Biochimica et Biophysica Acta, 309 (1973) 75–82

BBA 66898

DIFFERENTIATION OF HOMOLOGOUS FORMS OF HEPATIC MICROSOMAL UDP-GLUCURONYLTRANSFERASE

II. CHARACTERIZATION OF THE BILIRUBIN CONJUGATING FORM

D. A. VESSEY, J. GOLDENBERG AND D. ZAKIM

Division of Molecular Biology and the Department of Medicine, Veterans Administration Hospital, San Francisco, Calif. 94121, and the Department of Biochemistry and Biophysics, and the Department of Medicine, University of California, School of Medicine, San Francisco, Calif. 94122 (U.S.A.)

(Received November 21st, 1972)

SUMMARY

The glucuronidation of bilirubin by UDPglucuronyltransferase (EC 2.4.1.17) was investigated using a kinetic assay. The role of albumin in the assay was studied at concentrations of bilirubin both above and below its limit of solubility. In assays saturated with respect to bilirubin, albumin was almost without affect on initial rates. At concentrations of bilirubin below its limit of solubility, albumin was inhibitory. Thus, the bilirubin–albumin complex is not a substrate for the enzyme. In assays containing saturating concentrations of bilirubin, the rate of glucuronidation was influenced by conditions which "salt in" bilirubin. Using a bisubstrate kinetic analysis, the dissociation constant for the enzyme–bilirubin complex was determined to be 12–18 μM. The enzyme was shown to be stimulated by the allosteric effector UDP-*N*-acetylglucosamine which caused an increase in the apparent affinity of the enzyme for UDPglucuronic acid. The bilirubin conjugating form of UDPglucuronyltransferase showed the same general behavior toward sulfhydryl reagents and perturbers of the lipid environment as previously studied forms. However, because of differences in the response of the rate of conjugation of bilirubin to metals, UDP-*N*-acetylglucosamine, and sulfhydryl reagents, it has been concluded that the bilirubin conjugating enzyme differs from the *p*-nitrophenol, *o*-aminophenol and *o*-aminobenzoate forms of UDPglucuronyltransferase.

INTRODUCTION

UDPglucuronyltransferase (EC 2.4.1.17) is a tightly-bound microsomal enzyme which detoxifies a wide range of compounds by catalyzing the synthesis of glucuronide derivatives. The most important endogenous substrate for the enzyme(s) is bilirubin. Assays of UDPglucuronyltransferase with bilirubin as glucuronyl acceptor are technically difficult, however, and for this reason most laboratories have studied

the enzyme with substrates for which the assay technique is more reliable. In view of recent evidence indicating that there are multiple substrate specific forms of UDPglucuronyltransferase[1,2] and also because of considerable indirect evidence suggesting that bilirubin is glucuronidated by a form of UDPglucuronyltransferase[3–5] which is distinct from those delineated to this time, results obtained in studies of UDPglucuronyltransferase with aglycones other than bilirubin may not apply to the form of the enzyme responsible for the synthesis of bilirubin glucuronide.

A second problem associated with previous investigations of the glucuronidation of bilirubin is that in direct studies of the reaction, bilirubin has been added either as a bilirubin–albumin complex or dissolved in a non-polar solvent[4] in order to increase its transport or solution. It has not been established whether the bilirubin–albumin complex is a substrate for UDPglucuronyltransferase; and it is known already that non-polar solvents can alter the properties of this enzyme[4,6]. In order to resolve some of the uncertainties concerning the properties of the enzyme responsible for the synthesis of bilirubin glucuronide, the kinetic parameters of UDPglucuronyltransferase with bilirubin as substrate were determined. The effects of treatment of this enzyme with sulfhydryl group reagents and UDP-*N*-acetylglucosamine were examined in order to compare regulatory properties for bilirubin conjugation with those for other forms of UDPglucuronyltransferase which are known to glucuronidate *o*-amino- and *p*-nitrophenols.

MATERIALS AND METHODS

Uridine diphosphoglucuronic acid, uridine diphospho-*N*-acetylglucosamine and bovine serum albumin Type F were obtained from Sigma Chemical Co. Bilirubin was purchased from Pfanstiel.

