METHODS OF
BIOCHEMICAL ANALYSIS

Edited by **DAVID GLICK**
Stanford University Medical School
Stanford, California

VOLUME 21

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PREFACE

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever-expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need in order for material achievement to keep in sight of the advance of useful ideas.

The volumes in this series are designed to try to meet the need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological, and if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well-established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.

The general plan followed in the organization of the individual chapters

is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analysis.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the Editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details, a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who either have originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Borad and the Editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

DAVID GLICK

CONTENTS

Techniques for the Characterization of UDP-Glucuronyltransferase, Glucose-6-Phosphatase, and Other Tightly-Bound Microsomal Enzymes

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I. INTRODUCTION

Since the isolation and identification of microsomes (1,2) relatively little progress has been made in characterizing most of the enzymatic systems contained within these structures. Problems of multiplicity, substrate specificity, mechanism of action, and dynamics of regulation remain unresolved. Although the classical technique for careful study of an enzyme begins with purification, the problem of removing microsomal enzymes from their attachment to membranes has contributed to the difficulties in working with these enzymes. It has also become apparent recently that the catalytic properties of many tightly bound microsomal enzymes depend on interactions with their microsomal environments (3–10). Hence the proper study of microsomal enzymes actually requires that they be characterized in experiments with intact microsomes. Investigators therefore face problems of experimental design which do not arise or are avoided easily when working with unbound cytoplasmic enzymes. For example, microsomal enzymes exist in a heterogeneous particle containing enzymes which may metabolize products and substrates in pathways other than the one due to the enzyme of interest. There are also problems in the preparation and storage of microsomes since physical or chemical agents which alter the microsomal lipids,

such as endogenous phospholipase A, can modify the properties of membrane-bound enzymes. The dietary history, hormonal balance, and age of animals also may influence the kinetic parameters of microsomal enzymes. In addition, substrates for many microsomal enzymes have limited solubility in H_2O, or are amphipathic and activate or inactivate microsomal enzymes because of nonspecific effects on the microsomal membrane.

It is not the purpose of this review to cover currently available techniques for the assay of microsomal enzymes in an encyclopedic way. Rather, emphasis is placed on the problems encountered in characterizing the properties of tightly bound enzymes in liver microsomes by assaying in the presence of a complex mixture of other microsomal enzymes, and on the ways in which most of these difficulties can be dealt with and meaningful assays for many microsomal enzymes developed. The assays of UDP glucuronyltransferase and glucose-6-phosphatase are presented in more detail, since these two tightly bound microsomal enzymes have been studied most extensively from the points of view of the number of separate species of protein needed to account for homologous reactions with different substates, the regulatory importance of protein-phospholipid interactions, and the determination of exact kinetic constants and kinetic mechanisms. The detailed descriptions of assay techniques for UDP glucuronyltransferase and glucose-6-phosphatase have general applicability to the problems likely to be encountered in examining the properties of other tightly bound microsomal enzymes. In addition to assay techniques, the closely related problems of preparation, storage, and subfractionation of microsomes, the techniques for studying the effects of treatment with phospholipases and detergents on the properties of tightly bound microsomal enzymes, and substrate forms are discussed in detail.

II. SPECIFIC ASSAYS OF MICROSOMAL ENZYMES

1. UPD-Glucuronyltransferase

A. BACKGROUND

Excretion of exogenous compounds as sugar conjugates was observed more than 100 years ago (11) in studies which led eventually to the elucidation of glucuronic acid as the conjugated sugar derivative. Work with intact organs, tissue slices, and homogenates, as well as the availability of ^{14}C-labeled sugars, established that glucose was the precursor of the glucuronic acid and that an "active factor," later shown to be UDP-glucuronic acid, was required for glucuronide synthesis in liver homogenates. It is now known that a variety of compounds are metabolized according to [1]:

$$\text{UDP-GA} + \text{RH} = \text{R-GA} + \text{UDP} \qquad [1]$$

where RH is an organic acid, a phenol, or an amine. Reaction [1], catalyzed by UDP glucuronyltransferase, is important for the detoxification of pharmacologic agents and several endogenously produced compounds in the microsomal fraction of the cell; activity is highest in liver but is present also in skin, kidney, intestinal mucosa, and some endocrine organs (12).

B. CHOICE OF AGLYCONE FOR ASSAY OF UDP GLUCURONYLTRANSFERASE

How many species of UDP glucuronyltransferase exist in liver microsomes is not known, but it is certain that all *O*-glucuronides are not synthesized by a single enzyme. Evidence obtained in this laboratory from kinetic studies of UDP glucuronyltransferase (13) and the properties of the —SH groups of this enzyme indicate that *o*-aminophenol and *p*-nitrophenol are glucuronidated by different enzymes, and it is clear that *o*-aminobenzoate does not share a common aglycone binding site with either of the other substrates. Therefore, assays of UDP glucuronyltransferase conducted with these glucuronyl acceptors, and probably bilirubin as well, do not measure the activity of the same enzyme. On the other hand, it is not known at this time what other substrates, if any, are glucuronidated by the *p*-nitrophenol, *o*-aminophenol, and *o*-aminobenzoate metabolizing forms of UDP glucuronyltransferase, and in general how many substrate-specific forms of UDP glucuronyltransferase exist. Although a discussion of the technical aspects of the problem of multiplicity is beyond the scope of this review, it should be stressed that with aglycones other than those listed above one cannot be certain what UDP glucuronyltransferase enzyme is being assayed.

a. *p*-Nitrophenol. *p*-Nitrophenol at alkaline pH has an absorption maximum at 400 nm which is lost on formation of the glucuronide; the assay with this substrate is based on the disappearance of *p*-nitrophenol as measured by the decrease in optical density at 400 nm.

REAGENTS. 1. *Sodium phosphate buffer*, 0.25*M*, pH 7.1.
2. *UDP-glucuronic acid*, ammonium salt, 0.05*M*, pH 7.1.
3. *p-Nitrophenol*, 0.002*M*.
4. *Trichloroacetic acid*, 0.1*M*.
5. *Potassium hydroxide*, 10*N*.

Procedure. For determination of activity at a single set of substrate concentrations the following final concentrations of reagents are convenient: $2 \times 10^{-4}M$ *p*-nitrophenol (0.05 ml), $5 \times 10^{-3}M$ UDP-glucuronic acid (0.05 ml), 0.05*M* phosphate buffer (0.10 ml), and 0.5 to 1.0 mg of microsomal protein in a final volume of 0.5 ml. Tubes are warmed to 37°, and the reaction is started by the addition of microsomes. After rapid mixing, a 0.1-ml aliquot of the reacion mixture is removed immediately and added

to 2.0 ml of 0.1*M* trichloroacetic acid (TCA). This sample is the blank and should be determined separately for each assay. Serial aliquots of 0.10 ml are removed 4, 8, and 12 min after the addition of enzyme and similarly deproteinized by addition to 2.0 ml of TCA. After brief centrifugation to remove denatured protein, the supernatants are decanted into tubes containing 0.05 ml of 10*N* KOH, which raises the pH to >10.0, and the optical density is determined at 400 nm. The extinction coefficient for *p*-nitrophenol at pH >10 is 1.81×10^4 cm^2/mole. Because the rate of disappearance of substrate is used to follow the course of the reaction, accurate pipetting is essential if quantitatively good data are to be obtained. Hence, the 0.1-ml aliquots should be removed from the reaction mixture with micropipettes. Also, we have found that the use of "Repipettes" (Labindustries, Berkeley, Calif.) provides the most convenient and reproducible method of accurately dispensing 2.0 ml of TCA.

The timing of the removal of serial aliquots from the reaction mixture can be adjusted according to the activity and amount of enzyme added; with untreated guinea pig microsomes and the concentrations of substrate specified above, rates of optical density change of about 0.020 per 4 min are observed, and the assay is linear with time. Hence, a single-point assay can be used. However, at lower concentrations of *p*-nitrophenol or UDP-glucuronic acid, assays are not linear with time, and serial time points must be used to estimate initial rates of activity. With rat liver microsomes linearity is not maintained even at relatively high concentrations of substrates, since these microsomes contain a highly active nucleotide pyrophosphatase which consumes UDP-glucuronic acid at a rapid rate, depleting the substrate concentration in the UDP glucuronyltransferase reaction. With rat microsomes, therefore, several time points always must be used to estimate initial rates of activity. The reaction rate varies little in the pH range of 7.0–7.8. At pH 8.0 and above, UDP glucuronyltransferase is activated irreversibly, activation being maximal at pH 10.5 (5). At pH values below 7.0 the assay cannot be used since in the presence of low concentrations of *p*-nitrophenol a chromophore, not precipitated by TCA and absorbing at 400 nm, is released from the microsomes. This chromophore is released also in the pH range 7.0–8.0, but not to a significant extent, at *p*-nitrophenol concentrations less than 0.6m*M*. Assay at pH less than 7 is also complicated by anomalous kinetics, since UDP glucuronlytransferase is activated by the phenolate form of *p*-nitrophenol (14). This complication also restricts the upper limit of concentrations of *p*-nitrophenol to 0.6m*M* in the pH range 7.0–8.0.

