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THE PARTIAL PURIFICATION OF PARA-NITROPHENOL: UDP-GLUCURONYLTRANSFERASE FROM RABBIT SMALL INTESTIONAL MICROSOMES

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THE PARTIAL PURIFICATION OF PARA-NITROPHENOL: UDP-GLUCURONYLTRANSFERASE FROM RABBIT SMALL INTESTINAL MICROSOMES

By

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ABSTRACT

THE PARTIAL PURIFICATION OF PARA-NITROPHENOL:
UDP-GLUCYRONYLTRANSFERASE FROM RABBIT SMALL INTESTINAL MICROSOMES.

By

Wanda Woodward Broderick

The purification of rabbit small intestinal microsomal p-nitrophenol: UPD-glucuronyltransferase might resolve many questions about the enzyme including its substrate specificty and heterogeneity. A procedure for the partial purification of p-nitrophenol: UDP-glucuronyltransferase from rabbit small intestinal microsomes was established, involving the preparation of intestinal microsomes, the solubilization of p-nitrophenol: UDP-glucuronyltransferase from these microsomes, and the DEAE column chromatography of the solubilized enzyme. This procedure was part of a scheme to purify the enzyme to homogeneity. Microsomes were obtained from the whole small intestine, which had an average p-nitrophenol: UDP-glucuronyltransferase activity of 24 nmoles p-nitrophenol conjugated per minute per mg of protein, and which were stable for six months when stored at -20°C. The microsomal solubilization process using octylglucoside increased the apparent p-nitrophenol: UDP-glucuronyltransferase specific activity approximately two fold, with a 75% recovery of enzyme activity. DEAE chromatography of the solubilized protein resulted in a 10 fold increase in p-nitrophenol: UDP-glucuronyltransferase specific activity over that of the microsomes.

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LIST OF ABBREVIATIONS

BHT	butylated hydroxtoluene
βME	β-mercaptoethanol
BSA	bovine serum albumin
cpm	counts per minute
DEAE	diethylamino ethyl
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetric acid
GT	glucuronyltransferase
i.p.	intraperitoneal
3-MC	3-methylcholanthrene
PAGE	polyacrylamide gel electrophoresis
PB	phenobarbital
PBB	polybrominated biphenyls
PEG	polyethylene glycol
pNP	p-nitrophenol
PNPGT	p-nitrophenol:UDP-glucuronyltransferase
SDS	sodium dodecyl sulphate
Tris	tris (hydroxymethyl) aminomethane
UDP	uridine-5-diphosphate
UDPGA	uridine diphospho-glucuronic acid
UDPGT	UDP glucuronyltransferase

INTRODUCTION

Man is exposed to a wide variety of environmental xenobiotics. The cytotoxicity of a xenobiotic depends in part on its metabolism. Since many xenobiotics are nonpolar and lipid soluble, it is advantageous to convert such xenobiotics into more water soluble compounds, thus facilitating their excretion and limiting the duration of their effect. In order to make them less lipid soluble, many xenobiotics are oxidized, reduced, or hydrolyzed by mammalian enzyme systems. The altered compounds may then be acted upon by transferases which catalyze the conjugation of these compounds with glucuronic acid, glycine, glutathione, or sulfate. Since the resulting conjugates are acidic and are ionized at physiological pH, they are water soluble and readily excreted into the urine and bile.

The most common types of conjugates formed with xenobiotics are β-glucuronides. Uridine diphosphate glucuronyl-transferase (UDP-glucuronate-glucuronosyltransferase [acceptor unspecific] E.C.2.4.1.17) catalyzes the formation of β-glucuronides by transferring the D-glucuronic acid of uridine diphospho-glucuronic acid (UDPGA) to many structurally different aglycones such as phenols and alcohols (1), carboxylic acids (2), amines (3), thiol derivatives (4), and acetylenic derivatives (5). Endogenous compounds, such as bile acids (6), many steriod hormones (7-9), thyroxine (10), and catecholamines (11) are

also glucuronidated. The anionic carboxylic acid group of the resulting conjugate facilitates excretion.

Some substrates of UDP-glucuronlytransferase (UDPGT) are the products of the mixed-function oxidase systems. These electron transport systems, located in the endoplasmic reticulum, are responsible for the oxidative metabolism of steroids and fatty acids, as well as xenobiotics. They consist of NADPH-cytochrome P-450 reductase, lipid, and multiple forms of the terminal oxidase, cytochrome P-450. Many compounds, once oxidized to hydroxylated derivatives by these systems, may then serve as substrates for UDPGT. Furthermore, epoxides, sometimès formed by the mixed-function oxidase system, may be converted to hydroxy compounds by epoxide hydrolase. While the hydroxylated compounds are usually more water soluble than their parent compound, glucuronidation of these hydroxlated derivatives greatly increases their solubility. Glucuronidation almost always results in detoxification and may be of great importance in the removal of carcinogenic, mutagenic, and toxic intermediates (12).

While in most instances glucuronidation results in the detoxification and excretion of xenobiotics, there are a few instances where glucuronidation results in more reactive xenobiotics. For example, phenacetin (13), and the carcinogens, safrole (4-allyl-1,2-methylenedioxybenzene) (14), and 2-acetyl-aminofluorene (15), are hydroxylated and then glucuronidated. The glucuronides of these compounds bind covalently to tissue macromolecules more readily than their unconjugated parent compounds.

Mammalian UDP-glucuronyltransferase activity is located in the endoplasmic reticulum of most tissues of the body including kidney (16), lung (17), skin (18), spleen, brain, heart, thymus (19), and the gastrointestinal mucosa (20). After homogenization, activity is recovered in the microsomal fraction. UDPGT activity has also been found in the nuclear envelope (21). While the liver plays an important role in glucuronidation, intestinal glucuronidation is important as well because the intestine is one of the sites of entry of many xenobiotics into the body. UDPGT and the other xenobiotic metabolizing enzymes found in the intestine, serve as a first line of defense against xenobiotics in the body.

Intestinal glucuronidation may have a significant effect on the overall metabolism, disposition, and pharmacokinetics of many xenobiotiotic and endogenous compounds metabolized by the liver. The fate of these compounds is influenced by the intestinal metabolism through the enterohepatic circulation. Hepatocytes excrete glucuronides of xenobiotic and endogenous compounds into the blood or the bile. Glucuronides in the bile enter the intestinal lumen where they may be hydrolyzed by bacterial β-glucuronidase. These metabolites, which have access to the large surface area of the intestine, may once again become glucuronidated. This process in which glucuronides are broken down and reformed may have a pronounced effect on the reactivity and half-life of many xenobiotics and their metabolites (22). For instance, after an intraperitoneal injection of phenobarbital or progesterone, they are excreted in the bile as

glucuronides. Saccharolactone, a potent inhibitor of β-glucuronidase, has been shown to shorten the pharmacological action of these drugs (23).

The intestinal microsomal mixed-function oxidase systems which are instrumental in xenobiotic metabolism are similar in structure to those in the liver. Cytochrome P-450 dependent polycyclic aromatic hydrocarbon hydroxylase activity has been demonstrated in the gastrointestinal wall of man, rabbit, rat, guinea pig, mouse, and hamster. Wattenberg's extensive work on benzo(a)pyrene hydroxylase activity in rat indicated that the intestinal mixed-function oxidase system plays an important role in the detoxification of arylhydrocarbons (24-28). Chhabra and Fouts (29) demonstrated both biphenyl and benzo(a)pyrene hydroxylase activities in the intestinal microsomes of rabbit, guinea pig, rat, and mouse. In some species, cytochrome P-450, and NADPH-cytochrome P-450 reductase were found in relatively high levels. In rabbit intestinal microsomes the cytochrome P-450 content per mg of microsomal protein was 33% that of the liver, while the NADPH-cytochrome P-450 reductase specific activity was 75% that of the liver (29).

Some of the hydroxylated aromatic products of the intestinal mixed-function oxidase system have been shown to be glucuronidated by the intestinal mucosa (30). Naphthalene, for instance, is converted by the mixed-function oxidase system to 1-naphthol, which in turn is rapidly glucuronidated (31). Aniline is hydroxylated by the mixed-function oxidase system to o-aminophenol which may may then serve

as a substrate for intestinal UDPGT (32). Hydroxlylated metobolites of 7-ethoxycoumarin (33), perazine (34), and biphenyl (35) have also been shown to be effectively glucuronidated in the small intestine.

Glucuronidation in the small intestine is also significant because it reduces the effect of many orally administered drugs. As an example, morphine, when administered orally, has diminished analgesic effect when compared to its effect when administered by another route (36). Morphine and several other phenolic drugs have been reported to be poorly absorbed when taken orally while their o-methylated congeners are absorbed to a greater extent (37).

The Modulation of UDPGT Activity:

Many questions about UDPGT, both *in vivo* and *in vitro*, remain to be resolved. Among these questions is how UDPGT activity is modulated *in vivo* and *in vitro*.

It is not known how UDPGT activity is regulated *in vivo*. Experiments comparing the *in vivo* with the *in vitro* hepatic or intestinal microsomal activity indicate that for all species studied, UDPGT activity is not fully expressed (38-40). "Latent" is an operational term used to describe the native form of the enzyme; that form found *in vivo* or found in tissue slices which have not been exposed to membrane perturbants. The activity of UDPGT in tissue slices may be increased by exposure to UDP-N-acetylglucosamine which enhances the affinity and specificity of the enzyme for UDPGA, thus making the enzyme more efficient (41). It has not been determined

whether or not UDP-N-acetylglucosamine regulates enzyme activity *in vivo*.

The mechanism by which UDPGT activity is enhanced *in vitro* is unknown. This enhancement of UDPGT activity *in vitro* by mechanical or chemical means is termed "activation". The activation of UDPGT by membrane perturbing agents is most commonly explained by assuming the breakdown of permeability barriers in the modified membranes (42-44). Vessey and Zakim proposed that activation might also arise from changes in the lipid-protein interaction (45).

The activity of hepatic microsomal UDPGT *in vitro* reflects the manner in which the microsomes are treated. A variety of conditions and agents which effect the integrity of the microsomal membrane to which UDPGT is bound have a pronounced effect on the activity of the enzyme. Studies on the effects of phospholipases, detergents, trypsin, freezing, and ultrasonication indicate that altering the microsomal environment has a marked effect on hepatic UDPGT activity. With membrane disruption, a biphasic activation-inactivation of enzyme activity is often observed. Zakim and Vessey (46) were able to activate rat, guinea pig, and rabbit microsomal UDPGT with phospholipase A. Continuation of phospholipase A treatment, after peak activity was reached, resulted in a decline in UDPGT activity. While ultrasonication of guinea pig microsomes initially activated UDPGT, prolonged ultrasonication produced a decrease in UDPGT activity (47). Low levels of the anionic detergent, deoxycholate, and the nonionic detergent, Triton X-100, activate guinea pig, mouse, and rat microsomal

UDPGT (48-49), although loss of activity is observed at high concentations of detergent.

UDPGT activity may vary not only with the way in which microsomes are treated, but may also vary with the organ from which the microsomes are obtained. Comparative studies on naphthol glucuronidation with intestinal loops *in vivo* and with mucosal homogenates *in vitro* suggests that intestinal UDPGT is latent in the intact tissue, as is the liver enzyme, and can be effected by a variety of membrane perturbing treatments (50). Aitio (51) studied the effect of phospholipase C, digitonin, and trypsin on UDPGT activity in rat liver and small intestinal microsomes. Digitonin had a negligible effect on intestinal UDPGT while it greatly enhanced liver activity. Although hepatic UDPGT was activated by trypsin and phospholipase C treatments, intestinal UDPGT activity was depressed. Del Villar *et al.* (52) demonstrated a difference in response of intestinal and hepatic microsomes to Triton X-100. The intestinal microsomal UDPGT might be activated to a greater extent than the hepatic enzyme and therefore respond differently to various membrane perturbing treatments. Since trypsin, phospholipase C, and surface active agents, like bile salts, are constituents of the small intestinal lumen, UDPGT might be spontaneously activated during the microsomal isolation procedure by these endogenous surface active agents (50).

Zakim and Vessey (53) and Wisnes (54) investigated the kinetic properties of hepatic *p*-nitrophenol:glucuronyltransferase (PNGT) activated by various membrane perturbing treatments. The activated

form of the enzyme was inhibited by UDP and UDP-sugars, had a decreased affinity for UDPGA and p-nitrophenol, and was insensitive to UDP-N-acetylglucosamine.

It has been proposed (55) that the altered kinetic properties of PNPGT, after treatment with membrane perturbants, are due to an altered enzyme conformation brought about by changes in the lipid environment. When Gorski and Kasper (56) separated 98% of the phospholipid from rat liver microsomal protein, using gel filtration, PNPGT activity was reduced to 0-6% of its original activity. By incubating the lipid depleted PNPGT with liposomes derived from microsomes, 30 to 44% of the original activity was restored. Besides mixed microsomal lipid, phosphatidylcholines, both synthetic and natural, restored PNPGT activity. Tukey et al. (57) found that phosphatidylcholine and lysophosphatidylcholine were most effective in restoring the activity of partially purified rabbit liver PNPGT which had been depleted of lipid. Burchell and Hallinan (58) were able to activate rat liver PNPGT, purified to homogeneity and containing very little phospholipid, 40-100% by incubation with phosphatidyl - and lysophosphatidyl - choline. With the addition of phosphatidyl - and lysophospha - tidyl-choline to lipid depleted guinea pig liver PNPGT, Erickson et al. (59) achieved a 50 fold stimulation of PNPGT activity. The length and degree of unsaturation of the acyl chains appeared to have a significant influence on the reactivation of PNPGT. Phosphatidylserine and phosphatidylethanolamine did not reactivate the enzyme.

The Heterogeneity of UDPGT:

There are several indications that UDPGT consists of a heterogeneous group of enzymes. Some glucuronyltransferase activities have been separated from each other during the course of purification (60-64). Recent work suggests that at least two forms of glucuronyltransferase exist in the rat which differ in tissue distribution, inducibility by xenobiotics and glucocorticoids, substrate specificity and perinatal development.

Hepatic glycuronyltransferase activity towards several groups of substrates is differentially induced. Many microsomal drug metabolizing enzymes are induced preferentially by the model inducer, 3-methylcholanthrene, while other enzymes are induced preferentially by another model inducer, phenobarbital. Glucuronyltransferase activity toward one group of substrates, which includes p-nitrophenol, 1-naphthol, and 2-aminophenol, is induced by 3-MC (65). Glucuronidation of another group of substrates, which includes morphine, phenolphthalein, and chloramphenicol, is induced by PB (65-66). Neither PB or 3-MC induce steriod glucuronidation (61).

Hepatic glucuronidation of different substrates follows different developmental patterns (67-68). The activity towards that group of substrates which is induced by 3-MC reaches adult levels during the late fetal stage of development, while that activity which is induced by PB, does not reach adult levels until 2 days after birth. Wishart (67) has termed these two groups of substrates "late fetal" and "neonatal". The "late fetal" substrates include 1-naphthol, 2-aminophenol, and p-nitrophenol, corresponding to that group of

substrates whose conjugation is induced by 3-MC; while the "neonatal" group inculdes phenolphthalein, morphine, bilirubin, and estradiol. Conjugation of this group of substrates is induced by PB. Having demonstrated that the synthetic glucocorticoid, dexamethasome, precociously induces transferase activity towards the "late fetal" group, but not to the "neonatal" group, Wishart proposed that fetal glucocorticoids may induce transferase activity towards the "late fetal" group. The activity towards the "neonatal" group may be unresponsive to glucocorticoids or may require an additional endogenous agent(s) for induction to occur.

The "neonatal" and "late fetal" group of substrates have have different tissue distribution (69). Transferase activity towards the "late foetal" group of substrates is ubiquitous, being found in such tissues as the liver, kidney, small intestine, lung, skin, and spleen, while activity towards the "neonatal" group of substrates is found primarily in the liver and intestine, being undetectable or barely detectable in other tissues.

It has been proposed that the substrate specificity of the various forms of transferase is based, in part, on the size and shape of the substate (66). According to the hypothesis developed by Wishart et al., that form of glucuronyltransferase, inducible by 3-MC, whose activity reaches adult levels at the late foetal stage of development, is specific for substrates which are planar. That glucuronyltransferase inducible by PB, whose activity reaches adult

levels neonatally, is specific towards phenolic compounds with bulky *para* substituents.

This proposal is supported by Wishart's studies on the perinatal development of the glucuronidation of a series of phenols substituted with alkyl groups in the *para* position. The glucuronidation of phenols which had relatively small alkyl groups in the *para* position attained adult levels during the late foetal stage of development, while glucuronidation of those phenols which had bulky alkyl groups attained adult levels neonatally. The same developmental pattern was demonstrated for a series of phenols with alkyl substituents in the *meta* or in the *ortho* position.

While studies on the perinatal development, induction, and tissue distribution of rat liver glucuronyltransferase have provided evidence for the functional heterogeneity of UDPGT, evidence for the true molecular heterogeneity of UDPGT has been provided by the purification of UDPGT. Early attempts at the purification of UDPGT from liver microsomes were hindered by enzyme instability and inadequate solubilization, but recent techniques have been more successful in overcoming these problems. Using DEAE- and UDP-affinity chromatography, in the presence of nonionic detergents, several investigators have been able to separate and purify to apparent homogeneity a hepatic glucuronyltransferase with activity towards small phenolic substrates from a glucuronytransferase with activity towards bulky substrates. Burchell *et al.* have purified rat liver bilirubin GT from rat liver *p*-nitrophenol GT (62). The purified rat liver PNPGT had

activity towards 1-naphthol, p-nitrophenol, and o-aminophenol in approximately the same ratio as in the liver homogenate suggesting that a single microsomal enzyme is responsible for the glucuronidation of these substrates (70-71). This enzyme also had slight activity towards morphine. Bock et al. have purified morphine GT and PNPGT from the rat liver (61). While the purified PNPGT had no detectable activity towards morphine and testosterone, the purified morphine GT had no detectable activity towards p-nitrophenol. Tukey et al. have purified, to apparent homogeneity, estrone GT and p-nitrophenol GT, from the rabbit liver (63-64). The purified estrone GT showed no activity towards PNPGT while the PNPGT had some activity towards estrone. Recently rat liver testosterone GT was purified by Matern et al. This purified transferase had no detectable activity towards estrone, bilirubin, p-nitrophenol or morphine (72).