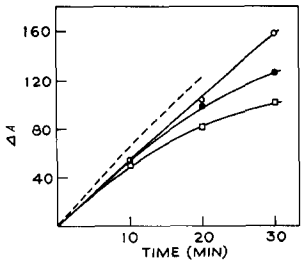
Microsomes from guinea pig and rat liver were prepared and stored as previously described[7]. The purification of phospholipase A from *Naja naja* venom and conditions for phospholipase digestion of microsomes is described in detail elsewhere[7,8].

The activity of UDPglucuronyltransferase with bilirubin as the aglycone was assayed using the colorimetric analysis for bilirubin glucuronide developed by Van Roy and Heirwegh[6]. Details of the assay have been discussed previously[7].

RESULTS

Bilirubin–albumin complex as a substrate for UDPglucuronyltransferase

One major difficulty in the assay of bilirubin glucuronidation is the limited solubility of bilirubin in water. In order to increase the substrate availability of bilirubin, it has been common to complex it to albumin[9] or another suitable protein fraction. The effects of variable amounts of albumin on the rate of reaction at a fixed concentration of bilirubin which exceeded its solubility are presented in Fig. 1. Albumin was not required for activity in agreement with the study by Wong[10]; and initial rates of reaction were independent of the concentration of albumin. However, albumin did modify the stability of UDPglucuronyltransferase. The commonly used technique of adding variable amounts of a bilirubin–albumin complex to assays, and removing a single time point sample therefore produces a false dependence of reaction rate on the concentration of bilirubin–albumin complex. Moreover, the albumin–



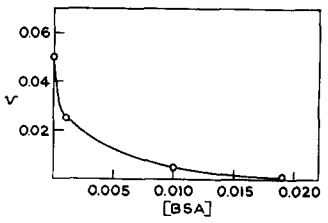
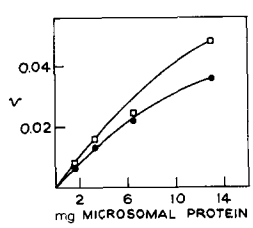


Fig. 1. Time course of bilirubin conjugation at various concentrations of albumin for assays containing an amount of bilirubin which is above its solubility. Assays contained, 0.1 M phosphate buffer (pH 7.6), 20 mM UDPglucuronic acid, 5 mM Mg^{2+}, 10 μmoles bilirubin and 10 mg guinea pig microsomal protein in a reaction volume of 2.5 ml. The concentrations of albumin in the assays were: 0.3% (○), 0.03% (●), 0.003% (□) and 0 (– – –).

Fig. 2. Initial rates of bilirubin conjugation as a function of the concentration of bovine serum albumin (BSA) for assays containing a concentration of bilirubin which is below its limit of solubility. Assays contained 0.1 M phosphate buffer (pH 7.6), 2 mM UDPglucuronic acid, 0.032 mM bilirubin, 5 mM Mg^{2+} and 4 mg/ml guinea pig microsomal protein. Rates are expressed as nmoles of bilirubin conjugated per min per mg of microsomal protein.

bilirubin complex may not be a substrate for UDPglucuronyltransferase. This conclusion was strengthened by the observation that albumin inhibits glucuronidation at concentrations of bilirubin below its limit of solubility (Fig. 2). The mechanism of this inhibition is presumably *via* the binding of bilirubin to albumin.

In view of the apparent importance of the amount of free bilirubin for enzyme activity and the possibility that microsomal protein could bind bilirubin in a non-specific manner, assays were constructed to test the proportionality between reaction rate and the concentration of microsomal protein. Proportionality curves were determined at a concentration of bilirubin above and below the limits of solubility. The data are reasonably linear up to protein concentration of about 4 mg/ml (Fig. 3). The non-linearity which develops at higher protein concentrations is similar for sub-saturating and saturating concentrations of bilirubin. Thus, the non-linearity for sub-



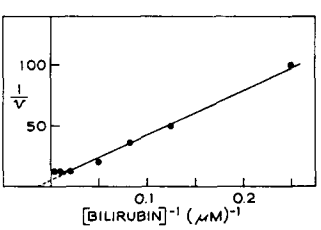


Fig. 3. Initial rates of bilirubin conjugation as a function of microsomal protein added to the assays for both a sub-saturating and saturating concentration of bilirubin. Assays contained 0.1 M phosphate buffer (pH 7.6), 10 mM UDPglucuronic acid, 4 mM Mg^{2+} and the indicated concentrations of guinea pig microsomal proteins in 2.5 ml assay volume. The bilirubin concentration was 0.020 mM (●) in the sub-saturating assay. To the other assay was added 0.15 μmoles of bilirubin (□).