Increasing the concentration of phosphate to greater than 0.10*M* inhibits UDP glucuronyltransferase assayed with *p*-nitrophenol. This effect may be a general action of salts since the enzyme is inhibited also by NaCl at concentrations greater than 0.2*M*.

Effect of Mg^{2+}. Addition of Mg^{2+} enhances the activity of UDP glucuronyltransferase, primarily by increasing the activity at V_{max}, but glucuronidation of *p*-nitrophenol does not require Mg^{2+}. Probably EDTA complexes endogenous heavy metals in the microsomes, since it decreases the activity with *p*-nitrophenol as glucuronyl acceptor. If activity is measured in the presence of Mg^{2+}, EDTA should be added to the TCA tubes to give a final concentration of $5 \times 10^{-3}M$ in order to prevent precipitation of $Mg(OH)_2$ when the pH is raised to > 10.0. Unless the specific effects of Mg^{2+} on the activity of UDP glucuronyltransferase are to be studied with *p*-nitrophenol as aglycone, it is best not to include Mg^{2+} in the assays. In the presence of Mg^{2+}, primary double reciprocal plots of $1/v$ versus [UDP-glucuronic acid] are not linear over a range of concentrations of UDP-glucuronic acid of 2.5 to $40 \times 10^{-3}M$, whereas they are linear in the absence of Mg^{2+}.

Interpretation of Data. Although the method outlined above is given for a single set of substrate concentrations, it should be made clear that the rates as measured are far from those prevailing at saturating concentrations of substrates. These values can be obtained only graphically, since it is not practical to use saturating concentrations of UDP-glucuronic acid or *p*-nitrophenol. With *p*-nitrophenol as aglycone, K_{UDPGA} is $1.2 \times 10^{-2}M$ with guinea pig liver microsomes as the source of the enzyme. Also, at concentrations of UDP-glucuronic acid greater than $6 \times 10^{-2}M$ there is substrate inhibition. As mentioned above, *p*-nitrophenol at relatively high concentrations has several nonspecific effects on the properties of UDP-glucuronyltransferase and the microsomes.

The data in Figures 1 and 2 illustrate the method for measuring activity at V_{max} for UDP glucuronyltransferase with *p*-nitrophenol as aglycone. Initial rates of activity are determined as a function of the concentration of UDP-glucuronic acid at several different fixed concentrations of *p*-nitrophenol. The intercepts on the $1/v$ axis of the primary double reciprocal plots (Figure 1) are replotted versus the reciprocals of the concentration of the fixed substrate (Figure 2). The intercept on the $1/v$ axis of the secondary plot is $1/V_{max}$.

The data presented in Figures 1 and 2 were obtained with concentrations of UDP-glucuronic acid greater than 2.5 mM. At concentrations below this level, plots of $1/v$ versus 1/[UDP-glucuronic acid] are not linear but bend concave downward. Thus, rates of glucuronidation are greater at low concentrations of UDP-glucuronic acid than would be anticipated by extrapolation of the rate data obtained at high concentrations of UDP-glucuronic acid. Careful analyses of the data indicate that the most likely explanation for non-linearity in double reciprocal plots for v as a function of the concentration of UDP-glucuronic acid is negative cooperativity in the sequential

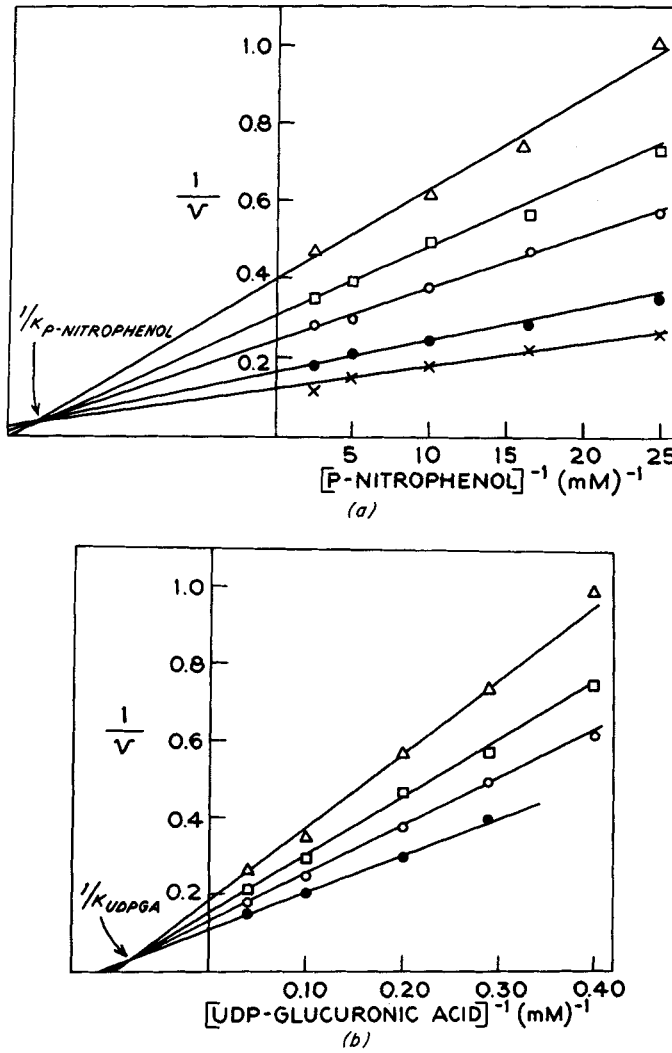

Figure 1. Determination of kinetic parameters of UDP-glucuronyltransferase. Initial rates of UDP-glucuronyltransferase were determined and plotted in double reciprocal form. (*a*) Rate as a function of the concentration of *p*-nitrophenol at several fixed concentrations of UDP-glucuronic acid: 2.5m*M* (△); 3.5m*M* (□); 5m*M* (○); 10m*M* (●); 25m*M* (×). (*b*) Rate as a function of the concentration of UDP-glucuronic acid at fixed concentrations of *p*-nitrophenol: 0.04m*M* (△); 0.06m*M* (□); 0.1m*M* (○); 0.2m*M* (●).

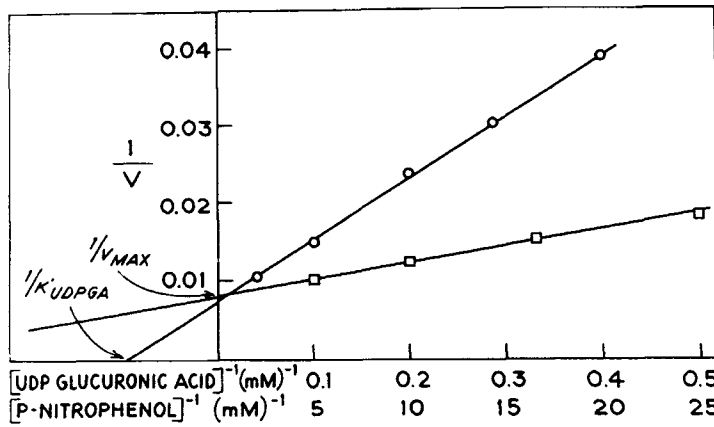

Figure 2. The intercepts on the $1/v$ axis in Figure 1 are replotted against 1/[UDP-glucuronic acid] (○) and 1/[*p*-nitrophenol] (□) in order to obtain $1/V_{max}$.

binding of UDP-glucuronic acid to UDP glucuronyltransferase (14a, 14b). In the presence of added Mg^{2+} double reciprocal plots are non-linear even at high concentrations of UDP-glucuronic acid probably for the same reason.

For an enzyme fulfilling the criteria of a Michaelis-Menten kinetic model, the intersection of the family of primary double reciprocal plots is the K_m for the variable substrate when it is the first substrate bound to the enzyme. The intercept on the 1/[s] axis of the secondary plot is the K_m when this substrate is bound second. More precise estimates of K_m for binding to free enzyme can be obtained by determining the ratio of secondary replots of the slopes and intercepts of the data in Figure 1 (14c). Obviously this type of straight forward interpretation of secondary plots is not possible with UDP glucuronyltransferase because of apparent homotropic cooperativity in substrate binding. Nevertheless, the K's determined above do reflect a real property of the enzyme. The exact physical meaning of these constants depends on the kinetic mechanism of the enzyme under study. For UDP glucuronyltransferase, which has a rapid-equilibrium, random-order mechanism (14), K_m is the enzyme-substrate dissociation constant for the binding of substrate to the *nth* subunit of the enzyme. It is especially important to emphasize that studies of activity during induction of the enzyme or development of *p*-nitrophenol conjugating activity in fetal animals should be based on measurements of activity at V_{max}; changes in activity which are based on rates of reaction at a single set of substrate concentrations cannot delineate differences in the binding of substrates or in the catalytic rate constant of the enzyme.

b. *o*-Aminophenol. Assays with this aglycone are based on the fact that *o*-aminophenylglucuronic acid can be diazotized selectively in the presence of unreacted *o*-aminophenol by careful control of the pH of the diazotization reaction, the conditions for which were established by Levvy and Storey (15). The diazotized *o*-aminophenylglucuronide is then complexed with *N*-(-naphthyl)ethylenediamine dihydrochloride. The product of this reaction is measured at its absorption maximum; the extinction coefficient for the coupled product is 2.9×10^4 cm²/mole at 555 nm.