Tukey et al. concluded that the estrone and p-nitrophenol glucuronytransferases were definitely different proteins based on their physical properties (64). Both enzymes had subunit molecular weights of 57,000 and gel chromatography indicated that both enzymes existed as tetramers with an apparent molecular weight of 230,000. However, with polyacrylamide isoelectric focusing, PNPGT had an isoelectric point of 6.8, while estrone GT had an isoelectric point of 7.6. Amino acid analysis of the purified enzymes revealed that PNPGT contained 53% hydrophobic amino acids, while estrone GT contained 60% hydrophobic amino acids. Furthermore, limited proteolysis, in the presence of SDS,

produced distinct differences in the peptide map composition of the two enzymes.

Richard Jagger and Steven Aust at Michigan State University have studied the activity of rabbit intestinal UDP-glucuronyltranferases towards several substrates. At this time, it is not known whether one transferase or several transferases are responsible for the glucuronidation of these substrates. Nor is it known whether the intestinal transferase(s) and the liver transferases which glucuronidate these substrates, are identical proteins.

Knowledge of substrate specificity would enhance the understanding of drug interactions and of the toxicity of xenobiotics. For instance, ingestion of significant amounts of a substance which is a substrate for PNPGT might limit the glucuronidation of other PNPGT substrates. Such substrates might remain in the intestinal lumen longer than they would otherwise. Glucuronidation of substrates brought to and from the intestine through enterohepatic circulation might also be effected.

Little is known on the substrate specificity of intestinal PNPGT(s) nor is it known whether liver and intestinal PNPGT are identical proteins. Purifying PNPGT(s) from rabbit intestinal microsomes would help clarify these and other questions.

UDPGT has yet to be purified from the small intestine.

The work in Dr. Aust's lab was undertaken to establish procedures for the purification of rabbit intestinal microsomal PNPGT. The purification of PNPGT requires a procedure for the large scale preparation of rabbit intestinal microsomes with stable PNPGT activity,

as well as a non-denaturing procedure for the solubilization of PNPGT. Once PNPGT is purified to homogeneity, its kinetic properties, physical properties, and substrate specificity can be better studied.

METHODS AND MATERIALS

Material Sources:

β-Glucuronidase, DEAE-Sephacel, p-nitrophenol (spectrophotomeric grade), phenophthalein, UDP (sodium salt), UDPGA (ammonium salt, 98% grade), UDP-N-acetylglucosamine, butylated hydroxytoluene, β -mercaptoethanol, heparin (soduim salt, Sigma Grade I), chicken ovomucoid trypsin inhibitor (Type II-O), egg yolk phosphatidylcholine crude soybean phoshatidylcholine, chlorhexidine, Dextran Blue 2000, Brilliant Blue R, sodium dodecyl sulfate, phenylmethylsulfonylfluoride, polyethylene glycol (approximate weight 400), bovine serum albumin (98% fatty acid free) Lubrol WX, and Tris base were obtained from Sigma Chemical Company, St. Louis, Missouri. β-Naphthaflavone was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Phenobarbital (sodium U.S.P.) was purchased from Merck and Company Inc., Rahway, New Jersey. Firemasters PBB, manufactured by Michigan Chemical Corporation, St. Louis, Michigan, was obtained from the Michigan Department of Agriculture, Lansing, Michigan. 3-MC, acrylamide, methylenebisacrylamide and tetramethylenediamine were purchased from Eastman Organic Chemicals, Rochester, New York. Octyl-β -D-glucopyranoside, and saccharo-1,4-lactone were obtained from Calbiochem-Behring Corporation, La Jolla, California. Triton X-100 (scintillation grade) and Aquasol 2 were purchased from New England Nuclear, Boston, Massachusetts. C^{14}octylglucoside was synthesized

by Paul Rosevear of Michigan State University. Emulgen 911 was a gift from the Kao-Alto Company, Tokyo, Japan. Renex 690 was a gift from Janice Lacedo of ICI, Wilmington, Delaware. Cholate and deoxycholate were recrystalized from ethanol.

All other chemicals were reagent grade.

Animals

Animals, outbread male Sprague-Dawley rats and New Zealand White rabbits were purchased from Spartan Research Animals, Inc., Haslett, Michigan. The rabbits ranged in weight from 2 to 5 kilograms and the rats from 225 to 250 grams. Water and Purina chow were given ad libitum.

For enzyme induction studies, rabbits were pretreated with PBBs, or with β-naphithaflavone, or with phenobarbital.

β-Naphthaflavone was dissolved in polyethylene glycol (MW 400) at 37°C, with vigorous stirring, to a concentration of 60mg/ml. The 37°C solution was immediately injected into the rabbits. Rabbits were injected i.p. with 80 mg/kg. Control rabbits were injected with polyethylene glycol. The rabbits were sacrificed 40 hours after they were injected.

Firemasters PBBs were dissolved in polyethylene glycol (MW 400) in a boiling water bath for several hours, to a concentration of 45mg/ml. The solution was equilibrated to ambient temperature. Rats and rabbits were injected with 90 mg/kg 7 days prior to sacrifice. Control rabbits and rats were injected with 2 ml/kg polyethylene glycol.

A 0.2% solution of phenobarbital in distilled water was adjusted to pH 7.5-7.8 with concentrated HCl. The solution was diluted 1:1 with tap water and given to rabbits ad libitum for 7 days prior to sacrifice.

Isolation of Intestinal Microsomes

Rat and rabbit microsomes were isolated by a method modified from that of Stohs et al. (73). Rabbits were sacrified by a blow to the head, rats by decapitation. The rabbit small intestine was immediately excised from the pylorus to 5 cm proximal to the ileocecal valve. The rat small intestine was excised to the first 25 cm of the duodenum. The intestinal lumen was washed vigorously with an ice cold solution containing 20 mM Tris-HCl pH 7.4, 20% glycerol, 154 mM KCl, and 5 mM EDTA. Excess fat was trimmed from the outer intestinal wall and the intestine was then placed in the above solution. Four ml of this solution was used per gm wet weight of small intestine. To this solution was added chicken ovomucoid trypsin inhibitor (6 mg/gm wet wt. small intestine), and 5 U/ml heparin. The intestine was minced with scissors and homogenized in a glass Teflon Potter-Elvehjem homogenizer at 500 rpm and immediately centrifuged at 10,000 g for 20 minutes. The supernatant was poured through four layers of cheese cloth and centrifuged at 105,000 g for 90 minutes. The microsomal pellet was washed once by suspension and recentrifugation in three volumes of isotonic KCl. The final pellet was suspended in 50 mM Tris-HCl, 50% glycerol, 2uM butylated hydroxytoluene pH7.4, to a protein concentration of 40 to 50 mg/ml and stored under argon at -20°C.

Assay of PNPGT Activity

Rabbit intestinal PNPGT activity, unless otherwise stated was assayed by a modification of Lucier's colorimetric assay (74). From 0.25 to 1.0 mg of protein was assayed in 1.4 ml of an assay mixture containing 0.15 M Tris-HCl, 2.0 mM p-nitrophenol, 10 mM $MgCl_2$, and 3 mM UDPGA. When assaying microsomes, the assay mixture also contained 0.013% (w/v) Triton X-100 per mg of protein. The assay mixture was preincubated for 5 minutes at 37°C. With the addition of UDPGA, the reaction was initiated. Substrate dissappearance was monitored continuously at 425 nm with a Cary 219 double beam spectrophotometer on the reverse beam mode with a 2.0 nm slit width and using cuvettes 0.2 cm wide. The reference cuvette contained the assay mixture without UDPGA. The extintion coefficient for p-nitrophenol at 425 nm in 0.15 M Tris-HCl, 10 mM MgCl2, 3 mM UDPGA, pH 7.4 at 37°C is 9.5 $uM^{-1}cm^{-1}$.

Rat intestinal PNPGT activity was assayed by a modification of the colorimetric method developed by Grote et al. (75). 1.5 mg of protein was assayed in 0.5 ml of an assay mixture containing 0.05 M Tris-HCl pH 7.4, 0.15 M KCl, 0.05% Triton X-100, 10 mM $MgCl_2$, 0.6 mM p-nitrophenol, and 5.0 mM UDPGA. After preincubation at 37°C, the reaction was started by adding UDPGA to the reaction mixture and stopped by adding 50ul aliquots of the reaction mixture to 1.0 ml of 2% trichloroacetic acid (w/v). After standing in trichloroacetic acid for 10 minutes, the assay mixture was centrifuged for 10 minutes at 2000 g

and the supernatant made more alkaline with the addition of 50ul of 5 N KOH. The absorbance was measured at 403 nm.

Assay of Phenolphthalein GT

Rabbit intestinal phenophthalein GT activity was assayed by a modification of a method developed by Wisnes (76). One mg of protein was assayed in 0.5 ml of an assay mixture containing 75 mM Tris-Maleate pH 7.4, 20 mg/ml BSA, 10 mM $MgCl_2$, 0.45 mM phenolphthalein and 4.3 mM UDPGA. After preincubation at 37°C, the reaction was started by adding UDPGA to the reaction mixture and stopped by adding 100 ul aliquots of the reaction mixture to 400 ul of 0.5 M glycine buffer pH 10.4. The reduction in absorbance at 550 nm was read immediately with a Cary 219 double beam spectrometer on the reverse beam mode with a 0.75 nm slit width. The extinction coefficient for phenolphthalein at 550 nm in 0.5M glycine buffer pH 10.4 is $1.3 \times 10^2 uM^{-1}cm^{-1}$.

Protein and Phosphate Assays:

Protein concentrations were estimated as described by Lowry et al. (77), using BSA standards.

Phosphate determinations were performed by the method of Bartlett (78).

Solubilization of Microsomes and DEAE Chromathography:

All steps were performed at 4°C. All buffers were deaerated and flushed with argon.

Solubilization of Intestinal Microsomes:

Unless otherwise stated, the rabbit intestinal microsomes were solubilized using β-D-octylglucoside. Microsomes were suspended to a

protein concentration of 10 mg/ml in 50 mM Tris-HCl pH 8.0, 1% octylglucoside, 20% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM βME, and 0.002% (v/v) Chlorhexidine. The suspension was sonicated, (4 x 20 s.) using a Branson sonifier with a microtip at a power setting of 6 (7 1/2 amperes), and centrifuged at 105,000 g for 2 1/2 hours.

DEAE-Sephacel Chromatography:

The 105,000 g supernatant was diluted 1:2, stirring gently but constantly, with 20% glycerol, 0.4% octylglucoside, and applied to a DEAE-Sephacel column (1:20, width:height) previously equilibrated with 50 mM Tris-HCl pH 8.0, 0.6% octylglucoside, 20% glycerol, 1 mM EDTA, 1 mM βME, and 0.002% Chlorhexidine. Five mg of protein was applied per ml of DEAE-Sephacel at a flow rate of 12 ml/h. The column was then washed with 2 column volumes of equilibration buffer. PNPGT activity was eluted with a linear 0 to 0.05 M KCl gradient in 4 volumes of equilibration buffer. The void volume was determined using Dextran Blue 2000.

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis:

Discontinous sodium dodecylsulfate-polyacrylamide slab gel electrophoresis was performed according to the method of O'Farrell (79). A 4.75% stacking gel and a 7.5% running gel was used. After electrophoresis, gels were stained with Brillian Blue R. The molecular weight markers run in parallel with all membrane protein samples were ovalbumin, glutamate dehydrogenase, catalase, and bovine serum albumin.

Extraction of Microsomal Lipid:

The extraction of lipid from intestinal microsomes was performed by the method of Folch et al. (80). All steps were carried out at 4°C. All buffers were flushed with nitrogen. Three grams of intestinal microsomes were extracted with 200 ml of 2:1 chloroform-methanol (v/v) and the insoluble material removed by filtration. The lipid extract was washed four times with a solution containing chloroform, methanol, and Folch salt solution in respective proportions of 3:48:47 by volume. The upper phases from the first three washes were discarded. The washed lipid extract was dried by rotary vacuum evaporation. The lipid was dissolved in 5 ml of 2:1 chloroform methanol and stored at -20°C under argon.

Preparation of Liposomes:

Aqueous suspensions of lipid were prepared by sonication at 4°C. An aliquot of lipid solution was transferred to a thin walled plastic tube and dried under nitrogen. Deaerated 0.15 M Tris-HCl pH 7.4 was added to the lipid, the tube capped under argon, and the suspension vigorously vortexed. The tube was then placed in an opening of a 3 necked vessel filled with water. The microtip of the Branson sonifier was placed in another opening of the vessel. The lipid suspension was sonicated (4X20 s) at a power setting of 6 (7 1/2 amperes).

Enzyme Incubation with Liposomes:

Fractions from the DEAE-Sephacel column were added to the liposomal suspensions with vigorous vortexing and left standing for six hours at 4°C. The protein-lipid preparations were then assayed for PNPGT activity by a method modified from that of Lucier et al. (74).

RESULTS

Microsomal Preparation:

The procedure used for the preparation of rabbit microsomes was a modification of the method developed by Stohs *et al.* (73) for the isolation of rat intestinal microsomes with stable cytochrome P-450.

Rabbit intestinal microsomes, prepared using the modified method, had a specific activity of 24 $\pm$ 2.1 nmoles *p*-nitrophenol conjugated per minute per mg of microsomal protein, with a range in specific activity of 21 to 27. The average microsomal protein yield from one small intestine was 320 mg (6mg/g wet weight), to give an average of 7680 total units of PNPGT activity.

Microsomal PNPGT activity was stable for at least 6 months, when the microsomes were stored under agron at -20°C.

The effects of changing various aspects of Stohs' isolation procedure on the yield of PNPGT activity was studied. Increasing the concentration of glycerol in the homogenizing medium above the 20% (v/v) used in Stohs' method, decreased the total units of activity (Table 1). In Stohs' procedure, microsomes are isolated from the upper villous layer of the mucosa. A comparision between microsomes taken from the whole wall, with those from the mucosal lining, showed that mucosal microsomes had a 30% higher specific activity. However 20% more total units of PNPGT activity were obtained by isolating microsomes from the whole wall (Table 2). Stohs derived microsomes from rats which were not fasted. When rabbits were fasted for 24 hours

TABLE 1. THE EFFECT OF THE GLYCEROL CONCENTRATION OF THE HOMOGENIZING MEDIUM ON INTESTINAL MICROSOMAL UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS p-NITROPHENOL.

% Glycerol (v/v)	PNPGT Specific Activity nmole p-intropenol conjugated/min per mg	Total PNPGT Activity nmole p-nitrophenol conjugated/min
0	9.1	2000
10	9.6	1860
20	9.1	1640
30	10.2	1470

PNPGT activity was assayed by the method of Lucier et al. (74) as described under "Methods".

TABLE 2. THE LOCALIZATION OF UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS *p*-NITROPHENOL IN RABBIT SMALL INTESTINE.

	PNPGT Specific Activity nmole *p*-nitropenol conjugated/min per mg	Total PNPGT Activity nmole *p*-nitrophenol conjugated/min
Whole Intestine	9.13 ± 1.8	1100 ± 70
Mucosal Lining	13.4 ± 2.1	950 ± 172

The mucosal lining of the small intestine was obtained by scraping the everted small intestine with the edge of a glass slide. PNPGT activity was assayed by the method of Lucier *et al.* (74) as described under "Methods".

prior to sacrifice these rabbits had slightly higher PNPGT specific activities than microsomes from nonfasted rabbits (Table 3), but more total units of microsomal PNPGT activity were obtained from nonfasted rabbits. Stohs only used rats which were male. With rabbits, microsomes had a similar PNPGT specific activity whether derived from male or female animals (Table 4).

In an effort to increase the specific activity and yield of PNPGT, rabbits were treated with several chemicals, known to induce enzymes for the metabolism of xenobiotics, prior to sacrifice. Pretreatment of rabbits with 3-MC type inducers β-naphthaflavone or PBBs did not increase microsomal PNPGT specific activity or yield (Table 5). Likewise, pretreatment of rabbits with phenobarbital did not increase yield (Table 6). Unlike rabbits, pretreatment of rats with PBBs

resulted in increased PNPGT specific activity. Rat microsomes, obtained from the 25 cm distal to the stomach pylous of intestines of rats not pretreated with PBBs, had a PNPGT specific activity of 25 nmoles p-nitrophenol conjugated per minute per mg of microsomal protein, while microsomes obtained from rats which were pretreated had a specific activity of 42.

The effect of changing several chemicals in the homogenizing medium used in Stohs' isolation procedure on the stability of microsomal PNPGT was examined. Stohs' method used soybean trypsin inhibitor to increase the stability of rat cytrochome P-450 during the microsomal preparation. In the of intestinal microsomes, it was found that the addition of chicken ovomucoid trypsin inhibitor, along with

TABLE 3. THE GLUCURONIDATION OF p-NITROPHENOL BY INTESTINAL MICROSOMES FROM FED AND FASTED RABBITS.

Treatment	PNPGT Specific Activity nmole p-intropenol conjugated/min per mg	Total PNPGT Activity nmole p-nitrophenol conjugated/min
Fasted	9.6	1210
Fed	8.2	1600

Fasted rabbits were given water ad libitum but no food for 24 hours prior to sacrifice. PNPGT activity was assayed by the method of Lucier et al. (74) as described under "Methods."

TABLE 4. SEX DIFFERENCES IN THE CONJUGATION OF p-NITROPHENOL BY INTESTINAL MICROSOMAL UDP-GLUCURONYLTRANSFERASE.

Sex	PNPGT Specific Activity nmole p-intropenol conjugated/min per mg	Total PNPGT Activity nmole p-nitrophenol conjugated/min
Female	13.0 ± 1.2	1320 ± 92
Male	11.2 ± 0.8	1010 ± 81

PNPGT activity was assayed by the method of Lucier et al. (74) as described under "Methods".