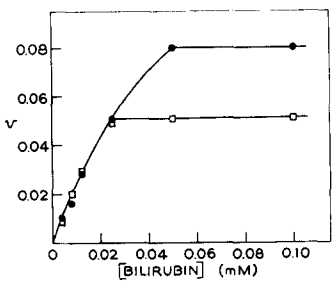
Fig. 4. Double-reciprocal plots of initial rates of bilirubin conjugation as a function of bilirubin concentration. Assays contained 0.1 M phosphate buffer (pH 7.6), 5 mM UDPglucuronic acid, 2 mM Mg^{2+} and 5 mg/ml rat liver microsomal protein. Reaction velocities are expressed as in Fig. 2.

saturating concentrations of bilirubin does not seem to be due to binding of soluble bilirubin by the microsomes.

Kinetic parameters of UDPglucuronyltransferase with bilirubin as aglycone

Since the data in Figs 1 and 2 indicate that free bilirubin is the preferred substrate for the glucuronidation reaction, as compared with an albumin–bilirubin complex, the rate of glucuronidation was studied as a function of the concentration of free bilirubin. These data, plotted in double reciprocal form, reveal typical Michaelis–Menten kinetics until the bilirubin concentration reaches the limits of its solubility (Fig. 4). At concentrations above its limit of solubility additional bilirubin has no effect on the reaction rate. It is of interest that colloidal bilirubin has no adverse effect on the activity of UDPglucuronyltransferase.

It is clear from the data in Fig. 4 that a potential complication in the assay of UDPglucuronyltransferase with bilirubin as aglycone is the effect of assay conditions on the solubility of bilirubin. For example, Burnstine and Schmid[11] have shown that



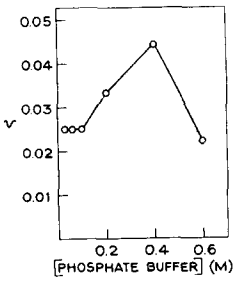
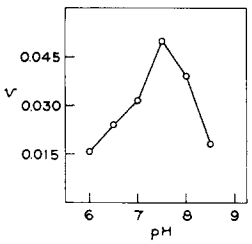


Fig. 5. Initial rates of bilirubin conjugation as a function of bilirubin concentration in the presence or absence of Mg^{2+}. Assays contained 0.1 M phosphate buffer (pH 7.6), 10 mM UDPglucuronic acid and 5 ml/mg guinea pig microsomal protein either in the presence of 2 mM Mg^{2+} (●) or the absence of Mg^{2+} (□). Rates are expressed as in Fig. 2.

Fig. 6. Initial rates of bilirubin conjugation as a function of the phosphate buffer concentration. Assays contained 12 mM UDPglucuronic acid, 0.016 mM bilirubin, 5 mM Mg^{2+} and 5 mg/ml guinea pig microsomal protein. Rates of reaction are expressed as in Fig. 2.

the solubility of bilirubin in aqueous systems is enhanced by increasing concentrations of salt or by increasing pH. Thus, changes in salt concentration, as for example the concentration of buffer or Mg^{2+}, or in pH, could influence activity indirectly by modifying the solubility of bilirubin. Therefore, the possible effects of salt concentration were examined by comparing activity in the presence and absence of Mg^{2+} at subsaturating and supersaturating concentrations of bilirubin. At subsaturating concentrations of bilirubin, Mg^{2+} (5 mM) has no effect on the rate of bilirubin conjugation. On the other hand, because Mg^{2+} increases the solubility of bilirubin it appears to activate the rate of glucuronidation of bilirubin at saturating concentrations of aglycone (Fig. 5). Phosphate buffer also increased bilirubin solubility. Moreover, at high concentrations of P_i it increased activity even at concentrations of bilirubin below its limit of solubility (Fig. 6). The mechanism of this activation is unclear, but the data in Fig. 6 show that 0.1 M is the highest usable concentration of phosphate buffer for studies of "native" enzyme. Activity as a function of pH was studied also



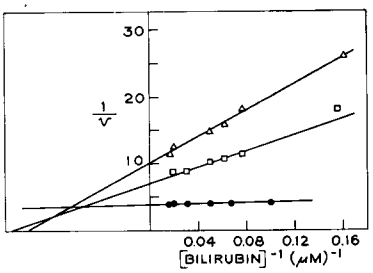


Fig. 7. Initial rates of bilirubin conjugation as a function of assay pH. Assays were constructed as in Fig. 6 with 0.1 M phosphate buffer. Rates of reaction expressed as in Fig. 2.