REAGENTS.
1. *Sodium phosphate* buffer, 0.25*M*, pH 7.6.
2. *UDP-glucuronic acid*, ammonium salt, 0.05*M*, pH 7.6.
3. *o-Aminophenylglucuronide* (Koch-Light Laboratories, Colnbrook, England).
4. *o-Aminophenol*, 0.002*M*, containing 2 mg/ml ascorbate, pH 7.0. The *o*-aminophenol solution should be prepared fresh each week by sublimation and stored at −20°.
5. *Ascorbate*, 2 mg/ml, pH 7.0.
6. *Trichloroacetic acid-sodium phosphate*, 1*M*, pH 2.0, mixed daily from solutions of TCA, 2*M*, pH 2.0, and sodium phosphate, 2*M*, pH 2.0.
7. *Sodium nitrite*, 0.05% (w/v).
8. *Ammonium sulfamate*, 0.5% (w/v).
9. *N-(1-naphthyl)ethylenediamine dihydrochloride.* Add 47.6 ml water to 55 mg in preweighed vials (Sigma).

Procedure. For assays at a single set of substrate concentrations pipette 0.2 ml *o*-aminophenol (final concentration $2 \times 10^{-4}M$), 0.2 ml UDP-glucuronic acid (final concentration $5 \times 10^{-3}M$), 0.4 ml phosphate buffer, and enough H_2O to produce a final volume of 2.0 ml. Allow the mixture to come to thermal equilibrium at 37°, and start the reaction by adding 1 to 2 mg of microsomal protein. At 5-min intervals during the course of the reaction transfer 0.5-ml aliquots to 0.5 ml of the TCA-sodium phosphate reagent. A single zero-time sample can serve as blank for a series of assays.

After removal of precipitated protein from the mixtures by centrifugation and decantation, add 0.1 ml sodium nitrite to each tube; shake and allow to stand at least 5 min. Add 0.1 ml ammonium sulfamate and, after 5 min, 0.1 ml *N*-(1-naphthyl)ethylenediamine dihydrochloride. Incubate the tubes in the dark at 25° for 2 hr; then read the optical density at 555 nm. Because of variability in the color yield from day to day, standards of *o*-aminophenylglucuronide should be run with each set of assays. With guinea pig liver microsomes the reaction is linear with time under these conditions. When microsomes contain nucleotide pyrophosphatase activity, or when the concentrations of substrates are reduced, the initial rates of activity must be

estimated by extrapolation to zero time of serial estimates of the *o*-aminophenylglucuronic acid synthesized. We have observed that in some situations the time for maximal color development may be variable, and should be determined if the assay system specified above is modified. The final pH of the diazotization mixture must be between 2.1 and 2.3 (16). At pH values less than 2.0 the aglycone, as well as the glucuronide, will be diazotized, giving spuriously high rates of glucuronidation. At pH levels greater than 2.3, diazotization of the glucuronide will be inhibited with consequent falsely low reaction rates. The pH of the TCA-phosphate reagent and the final pH of the diazotization mixture should be checked daily, and adjustments made in the pH of the stock TCA-phosphate in order to maintain the final pH of the mixture, after addition of the assay aliquot, in the desired range.

Substitution of amine-containing buffers for phosphate is not recommended, since we have found that even small amounts of these substances interfere with the diazotization. The effect of high concentrations of salt on the glucuronidation of *o*-aminophenol has not been investigated. If the amount of *o*-aminophenol added to assay tubes is less than that specified above, additional ascorbate is needed to maintain a constant concentration of this compound, since ascorbic acid is added to prevent oxidation of the *o*-aminophenol.

Effect of Mg^{2+}. As with *p*-nitrophenol, the UDP glucuronyltransferase responsible for the glucuronidation of *o*-aminophenol is enhanced by Mg^{2+}, though there is no absolute dependence on Mg^{2+}. The effect of Mg^{2+} is on activity at V_{max}. The addition of EDTA decreases the rate of glucuronidation of *o*-aminophenol.

Interpretation of Data and Limitations of the Method. Rates of *o*-aminophenol glucuronidation measured at a single set of substrate concentrations do not reflect maximum rates, and comparisons of activities under different experimental conditions should be based on determinations at V_{max} so that effects on the amount, catalytic constants, and binding affinity for substrates can be resolved. There are, however, more limitations on the estimate of V_{max} with *o*-aminophenol than with *p*-nitrophenol. Not only does *o*-aminophenol activate UDP glucuronyltransferase at high concentrations (14) but also plots of $1/v$ versus $1/[\text{UDP-glucuronic acid}]$ are nonlinear; and relatively high concentrations of UDP-glucuronic acid (greater than $15 \times 10^{-3}M$) are inhibitory. A detailed consideration of the causes of the anomalous kinetic behavior, is beyond the scope of this review, but it again seems to reflect negative cooperativity in the binding of UDP-glucuronic acid. It is possible to obtain good estimates of maximal activity in the same manner as with *p*-nitrophenol, with careful selection of substrate concentrations. The concentration of *o*-aminophenol should be kept below $2 \times 10^{-4}M$, and the concentration of UDP-glucuronic acid in the range of $3\text{–}15 \times 10^{-3}M$.

c. *p*-Aminophenol. The glucuronidation of this compound can be studied in the same way as that of aminophenol, with only minor modifications. The maximum absorbance of the coupled glucuronide occurs at 540 nm for *p*-aminophenylglucuronide; as with *o*-aminophenylglucuronide, the final pH during diazotization must be kept between 2.1 and 2.3. Maximum color development at 25° requires 1.5 hr.

d. *o*-Aminobenzoate. This assay differs from the others in that, after the reaction is completed, unreacted aglycone is extracted into an organic phase. The glucuronide remains in the aqueous phase and is measured directly by diazotization and coupling with *N*-(1-naphthyl)ethylenediamine dihydrochloride.

REAGENTS. 1. *Sodium phosphate*, 0.25*M*, pH 7.6.
2. *UDP-glucuronic acid*, ammonium salt, 0.02*M*, pH 7.6.
3. *o-Aminobenzoate*, 0.002*M*, pH 7.6.
4. *Trichloroacetic acid*, 0.22*M*.
5. *Sodium phosphate*, 0.5*M*, pH 3.9.
6. *Sodium phosphate*, 1.0*M*, pH 1.0.
7. *Anhydrous ether.*
8. *Sodium nitrite*, 0.05% (w/v).
9. *Ammonium sulfamate*, 0.1% (w/v).
10. *N-(1-naphthyl)ethylenediamine dihydrochloride;* 55 mg in a preweighed vial (Sigma) is dissolved in 47.6 ml water.

Procedure. The reaction mixture contains 0.1 ml sodium phosphate, pH 7.6, 0.2 ml UDP-glucuronic acid (final concentration $5 \times 10^{-3}M$), 0.1 ml *o*-aminobenzoate (final concentration $5 \times 10^{-4}M$), and 2 mg microsomal protein in a final volume of 0.8 ml. After equilibration at 37°, the reaction is started by addition of microsomes. Aliquots of 0.2 ml are removed 5, 10, and 15 min after initiation of the reaction, and added to 0.3 ml 0.22*M* TCA. A single zero-time sample (blank) is sufficient. After centrifugation to remove precipitated protein, the supernatants are decanted into 0.3 ml sodium phosphate, pH 3.9. This mixture is extracted 3 times with anhydrous ether, and the ether layer discarded. Sodium phosphate, 0.1 ml, pH 1.0, is added to the remaining aqueous phase, and the glucuronide is diazotized and coupled with *N*-(1-naphthyl)ethylenediamine dihydrochloride, as for *o*-aminophenol. Color yield is maximal in 1 hr, and optical density is determined at 555 nm. Previous workers hydrolyzed the glucuronide in acid after separation of unreacted aglycone before diazotization, but we have found this step to be unnecessary.

The properties of the glucuronidation reaction have not been investigated as extensively with *o*-aminobenzoate as with *p*-nitro- and *o*-aminophenols. It is known, however, that Mg^{2+} and EDTA have qualitatively identical

effects on the rate of glucuronidation of all three substrates. On the other hand, *o*-aminobenzoate differs from the two phenolic substrates in that at high concentrations it has no nonspecific activating effect on the rate of its own metabolism. As with the phenols, however, reaction rates at any single set of substrate concentrations are not a good measure of activity at V_{max}, and graphical methods should be used for the determination of this value and other kinetic constants.

e. Bilirubin. Under suitable conditions bilirubin glucuronide can be diazotized selectively in the presence of bilirubin. The procedure described below for diazotization is basically that of Van Roy and Heirwegh (16).

REAGENTS. 1. *Potassium phosphate*, 0.1*M*, pH 7.5.
2. *Potassium phosphate*, 1.0*M*, pH 2.6.
3. *UDP-glucuronic acid*, 0.05*M*, pH 7.5.
4. *Bilirubin*, $2.5 \times 10^{-3}M$ in 0.01 *N* sodium hydroxide containing 0.002*M* EDTA. This reagent is prepared in the dark just before use.
5. *HCl*, 0.15*M*.
6. *Sodium nitrite*, 0.5% (w/v).
7. *Ammonium sulfamate*, 0.7% (w/v).
8. *Ethylanthranilate.*
9. *2-Pentanone.*
10. *Butyl acetate.*
11. *Ascorbate*, 40 mg/ml, prepared just before use.