TABLE 5. THE EFFECT OF -NAPHTHAFLAVONE OR PBBs ADMINISTRATION ON THE PNPGT ACTIVITY OF RABBIT INTESTINAL MICROSOMES.

Treatment	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg	Total Protein (mg)	Total PNPGT Activity nmole p-nitrophenol conjugated/min
None	9	360	3200
PBBs	10	200	2000
β-Naphthaflavone	9	235	2110

Rabbits were injected i.p. with 80 mg/kg β-naphthaflavone 40 h prior to sacrafice, or with 90 mg/kg PBBs 7 days prior to sacrifice as described under "Methods." The PNPGT activity was assayed by the unmodified method of Lucier et al. (74).

TABLE 6. THE EFFECT OF PHENOBARBITAL ADMINISTRATION ON THE ION ON THE PNPGT ACTIVITY OF RABBIT INTESTINAL MICROSOMES.

Treatment	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg	Total Protein (mg)	Total PNPGT Activity nmole p-nitrophenol conjugated/min
None	9.5 ± 1.8	330 ± 80	3100
Phenobarbital	12.4 ± 2.8	300 ± 75	3720

0.1% Phenobarbital was given to rabbits in their drinking water *ad libitum* for 7 days prior to sacrifice as described under "Methods." PNPGT was assayed by the unmodified method of Lucier *et al.* (74).

EDTA, to the homogenizing medium increased the stability of rabbit intestinal microsomal PNPGT. Microsomes prepared with a homogenizing medium containing 0.1 mM phenylmethysulfonylfluoride had half the specific activity of those microsomes prepared using chicken ovomucoid trypsin inhibitor.

The distribution of PNPGT activity of microsomes obtained from rabbit small intestine is shown in figure 1. The duodenum had the highest specific PNPGT activity while microsomes obtained from the ileum had one third the activity of microsomes obtained from the duodenum.

Rabbit small intestinal microsomes had an average phenolphthalein GT activity of 0.14 nmoles phenophthalein conjugated per minute per mg of microsomal protein.

Assays for PNPGT and Phenolphthalein GT Activity:

The enzyme assay for rat intestinal PNPGT modified from Grote's assay for rat liver microsomal PNPGT, was apparently linear for 10 minutes when 5.0 mM UDPGA was used.

A continuous enzyme assay was developed for rabbit intestinal PNPGT by modifying Lucier's discontinuous PNPGT assay (74). When rabbit intestinal microsomes were assayed by Lucier's unmodified method, the apparent PNPGT specific activity, was half that seen when these microsomes were assayed by the modified method which uses higher concentrations of UDPGA and p-nitrophenol. The modified enzyme assay was linear for 15 minutes and between 0.25 to 2 mg of microsomal protein (Figure 2) per 1.4 ml of incubation mixture. In the absence of

Figure 1. THE DISTRIBUTION OF MICROSOMAL PNPGT ACTIVITY ALONG THE SMALL INTESTINE OF THE RABBIT.

Rabbit small intestines were cut into 50 cm segments and microsomes were derived from these segments as described under "Methods." PNPGT activity was assayed by the unmodified method of Lucier et al. (74).

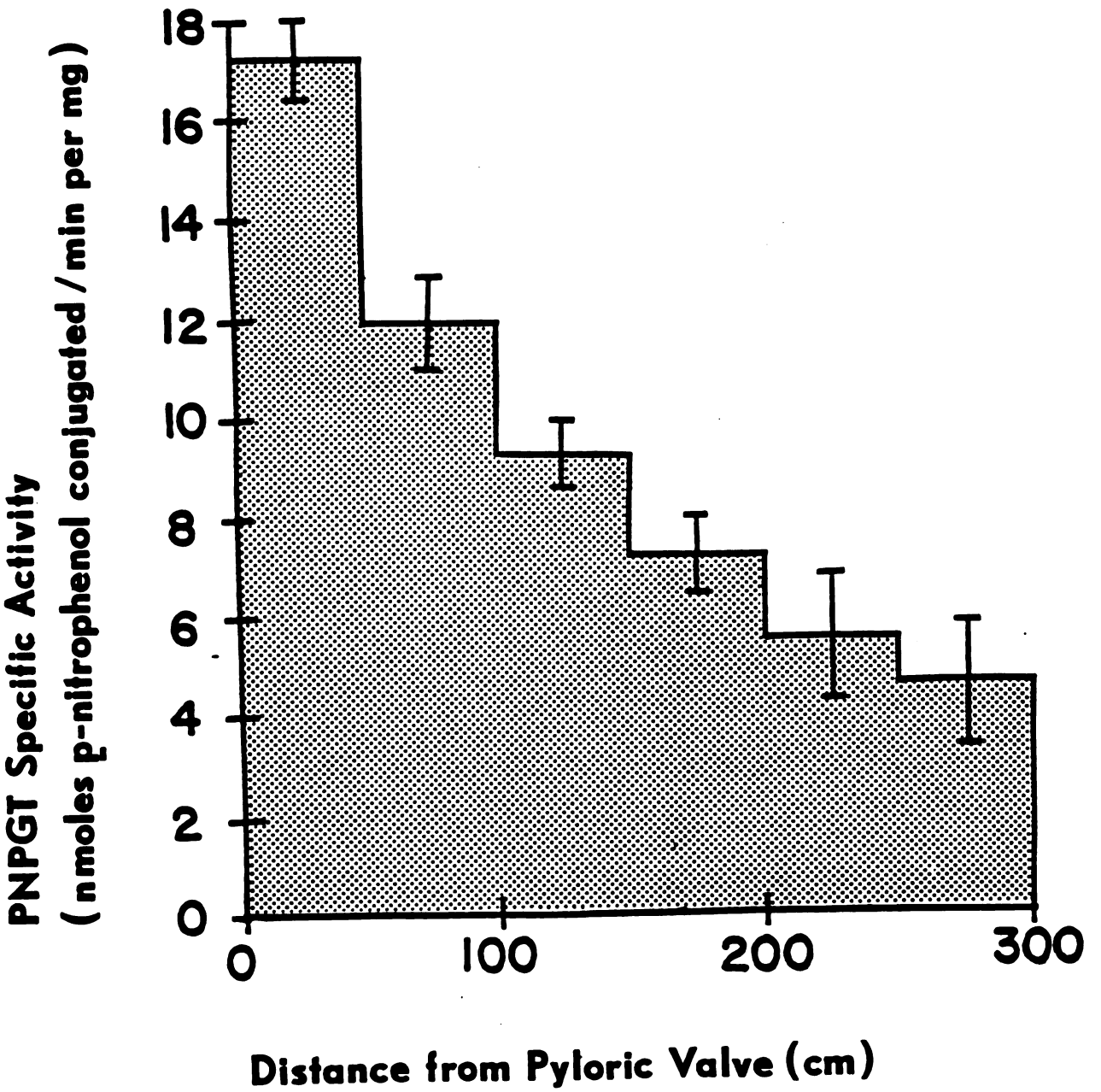
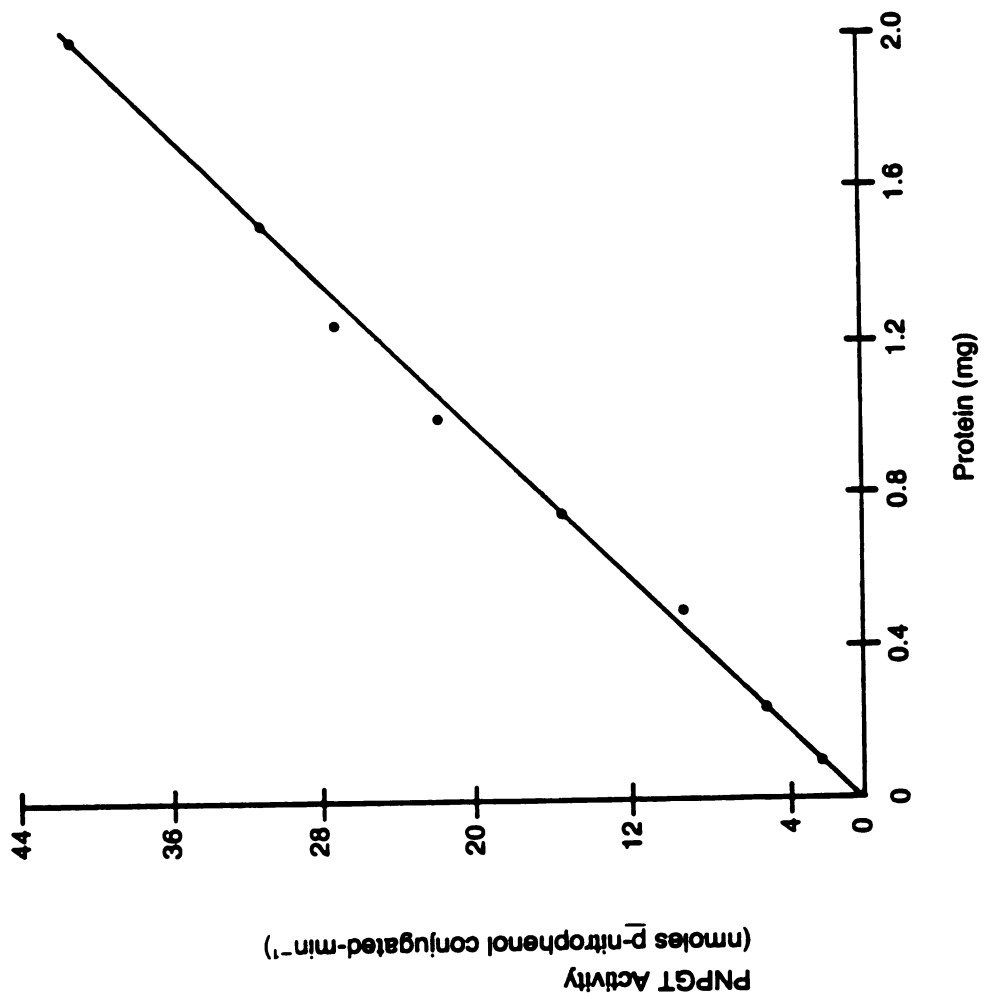

PNPGT Specific Activity
(nmoles p-nitrophenol conjugated / min per mg)
18
16
14
12
10
8
6
4
2
0
0
100
200
300
Distance from Pyloric Valve (cm)

Figure 2. RABBIT INTESTINAL MICROSOMAL PNPGT ACTIVITY AS A FUNCTION OF MICROSOMAL PROTEIN IN THE PNPGT ASSAY.

PNPGT was assayed as described under "Methods" using a procedure which was a modification of the assay developed by Lucier et al. (74).


PNPGT Activity
(nmoles p-nitrophenol conjugated-min⁻¹)
44
36
28
20
12
4
0
0
0.4
0.8
1.2
1.6
2.0
Protein (mg)

$MgCl_2$, the enzyme reaction was approximately half as fast.

The PNPGT assay was a spectrophotometric assay which involved monitoring the disappearance of the chromophore, p-nitrophenol, at 425 nm with the formation p-nitrophenol-glucuronide. The addition of saccharo-1,4-lactone, a potent inhibitor of intestinal β-glucuronidase, an enzyme which hydrolyzes p-nitrophenol-glucuronide, to the enzyme assay mixture, at concentrations of 0.5, 1.0, and 5.0 mM did not increase the apparent PNPGT specific activity.

In order to confirm that the decrease in absorbance at 425 nm was due to the formation of p-nitrophenol-glucuronide, β-glucuronidase was added to the incubation mixture after the reaction had proceeded for an hour. The addition of -glucuronidase resulted in a rapid increase in the absorbance at 425 nm.

The effect of incubating microsomes with various detergents on the activity of PNPGT was examined. The enzyme was activated by Lubrol WX, Emulgen 911, octylglucoside, Renex 690, and inhibited by them at high concentrations (Figures 3 and 4). Cholate, Brij 56, and dexycholate were inhibitory even at low concentrations (Figure 5).

Wisnes developed a discontinuous enzyme assay for phenolphthalein GT (76) in rat liver homogenate. The assay was a spectrophotometric assay which involved monitoring the disappearance of the chromophore, phenolphthalein, at 555nm. In assaying for rabbit intestinal microsomal phenolphthalien GT, this assay was modified and was apparently linear for 10 minutes and between 0.5 to 2 mg of microsomal protein per ml of incubation mixture. The addition of 10 mM $MgCl_2$ to

Figure 3. THE EFFECT OF FIVE NONIONIC DETERGENTS ON THE PNPGT ACTIVITY OF RABBIT INTESTINAL MICROSOMES.

Intestinal microsomes were assayed by the method of Lucier et al. (74) as described under "Methods", except that the indicated type and final concentration of detergent were used.

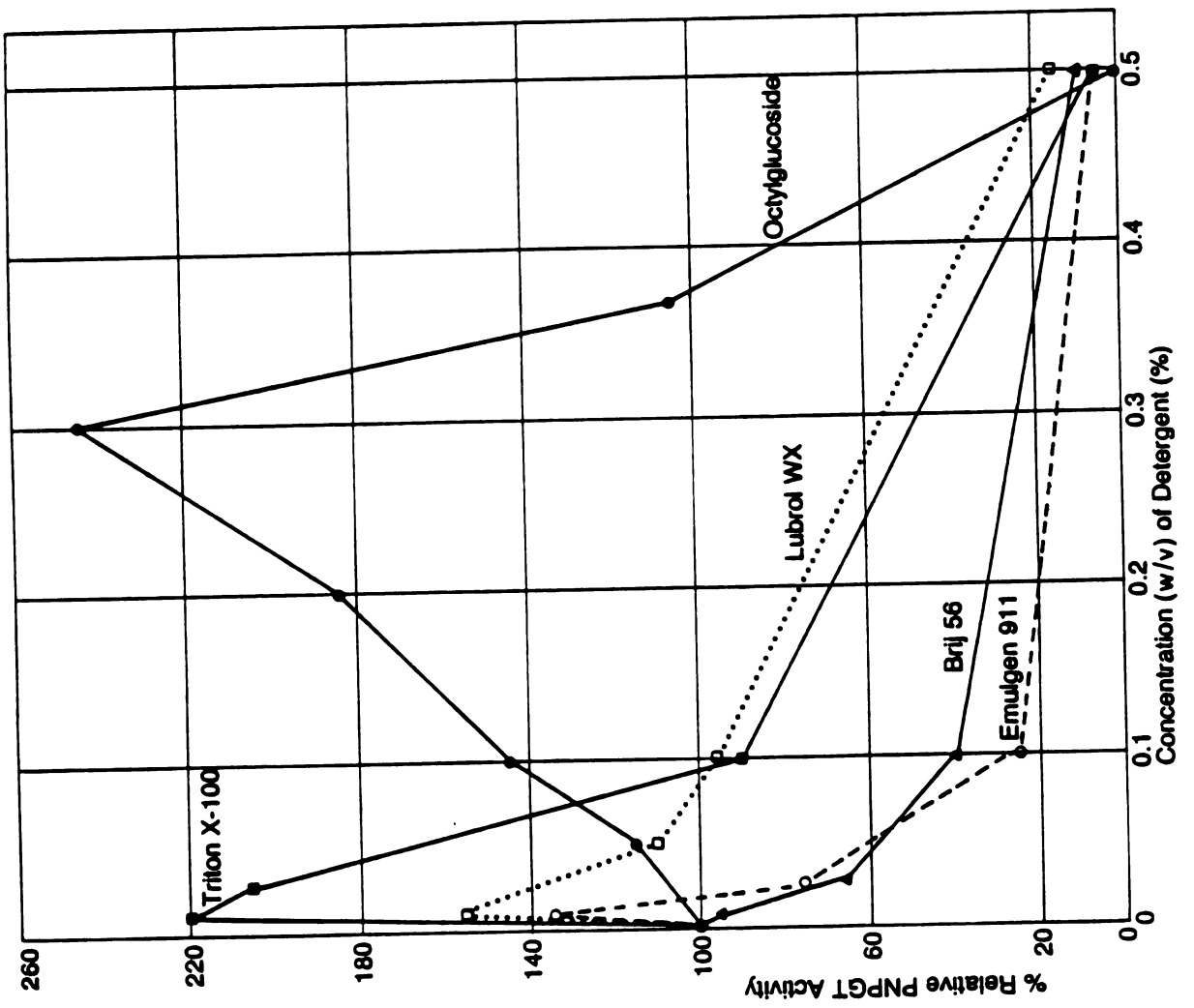

% Relative PNPGT Activity
260
220
180
140
100
60
20
0
0
0.1
0.2
0.3
0.4
0.5
Concentration (w/v) of Detergent (%)
Octylglucoside
Lubrol WX
Brij 56
Emulgen 911
Triton X-100

Figure 4. THE EFFECT OF THE NONIONIC DETERGENTS, RENEX 690 AND TRITON X-100, ON THE PNPGT ACTIVITY OF RABBIT INTESTINAL MICROSOMES.

Intestinal microsomes were assayed by the method of Lucier et al. (74) as described under "Methods", except that the indicated type and final concentration of detergent were used.