Fig. 8. Double-reciprocal plots of initial rates of bilirubin conjugation as a function of bilirubin concentration at three different fixed concentrations of UDPglucuronic acid. Assays as in Fig. 4 at UDPglucuronic acid concentrations of 6.0 (△), 12.0 (□) and 25 mM (●). Rates are expressed as in Fig. 2.

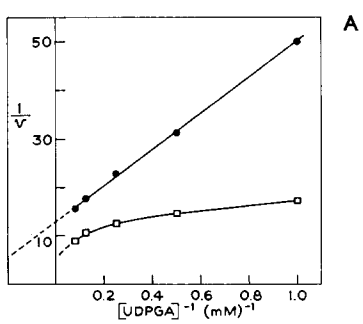
at concentrations of bilirubin below saturating for the lowest pH used. With these assay conditions there is a sharp optimum at pH 7.5 (Fig. 7), which reflects the direct action of pH on the enzyme.

Kinetic analysis

Many investigators have attempted to determine the kinetic constants but utilized single substrate kinetics at a presumed saturating concentration of second substrate. It is clearly impossible to saturate the enzyme with bilirubin due to its low solubility and the failure of the albumin–bilirubin complex to serve as substrate for the enzyme. It is also not practical to use saturating concentrations of UDP-glucuronic acid (Fig. 8). Bisubstrate kinetic analysis yields a $K_{\text{bilirubin}}$ of approx. 15 μM and $K_{\text{UDPglucuronic acid}}$ of 10 mM for the binding of substrates to free enzyme.

Regulation of bilirubin: UDPglucuronyltransferase

With *p*-nitrophenol, *o*-aminophenol, or *o*-aminobenzoate as aglycones, UDP-



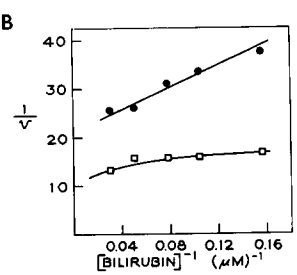
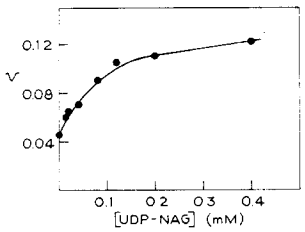


Fig. 9. Effect of UDP-*N*-acetylglucosamine on double-reciprocal plots of initial rates as a function of substrate concentration. A. Assays contained variable concentrations of UDP-glucuronic acid, (UDPGA) 0.020 mM bilirubin, 0.1 M phosphate buffer (pH 7.6), 4 mM Mg^{2+} and 4 mg/ml of guinea pig microsomal protein in either the presence (□) or absence (●) of 0.5 mM UDP-*N*-acetylglucosamine. B. Assays contained variable concentrations of bilirubin, 4.0 mM UDP-glucuronic acid, 0.1 M phosphate buffer (pH 7.6), 4 mM Mg^{2+} and 4 mg/ml of guinea pig microsomal protein in either the presence (□) or absence (●) of 0.5 mM UDP-*N*-acetylglucosamine.



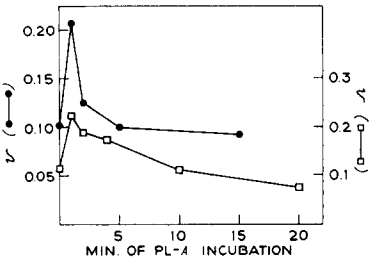


Fig. 10. Initial rates of bilirubin conjugation as a function of the concentration of UDP-*N*-acetylglucosamine. Assays contained 0.1 M phosphate buffer (pH 7.6), 1 mM UDPglucuronic acid, 0.032 mM bilirubin, 4 mM Mg^{2+} and 5 mg/ml guinea pig microsomal protein. Rates are expressed as in Fig. 2. UDP-NAG, UDP-*N*-acetylglucosamine.