Procedure. Working in the dark, add 0.25 ml potassium phosphate, pH 7.5, 0.03 ml bilirubin (final concentration $3 \times 10^{-5}M$), 10 mg of microsomal protein, and sufficient water so that the final volume will be 2.5 ml. After equilibration at 37°, start the reaction by addition of 0.25 ml UDP-glucuronic acid (final concentration $5 \times 10^{-3}M$). Immediately remove a 0.5-ml aliquot as the zero-time sample (blank), and add to a glass-stoppered centrifuge tube on ice containing 0.3 ml potassium phosphate, pH 2.6. Remove additional 0.5-ml aliquots 5, 10, and 15 min later and treat them similarly. Place the potassium phosphate-treated samples in a 25° bath for 10 min.

During this time the diazo reagent should be prepared by adding 0.1 ml of ethylanthranilate to 10 ml of 0.15*M* HCl, followed by 0.30 ml of the sodium nitrite solution; 0.1 ml of 0.7% ammonium sulfamate is added, and the diazo reagent allowed to stand for 3 min more. After preparation, 0.5 ml of diazo reagent is added to each potassium phosphate tube and the samples are warmed for 20 min at 25°. At the end of this incubation, the tubes are removed from the water bath, and 4 ml of ascorbate is added to each,

followed by 3 ml of a mixture of 2-pentanone and butyl acetate (17/3, v/v). The tubes are then shaken vigorously, and the phases separated by centrifugation. The optical density of a portion of the upper, organic phase, which contains the coupled bilirubin glucuronide, is measured at 530 nm. The extinction coefficient for the coupled bilirubin glucuronide is 44.4×10^3 cm^2/mole. Note that all operations should be carried out in the dark, and optical density should be determined promptly.

The bilirubin assay is complicated by the limited solubility of the glucuronyl acceptor, a problem which does not arise with the phenols or *o*-aminobenzoate. In several previously described assay methods bilirubin was added as a bilirubin-albumin complex in order to make the bilirubin more "soluble." No direct measurements were made, however, of the concentration of bilirubin in true solution as a function of different ratios of bilirubin to albumin. Studies in this laboratory indicate in fact that at concentrations of bilirubin less than its limit of solubility the addition of albumin decreases the rate of synthesis of bilirubin glucuronide. Also, if data are based on measurement of initial rates of activity, the rate of formation of bilirubin glucuronide is independent of the amount of bilirubin-albumin complex when the ratio of bilirubin to albumin is constant.

With the conditions given above, the concentration of bilirubin is at the limit of its solubility, $3 \times 10^{-5}M$. Solubility can be increased to a limited extent by increasing the pH or the concentration of salt (17). As long as the concentration of bilirubin added to the assays is less than the limit of its solubility, the enzyme shows typical Michaelis-Menten kinetics for dependence of the rate of glucuronidation on the concentration of bilirubin

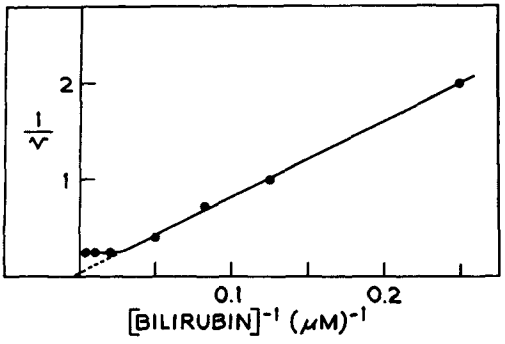

Figure 3. The rate of UDP glucuronyltransferase as a function of variable concentrations of bilirubin. Initial rates of synthesis of bilirubin glucuronide were determined with the indicated amounts of bilirubin and 5.0m*M* UDP-glucuronic acid. Rates are expressed as optical density change per minute per milligram protein.

(Figure 3). At bilirubin concentrations greater than saturating there is, as expected, no dependence of rate on the concentration of bilirubin.

The addition of Mg^{2+} has no direct effect on the enzyme catalyzing the synthesis of bilirubin glucuronide. At greater than saturating concentrations of bilirubin, Mg^{2+} increases the rate of glucuronidation by increasing the solubility of the bilirubin. Potassium phosphate also enhances the solubility of bilirubin, but in addition, at relatively high concentrations, directly increases the rate of synthesis of bilirubin glucuronide via what appears to be an independent effect on the enzyme. For this reason, the concentration of potassium phosphate in the assay system should be kept constant and preferably below 0.2*M*.

As with the other substrates, maximal rates of synthesis of bilirubin glucuronide cannot be estimated from assays at a single set of substrate concentrations. Values can be estimated by extrapolation to V_{max}, as described in the previous discussion, although this determination is inherently less accurate because of the limited solubility of bilirubin.

f. Phenolphthalein. In addition to the methods outlined above, other substrates have been utilized for measuring UDP glucuronyltransferase. Experience in this laboratory with one commonly used compound, phenolphthalein, has shown it to be a less suitable substrate than the others discussed. The reason for this is extensive binding of the substrate by the microsomal membrane. This binding not only complicates the problem of kinetic studies but may be associated also with changes in the properties of the microsome. Unless it can be shown that phenolphthalein is glucuronidated by a separate UDP glucuronyltransferase, for which there is no other known substrate, there appears to be no need for using this compound as a glucuronyl acceptor.

g. Assays Using ^{14}C-Labeled Glucuronyl Acceptors. Lucier et al. (18) have described a simple technique which should be useful in the study of the glucuronidation of a number of compounds. This technique is based on the difference in the solubilities of relatively apolar aglycones and their more polar glucuronides in aqueous and organic solvents. The method has been used with $^{14}C_4$-testosterone, ^{14}C-1-naphthol, and ^{14}C-dieldrin as aglycones, but should have general application for any aglycone which can be separated quantitatively from its glucuronide via solvent extraction.

REAGENTS. 1. *$^{14}C_4$-testosterone* dissolved in benzene.
2. *^{14}C-1-naphthol* dissolved in benzene.
3. *UDP-glucuronic acid.*
4. *Tris-HCl*, pH 7.4, 0.05*M*.

Procedure. One of the labeled substrates is added to a liquid scintillation counting vial, and the solvents are evaporated under a stream of nitrogen. Tris (1.0 ml, 0.05*M*) and UDP-glucuronic acid to give a final concentration of $5 \times 10^{-3}M$ are then added. The vial is warmed to 37°, and the reaction started by the addition of 0.4 to 2.0 mg of microsomal protein. After a 6-min incubation under nitrogen, the reaction is stopped by adding 10 ml of a nonaqueous scintillation fluid (18). Unreacted aglycone is partitioned into the scintillation fluid and is measured by liquid scintillation counting after removal of the aqueous phase. More accurate results probably could be obtained by direct counting of the glucuronide products in the aqueous phase, although this would be more time consuming. Any glucuronyl acceptor could be used in this type of assay if appropriate solvent systems could be found for complete partition of aglycone into the organic phase while retaining the glucuronide in the aqueous phase.

C. ASSAYS OF UDP GLUCURONYLTRANSFERASE BASED ON THE REVERSE REACTION

REAGENTS. 1. *UDP*, 0.05*M*, pH 7.1.
2. *Sodium phosphate buffer*, 0.25*M*, pH 7.1.
3. *Saccharic acid–1,4-lactone*, 0.05*M*.
4. *p-Nitrophenylglucuronide*, 0.05*M*, pH 7.1.
5. *Trichloroacetic acid*, 0.1*M*.
6. *Potassium hydroxide*, 10*N*.

Procedure. Add 0.05 ml of UDP (final concentration $5 \times 10^{-3}M$), 0.05 ml saccharic acid–1,4-lactone (final concentration $5 \times 10^{-3}M$), 0.1 ml *p*-nitrophenylglucuronide (final concentration 0.01*M*), 0.1 ml sodium phosphate buffer, and sufficient H_2O to give a final volume of 0.5 ml. Allow the mixture to come to 37°, and start the reaction by adding 1 mg of microsomal protein. Immediately, and 2, 4, and 6 min later, remove 0.1-ml aliquots with a micropipette and add to centrifuge tubes containing 2.0 ml of TCA. After centrifugation to remove precipitated protein, decant into tubes containing 0.05 ml of 10*N* potassium hydroxide, mix, and determine the optical density at 400 nm.

Measurement of reaction rates in the reverse direction at a single set of substrate concentrations yields no better estimates of activity at V_{max} than those obtained in the forward direction, since K_{UDP} and $K_{p\text{-}NPGA}$ are very large, and in some species, such as the guinea pig, the decline in reaction rate with time is quite fast because of rapid destruction of UDP by an interfering enzyme. On the other hand, large concentrations of glucuronide do not alter the properties of the microsome; and the rate of the reverse reaction in rat and beef liver microsomes, which destroy UDP-glucuronic

acid via an alternative reaction, but not UDP, is faster than that of the forward reaction. An additional advantage of the back reaction is that low reaction rates can be measured more accurately because product formation is measured directly. Saccharic acid–1,4-lactone is added to the assay in order to inhibit β-glucuronidase, which interferes with estimates of the rate of the reverse reaction by hydrolyzing O-glucuronides. The lactone has no direct effect on the activity of UDP glucuronyltransferase. With 0.01*M* *p*-nitrophenylglucuronic acid and 5×10^{-3} *M* saccharic acid–1,4-lactone there is no production of *p*-nitrophenol in the absence of UDP. Thus far, the reverse reaction has been found useful only with *p*-nitrophenylglucuronide as substrate; we have not been able to demonstrate UDP-dependent hydrolysis of *o*-aminophenylglucuronide or phenolphthalein glucuronide.