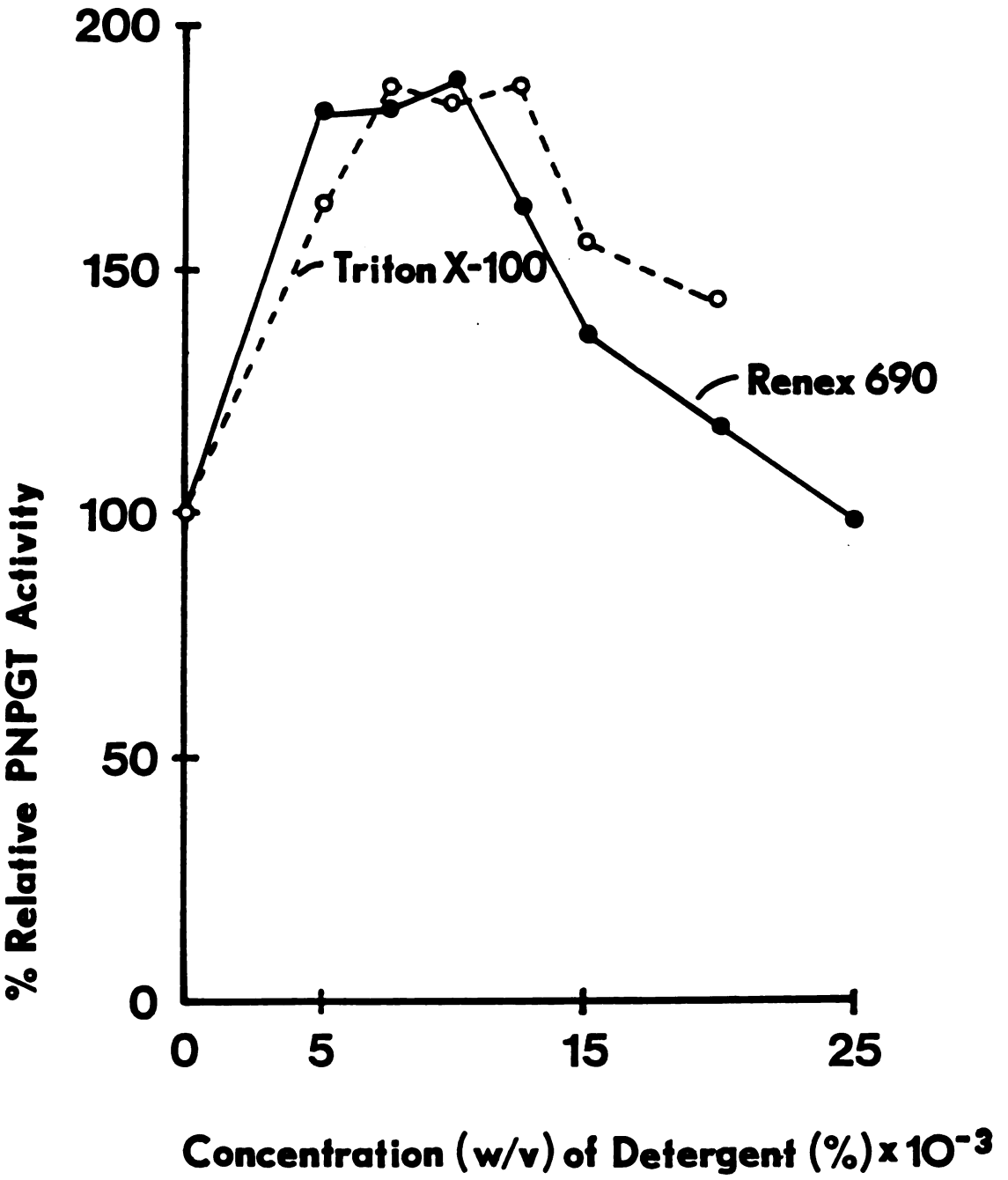
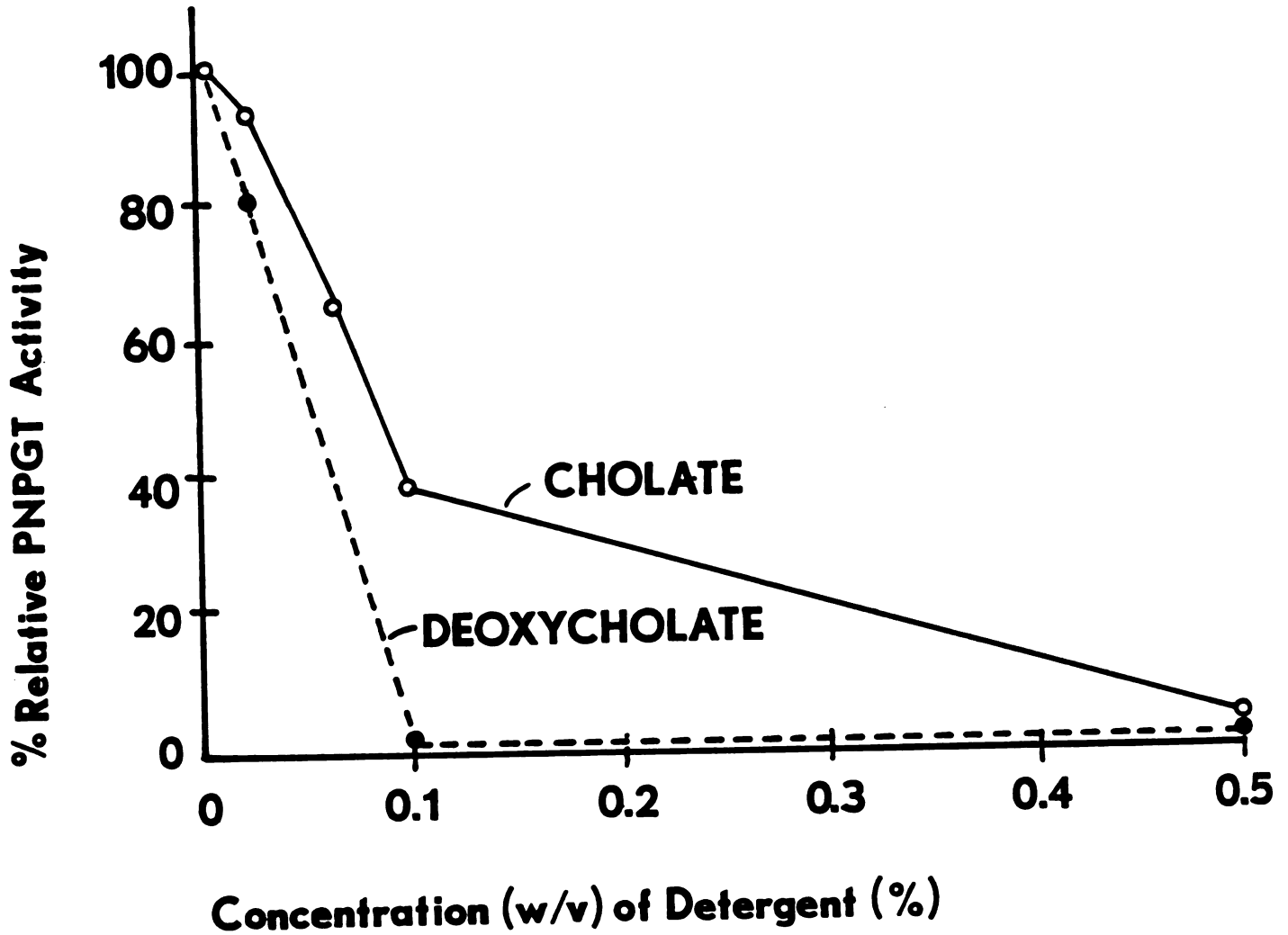

200
150
100
50
0
0
5
15
25
Triton X-100
Renex 690
% Relative PNPGT Activity
Concentration (w/v) of Detergent (%) x 10⁻³

Figure 5. THE EFFECT OF THE CHOLATE AND DEOXYCHOLATE ON THE PNPGT ACTIVITY OF RABBIT INTESTINAL MICROSOMES.

Intestinal microsomes were assayed as described under "Methods", except that the indicated type and concentration of detergent were used.


100
80
60
40
20
0
% Relative PNPGT Activity
CHOLATE
DEOXYCHOLATE
0
0.1
0.2
0.3
0.4
0.5
Concentration (w/v) of Detergent (%)

the incubation mixture had no effect on the reaction rate.

The effect of nonionic detergents on the apparent activity of phenolphthalein GT differed from their effect on the activity of PNPGT. Unlike PNPGT, phenolphthalein GT was not activated by relatively low levels of octylglucoside. At levels greater than 0.55% (w/v), the rate of change of absorbance at 555 nm decreased. Phenolphthalein was insoluble when concentrations of Triton X-100 as low as 0.001% (w/v) were added to the incubation mixture.

The kinetic properties of rabbit intestinal microsomal PNPGT were studied. The apparent K_m of microsomal PNPGT for p-nitrophenol was 0.3 mM at a UDP-glucuronic acid concentration of 3 mM, (Figure 6) and for UDP-glucuronic acid was 0.2 mM at a p-nitrophenol concentration of 2 mM (Figure 7). Five mM UDP-N-acetylglucosamine had no effect on the detergent activated enzyme.

Solubilization Studies:

Because the instability of glucuronyltransferase hindered its characterization, the solubilization of rabbit intestinal PNPGT with the relatively mild nonionic detergents - Lubrol WX, Emulgen 911, and octylglucoside - was examined. In order to solubilize microsomal protein, buffered detergent was added to the microsomes and the resulting suspension was sonicated and centrifuged.

Lubrol WX and Emulgen 911 were used in the initial solubilization studies. Table 7 shows the result of this experiment. After treatment with 0.5% Lubrol WX, 19% of the original PNPGT activity was detected in the 2 1/2 h, 180,000 x g supernatant. Increasing the Lubrol WX

Figure 6. DOUBLE RECIPROCAL PLOTS OF INITIAL RATES OF RABBIT INTESTINAL MICROSOMAL UDP-GLUCURONYTRANSFERASE AS A FUNCTION OF VARYING CONCENTRATIONS OF p-NITROPHENOL AT DIFFERENT FIXED CONCENTRATIONS OF UDP-GLUCURONIC ACID.

Each point is based on initial rate determination of UDP-glucuronyltransferase activity in microsomes as described under "Methods" and expressed as mmoles of p-nitrophenol conjugated per min per mg of microsomal protein. The concentrations of UDP-glucuronic acid were 0.5mM (□), 1.5mM(○), 3mM(●), and 6mM(▲).

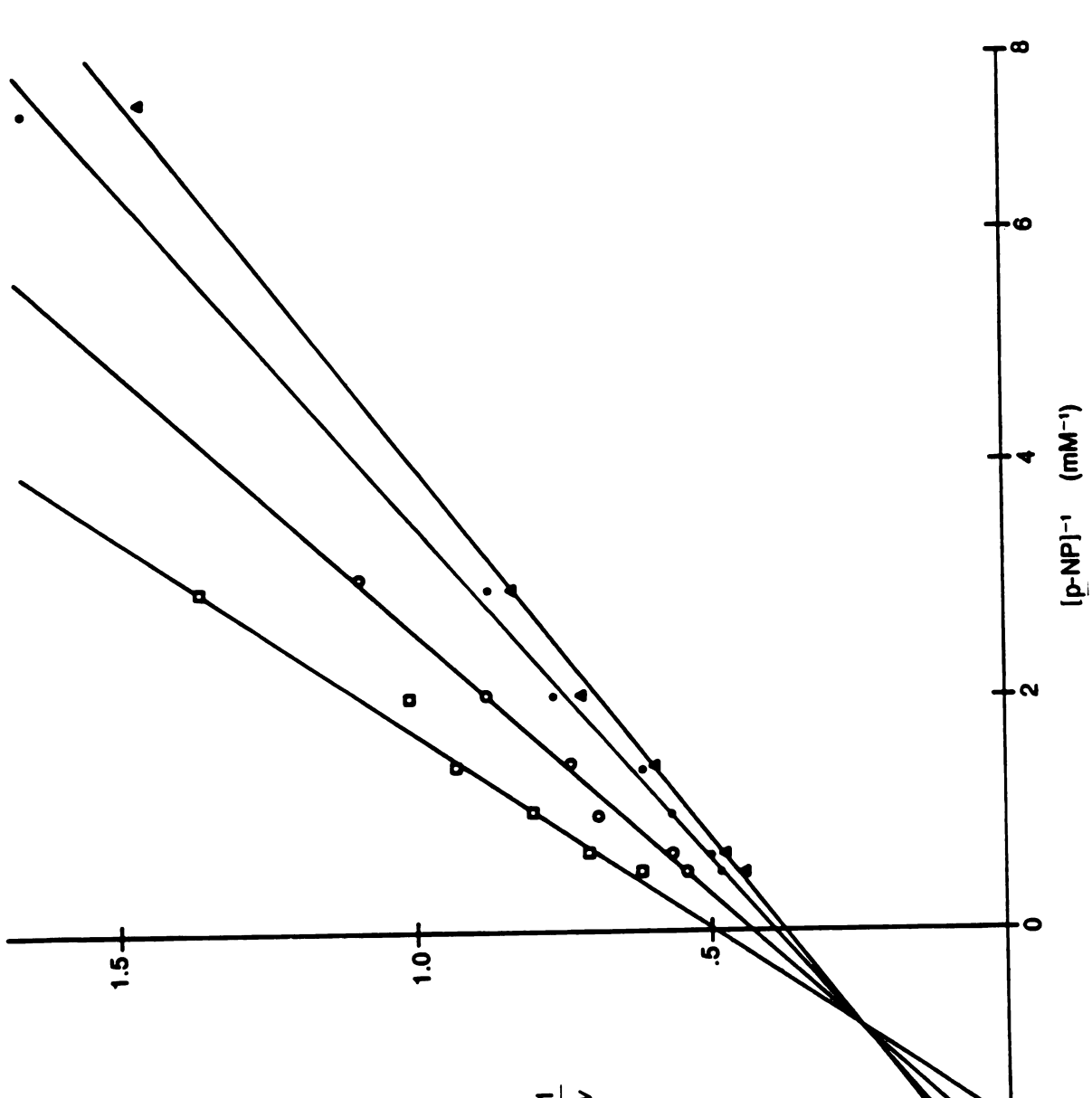

1.5
1.0
.5
0
-2
2
4
6
8
v^{-1}
$[p\text{-NP}]^{-1}$ (mM^{-1})

Figure 7. DOUBLE RECIPROCAL PLOT OF INITIAL VELOCITY AGAINST VARIABLE UDP-GLUCURONIC ACID CONCENTRATIONS IN RABBIT INTESTINAL MICROSOMES.

Each point is based on initial rate determination of UDP-glucuronyltransferase activity as described under "Methods" and expressed as nmoles of p-nitrophenol conjugated per min per mg of microsomal protein. The concentration of p-nitrophenol was 2.0 mM.

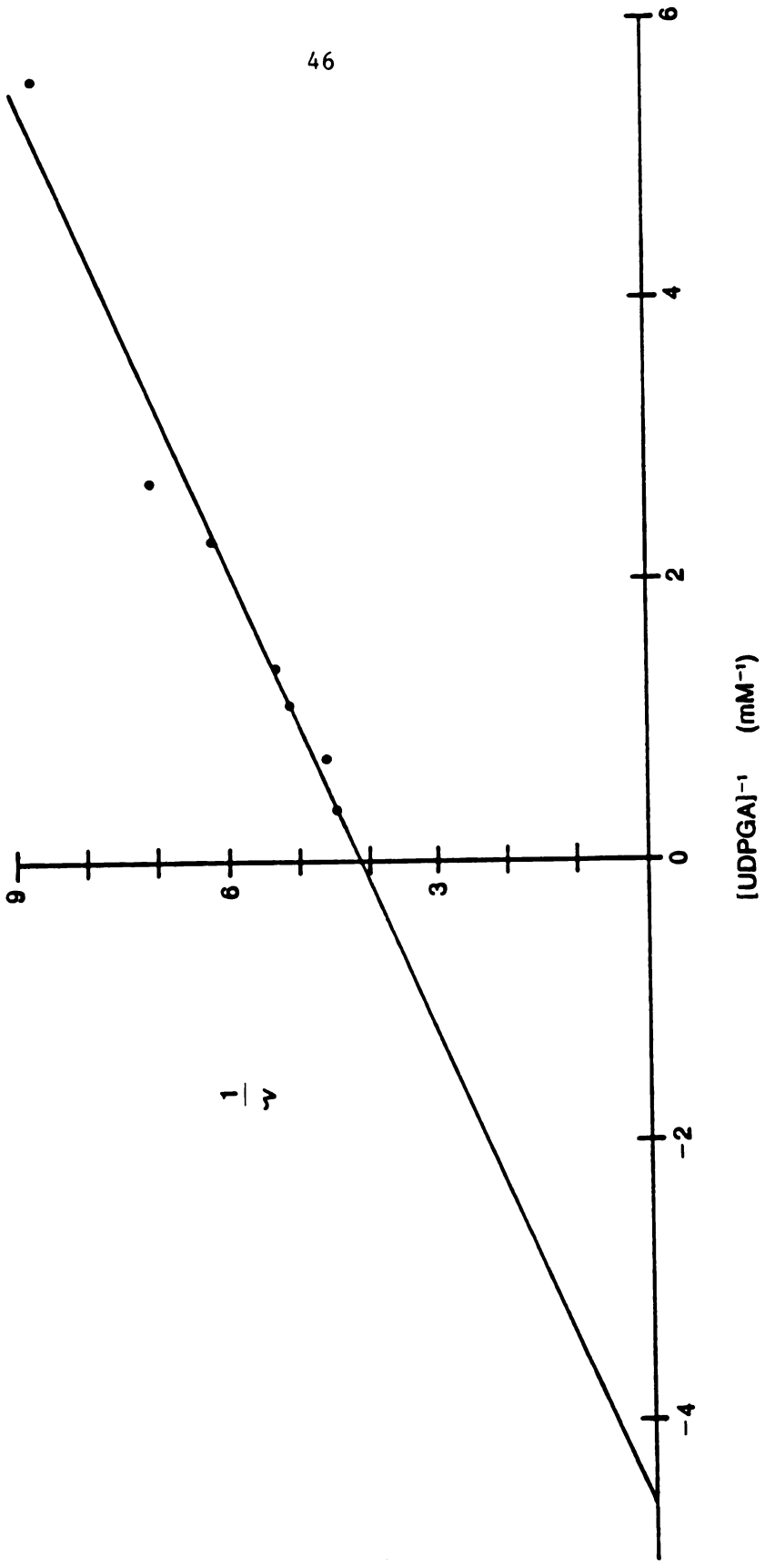

9
6
3
0
-2
-4
2
4
6
[UDPGA]$^{-1}$ (mM^{-1})
$\frac{1}{v}$

TABLE 7. THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL PNPGT WITH NONIONIC DETERGENTS.

