0.04	39.76	0.10	6.99	-0.14
Galactose	*1.0	42.98	0.10	38.47	0.04	0.91	-9.00
	5.0	45.19	0.14	40.25	0.10	4.99	-0.20
	10.0	45.07	0.19	34.93	0.12	10.23	+2.30
Xylose	*1.0	45.62	0.28	39.33	0.10	0.97	-3.00
	5.0	45.74	0.09	39.55	0.19	5.30	+6.00
	10.0	44.98	0.18	32.60	0.16	9.53	-4.70

* 5-ml aliquots of sample utilized

CHAPTER IV

SUMMARY AND CONCLUSIONS

I have outlined a procedure, based on the Tollens naphthoresorcinol reaction for the quantitative determination of total and conjugated glucuronic acid in pure and biological systems. The procedure is six times more sensitive than Fishman's method and eight times more sensitive than Nir's method. The precision of the MNR procedure in serum and urine is $\pm$ 0.3 μg per 3.0 ml (maximum) and the error limits in the optimum concentration range are $\pm$ 5%. The method provides increased accuracy due to the selective elimination of the glucose interference by glucose oxidase and specific degradation of free glucuronic acid for the determination of conjugated glucuronic acid. The procedure is flexible and not as susceptible to changes due to variations in sample handling as are other procedures (62). The reagent is stable for a period of a week. The procedure is reproducible and very little change occurs in the results from various MNR preparations and matrices under investigation. The time required for a complete analysis is four hours. This study clarifies the accuracy and reproducibility of the naphthoresorcinol reagent which

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seems to be neglected in previous work. The molar

absorptivity of the reagent under the MNR conditions is

30.1×10^3 and 16.5×10^3 depending on the heating period.

The optimum concentration for the MNR procedure at 90 min

and 30 min heating are 3.5 to 16 µg per 3.0 ml and 5 to

25 µg per 3.0 ml, respectively. As little as 0.25-ml

samples of serum can be analyzed by this procedure by

the method of standard addition described earlier in the

text. It is suggested, however, that the lower concentra-

tion limit be maintained at 1 µg per ml. Therefore, one

can utilize 1.0, 0.5, and 0.25-ml samples depending on

the availability of sample and final concentration of

glucuronic acid being determined.

N-Bromosuccinimide has been employed as a reagent

for the quantitative determination of glucose, galactose,

and xylose in pure solutions. The reactions are carried

out at room temperature for 15 minutes. The errors

involved are less than $\pm$ 6% at the milligram level. The

time required for a complete determination is 45 minutes

and the only standard solution required is sodium thiosulfate.

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