2. Glucose-6-Phosphatase

A. BACKGROUND

It has been shown recently that glucose-6-phosphatase is a multifunctional enzyme which acts as a phosphohydrolase and phosphotransferase. Although the enzyme has not been purified, a large body of kinetic evidence suggests that the two types of reaction are catalyzed by a single protein (19–21). The mechanism of reaction involves the formation of a covalently bound enzyme-P_i intermediate which reacts with a variety of P_i acceptors, such as water (phosphohydrolase function) or glucose (phosphotransferase function).

$$\text{Glucose-6-P} + \text{E} \rightarrow \text{E-P}_i + \text{glucose} \quad [2]$$

$$\text{PP}_i + \text{E} \rightarrow \text{E-P}_i + \text{P}_i \quad [3]$$

$$\text{E-P}_i + \text{H}_2\text{O} \rightarrow \text{E} + \text{P}_i \quad [4]$$

$$\text{E-P}_i + \text{glucose} \rightarrow \text{E} + \text{glucose-6-P} \quad [5]$$

A detailed review of the properties of the glucose-6-phosphatase catalyzed reaction is now available (19). In the methods described below, procedures are given for estimating the rate of the phosphohydrolase reaction with glucose-6-P and PP_i as substrates and the phosphotransferase reaction with PP_i and glucose as substrates.

B. ASSAY OF PHOSPHOHYDROLASE ACTIVITY

a. Hydrolysis of Glucose-6-Phosphate

REAGENTS. 1. *Sodium acetate* buffer, 1.0*M*, pH 5.75.
2. *Glucose-6-P*, 0.4*M*, pH 5.75.
3. *Trichloroacetic acid*, 10% (w/v).

4. *Ammonium molybdate*, 1.6% (w/v) in 1*N* sulfuric acid.
5. *Ferrous sulfate*, 2.5 g per 25 ml 0.15*N* sulfuric acid, prepared fresh each day.

Procedure. Pipette into a test tube 0.1 ml sodium acetate buffer, 0.2 ml glucose-6-P (final concentration $8 \times 10^{-2}M$), and enough water so that the final volume will be 1.0 ml. After equilibrium at 37°, start the reaction by adding 1 mg of microsomal protein. Stop the reaction, usually after a 5-min incubation, by adding 0.5 ml TCA. Remove precipitated protein by centrifugation, and add 0.5 ml of supernatant to 5.0 ml of ammonium molybdate solution. Then add 0.8 ml of ferrous sulfate solution, and read the optical density at 660 nm after shaking.

With this procedure for the determination of phosphorus, maximum color intensity develops almost instantaneously, is stable for at least 1 to 2 hr, and is linear with phosphorus concentrations up to an optical density of 1.00. The rate of glucose-6-P hydrolysis is linear with time for at least 20 min with untreated microsomes. Assays at concentrations of glucose-6-P of 0.08*M* are nearly equal to activity at V_{max}, since $K_{G\text{-}6\text{-}P}$ is approximately $5 \times 10^{-3}M$.

With liver preparations we have found it sufficient to run only a reagent blank as control. In some tissues, however (e.g., intestinal mucosa), there is appreciable hydrolysis of glucose-6-P because of the action of nonspecific phosphatases. In this instance the rate of phosphorus release by the nonspecific phosphatase can be estimated by utilizing β-glycerophosphate as substrate, or by measuring the rate of hydrolysis of glucose-6-P after destruction of glucose-6-phosphatase by heating microsomes for 30 min at 37° (22). Standard curves for the phosphorus assay are determined as above, using 1.3613 g of KH_2PO_4 per liter, which gives a phosphorus concentration of 10 μmoles/ml.

In untreated microsomes glucose-6-phosphatase has a broad pH optimum between 5.5 and 6.5. Although the shape of the pH-activity curve shifts after treatment of the microsomes with NH_4OH and detergents (23,24), the pH optimum remains close to 6.0 and the curve is flat in this region of the pH scale. Hence pH 5.75 is suitable for the assay of glucose-6-phosphatase in all preparations.

b. Hydrolysis of Pyrophosphate

REAGENTS. 1. *Sodium acetate* buffer, 1.0*M*, pH 5.75.
2. *Sodium pyrophosphate*, 0.2*M*, pH 5.75.
3. *Trichloroacetic acid*, 10% (w/v).
4. *Sodium acetate* buffer (0.1*M* acetic acid, 0.025*M* sodium acetate), pH 4.0.
5. *Ascorbate*, 0.5% (w/v).
6. *Ammonium molybdate*, 7.6% (w/v) in 0.05*N* sulfuric acid.

Procedure. Pipette into a test tube 0.1 ml sodium acetate buffer, pH 5.75, 0.2 ml sodium pyrophosphate (final concentration 0.04*M*), and enough H_2O for a final volume of 1.0 ml. After equilibration at 37°, start the reaction by adding 1 mg microsomal protein. Stop the reaction 5 min later by the addition of 0.5 ml TCA, and determine the inorganic phosphorus on a 0.5-ml portion of the protein-free supernatant by the method of Lowry and Lopez (25).

The aliquot from the reaction mixture is added to 5 ml of sodium acetate buffer, pH 4.0, making certain that the final pH of the resulting mixture is between 3.5 and 4.2. After addition of 2.5 ml of H_2O, 0.5 ml of ascorbate and then 0.5 ml of ammonium molybdate are added. Optical density is determined at 660 nm, against a reagent blank, 10 min after the addition of ammonium molybdate. In comparison with the original method of Lowry and Lopez, it is necessary to add larger amounts of ammonium molybdate because of the high concentration of pyrophosphate. The phosphorus assay is linear to at least 2.0 μmole phosphate per assay. Pyrophosphatase activity is equal to one half of the amount of phosphate produced. Standard curves for phosphate should be determined, using the above conditions in the presence of pyrophosphate.

As with the glucose-6-phosphatase activity, there is a relatively broad pH optimum for the cleavage of pyrophosphate in untreated rat liver microsomes with an optimum at pH 5.75 (23). This peak becomes sharp and is shifted to pH 5.0 after treatment of microsomes with NH_4OH or with detergents (24). With untreated beef liver microsomes the pH optimum is 5.25 and shifts to 5.75 after the microsomes are treated with phospholipase A. The pH for assays of pyrophosphatase activity thus depends on the source of the enzyme and its previous treatment.

C. PP_i-GLUCOSE PHOSPHOTRANSFERASE ACTIVITY

REAGENTS.
1. *Sodium acetate* buffer, 1.0*M*, pH 5.50.
2. *Sodium pyrophosphate*, 0.2*M*. pH 5.50.
3. *Glucose*, 1.0*M*.
4. *NADP*, 12.5 mg/ml.
5. *Glucose-6-phosphate dehydrogenase*, 5 mg/ml (Sigma, Type VII from baker's yeast. Very low to absent contamination with 6-phosphogluconate dehydrogenase).
6. *Tris-HCl* buffer, 1.0*M*, pH 8.0.

Procedure. Sodium acetate (0.1 ml), sodium pyrophosphate (0.4 ml), glucose (0.4 ml), and H_2O to a final volume of 1.0 ml are brought to 37°. The reaction is started by adding 1 mg of microsomal protein and is stopped 5 min later by heating the tubes for 3 min in a boiling-water bath. Precipi-

tated protein is removed by centrifugation, and the amount of glucose-6-P synthesized measured enzymatically by adding 0.025- to 0.1-ml aliquots of supernatant to a quartz cuvette containing 0.1 ml Tris, pH 8.0, 0.025 ml NADP, 0.002 ml glucose-6-phosphate dehydrogenase, and H_2O to give a final volume of 1.0 ml. The increase in optical density at 340 nm due to the reduction of NADP to NADPH is a direct measure of the glucose-6-P in the sample, which is calculated using an extinction coefficient for NADPH of $6.22 \times 10^6/cm^2/mole$. Zero-time samples, after addition of microsomes, serve as the blank. Carbamyl-P, a variety of acyl-P compounds, CTP, CDP, deoxy-CTP, ATP, ADP, GTP, GDP, and IDP can also serve as P_i donor in the phosphotransferase reaction (19). Assays can be conducted with these compounds by substituting 30 μM of each for the PP_i. The pH of each phosphate compound should be brought to the pH of the final reaction mixture before addition to the assay.

The nucleotide-sugar transferase reaction is severely constrained in untreated microsomes. Measurement of this reaction requires that the microsomes be treated with detergent, or that detergent be added to the assay tubes. About 1 mg of cholate or deoxycholate should be added per 4 mg of microsomal protein. The amount needed for maximum rates of reaction will vary with the preparation and can be determined by titration of the microsomes with detergent.