Detergent	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
	super.	ppt.	super.	ppt.	super.	ppt.
NONE	0	7	0	87	5	77
LUBROL WX						
0.05%	0	6	0	81	4	76
0.10%	0	6	0	81	4	76
0.50%	12	5	16	62	19	57
1.00%	0	4	0	50	16	55
EMULGEN 911						
0.05%	0	6	0	81	5	76
0.10%	0	4	0	47	7	69
0.50%	7	2	14	25	16	52
1.00%	0	1	0	16	17	43

10 mg microsomal protein was mixed at 4°C in 1 ml of 50 mM Tris-Ac pH 7.6, 25% (v/v) glycerol, 0.5mM DTT ± detergent. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 x g for 2 1/2 hours. PNPGT was assayed by the method of Lucier *et al*. (74).

concentration to 1% did not increase the amount of enzyme activity in the supernatant. Using Emulgen 911, instead of Lubrol WX, resulted in a lower recovery of total PNPGT activity. This lower recovery might have been due to the presence of peroxides in the Emulgen. The 1.0% Emulgen solution was found to be more than 3 mM in peroxide.

The effect of protein concentration, pH, ionic strength, and temperature on the solubilization of PNPGT activity with Lubrol WX was examined. Lowering the protein to detergent ratio did not increase the percentage of initial enzyme activity recovered in the supernatant. At protein concentrations of 2.5 mg/ml (0.5% ( ) Lubrol WX), PNPGT activity was not detected in the supernatant (Table 8). Increasing the pH of the detergent solution, while keeping the protein concentration at 10 mg/ml, did not increase the supernatant recovery of PNPGT activity (Table 9). No increase in the recovery of PNPGT activity in the supernatant was observed after incubating microsomes for half an hour in detergent solution at either 10°C or 22°C (Table 10). Increasing the ionic strength of the solubilization buffer did not improve solubilization (Table 11).

Mild sonication, however improved the recovery of PNPGT activity in the supernatant. If the microsomal detergent suspension was sonicated with a Bronson sonifier (4x20 sec) with a microtip set at 7 1/2 amperes, and centrifuged for 2 1/2 hours at 180,000 x g, 63% of the initial activity was recovered in the supernatant (Table 12).

Under similar conditions, with either Lubrol WX, or with octylglucoside, about 50-60% of the microsomal PNPGT activity was

TABLE 8. THE EFFECT OF PROTEIN CONCENTRATION ON THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL PNPGT WITH LUBROL WX.

Initial Protein Concentration (mg/ml)	Detergent	PNPGT Specific Activity nmoles p-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
		super.	ppt.	super.	ppt.	super.	ppt.
10	None	0	7.4	0	61	5	73
	0.5% Lubrol WX	9.0	9.8	20	55	20	50
5.0	None	0	9.5	0	67	5	62
	0.5% Lubrol WX	0	7.9	0	53	20	60
2.5	None	0	9.3	0	65	0	62
	0.5% Lubrol WX	0	9.7	0	48	93	44

Microsomal protein was mixed at 4°C in 50 mM Tris-Ac pH 7.6, 25% (v/v) glycerol, 0.5 mM DTT, ± 0.5% Lubrol WX. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours. PNPGT was assayed by the method of Lucier *et al.* (74).

TABLE 9. THE EFFECT OF pH ON THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL PNPGT WITH LUBROL WX

pH of Tris-Ac	Detergent	PNPGT Specific Activity nmoles p-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
		super.	ppt.	super.	ppt.	super.	ppt.
7.6	None	0	10.4	0	71	5	65
	0.5% Lubrol WX	8.3	9.0	20	58	23	61
8.0	None	0	9.0	0	65	5	68
	0.5% Lubrol WX	6.9	9.0	19	52	26	55
8.5	None	0	9.5	0	63	8	63
	0.5% Lubrol WX	6.4	10.2	19	56	28	52

10 mg/ml microsomal protein was stirred at 4°C in 50 mM Tris-Ac buffer of the indicated pH 25% (v/v) glycerol, 0.5 mM DTT, ± 0.5% Lubrol WX. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours at 4°C. PNPGT was assayed by the method of Lucier *et al.* (74).

TABLE 10. THE EFFECT OF TEMPERATURE ON THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL PNPGT WITH LUBROL WX.

Temperature	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
	super.	ppt.	super.	ppt.	super.	ppt.
4°C	7.3	8.8	16	52	18	52
10°C	7.1	8.5	16	52	19	51
22°C	11.4	8.4	19	48	18	52

10 mg/ml microsomal protein was stirred at the indicated temperatures in 50 mM Tris-Ac pH 7.6, 25% (v/v) glycerol, 0.5 mM DTT, ± 0.50% Lubrol WX. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours at 4°C. PNPGT was assayed by the method of Lucier et al. (74).

TABLE 11. THE EFFECT OF IONIC STRENGTH ON THE SOLUBILIZATION OF INTESTINAL MICROSOMAL PNPGT WITH LUBROL WX.

Molarity of Tris Ac-Buffer	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
	super.	ppt.	super.	ppt.	super.	ppt.
0.05 M	9.3	10	25	56	27	56
0.10 M	9.3	9.3	27	51	29	55
0.20 M	9.6	9.5	27	53	28	56

10 mg/ml microsomal protein was stirred at 4°C Tris-Ac pH 7.6, 25% (v/v) glycerol, 0.5 mM DTT, ± 0.5% Lubrol WX. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours at 4°C. PNPGT was assayed by the method of Lucier *et al.* (74).

TABLE 12. THE EFFECT OF SONICATION ON THE SOLUBILIZATION OF INTESTINAL MICROSOMAL *p*-NITROPHENOL UDP-GLUCURONYLTRANSFERASE WITH LUBROL WX.

Treatment	PNPGT Specific Activity nmole *p*-nitrophenol conjugated/min per mg		Total PNPGT Activity % of original microsomal total PNPGT activity		Total Protein % of original microsomal total protein	
	super.	ppt.	super.	ppt.	super.	ppt.
No sonication	9.0	9.5	21	55	23	57
Sonication	13.8	4.9	63	20	45	40

10 mg/ml microsomal protein with a PNPGT specific activity of 11, was mixed at 4°C 50 mM Tris-Ac pH 7.6, 25% (v/v) glycerol, 0.5 mM DTT, 0.5% Lubrol WX. The microsomal-detergent suspension was sonicated, (4x20s) using a Branson sonifier with a Supernatant (super.) and percipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours at 4°C. PNPGT was assayed by the method of Lucier *et al.* (74).

recovered in the supernatant (Table 13). Octylglucoside was used in subsequent solubilization studies because it was readily dialyzable (Figure 8), had a defined composition, had a reduced absorption at 280nm allowing for more sensitive uv monitoring of protein, and it gave more consistent results than Lubrol WX. Fifty percent of the microsomal PNPGT activity was recovered in the supernatant when microsomes were treated with 1.0% (34 mM) octylglucoside in this buffer at pH 7.5 (Figure 9). The recovery of PNPGT activity was improved by treating the microsomes with a more alkaline octylglucoside solution (Table 14). An even greater recovery of PNPGT activity was obtained by increasing both the pH and the ionic strength of the octylglucoside solution (Table 14). With 34 mM (1.0%) octylglucoside in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 70% of the original microsomal PNPGT activity (Figure 9) and 31% of the original microsomal protein was recovered in the supernatant. The PNPGT activity, in a supernatant containing EDTA and ME was stable for 12 hours at 4°C and had a half life of 3 days. In the absence of EDTA, PNPGT activity had a half life of 12 hours.

Less than half the initial microsomal phenolphthalein GT activity was detected in the 34 mM octylglucoside microsomal suspension. After centrifugation, little or no activity was seen in the supernatant or the pellet.

DEAE Chromatography:

In preliminary experiments, the binding of octylglucoside solubilized PNPGT to DEAE-Sephacel at pH 7.5 was studied. At this pH,

TABLE 13. THE SOLUBILIZATION OF INTESTINAL MICROSOMAL PNPGT WITH LUBROL WX AND OCTYLGLUCOSIDE.

Detergent	PNPGT Specific Activity		Total PNPGT Activity		Total Protein	
	nmole p-nitrophenol conjugated/min per mg		% of original microsomal total PNPGT activity		% of original microsomal total protein	
	super.	ppt.	super.	ppt.	super.	ppt.
Lubrol WX 0.50%	15	4	62	18	44	36
Ochylglucoside 1.0%	13	4	50	20	40	38

10 mg/ml microsomal protein with a specific PNPGT activity of 11, was mixed at 4°C 50 mM Tris-HCl pH 7.5, 20% (v/v) glycerol, 0.5 mM DTT, detergent. The microsomal detergent suspension was sonicated, using a Branson sonifier (4x20sec) with a microtip at 7 1/2 amperes. Supernatant (super.) and precipitate (ppt.) fractions were prepared by ultracentrifugation at 180,000 X g for 2 1/2 hours. PNPGT was assayed by the method of Lucier et al. (74).

Figure 8. THE DIALYSIS OF OCTYLGLUCOSIDE.

10 mg/ml of microsomal protein in a Tris buffer containing 1.0% octylgucoside was dialyzed against 500 volumes of the same buffer without octylglucoside

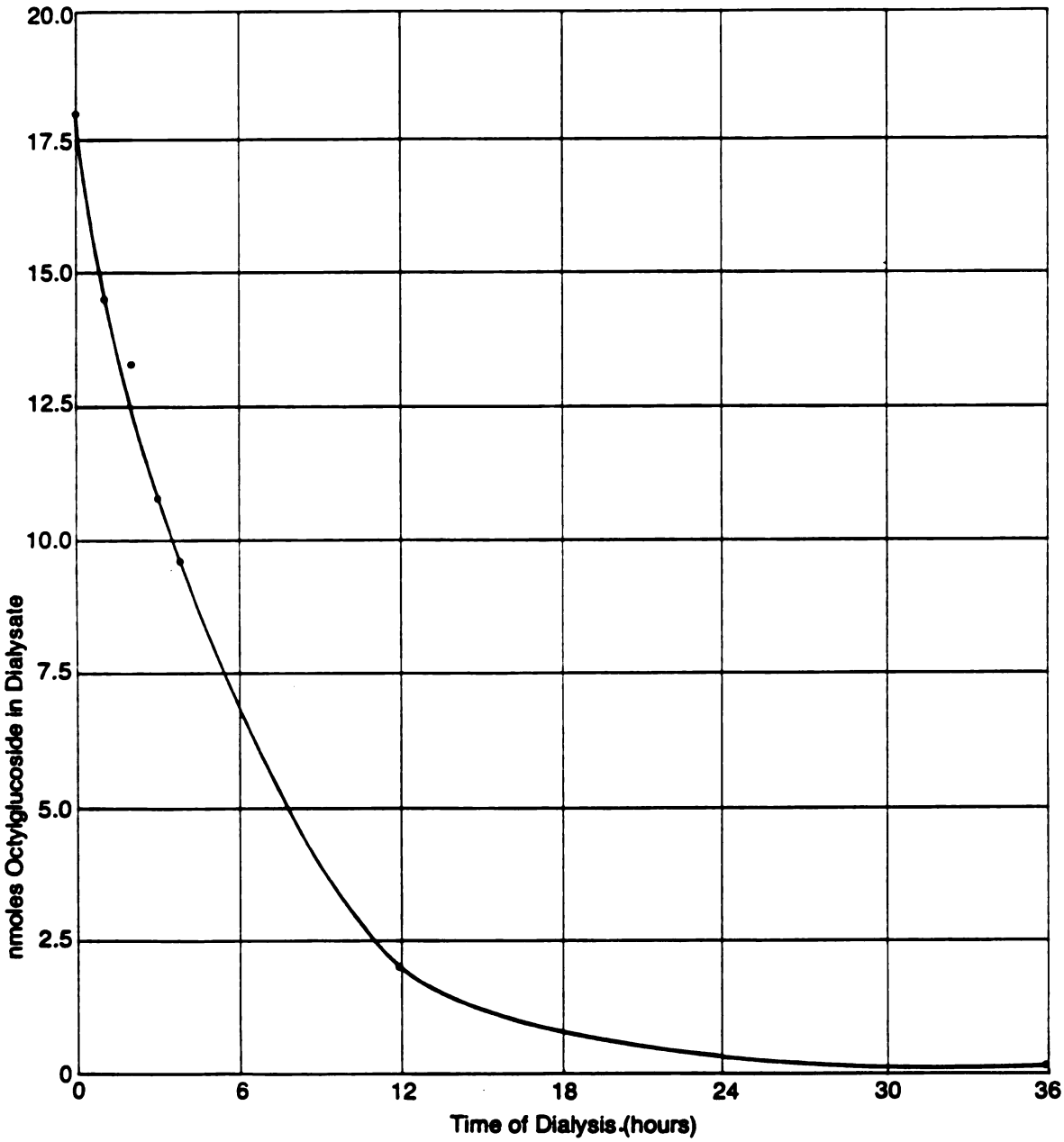

20.0
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0
nmoles Octylglucoside in Dialysate
0
6
12
18
24
30
36
Time of Dialysis.(hours)

Figure 9. THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS p-NITROPHENOL AT VARYING CONCENTRATIONS OF OCTYLGLUCOSIDE.

10 mg per ml microsomal protein was mixed at 4°C in varying concentrations of octylglucoside and 50 mM Tris-HCl pH 7.5, 20% (v/v) glycerol (●) or 50mM Tris-HCl pH 8.0, 100mM kCl, 20% (v/v) glycerol (0) as described under "Methods". Supernatant (super) fractions were prepared by centrifugation at 105,000 X g for 2 1/2 hours.

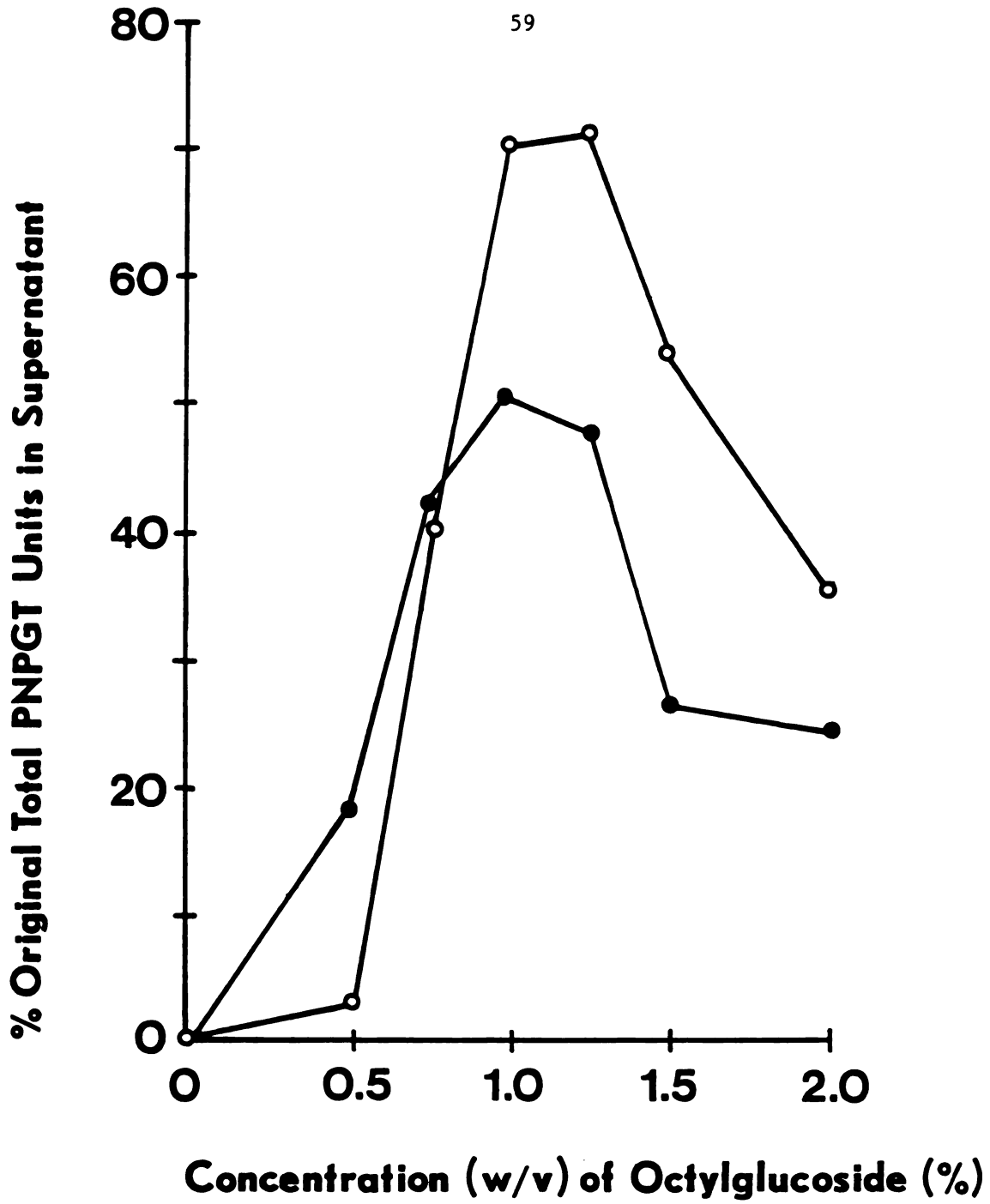

80
60
40
20
0
0
0.5
1.0
1.5
2.0
% Original Total PNPGT Units in Supernatant
Concentration (w/v) of Octylglucoside (%)

TABLE 14. THE EFFECT OF pH AND IONIC STRENGTH ON THE SOLUBILIZATION OF RABBIT INTESTINAL MICROSOMAL PNPGT WITH OCTYLGLUCOSIDE

Treatment	PNPGT Specific Activity nmole p-nitrophenol conjugated/min per mg	Total PNPGT Activity % of original microsomal total PNPGT activity	Total Protein % of original microsomal total protein
-KCl pH 7.4	51	50	24
+KCl	49	61	30
-KCl pH 7.6	50	53	26
-KCl	48	69	34
-KCl pH 7.8	45	61	29
+KCl	51	66	31
-KCl pH 8.0	50	63	29
+KCl	55	70	31

10 mg/ml microsomal protein with a PNPGT specific activity of 24 nmoles p-nitrophenol conjugated/min per mg was mixed at 4°C 50 mM Tris-HCl, 20% (v/v) glycerol, 1mM ME, ± 100mM kCl, 0.002% Chlorhexidine, and 1.0% octylglucoside. Supernatant fractions were prepared by centrifugation at 105,000 X g for 2 1/2 hours and assayed for PNPGT activity and protein as described under "Methods".