Fig. 11. The effect of phospholipase A on initial rates of bilirubin conjugation. Rat microsomes (●) and guinea pig microsomes (□) were treated with phospholipase A (PL-A) at 23 °C in the presence of 0.05 M Tris buffer (pH 8.0) at a microsomal to phospholipase protein ratio of 100:1 for the times indicated. Aliquots of the incubation mixture were removed and assayed in the presence of 5 mM EDTA with 12 mM UDPglucuronic acid and saturating concentrations of bilirubin. Rates are expressed as in Fig. 2.

N-acetylglucosamine stimulates the activity of UDPglucuronyltransferases by increasing the affinity of the enzyme for UDPglucuronic acid[12]. This is also the case with bilirubin as glucuronyl acceptor (Fig. 9). In fact, in the presence of UDP-*N*-acetylglucosamine the rate of glucuronidation of bilirubin tends to become independent of the concentrations of either substrate (Fig. 9).

The activities of UDPglucuronyltransferases with *p*-nitrophenol, *o*-aminophenol and *o*-aminobenzoate as aglycones are sensitive to alterations in the lipid phase[2,8]. We have examined this property of the enzyme with bilirubin as substrate by determining the effect of treatment with phospholipase A on enzymatic activity. The data in Fig. 11 show the time course for the effects of treatment with phospholipase A on activity for both rat and guinea pig microsomes and reveal that the maximum potential activity with bilirubin as aglycone, which is constrained in native microsomes, is increased by treatment of microsomes with phospholipase A.

Another general characteristic of UDPglucuronyltransferases is their sensitivity to organic mercurials. There are three distinct types of sulfhydryl groups which influence the rate of glucuronidation of *o*-amino- and *p*-nitrophenols and *o*-aminoben-

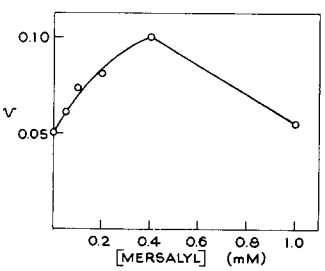


Fig. 12. The effect of mersalyl on initial rates of bilirubin conjugation. Rat microsomes at a final protein concentration of 10 mg/ml in 50 mM Tris buffer (pH 7.6) were incubated for 2 min at 23 °C with the indicated concentration of mersalyl and then assayed in the presence of 12 mM UDPglucuronic acid, 5 mM Mg^{2+} and 0.02 mM bilirubin. Activity is expressed as in Fig. 2.

zoic acid[13]. Two of these, Types 2 and 3, also effect the glucuronidation of bilirubin as evidenced by the biphasic titration curve of organic mercurials (Fig. 12). The stimulation of activity at low concentrations of mersalyl is attributed to the titration of the Type 2 sulfhydryl and the decline in activity seen at concentrations of mersalyl above 0.4 mM results from the titration of the Type 3 sulfhydryl.

DISCUSSION

Assay of UDPglucuronyltransferase with bilirubin as aglycone

The bilirubin metabolizing form of UDPglucuronyltransferase illustrates many of the problems associated with the assay of membrane-bound enzymes which have substrates with low solubility in water. Previous methods for enhancing the solubility of bilirubin in assays of activity of UDPglucuronyltransferase are not needed and often produce spurious results. For example, it has been reported previously that Mg^{2+} increases the rate of synthesis of bilirubin glucuronide[6,9,10], but in carefully controlled experiments this effect of Mg^{2+} results from an enhancement of the solubility of bilirubin. Mg^{2+} has no direct effect on the activity of the bilirubin metabolizing form of UDPglucuronyltransferase. Similarly, many of the previous estimates of pH–activity curves of this enzyme are erroneous because variations in pH not only affect the enzyme directly, but also influence the solubility of bilirubin. Correct pH–activity curves can be obtained, however, with assays containing less than a saturating concentration of bilirubin (Fig. 7).

Regulation of the bilirubin metabolizing form of UDPglucuronyltransferase

UDP-*N*-acetylglucosamine enhances the affinity of UDPglucuronyltransferase for UDPglucuronic acid; and in the presence of UDP-*N*-acetylglucosamine rates of glucuronidation are measured easily for concentrations of UDPglucuronic acid as small as 0.1 mM. As seen in Fig. 8, prior binding of UDPglucuronic acid increases the affinity of the enzyme for bilirubin; and either because of its effect on affinity for UDPglucuronic acid or directly on the binding of bilirubin, UDP-*N*-acetylglucosamine increases the apparent affinity of the enzyme for bilirubin. As a result, the rate of synthesis of bilirubinglucuronide is essentially independent of the concentration of either substrate for assays carried out in the presence of UDP-*N*-acetylglucosamine. Since the solubility of free bilirubin within the cytosol is adequate to maintain high rates of synthesis of bilirubin glucuronide, there is no need to postulate *in vivo* "solubilization" of bilirubin by protein carriers as necessary for enzyme function.