The problem of the selection of pH for assays of the phosphotransferase activity is greater than for the other activities of glucose-6-phosphatase in that the phosphotransferase has a sharp pH optimum at 5.50 in untreated beef liver microsomes (4), but a broad pH optimum between 4.80 and 5.20 in rat liver microsomes (21,23). In both species the pH optimum is shifted to more alkaline values by treatment of the microsomes with activating agents such as phospholipases (4,24).

III. INVESTIGATIONS OF PHOSPHOLIPID-PROTEIN INTERACTIONS

1. Background

As mentioned in the Introduction (Section I), the activities of several microsomal enzymes are constrained in some manner in the native microsome by the relationship between the membrane phospholipids and the enzyme protein. The evidence for this is that treatment with phospholipases or detergents can increase enzyme activity. Another sort of enzyme-phospholipid interaction is phospholipase-induced inactivation of some microsomal enzymes, which appears to be reversible on subsequent treatment of the phospholipase-treated form of the enzyme with phospholipid micelles. It is

thus apparent that studies of the effects of treatment with phospholipases, detergents, and phospholipids on the properties of microsomal enzymes are important for full characterization of some of these enzymes.

2. Phospholipase A

A. PURIFICATION OF PHOSPHOLIPASE A FROM NAJA NAJA VENOM (28)

Digestion of microsomes by proteolytic enzymes has been reported both to activate (26) and to inactivate (27) microsomal enzymes. Ideally, phospholipases should be free of proteolytic enzyme activities.

Procedure. Adjust the pH of a 10% (w/v) solution of crude *Naja naja* venom (Miami Serpentarium, Miami, Florida) to 3.5 to 3.7 with 1*N* sulfuric acid, and heat in a boiling-water bath for 5 to 10 min. After cooling the venom to room temperature, add 1*M* potassium phosphate to yield a final concentration of 0.05*M*, and adjust the pH to 7.6 with 3*M* NH_4OH. Remove precipitated protein by centrifugation at 0°, and chromatograph the supernatant on a column of Sephadex G-75 equilibrated with 0.05*M* potassium phosphate, pH 7.6. The V_0 of the column should be at least 30% greater than the volume of enzyme put on the column. Elute the column with 0.05*M* potassium phosphate, pH 7.6, and collect fractions equivalent to one fourth of the V_0.

Phospholipase A is eluted in the last portion of the excluded volume, and in the first few tubes of the included volume. The excluded portion contains the highest specific activity for phospholipase A as measured by the rate of release of fatty acids from an artificial substrate (29). The enzyme is stable almost indefinitely when frozen. Pure isoenzyme fractions can be obtained by isoelectric focusing of the purified phospholipase A preparation (29). Since there are differences in the substrate specificities of the isoenzymes of *Naja naja* venom (30), detailed studies with single isoenzyme forms of phospholipase A may offer advantages. There are, however, no data on this point as yet.

B. PURIFICATION OF PHOSPHOLIPASE A FROM *Crotalus adamanteus* VENOM (31)

Procedure. Adjust the pH of a 1% (w/v) solution of crude dried *Crotalus adamanteus* venom (Miami Serpentarium) to 9.0 with 0.1*N* potassium hydroxide, and remove the precipitated protein by centrifugation at 12,800*g* for 10 min at 4°. Adjust the pH of the supernatant to 7.0, and dialyze at 4° for at least 30 hr against 300 vols of $10^{-3}M$ EDTA and then 300 vols of $10^{-4}M$ EDTA for an additional 40 hr. Adjust the enzyme solution to pH 3.0, and heat at 90° for 5 min while stirring. Cool the solution in ice-cold water and adjust the pH to 7.4. Remove precipitated protein by centrifugation at

12,800*g* for 10 min at 4°. Dialyze the supernatant against 300 vols of potassium phosphate, 0.005*M*, pH 7.4, containing $10^{-3}M$ EDTA. Equilibrate DEAE-cellulose with the same buffer and pack into a column 1.8 × 37 cm. After passing buffer through the column overnight at 4°, apply the protein and collect fractions of about 25 to 30 ml each, at a flow rate of 50 ml/hr. The fractions are monitored by their absorption at 280 nm. When the breakthrough peak is reached and absorption at 280 nm returns to the base line, begin a linear gradient of 0.005 to 0.1*M* potassium phosphate, pH 7.4, containing $10^{-3}M$ EDTA. Two peaks with phospholipase A activity are resolved.

C. PROPERTIES OF PHOSPHOLIPASE A

Although we have observed no differences in the effects of phospholipase A from *Naja naja* and *Crotalus adamanteus* venoms on the catalytic properties of glucose-6-phosphatase and UDP glucuronyltransferase, this result may not apply to all microsomal enzymes, since the two phospholipases have differing specificities with synthetic phospholipid substrates and also have differing effects on the solubilization of mitochondrial NADH dehydrogenase (30). Phospholipase A from *Naja naja* venom has considerably greater activity with microsomes than that from *Crotalus adamanteus* on a milligram basis.

The pH activity curve of partially purified phospholipase A from *Crotalus adamanteus*, which is a mixture of two isoenzymes, is essentially flat from pH 4.5 to 9.5 (31). The pH optimum of phospholipase A from *Naja naja* venom is relatively sharp, for most of the isoenzymes studied, with a peak at approximately 7.8 (30). With a mixture of isoenzymes from *Naja naja* venom, activity at pH 7.0 or 8.5 is 25% of that at the optimum pH. Since treatment of microsomes with phospholipases can alter the stability of microsomal enzymes as a function of pH, selection of the phospholipase to be used on the basis of its pH-activity curve may be an important consideration in some experiments.

With synthetic substrates all phospholipases require an exogenous source of Ca^{2+} for activity. This is true also for the action of *Crotalus adamanteus* phospholipase A on microsomes, but the *Naja naja* variety of phospholipase A has full activity with liver microsomes in the absence of added Ca^{2+}. The hydrolysis of phospholipids by both enzymes is inhibited completely by $5.0 \times 10^{-3}M$ EDTA (5).

3. Phospholipase C: Preparations and Properties

Crude preparations of phospholipase C from filtrates of *Clostridium welchii* have generally been used without further purification. The enzymatic activity of this material has not been investigated in detail, but the filtrates

contain "toxins" which do not have "lecithinase" activity (32). All but the "lecithinase" activity can be destroyed, however, by heating for 10 min at 100°, pH 7.6, in a sealed ampoule. Approximately 50% of the phospholipase C is also lost during heating.

The effects of pure preparations of phospholipase C on the properties of microsomal membranes have not been studied. Two isoenzymes of phospholipase C can be purified from extracts of *Clostridium welchii* (33,34); sphingomyelin is the preferred substrate for one isoenzyme, and lecithin for the other (34). The pH optimum for the sphingomyelin-hydrolyzing isoenzyme is 7.8 to 8.8, and this form of phospholipase C is activated by Mg^{2+} but inhibited almost completely by $10^{-3}M$ Ca^{2+}. The rate of hydrolysis of lecithin is 9% of that for sphingomyelin in the presence of $10^{-3}M$ $MgCl_2$. The isoenzyme for which lecithin is the preferred substrate has a pH optimum at 7 and shows nearly equal activity with sphingomyelin or lecithin as substrate in the absence of Ca^{2+}. In the presence of $10^{-3}M$ Ca^{2+}, however, there is no activity with sphingomyelin. The addition of Mg^{2+} has no effect on the activity of this isoenzyme. Since all studies of microsomal enzymes reported to date have utilized phospholipase C treatment of microsomes in the presence of Ca^{2+}, the effects of phospholipase C on the properties of microsomal enzymes almost certainly reflect the effects of hydrolysis of microsomal lecithins.

4. Phospholipase D: Preparations and Properties

This enzyme, which has been found only in plants, is activated by Ca^{2+} and anionic detergents (35). The pH optimum is 5 to 6, with little activity below 2 or above 8.0. To date few studies of protein-lipid interactions in microsomal enzymes have been made with phospholipase D.

5. Treatment of UDP Glucuronyltransferase and Glucose-6-Phosphatase with Phospholipases

REAGENTS. 1. Partially purified *phospholipase A*, 10 mg/ml, from *Naja naja* or *Crotalus adamanteus* venom. *Phospholipase C* from *Clostridium welchii*, 10 mg/ml, prepared fresh each day.
2. *Calcium chloride*, $0.1M$.
3. *Tris-HCl*, $0.5M$, pH 8.0.
4. *Microsomes*, 20 mg/ml in $0.25M$ sucrose.
5. *EDTA*, $0.25M$.

Procedure. Microsomes (0.5 ml), Tris-HCl (0.1 ml), and H_2O (0.45 ml) are placed in the reaction tube; 0.025 ml $CaCl_2$ is added if phospholipase A from *Crotalus adamanteus* or phospholipase C is to be used. After equilibration at

25°, the reaction is started by addition of 0.005 ml phospholipase A or 0.1 ml phospholipase C. Aliquots are removed from the reaction mixture at ½, 1, 2, 5, and 10 min after addition of phospholipase, and placed in appropriate assay tubes containing EDTA at a final concentration of 0.005*M* to inhibit further action of phospholipase.