(Table 14). With 34 mM (1.0%) octylglucoside in 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 70% of the original microsomal PNPGT activity (Figure 9) and 31% of the original microsomal protein was recovered in the supernatant. The PNPGT activity, in a supernatant containing EDTA and ME was stable for 12 hours at 4°C and had a half life of 3 days. In the absence of EDTA, PNPGT activity had a half life of 12 hours.

Less than half the initial microsomal phenolphthalein GT activity was detected in the 34 mM octylglucoside microsomal suspension. After centrifugation, little or no activity was seen in the supernatant or the pellet.

DEAE Chromatography:

In preliminary experiments, the binding of octylglucoside solubilized PNPGT to DEAE-Sephacel at pH 7.5 was studied. At this pH, with a 50 mM Tris-HCl column buffer, 15% of the original PNPGT activity applied to the column was eluted immediately after the void volume and 15% of the activity was eluted with a linear 0 to 0.25 M KCl gradient. Column fractions immediately after the void volume, had apparent PNPGT specific activities higher than that of the solubilized protein applied to the column. Changing the pH of the column buffers from pH 7.5 to 7.4 or to pH 7.6 did not increase the PNPGT specific activity or yield of column fractions immediately after the void volume. To determine if the activity of column fractions eluted immediately after the void volume could be attributed to overloading the column, those fractions containing PNPGT activity were applied to another DEAE-Sephacel column equilibrated with 50 mM Tris-HCl pH 7.5. Activity was eluted

immediately after the void volume which contained the highest PNPGT activity were pooled and applied to a second DEAE-Sephacel column equilibrated with 20 mM Tris-HCL pH 7.5. Forty percent of the PNPGT applied to this column was eluted with a 0 to 0.15 M KCl gradient. The pooled column fractions from the KCl eluate had an apparent PNPGT specific activity 10 fold higher than that of the original microsomes, with an overall yield of 3% (Table 15).

In subsequent experiments octylglucoside solubilized rabbit intestinal microsomal protein was applied to a DEAE-Sephacel column equilibrated with 50 mM Tris-HCl pH 8.0. Rabbit intestinal microsomes were solubilized in octylglucoside buffered with 50 mM Tris-HCl pH 8.0, containing 100 mM KCl, and centrifuged at 105,000 xg for 2 1/2 hours. The chloride concentration of the 105,000 x g supernatant was approximately 145 mM. At this chloride concentration, PNPGT does not bind to DEAE-Sephacel equilibrated to pH 8.0. However, PNPGT does bind to DEAE-Sephacel at 45 mM chloride. Therefore, the supernatant was diluted to lower the ionic strength. When the 105,000 x g supernatant was diluted 1:2 with 20% glycerol to allow for the binding of PNPGT to DEAE, the solution became turbid, though there was no apparent loss in PNPGT activity. After centrigation of this solution for 2 1/2 hours, at 105,000 xg, 48% of the original PNPGT activity was in the pellet. Therefore, after microsomes were treated with octylglucoside, sonicated and centrifuged, the resulting supernatant was diluted 1:2 with 20% (v/v) glycerl containing enough octylglucoside to prevent turbidity.

TABLE 15. THE PARTIAL PURIFICATION OF RABBIT SMALL INTESTINAL UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS p-NITROPEHNOL AS SUBSTRATE USING SEQUENTIAL DEAE COLUMNS.

Purification Step	Total Protein (mg)	Specific Activity (units/mg protein)	Apparent Purification	Total Activity (units)[a]	Yield (%)
1. Microsomal Fraction	440	12	1.0	5280	100
2. Octylglucoside solubilization supernatant	154	17	1.7	2620	50
3. DEAE-Sephacel 50mM Tris-HCl pH 7.5 column wash	12	34	2.8	480	8
4. DEAE-Sephacel 20mM Tris-HCl pH 7.5 KCl eluate	1	120	10	134	3

al unit of activity represents 1 nmole p-nitrophenolglucuronide formed per min. PNPGT activity was assayed by the method of Lucier et al. (74). Results shown were obtained from the equivalent of 1 1/2 rabbit small intestines. Microsomes were suspended in 50 mM Tris HCl pH 7.5. The suspension was sonicated and centrifuged at 105,000 X g for 2 1/2 hours. The solubilized microsomal protein was applied to a (18.5 cmX 0.8cm) DEAE-Sephacel column (5.5 mg protein/ml Sephacel) equilibrated with : 50mM Tris-HCl pH 7.5, 0.5% octylglucoside, 20% glycerol (v/v), 1mM EDTA, 0.002% chlorhexidine at a flow rate of 9 ml/h. The (8.8cm x 0.3 cm) DEAE-Sephacel column equilibrated with: 20 mM Tris-HCl pH 7.5, 0.1% octylglucoside, 20% glycerol (v/v), 0.25 mM EDTA, 1mM ME, 0.002% Chlorhexidine, (v/v), at a flow rate 9 ml/h. The column was washed with 3 volumes of equilibration buffer. The PNPGT activity was eluted with 5 column volumes of equilibration buffer using a linear 0-0.15 M KCl salt-gradient.

The diluted supernatant was applied to a DEAE-Sephacel column equilibrated with 50 mM Tris-HCl buffer pH 8.0. Figure 10 shows the elution profile of this column. When DEAE column was equilibrated with a buffer containing 0.60% (w/v) octylglucoside, no PNPGT activity was detected immediately after the void volume. PNPGT activity was detected after the void volume when the column was equilibrated to 0.65% (w/v) octylglucoside (Figure 11, Table 16). With a linear 0 to 0.05 M KCl gradient, at pH 8.0, approximately 40% of the transferase activity applied to the column was eluted. The greatest total PNPGT activity was eluted with 0.02 M KCl. Fractions, which had an apparent PNPGT specific activity greater than 200 nmoles p-nitrophenol cojugated per minute per mg protein, were pooled. The PNPGT activity of the pool from the column equilibrated to 0.60% (w/v) octylglucoside, when stored at 4°C, had a half life of about two weeks. The pool from the column equilibrated to 0.65% (w/v) octylglucoside lost 25% of its original PNPGT activity in 24 hours.

No phenolphthalein GT activity was detected in the pooled fractions of the KCl eluate for the DEAE-Sephacel columns equilibrated to 0.60% (w/v) octylglucoside.

Table 15 shows the results of the solubilization and chromatography procedure.

Properties of PNPGT Eluted from the DEAE Column:

The kinetic properties of the PNPGT in the pooled fractions of the KCl eluate from the DEAE column, which was equilibrated to pH 8.0, were examined. Kinetic analyses yielded an apparent V max of 280 nmoles

Figure 10. DEAE-SEPHACEL COLUMN CHROMATOGRAPHY OF RABBIT INTESTINAL p-NITROPHENOL UDP-GLUCURONYLTRANSFERASE ACTIVITY AT pH 8.0, 0.60% OCTYLGLUCOSIDE.

Two hundred mg of solubilized microsomal protein were diluted 1:2 with 20% glycerol containing 0.40% octylglucoside, and applied to a DEAE-Sephacel column (1.35cm X 30cm) equilibrated to 0.60% octylglucoside. Appproximately 100 ml of buffer A was passed through the column before the introduction of a linear 0-0.05 M KCl gradient (--). Fractions (2ml/tube) were collected at a flow rate of 13 ml/h and assayed for UDP-glucuronyltransferase activity towards p-nitrophenol (●), and protein (0), as described under "Methods."

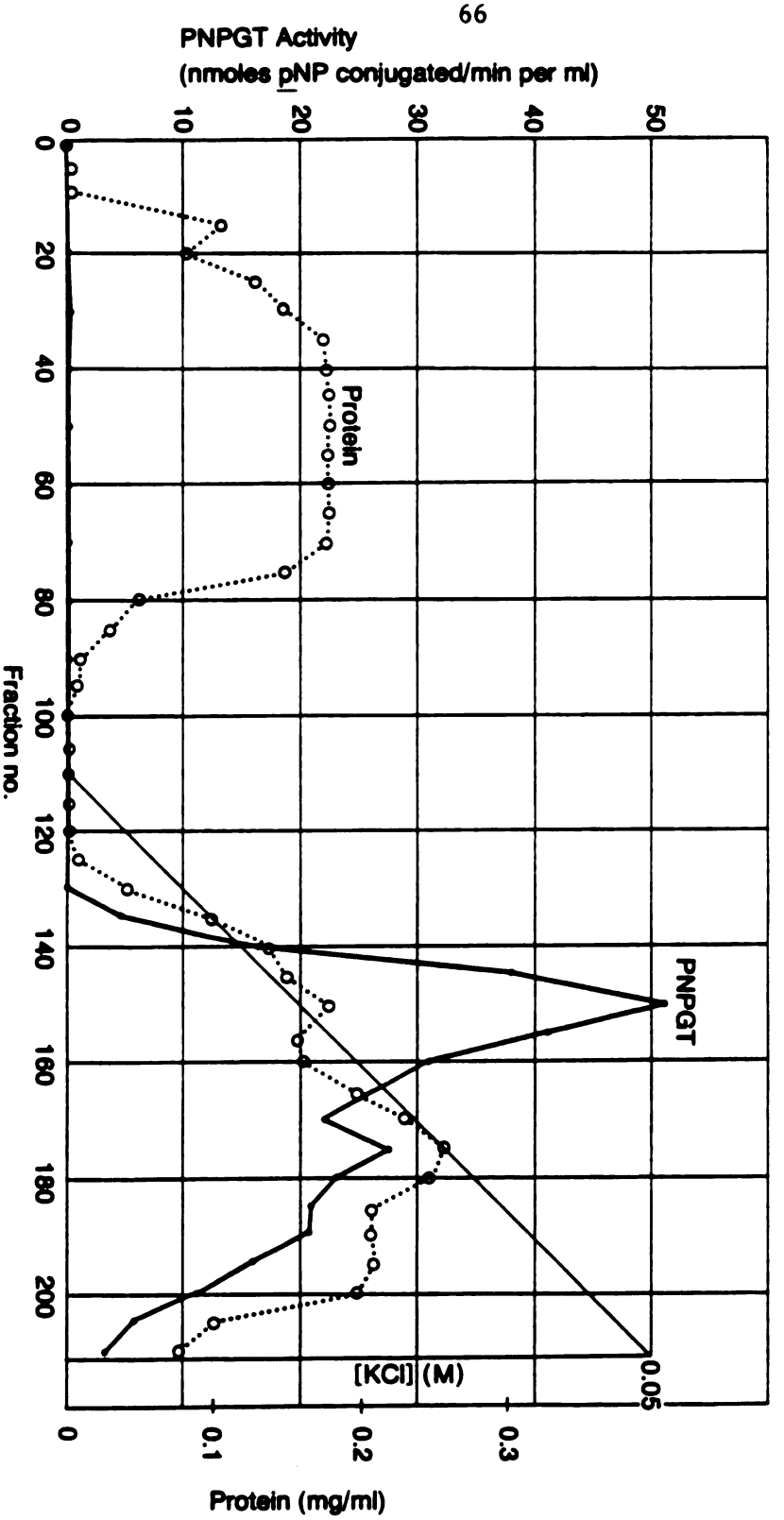

PNPGT Activity
(nmoles pNP conjugated/min per ml)
0
10
20
30
40
50
Protein
PNPGT
Fraction no.
0
20
40
60
80
100
120
140
160
180
200
[KCl] (M)
0.05
0
0.1
0.2
0.3
Protein (mg/ml)

Figure 11. DEAE-SPHACEL COLUMN CHROMATOGRAPHY OF RABBIT INTESTINAL p-NITROPHENOL:UDP-GLUCURONYLTRANSFERASE ACTIVITY AT pH 8.0, 0.65% OCTYLGLUCOSIDE.

Fifty mg of solubilized microsomal protein were diluted 1:2 with 20% glycerol (v/v) containing 0.48% octyglucoside. Approximately 30 ml of buffer A was passed through the column before the introduction of a linear 0 - 0.05 M KCl gradient (--). Fractions (0.7 ml/tube) were collected at a flow rate of 10 ml/h, and assayed for UDP-glucuronlytransfersae activity towards p-nitrophenol (●), protein (0),as described under "Methods".

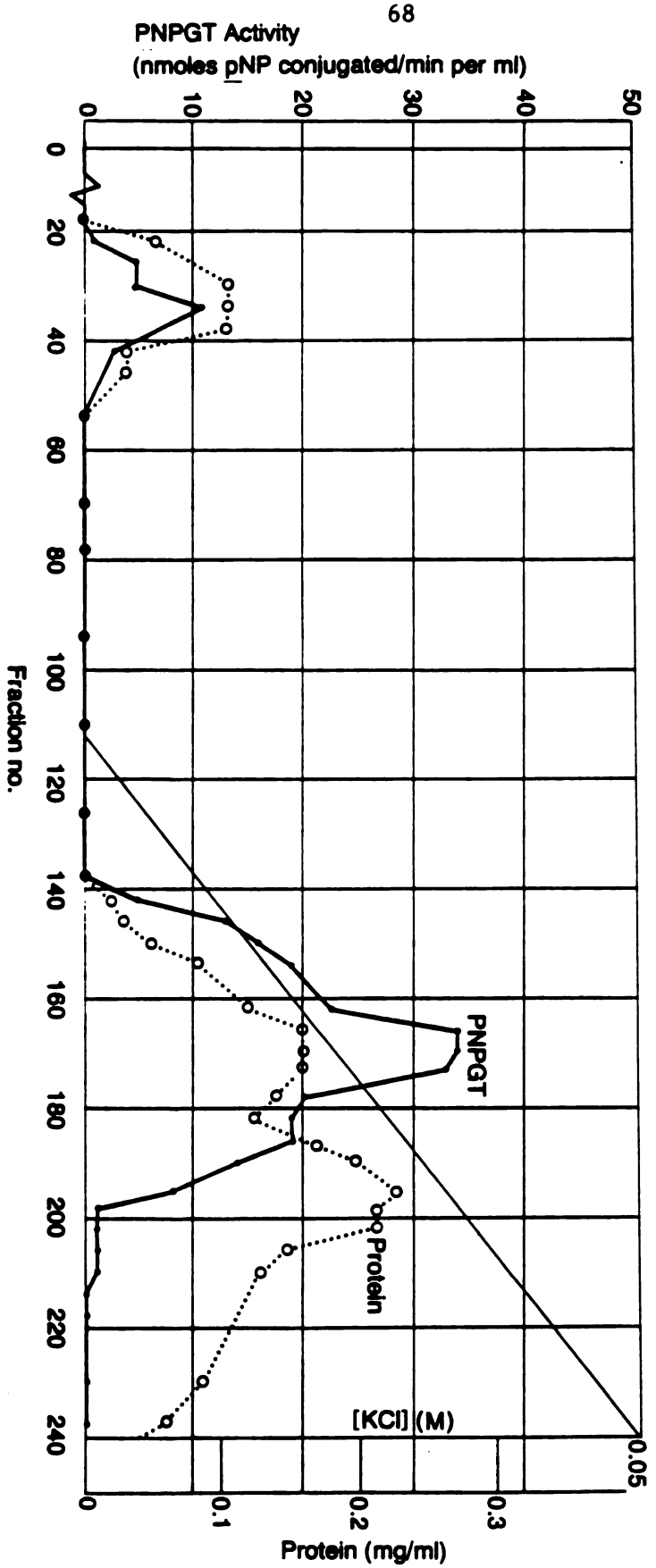

PNPGT Activity
(nmoles pNP conjugated/min per ml)
0
10
20
30
40
50
Fraction no.
0
20
40
60
80
100
120
140
160
180
200
220
240
PNPGT
Protein
[KCl] (M)
0.05
0
0.1
0.2
0.3
Protein (mg/ml)

TABLE 16. THE PARTIAL PURIFICATION OF RABBIT SMALL INTESTINAL UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS p-NITROPEHNOL AS SUBSTRATE.

Purification Step	Total Protein (mg)	Specific Activity (units/mg protein)	Apparent Purification	Total Activity (units)[a]	Yield (%)
1. Microsomal Fraction	152	27	1.0	4100	100
2. Octylglucoside solubilization					
supernatant	59	58	2.1	3440	84
3. DEAE-Sephacel					
column wash	0.3	100	3.7	30	1
KCl eluate	1.9	260	9.6	495	12

a 1 unit of activity represents 1 nmole p-nitrophenolglucuronide formed per minute. PNPGT activity was assayed by the modified method of Lucier et al. (74). The DEAE-Sephacel column (pH 8.0) was equilibrated to 0.65% octylglucoside. Results shown were obtained from the equivalent of half a rabbit small intestine.

p-nitrophenol conjugated per minute per mg of protein and an apparent Km for PNP of 0.2 mM at a UDPGA concentration of 3 mM (Figure 12). An apparent Km of 0.4 mM was obtained for UDPGA at a 2 mM PNP concentration (Figure 13). UDP had a strong inhibitory effect on PNPGT activity. When UDPGA was the variable substrate and the PNP concentration was fixed at 2.0 mM, UDP at concentrations between 0.5 and 10 mM produced inhibition competitive with UDPGA (Figure 13).

A sample of the pooled fractions, from the DEAE column KCl eluate, was tested with liposomes to determine if the addition of lipid would enhance the PNPGT activity. Neither intestinal liposomes nor egg yolk phosphatidylcholine in lipid to protein (w/w) ratios of 0.2, 1.2, 2.3, 5.8 and 12 had an effect on PNPGT specific activity. Crude soybean phospolipid at lipid to protein ratios of 0.5 to 15 also had no effect on PNPGT specific activity. After centrifugation of the phosphatidylcholine - enzyme suspension for one hour at 105,000 x g, 78% of the PNPGT activity was associated with the lipid pellet.