Relationship of bilirubin metabolizing form of UDPglucuronyltransferase to other substrate specific forms of this enzyme

As for all other forms of UDPglucuronyltransferase examined in this laboratory the activity of the bilirubin metabolizing form is sensitive to changes in its lipid environment, has at least two sulfhydryl groups which affect activity, and is subject to regulation by UDP-*N*-acetylglucosamine. Despite these similarities it is possible to distinguish between the bilirubin metabolizing and other forms of the enzyme. Thus, titration with UDP-*N*-acetylglucosamine reveals greater affinity for this compound by the bilirubin metabolizing form of UDPglucuronyltransferase than those responsible for the synthesis of *p*-nitrophenyl-, *o*-aminophenyl- and *o*-aminobenzoyl-

glucuronides. Similarly, the Type 2 sulfhydryl group of the bilirubin metabolizing enzyme is titrated by smaller concentrations of mersalyl than the Type 2 sulfhydryl in other UDPglucuronyltransferases. A third clear-cut difference between the UDPglucuronyltransferase synthesizing bilirubin glucuronide and the other species of this enzyme is in their response to metals. As shown in Fig. 5, Mg^{2+} has no direct effect on the kinetic behavior of UDPglucuronyltransferase in assays with bilirubin as aglycone whereas Mg^{2+} alters the affinity of the *o*-aminobenzoic, *o*-aminophenol and *p*-nitrophenol forms of the enzyme for UDPglucuronic acid. With the *o*-aminophenol and *p*-nitrophenol as aglycones Mn^{2+} and Ca^{2+} have effects similar to those of Mg^{2+}. With bilirubin as aglycone, Mn^{2+} or Ca^{2+} inactivate UDPglucuronyltransferase. The data suggest, therefore, that bilirubin is glucuronidated by a form of UDPglucuronyltransferase different from those metabolizing *p*-nitro- and *o*-aminophenols and *o*-aminobenzoate, but that all three enzymes are related closely.

ACKNOWLEDGEMENTS

The authors thank Dr Thomas P. Singer for his continued interest and support of this work. D. A. V. is a Fellow of the Giannini Foundation. This work was supported in part by grants from the United States Public Health Service (HE 10027) and the National Science Foundation (8248) to Dr Thomas P. Singer.

REFERENCES

1 Zakim, D. and Vessey, D. A. (1972) *Fed. Proc.* 31, 925
2 Zakim, D., Goldenberg, J. and Vessey, D. A. (1973) *Biochim. Biophys. Acta* 309, 67–74
3 Tomlinson, G. A. and Yaffee, S. J. (1966) *Biochem. J.* 99, 507–512
4 Dutton, G. J. (1966) in *Glururonic Acid, Free and Combined* (Dutton, G. J., ed.), pp. 185–299, Academic Press, New York
5 Wisnes, A. (1969) *Biochim. Biophys. Acta* 191, 279–291
6 Van Roy, F. P. and Heirwegh, K. P. M. (1968) *Biochem. J.* 107, 507–518
7 Zakim, D. and Vessey, D. A. (1972) in *Methods of Biochemical Analysis* (Glick, D., ed.), Vol. 21, in press, Interscience, New York
8 Vessey, D. A. and Zakim, D. (1971) *J. Biol. Chem.* 246, 4649–4656
9 Lathe, G. M. and Walker, M. (1958) *Biochem. J.* 70, 705–709
10 Wong, K. P. (1971) *Biochem. J.* 125, 27–35
11 Burnstine, R. C. and Schmid, R. (1962) *Proc. Soc. Exp. Biol. Med.* 109, 356–359
12 Vessey, D. A., Goldenberg, J. and Zakim, D. (1973) *Biochim. Biophys. Acta* 309, 58–66
13 Zakim, D. and Vessey, D. A. (1972) *Arch. Biochem. Biophys.* 148, 97–106
14 Keppler, D. O. R., Rudigier, J. F. M., Bischoff, E. and Decker, K. F. A. (1970) *Eur. J. Biochem.* 17, 246–253