There is no correlation between the rate of hydrolysis of microsomal phospholipids and phospholipase-induced modifications in the properties of glucose-6-phosphatase (4), and the time course for these effects are variable for different enzymes and in different species. The times used in the above method are satisfactory when working with glucose-6-phosphatase and UDP glucuronyltransferase, but it is essential to establish carefully for each enzyme and species the time course for the effects of treatment with phospholipases. If effects (especially, e.g., inactivation) appear to be immediate, the ratio of phospholipases to microsomal protein should be reduced in order to be certain that a rapid activation is not being misssed.

6. Properties of Phospholipase A-Treated Enzymes

It should be appreciated that treatment with phospholipase A may have mixed effects on the properties of microsomal enzymes. The data in Figure 4 are illustrative of this point. Microsomes were treated with phospholipase A and assayed for UDP glucuronyltransferase in the presence of EDTA at single time points under conditions which gave linear kinetics for untreated microsomes. The data suggest that treatment with phospholipase A has a biphasic effect on the activity of UDP glucuronyltransferase. That this is an incorrect conclusion is clear from the experiment presented in Figure 5, in which the initial rates of activity were determined at 23°. When this was done, treatment with phospholipase A is seen to have only an activating effect on UDP glucuronyltransferase. The phospholipase A-activated form

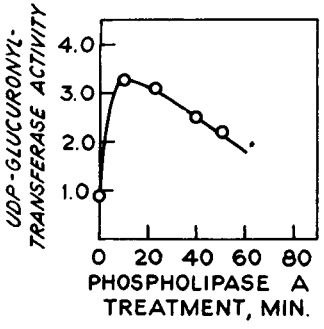

Figure 4. The effect of treatment with phospholipase A on the apparent activity of UDP-glucuronyltransferase. Microsomes from beef liver were treated with partially purified phospholipase A from *Naja naja* venom at 23° in 0.05*M* Tris, pH 8.0, at a microsomal phospholipase A protein ratio of 60:1. Assays were carried out at a *p*-nitrophenol concentration of 0.6m*M* and 3.0m*M* UDP-glucuronic acid. Assay time was 10 min. Rate is expressed as millimicromoles *p*-nitrophenol conjugated per minute per milligram protein.

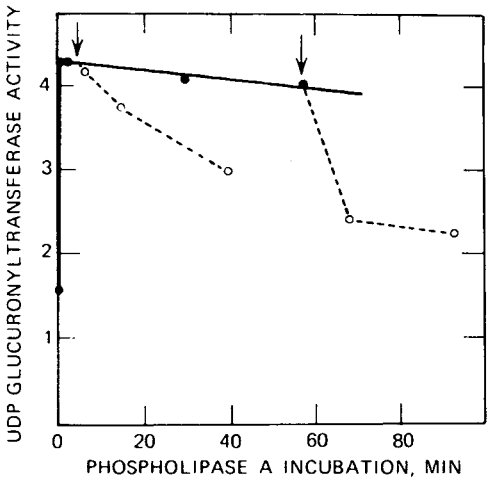

Figure 5. Effect of treatment with phospholipase A on the stability of UDP-glucuronyltransferase. Microsomes from beef liver were treated with phospholipase A as in Figure 4 at a microsome phospholipase A protein ratio of 25:1. (●). At the indicated times (arrows) aliquots were removed, made 5m*M* in EDTA in order to inhibit the further action of phospholipase A, and incubated at 37° (○). Reproduced from Vessey and Zakim (5).

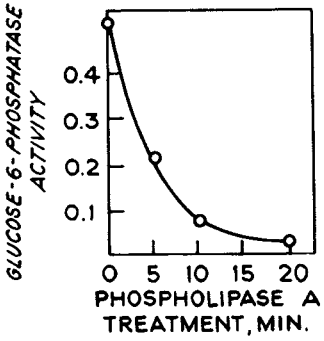

Figure 6. Effect of treatment with phospholipase A, as in Figure 4, on the activity of glucose-6-phosphatase in beef liver microsomes. Activity is expressed as micromoles phosphorus produced per minute per milligram microsomal protein.

of the enzyme, however, is unstable at 37° (dotted line in Figure 5). The effect appears to be biphasic in Figure 4 because conditions which gave zero-order kinetics for the untreated enzyme yield nonlinear kinetics for the phospholipase A-treated form of UDP glucuronyltransferase.

The importance of carefully designed studies of the effect of phospholipases and any other activators on stability is illustrated also in Figures 6 and 7. Treatment of beef liver microsomes with phospholipase A appears to inactivate glucose-6-phosphatase directly (Figure 6). As seen in Figure 7,

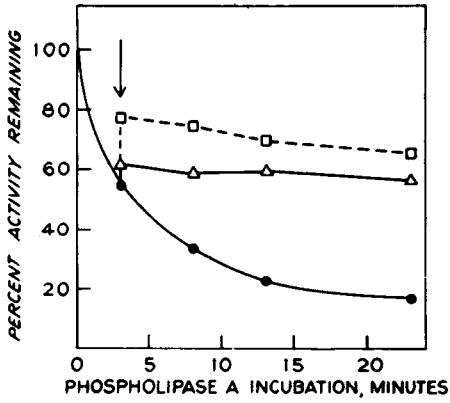

Figure 7. Effect of EDTA (●), EDTA plus albumin (□), or EDTA plus asolectin (△) on the activity of glucose -6-phosphatase in phospholipase A-treated beef liver microsomes. Microsomes were treated with phospholipase A, as in Figure 4, at a microsomal phospholipase A protein ratio of 50:1 At the arrow 5m*M* EDTA (final concentration), EDTA plus albumin, or EDTA plus asolectin was added to portions of the phospholipase A-treated microsomes and the incubations were continued at 23°. Reproduced from Zakim (4).

however, treatment with phospholipase A has no direct effect on the catalytic properties of glucose-6-phosphatase but only makes the enzyme unstable. In the absence of independent studies on stability, it might appear from selection of data in Figure 7 that treatment with phospholipase A inactivates glucose-6-phosphatase and that this effect is reversed by subsequent treatment with phospholipids. This conclusion, which has proved incorrect, was reached by Duttera et al. (35a) in studies which did not investigate the stability of phospholipase A-treated glucose-6-phosphatase (4).

Care should be taken also in interpreting the effects of phospholipase C on the properties of microsomal enzymes. The reason for this, as pointed out by Cater and Hallinan (36), is the presence of an endogenous microsomal acylhydrolase. It has been shown that the fatty acids released by the combined action of phospholipase C and endogenous diglyceride acylhydrolase make the phospholipase C-treated form of glucose-6-phosphatase unstable at 37°. To what extent presumed actions of phospholipase C on the properties of glucose-6-phosphatase and other microsomal enzymes result from these combined effects is unknown, since it is not possible to inhibit selectively the endogenous microsomal acylhydrolase.

With enzymes like UDP glucuronyltransferase, which is assayed at less

than saturating concentration of substrates, activation or inactivation could reflect changes in the binding affinity of substrates and/or the catalytic rate constant. For this reason the kinetic parameters of the activated and unactivated UDP glucuronyltransferases should be studied as in Section II, in order to determine the kinetic mechanism for changes in activity after treatment with phospholipases or other activating agents. Another important reason for determining the kinetic constants of microsomal enzymes after treatment with activating agents is that each agent may produce a different form of the enzyme. In the case of UDP glucuronyltransferase, activations by phospholipase A, Triton X-100, and alkaline pH appear to yield identical results if activity is determined at only one set of substrate concentrations; but the kinetic constants of the enzyme are different for each type of treatment. This is less of a problem with glucose-6-phosphatase since it is not too difficult to measure activity at close to V_{max} by using very high concentrations of glucose-6-P.

7. Activation by Detergents

The kinds of precautions important in investigating the effects of phospholipases on the properties of microsomal enzymes apply also to studies with detergents. Each enzyme should be titrated with a wide range of detergent concentrations, and the design of experiments should be such that changes in the stability of the enzyme are monitored at each stage of the study. Especially important in using detergents is careful control of the temperature, since treatment with detergents makes microsomal enzymes extremely labile.

IV. PREPARATION OF MICROSOMES

1. Choice of Homogenization Medium

Experience in this laboratory with liver microsomes has shown that unbuffered 0.25*M* sucrose is the most suitable homogenization medium. The pH of an unbuffered sucrose homogenate of microsomes is approximately 6. Buffering of this homogenate at pH values above 7.0 is to be avoided, since microsomes prepared in this manner can clump after freezing and thawing. High ionic strength or the addition of divalent cations to homogenizing media has a similar effect. In addition, Na^+ is reported to activate the endogenous phospholipase A of microsomes (37). The choice of homogenizing media depends also on the tissue studied. For example, optimal conditions for the isolation of sarcoplasmic reticulum are different from those for the preparation of liver microsomes (7,38).

2. Homogenization, Isolation, and Storage

For small-scale preparations we have homogenized in loose-fitting Potter-Elvehjem tubes. Preparations of good quality, biochemically and morphologically, can also be obtained after brief homogenization in a Waring blender, which we have used for large-scale preparations. Microsomes are then isolated by differential centrifugation. These microsomes are not completely stable on storage, however, in that at 0°, or even prolonged storage at −20°, the activity of glucose-6-phosphatase and UDP glucuronyltransferase increases slowly (39). The exact cause for the increasing activity of these two enzymes on storage is not completely clear, but changes observed in the kinetic properties of UDP glucuronyltransferase in beef microsomes on aging are similar to the effects of treatment with phospholipase A (5). Thus, after either addition of exogenous purified phospholipase A from *Naja naja* venom or storage, $K_{p\text{-nitrophenol}}$ increases as the activity increases. The theory that the increase in activity on storage results from the action of phospholipases is further supported by the fact that $5 \times 10^{-3}M$ EDTA partially inhibits spontaneous activation.