Egg yolk phosphatidylcholine liposomes did not increase the PNPGT activity of the DEAE column fractions immediately after the void volume.

Changes in the protein to octylglucoside ratio produced pronounced effects on the PNPGT activity of the pooled fractions immediately after the void volume of the DEAE column. At protein to octylglucoside ratios (w/w) below 0.03 PNPGT was completely inactivated (Figure 14).

Figure 12. DOUBLE RECIPROCAL PLOT OF INITIAL RATES OF UDP-GLUCURONYL-TRANSFERASE IN THE DEAE-SEPHACEL COLUMN KCl ELUATE AS A FUNCTION OF VARYING CONCENTRATIONS OF p-NITROPHENOL.

Each point is based on initial rate determination of UDP-glucurnyltransferase in the pooled fractions of the DEAE-Sephacel column (pH 8.0) KCl eluate as described under "Methods" and expressed as umoles of p-nitrophenol conjugated per min per mg of eluate protein. The concentration of UDP-glucuronic acid was 3mM.

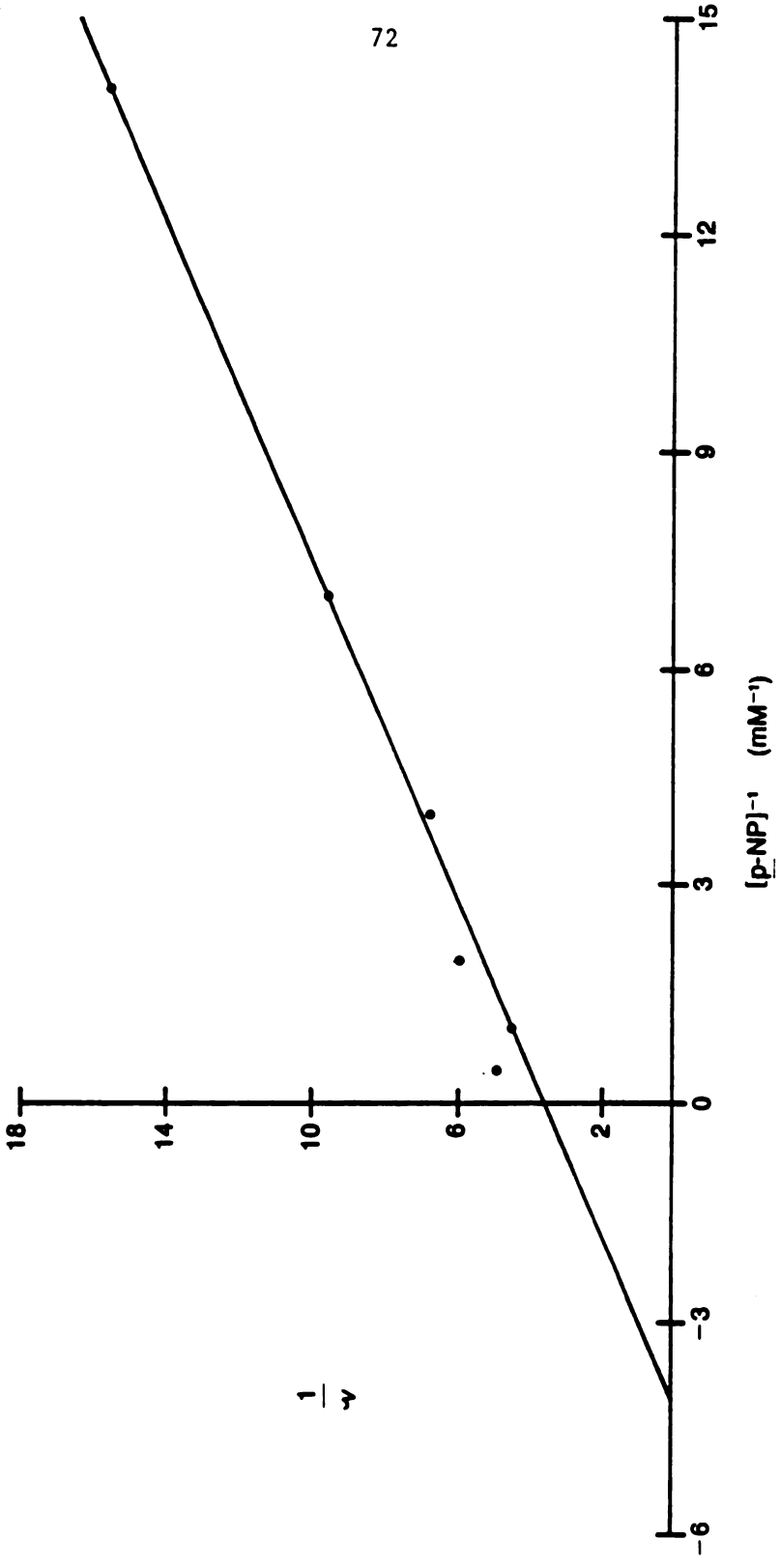

18
14
10
6
2
0
-3
-6
3
6
9
12
15
[p-NP]⁻¹ (mM⁻¹)
1/v

Figure 13. DOUBLE RECIPROCAL PLOTS OF INITIAL RATES OF UDP-GLUCURONYL-TRANSFERASE IN THE DEAE-SEPHACEL COLUMN KCl ELUATE AS A FUNCTION OF VARYING CONCENTRATIONS OF UDP-GLUCURONIC ACID. AT DIFFERENT FIXED CONCENTRATIONS OF UDP.

Each point is based on initial rate determination of UDP-glucuronyltransferease activity of the pooled fractions of a DEAE-Sephacel (pH 8.0) KCl eluate, as described under "Methods", and expressed as umoles of p-nitrophenol conjugated per min per mg of eluate protein. The concentration of p-nitrophenol was 2.0mM. The concentration of p-nitrophenol was 2.0mM. The concentrations of UDP were 0.5mM(0), 2.0mM (■), 4.0mM (▲).

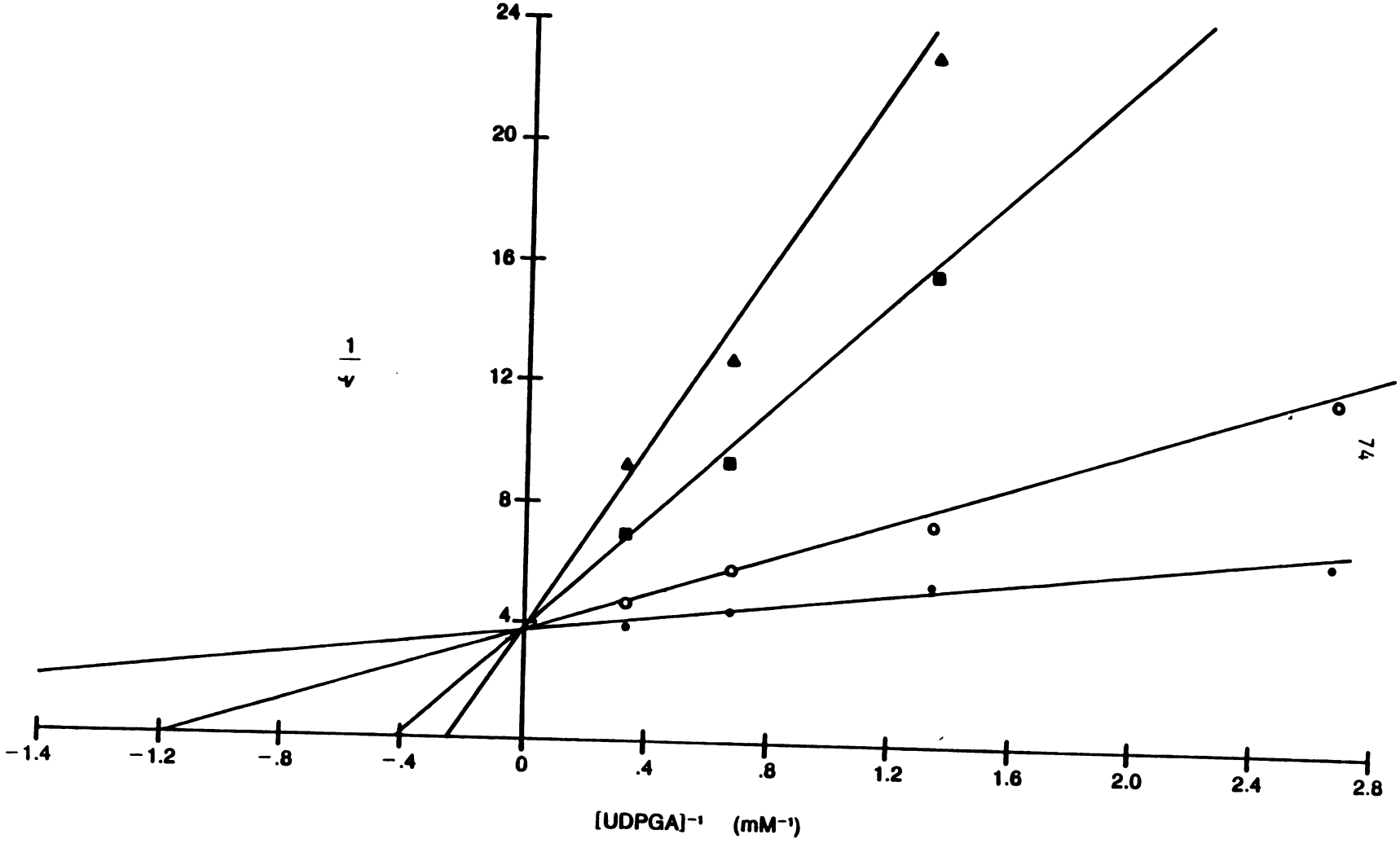
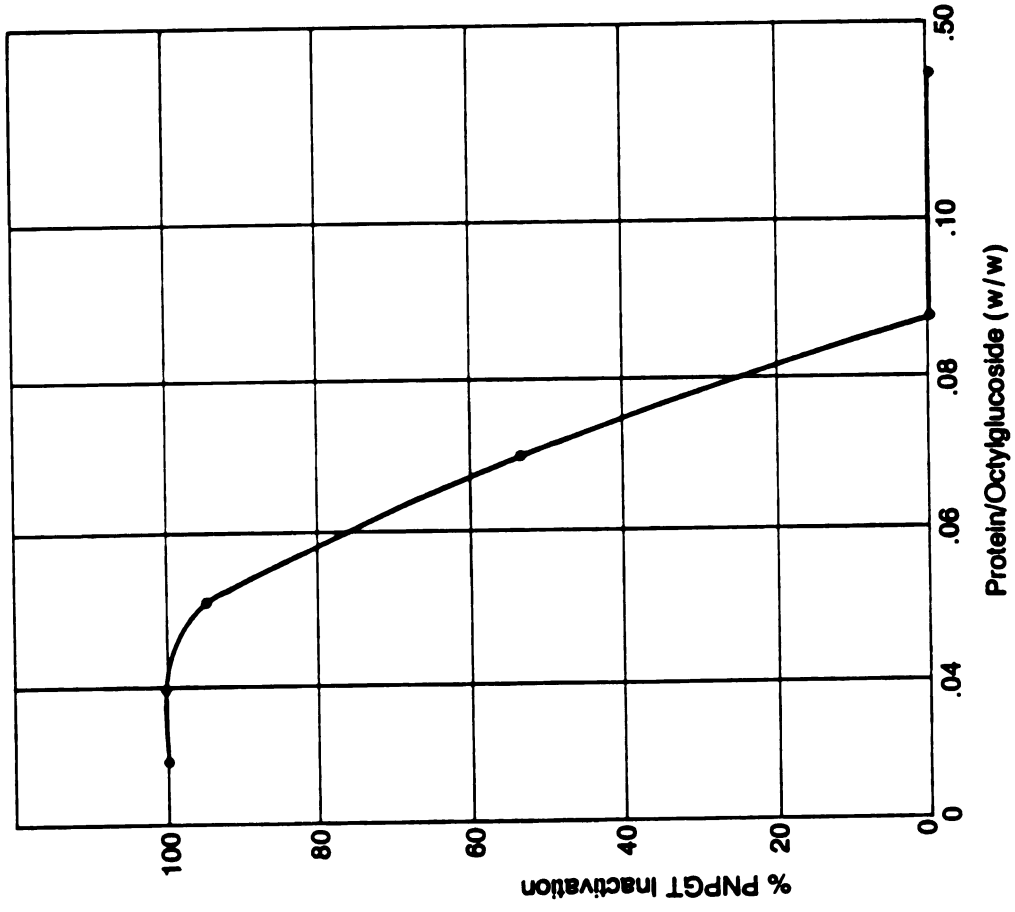

24
20
16
12
8
4
1/v
−1.4
−1.2
−.8
−.4
0
.4
.8
1.2
1.6
2.0
2.4
2.8
[UDPGA]⁻¹ (mM⁻¹)
74

Figure 14. THE INACTIVATION OF UDP-GLUCURONYLTRANSFERASE ACTIVITY TOWARDS p-NITROPHENOL AS A FUNCTION OF THE PROTEIN TO OCTYLGLUCOSIDE RATIO.

Octylglucoside in varying amounts was added to pooled fractions from a DEAE-Sephacel column, which were diluted with column buffer containing no octylglucoside, to varying concentrations of protein. The PNPGT activity was compared with the PNPGT ativity of undiluted pooled fractions, containing no additional octylglucoside. As a point of reference, these undiluted pooled fractions, were regarded as having no inactivation. The PNPGT activity and protein were assayed as described under "Methods".


% PNPGT Inactivation
0
20
40
60
80
100
Protein/Octylglucoside (w/w)
0
.04
.06
.08
.10
.50

When the protein to octylglucoside ratio of the pool of the KCl eluate from the DEAE column was increased two fold by ultrafiltration the solution became turbid. After centrifugation at 150,000 x g for 1 1/2 hours, the pellet had 37% of the original PNPGT activity while the supernatant had 7% of the original activity.

The pool of KCl eluate from the DEAE column could be diluted with equilibration buffer with no octylglucoside, without apparent turbidity or an apparent loss in PNPGT activity.

Gel Electrophoresis:

Figure 15 shows the polypeptides which were stained after the gel electorphoresis of various fractions from the purification procedures. The pattern produced from the supernatant of the solubilization process lacked two major bands which were in the patterns produced from the intesinal microsomes. Most of the polypeptide bands with molecular weights greater than 65,000 in the pattern from the intestinal microsomes were absent or not as darkly stained as those in the pattern produced from the supernatant. The pattern produced on the slab gel, from the pooled fractions of the DEAE column eluate had 5 major polypeptide bands. By comparison with standard proteins of known subunit molecular weight, these bands exhibited molecular weights estimated at 60,000, 55,000, 48,000 and 46,000, with a very broad band at 52,000. The pattern produced from the pooled fractions lacked most of the polypeptide bands with molecular weights greater than 65,000 present in the pattern from the intestinal microsomes. Polypeptide bands with estimated molecular weights of 60,000, 55,000 and 48,000

Figure 15. SDS DISC PAGE SLAB GEL.

A. 3-MC pretreated rat liver microsomes.

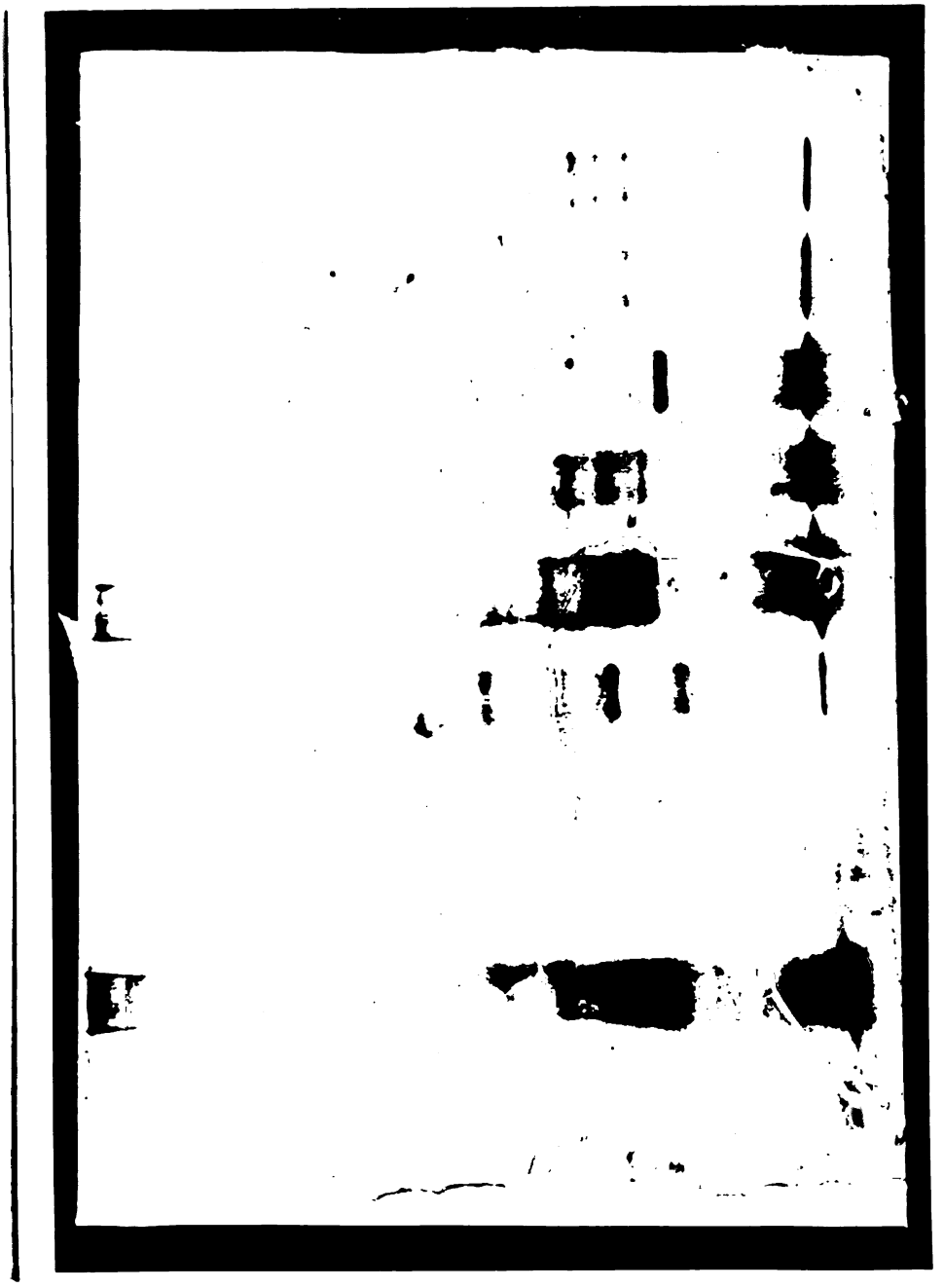
B. Rat liver microsomes.

