METHODS OF BIOCHEMICAL ANALYSIS

Volume 21

METHODS OF BIOCHEMICAL ANALYSIS

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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 m*M*. 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

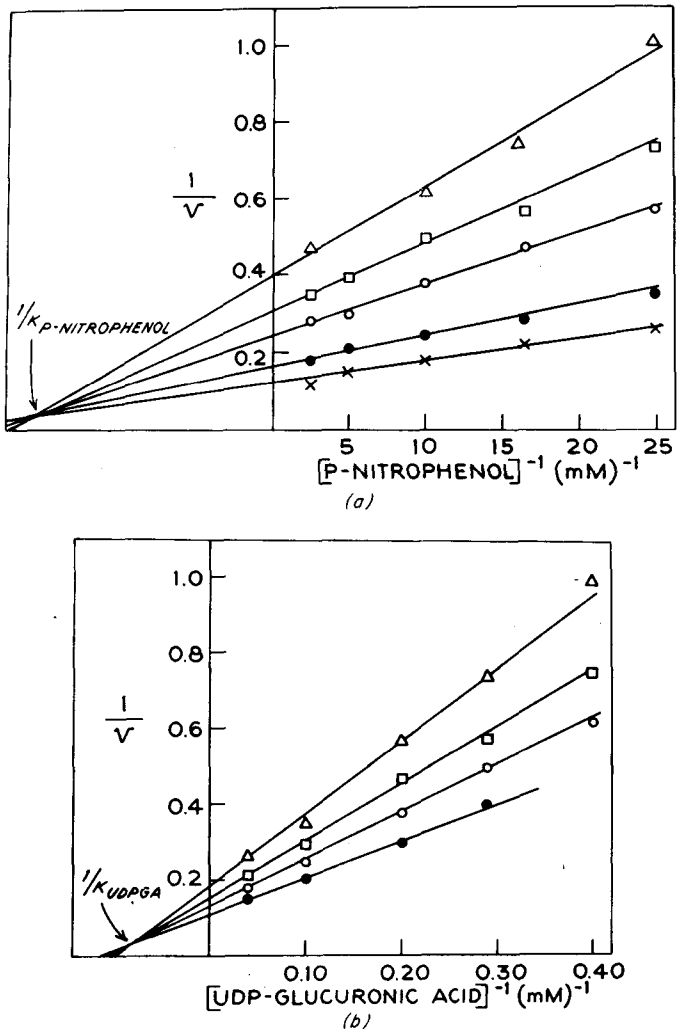

Figure 1. Determination of kinetic parameters of UDP-glucuronyltransferase. Initial rates of UDP-glucuronyltransferase were determined and plotted in double reciprocal form. (*a*) Rate as a function of the concentration of *p*-nitrophenol at several fixed concentrations of UDP-glucuronic acid: 2.5m*M* (△); 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* (●).

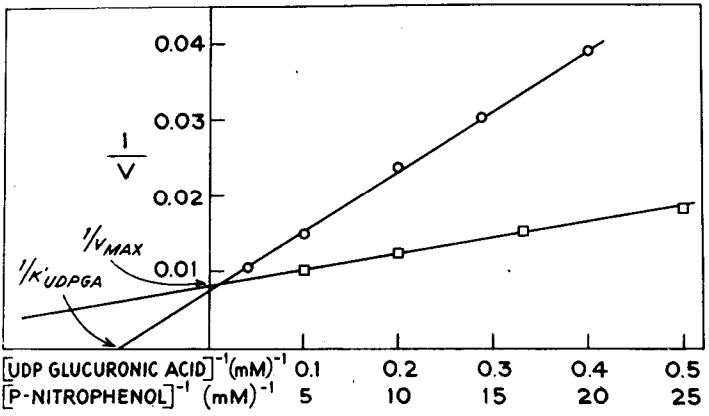

Figure 2. The intercepts on the $1/v$ axis in Figure 1 are replotted against $1/$[UDP-glucuronic acid] (○) and $1/$[*p*-nitrophenol] (□) in order to obtain $1/V_{max}$.

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/[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 $3\text{–}15 \times 10^{-3}M$.