Elevation of the pH of the microsomal homogenate to 7.0 slows down the rate of spontaneous activation; and since lysosomal phospholipases have a pH optimum in the acid range, it is likely that the source of phospholipase which leads to activation is contaminating lysosomes, rather than the endogenous phospholipase A of the microsomes. Although addition of EDTA or adjustment of the pH to the range of 7–8 tends to increase the stability of glucose-6-phosphatase and UDP glucuronyltransferase on storage, these methods are unsuitable for the reasons cited above. Alternatively, we have attempted to diminish the contamination of microsomes with lysosomes by including an extra centrifugation step. Thus the postmitochondrial supernatant is centrifuged at 10,000*g* for 20 min before the collection of microsomes at 100,000*g*. The microsomal pellet collected by centrifugation at 100,000*g* for 60 min is resuspended and centrifuged again at 10,000*g* for 20 min before preparation of the final pellet. The rate of spontaneous activation of UDP glucuronyltransferases is slower in this type of microsomal preparation than in microsomes collected after a single centrifugation of mitochondrial supernatant at 100,000*g*, and these preparations have a useful age of at about 2 weeks when stored at −20°. Once thawed, microsomal preparations should not be refrozen and used again since freezing and thawing alone alters their properties.

Although our experience is limited to studies of the properties of glucose-6-phosphatase and UDP glucuronyltransferase, it should be pointed out that the activities of several other microsomal enzymes can be modified by treatment *in vitro* with phospholipases, detergents, and organic solvents.

It is likely, therefore, that spontaneous activation of other microsomal enzymes will be observed when looked for.

The problems of spontaneous activation of tightly bound microsomal enzymes are not limited to difficulties in comparing experiments done on different days. Spontaneous activation can alter the stability of enzymes and, more important, the effects of experimental treatments on the properties of the enzymes. For example, Triton X-100 is a potent activator of UDP glucuronyltransferase assayed with *p*-nitrophenol as aglycone (5,39). After spontaneous activation, however, treatment with Triton X-100 becomes inhibitory (39). In fact, lack of attention to the potential difficulties caused by spontaneous activation of UDP glucuronyltransferase may have contributed to conflicting reports in the literature on the effects of treatment with phospholipase A on the activity of this enzyme (5,40).

3. Large-Scale Preparation

One of the difficulties encountered in attempts to purify microsomal enzymes is the length of time needed for the preparation of starting material. Although we have not investigated its value for all microsomal enzymes, a preparation of microsomes with high specific activity for glucose-6-phosphatase in high yield can be made with a Sharples centrifuge. Chunks of beef liver are homogenized in 4 vols of 0.25 sucrose for 40 sec in a Waring blender. The homogenate is strained through cheesecloth, and centrifuged in a Sharples centrifuge with a separation bowl producing a force of 65,000*g*, at a flow rate of 200 ml/min. The supernatant from this process is equivalent to that obtained after centrifugation for 10 min at 10,000*g*. The postmitochondrial supernatant is diluted by the addition of $\frac{1}{3}$ vol of 0.25*M* sucrose and recentrifuged in the Sharples with a flow rate of 80 ml/min. The sedimented microsomes obtained in this manner have 70% of the glucose-6-phosphatase activity present in the postmitochondrial supernatant. Examination of this fraction with an electron microscope shows that the preparation contains smooth and rough microsomes with relatively little contamination by other cellular components.

4. Subfractionation of Microsomes

The distribution of enzymes within microsomal membranes is not random. For example, liver microsomal UDP *N*-acetylglucosaminyl-, galactosyl-, and sialyltransferases, which account for the terminal trisaccharide unit of a variety of plasma glycoproteins, appear to be concentrated in the Golgi apparatus (41). Fouts and his coworkers have found that several NADPH-dependent drug-metabolizing enzymes are concentrated in smooth microsomes (42). On the other hand, UDP glucuronyltransferase activities with

p-nitro- and *o*-aminophenols as aglycones were found predominantly in rough microsomes from rabbit liver (43).

Not only is there an uneven distribution of enzyme activities within the microsomal membranes, but also the properties of tightly bound microsomal enzymes are not the same in all subfractions. The specific activity of glucose-6-phosphatase is greater in rough than in smooth microsomes, but after pretreatment of microsomes at alkaline pH or with deoxycholate the specific activities in rough and smooth microsomes increase and become identical (45). Apparently, the environment of the smooth microsomes imposes a greater constraint on the maximum potential activity of glucose-6-phosphatase than does that of the rough microsomes. Although the situation is more complex with UDP glucuronyltransferase, constraint on the activity of this enzyme is also different in rough and smooth microsomes when *p*-nitrophenol is used as aglycone.*

Rough and smooth microsomes may be separated in three different ways. The first technique, described by Moule et al. (45), makes use of a series of differential centrifugations in 0.88*M* sucrose. As pointed out by Dallner and Ernster (46), this method does not yield highly purified subfractions. Rothchild's method (52) separates rough and smooth microsomes on a discontinuous sucrose gradient, but the 8-hr centrifugation required introduces problems related to stability and spontaneous activation of microsomal enzymes. The third method, which is the simplest, was developed by Dallner and his coworkers (46,48); it is based on selective aggregation of the rough microsomal fraction by monovalent cations. The Cs^+ cation is used most often, though other members of group IA of the periodic table have a similar effect. Aggregation seems to depend on neutralization of the net negative surface charge of the rough fraction of microsomes and is reversible in all cases (46).

Cesium chloride to a final concentration of 0.015*M* is added to post-mitochondrial supernatant (4.5 ml) in 0.25*M* sucrose and layered over 2 ml of 1.3*M* sucrose containing 0.015*M* CsCl. After centrifugation for 90 min at 40,000 rpm in a Spinco No. 40.2 rotor, the rough microsomes sediment as a pellet and the smooth collect at the interface. The CsCl is removed by repelleting each fraction in 0.25*M* sucrose. Rotors other than the 40.2 can be used with the limitation that the tube-to-rotor angle be at least 34°; the method will not yield good separation of fractions with a Spinco No. 30 or 40 rotor. The conditions described are suitable only for liver and must be modified when other tissues are studied (46).

Smooth and rough microsomes can be separated further into subfractions. The smooth microsomes can be fractionated into two subgroups as a result of

* D. Zakim, D. A. Vessey, and J. Goldenberg, unpublished data.

selective aggregation of a portion of the smooth microsomes with $MgCl_2$ (48). The fluffy interfacial layer obtained after centrifugation in 0.015*M* CsCl is made 0.007*M* in $MgCl_2$ and layered over 1.15*M* sucrose-0.007*M* $MgCl_2$. Centrifugation for 45 min at 102,000*g* in a No. 40.2 rotor yields one fraction of smooth microsomes as a pellet and another at the interface. The specific activity of glucose-6-phosphatase is different in these two subfractions of smooth microsomes (49). Unfortunately, however, the aggregating effect of $MgCl_2$ cannot be reversed (46), and Mg^{2+} alters the kinetic properties of some microsomal enzymes (see Section II).

The rough microsomal fraction has been fractionated further by short periods of centrifugation on continuous sucrose density gradients in the range 1.17–1.25*M* (46). The activities of NADPH and NADH cytochrome *c* reductase and glucose-6-phosphatase are not distributed evenly in these subfractions.

Morre et al. (50) have prepared highly enriched fractions of Golgi apparatus by gentle homogenization of minced liver for 30 to 60 sec in a Polytron (Brinckmann Instruments, Westbury, N.Y.), which releases subcellular structures intact. Inclusion of a relatively high concentration of sucrose and 1% dextran in the homogenization medium seems to be required for good results. After centrifugation of whole homogenates at 2000*g* for 20 min, the Golgi are isolated on a discontinuous sucrose density gradient. Electron microscopy of purified fractions shows morphologically intact Golgi apparatus. Golgi membranes have been prepared also after homogenization of liver in a Potter-Elvejhem tube by zonal centrifugation or by single-step gradient centrifugation (51,52). It has been suggested that UDP galactose-*N*-acetylglucosamine galactosyltransferase, which is 100-fold concentrated in Golgi as compared to whole homogenates of liver, is a useful marker enzyme for this organelle (52).

5. Variability in the Properties of Microsomal Enzymes

An additional problem encountered in working with microsomal enzymes is a lack of constancy in their molecular properties during the life of an animal. It has been observed that the apparent K_{aniline} and $K_{\text{ethylmorphine}}$ of microsomal aniline hydroxylase and ethylmorphine demethylase, respectively, change continuously in male rats between 1 and 12 weeks of life (53). Whether these developmental changes result from a heterogeneity of aniline hydroxylases and ethylmorphine demethylases or from interactions between the enzyme and a changing microsomal environment is unclear. Nevertheless, care must be taken in comparing the molecular properties of microsomal enzymes in animals of different ages. Unfortunately, no useful data are available on changes in the kinetic characteristics of other microsomal enzymes.