C. Rabbit intestinal microsomes.

D. 105,000 x g supernatant from octylglucoside solubilization at pH 8.0 of rabbit intestinal microsomes.

E. Pooled fractions from KCl eluate of DEAE-Sephacel column pH 8.0, 0.60% octylglucoside

F. Molecular weight standards:
Ovalbumin M.W. 45,000; glutamate dehydrogenase M.W. 53,000; catalase M.W. 58,000; bovine serum albumin M.W. 66,000


F E D C B A

were darker in the pattern produced from the pooled fractions than in that produced from the supernatant. In turn, these bands were darker in the pattern produced from the supernatant than that from the microsomes.

DISCUSSION

The present work was undertaken to establish procedures for the purification of PNPGT from the rabbit small intestine. The first step in the purification scheme is the preparation of intestinal microsomes. A relatively simple procedure was developed for the large scale preparation of intestinal microsomes with stable PNPGT activity.

In these studies it was found that the activity of intestinal microsomal PNPGT measured in a particular assay mixture reflects the concentration and type of detergent in the assay mixture. From figures 3-5, it is apparent that a slight change in the concentration of a detergent can result in a dramatic change in intestinal microsomal PNPGT activity. When compared to other detergents, at the various concentrations studied, Triton X-100, Renex 690, and octylglucoside activated PNPGT to the greatest extent.

Illing and House (81) studied the effect of several detergents on rat liver UDP-glucuronyltransferase activity towards fixed concentrations of five phenolic acceptor substrates with widely varying octanol buffer (pH 7.4) partition coefficents. These phenolic substrates have been postulated to be conjugated by one UDP-glucuronyltransferase, "GT_1" (82). With some detergents the extent of activation was dependent on the substrate partition coefficient. While other detergents, such as Triton X-100, acted independently of this coefficient. Based on these findings, Illing and House (81) speculated that Triton X-100 might affect the active site of

"GT_1" (PNPGT) and thus might not be suitable as a solubilizing agent.

Intestinal microsomes with relatively high PNPGT specific activity were obtained from the duodenum. Intestinal microsomal PNPGT activity decreased rapidly from the duodenum to the ileum. This is in agreement with the findings of Aitio et al. (83). On the other hand, Lasker (84) reported that the capacity for the glucuronidation of the artificial steroid, diethylstilbesterol, was uniformily distributed throughout the small intestine. In light of Tukey's conclusion that in the liver the form of UDPGT which metabolizes the steroid, estrone, is distinct from the form which metabolizes p-nitrophenol, (64) it is possible that these two distinct forms of UDPGT exist in the intestine as well. The form which conjugates estrone might also conjugate diethyestilbesterol, and have a distribution pattern along the small intestine distinct from that of PNPGT.

Since the duodenum is the region of the small intestine in which the greatest absorption of dietary xenobiotics occurs, there would be a physiological need for greater xenobiotic metabolizing activity in the duodenum. Several enzymes, which like PNPGT are instrumental in the metabolism of xenobiotics, have a distribution along the length of the intestine, similar to that of PNPGT. Cytochrome P-450 dependent mixed function oxidase activity has been found to be substantially higher in the duodenum than in the ileum (85). Epoxide hyclrolase was reported to have a relatively higher activity in the duodenum (86). The activity of the cytoplasmic enzyme, glutathione-S-transferase, which

conjugates many xenobiotics with glutathione, was shown to be four fold higher in the duodenum than in the ileum (87). Even though the highest PNPGT specific activity is found in the duodenum, the entire length of the rabbit small intestine was used to obtain microsomes in order to increase the total enzyme yield. The specific activity of PNPGT was found to be greater in microsomes derived from the intestinal mucosa than those derived from the whole intestine. These findings are in agreement with the work of several investigators who by using mucosal scrapings of the small intestine have shown that the epthelium was the principal site for intestinal drug metabolism (88,89). Hoensch et al. found that the specific activity of the drug metabolizing microsomal mixed function oxidase system derived from the intestinal mucosa was higher in the upper villous cells than in the epithelial crypt cells (90). Although the specific activity of PNPGT was higher in microsomes obtained from the mucosa, in the partial purification of rabbit intestinal PNPGT, microsomes were obtained from the whole intestine. Microsomal preparations using mucosal scrapings were time consuming and yielded less PNPGT activity. For studies not involving the purification of a microsomal enzyme, the microsomal preparation procedure developed by Shirkey et al. might be suitable (91).

It was found that fasting rabbits for 24 hours resulted in a decrease in the total microsomal PNPGT activity, although the specific activity was slightly increased. Similar results were reported by Marselos et al. (92).

One common means of increasing the total activity of microsomal enzymes is to treat animals with certain chemicals, such as phenobarbital or 3-methylcholanthree, which induce the synthesis of the enzymes. Phenobarbital is one of many drugs that stimulate various pathways of metabolism in liver microsomes, whereas polycyclic aromatic hydrocarbons, such as 3-MC, stimulate a more limited number of enzymes (93). An inducer which functions about the same as 3-MC, but which is not a carcinogen, is β-naphthaflavone. An inducer with properties of both penobarbital and 3-MC is the Firemaster mixture of PBBs (94). Unlike phenobarbital whose inductive effect may be restriced to the liver (95), 3-MC has been shown to be a good inducer of rat duodenal PNPGT activity (96).

Pretreatment of rabbits with β-naphthaflavone or with Firemaster PBBs did not increase the total intestinal microsomal PNPGT activity. SDS polyacrylamide gel electrophoresis revealed that some proteins of the intestinal microsomes obtained from rabbits pretreated with PBBs were induced as the polypeptide pattern produced by these microsomes differed from that produced by the microsomes obtained from untreated rabbits. The apparent lack of induction might be attributed to species differences in the stimulation of intestinal enzymes. Treatment of rats with Firemaster PBBs induced rat duodenal PNPGT activity two fold but did not induce rabbit PNPGT. Likewise, treatment of rabbits with 3-MC did not increase intestinal mixed function oxidase activity, but did increase the intestinal mixed function oxidase activity of rats (97).

In these studies, when rabbits were pretreated with phenobarbital, the intestinal microsomal PNPGT total activity was not increased though the specific activity increased slightly. This parallels the findings of Aitio et al., who reported that phenobarbital, whether given in the drinkng water or given i.p., did not increase UDPGT activity (98), and parallels the findings of Bock et al. (99) as well.

Once a procedure for the isolation of intestinal microsomes had been established, a procedure was developed for the solubilization of rabbit intestinal PNPGT from intestinal microsomes. The selection of a detergent for the solubilization of PNPGT was based in part on its non-denaturative properties. Because it is a relatively mild detergent, octylglucoside was chosen for the solubilization of PNPGT. Another factor in choosing octylglucoside was that octylglucoside was readily dialyzable in the presence of membrane protein, confirming the previous work of Baron and Thompson (100). Being readily dialyzable, octylglucoside could be more readily removed from a purified PNPGT preparation which might be valuable in reconstitution studies with this preparation. Other nonionic detergents, such as Lubrol WX, would probably be more difficult to remove.

Sato et al. (101) purified rabbit liver microsomal PNPGT to apparent homogeneity with an overall yield of 10-15%, using the anionic detergent, sodium chloate. However, in these studies, sodium cholate was found to inactivate rabbit intestinal microsomal PNPGT even at very low concentrations.

Emulgen 911, a nonionic polyoxyethylene ether detergent, has been used for the successful purification of rabbit liver PNPGT (64). In these studies however, when rabbit intestinal PNPGT was solubilized with Emulgen 911, all PNPGT activity was lost within 12 hours. Some of this loss of activity may have been due to the 3 mM peroxide contamination in the detergent. It was found that 3 mM cumene peroxide resulted in a complete loss of PNPGT activity after one day. Peroxide contamination frequently occurs in nonionic polyoxyethylene ether detergents.

The two fold increase in intestinal PNPGT specific activity that was found upon solubilization of intestinal microsomes with octylglucoside indicated that the detergent was slightly selective in the extraction of PNPGT. This was also indicated by polyacrylamide gel electrophoresis, as the polypeptide pattern produced from the supernatant of the solubilization process lacked many of the bands produced from the unsolubilized intestinal microsomes.

Along with detergents, mechanical means can be used to aid in the solubilization of membrane proteins. Sonication is widely used to solubilize microsomal protein, even though it usually produces a heterogeneous mixture of membrane fragments (102).

The Purification Scheme

Successful purifications of rabbit and rat liver PNPGT have employed UDP-affinity chromatography (60-64, 72, 103). It is likely that UDP-affinity chromatography could be used successfully for the purification of rabbit intestinal PNPGT as well. It was found that UDP

is strongly inhibitory to rabbit intestinal PNPGT, and therefore it would be expected that PNPGT could bind well to the UDP meioty of an affinity resin. In the purifications of liver transferases (60-64, 72, 103), the total detergent solubilized microsomal protein was not applied to the UDP-affinity resin. Rather, a transferase preparation, partially purified by DEAE chromatography, was applied to the UDP-affinity resin. Gorski and Kasper (103) reported that better results were achieved when a partially purified transferase preparation was applied to the UDP-affinity resin. They speculated that this may have been because DEAE chromatography of the solubilized microsomal protein reduced the amount of nonspecific binding on the affinity gel and eliminated degradative enzymes which could remove the UDP moiety from the support. DEAE chromatography might, by separating different UDP-glucuronyltransferases, reduce the competition between these transferases for the UDP moiety and result in a more homogeneous transferase preparation. Thus, the use of DEAE chromatography as one of the first steps in the purification of rabbit intestinal PNPGT was studied.

That DEAE-chromatography of octylglucoside solubilized intestinal microsomes removed many of the contaminating and detrimental proteins is suggested by the increased apparent PNPGT specific activity, the enhanced PNPGT stability and the SDS PAGE gel of the pooled fractions from the DEAE column KCl eluate.

The Yield of PNPGT Activity After DEAE Chromatography

Although DEAE chromatography has been a useful step in the purification of rat and rabbit liver PNPGT, the yield of PNPGT activity has been relatively low. Both Burchell (62) and Tukey et al. (57) used DEAE columns equilibrated with Tris buffer pH 8.0, and eluted PNPGT with a KCl gradient. In their purification, about 16% of the PNPGT units applied to the DEAE column were eluted with the pooled fractions of the KCl eluate,with about a four fold increase in the specific activity.

In the partial purification of rabbit intestinal PNPGT, about 20% of the rabbit intestinal PNPGT units applied to a DEAE column equilibrated to pH 8.0, were eluted with the pooled fractions of the KCl eluate, with a five and a half fold increase in PNPGT specific activity.

The low yield of intestinal PNPGT activity in the KCl eluate after DEAE chromatography, might be due to the degradation, the inactivation, and/or the inhibition of PNPGT. Inactivation by octylglucoside might contribute to the low recovery of intestinal PNPGT activity. As shown in Figure 13, the octylglucoside to protein ratio can have a pronounced effect on PNPGT activity. Baron and Thompson (100) described that octylglucoside had a "critical denaturing concentration", a concentration above which the activity of an enzyme was extensively and irreversibly lost. Some fractions from the DEAE column had protein to octylglucoside ratios which could be inactivating. A few column fractions had protein to octylglucoside ratios less than the 0.03 (w/w) which was found to be completely inactivating for the PNPGT activity of

the fractions immediately after the void volume. Still, other fractions might have had protein to octylglucoside ratios which, while not large enough to have caused visible turbidity, could have been large enough to produce amorphous protein aggregation. Such aggregation, in turn, might have hindered the resolution of intestinal PNPGT by DEAE chromatography.

Partial delipidation of intestinal PNPGT might have contributed to the low yield of PNPGT either by promoting enzyme inactivation or by diminishing enzyme stability. It is not yet known if delipidation could lead to the inactivation of rabbit intestinal PNPGT. Rat liver PNPGT was reported by Jansen et al. to be irreversibly inactivated by delipidation (104). Several investigators have reported that delipidation of partially purified liver PNPGT preparations resulted in a marked diminuation of activity, which was then restored with the addition of certain classes of phospholipids, phosphatidylcholines being the most effective in the restoration of activity (105, 106). In studies with intestinal PNPGT, it was found that incubation of partially purified intestinal PNPGT with egg yolk phosphatidylcholine, crude soybean phospholipid, or intestinal microsomal lipid did not increase PNPGT activity. Still it can not be ruled out that some PNPGT activity from the DEAE-Sephacel column might have been lost due to inactiviation resulting from the partial delipidation of the enzyme. It is not yet known whether or not delipidation could lead to the diminished stability of rabbit intestinal PNPGT. It was found that the PNPGT activity of the solubilized intestinal microsomal preparation had

a half life of 12 hours. Thus some activity was lost during the application of the solubilized preparation to the DEAE column. Delipidation might have contributed to this instability. With rat liver PNPGT, Gorski and Kasper reported that the delipidation enzyme showed irreversible inactivation, the rate of which could be slowed by combining the enzyme with ethylene glycol and/or liposomes (106).

The low yield of rabbit intestinal PNPGT activity might have been due in part to some of the PNPGT remaining bound to the DEAE column. After PNPGT activity was eluted with a 0 to 0.05 M KCl gradient, more PNPGT, with a low specific activity, was eluted with a 0.05 to 0.3 M KCl gradient.

The Elution Profile of UDPGT Activity After DEAE Column Chromatography:

DEAE column chromatography of both liver and intestinal PNPGT produced similar elution profiles. With DEAE-cellulose column chromatography (pH 8.0) of both rabbit and rat liver PNPGT (957,62), a peak of PNPGT activity eluted at low KCl concentrations. These two peaks exhibited different glucuronidation activities towards alternate substrates. In the partial purification of rabbit intestinal PNPGT, a peak of rabbit intestinal PNPGT activity was eluted immediately after the void volume when the DEAE-column was equilibrated to .60%. The two peaks of PNPGT activity may be due to two p-nitrophenol: VDP-glucuronyltransferases which have protein structures which are distinct from each other or due to two forms of a single PNPGT, such as an active lipoprotein complex with both size and charge heterogeneity. Corski and Kasper (106) purified rat liver PNPGT which demonstrated

three isoelectric points upon isoelectric focusing but which produced a single polypeptide pattern upon SDS-polyacrylamide gel electrophoresis.

Phenolphthalein GT and PNPGT

Wishart (67) postulated that in the liver there was a UDP-glucronyltransferase which conjugated p-nitrophenol which was distinct from a glucuronyltransferase which conjugated phenolphthalein GT. During the purification procedure for rabbit intestinal PNPGT, there were several indications that there may indeed be two distinct forms of UDPGT. Phenolphthalein GT was inactivated by the octylglucoside solubilization procedure, while PNPGT was activated. No phenolphthalein GT was detected in the pooled fractions of the kCl eluate from the DEAE column which had a high PNPGT specific activity.

SUMMARY

A procedure for the partial purification of PNPGT from rabbit small intestinal microsomes was developed, which involved the preparation of intestinal microsomes, the solubilization of PNPGT from these microsomes and the DEAE column chromatography of the solubilized enzyme. This procedure was suitable for the first stages of a scheme to purify the enzyme to homogeneity.

Microsomes obtained from the first 250 cm of the small intestine distal to the stomach pylorus had an average PNPGT specific activity of 25 nmoles p-nitrophenol conjugated per minute per mg of protein, and were stable for six months when stored at 20°C.

The distribution of PNPGT in the intestine was studied. The highest PNPGT specific activity was in the duodenum. Microsomes obtained from mucosal scraping of the intestine had a higher PNPGT specific activity than microsomes from the whole intestine.

Microsomes derived from rabbits which were treated with penobarbital, β-naphthaflavone, or PBBs did not have a higher PNPGT total or specific activity than microsomes derived from untreated rabbits.

Octylglucoside was in the solubilization of microsomal PNPGT as it is a mild nonionic detergent which is readily dialyzable. Microsomal solubilization was enhanced by increasing the ionic strength and alkalinity of the solubilization buffer. With a pH 8.0 solubilization buffer with 145 mM chloride and 34 mM of the microsomal PNPGT activity was recovered in the 105,000 x g supernatant, with a two fold increase in the apparent specific activity.

The actylglucoside-microsomal preparation was diluted to lower the chloride concentration and applied to a DEAE column equlilibrated to pH 8.0. When the column was equilibrated to 0.65% octylglucoside, one peak of PNPGT activity was seen immediately after the void volume and another peak was eluted with approximately 0.02 MKCI. However, when the column was equilibrated to 0.60% the peak immediately after the void volume was not detected. Pooled column fractions from the KCl eluate had an average specific activity of 250 nmoles p-nitrophenol conjugated per minute per mg of microsomal protein.

Incubation of the pooled column fractions with phosphatidylcholine or with intestinal microsomal liposomes did not increse the PNPGT activity.

The protein to octylglucoside ratio was a critical factor. Below a certain level, PNPGT was completely inactivated, above a certain there was visible turbidity and presumably protein aggregation.

Phenolphthalein GT did not co-purify with PNPGT.

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