Biochemical Pharmacology, Vol. 25, pp. 1293–1297. Pergamon Press, 1976. Printed in Great Britain.

UDP-GLUCURONYLTRANSFERASE IN PERFUSED RAT LIVER AND IN MICROSOMES—III. EFFECTS OF GALACTOSAMINE AND CARBON TETRACHLORIDE ON THE GLUCURONIDATION OF 1-NAPHTHOL AND BILIRUBIN*

GENJI OTANI†, MAHMUD M. ABOU-EL-MAKAREM‡ and KARL WALTER BOCK
Institut für Toxikologie der Universität Tübingen

(*Received* 23 *September* 1975; *accepted* 21 *November* 1975)

Abstract—Galactosamine treatment (400 mg/kg, i.p., 4 hr) markedly decreased the level of UDP-glucuronic acid (UDPGA) and 1-naphthol glucuronidation in perfused liver. In contrast, bilirubin glucuronidation was not affected. In non-activated microsomes both 1-naphthol and bilirubin glucuronidation were dependent upon the concentration of UDPGA. In UDP-*N*-acetylglucosamine-activated microsomes, 1-naphthol glucuronidation remained dependent upon UDPGA whereas bilirubin glucuronidation tended to be independent of UDPGA.

Carbon tetrachloride treatment (5 ml/kg, *per os*, 24 hr) strongly decreased 1-naphthol glucuronidation in the intact liver without altering the level of UDPGA. Bilirubin glucuronidation was affected similarly but to a lesser extent. In contrast, 1-naphthol glucuronidation in liver microsomes was increased under these conditions. In the presence of UDP-*N*-acetylglucosamine and UDP, however, enzyme activity in microsomes from CCl_4-treated rats was lower than in control microsomes.

The results suggest a differential regulation of 1-naphthol and bilirubin glucuronidation and stress the importance of intracellular effectors for glucuronidation in the intact liver.

Glucuronidation is a major pathway by which the body inactivates and eliminates a wide variety of foreign chemicals as endogenous substances [1]. In previous papers [2, 3] methods have been described to study 1-naphthol and bilirubin glucuronidation in the intact liver. In the present report the level of UDP-glucuronic acid was altered by various treatments and the microsomal membrane structure was damaged by CCl_4 treatment in order to evaluate different factors influencing glucuronidation *in vivo*. The influence of these treatments on glucuronidation of 1-naphthol and bilirubin was studied. The effects observed in the intact liver were compared with properties of UDP-glucuronyltransferase (1-naphthol and bilirubin as substrates) in liver microsomes.

Part of this work has been presented in preliminary form [4].

MATERIALS AND METHODS

1-[1-^{14}C]naphthol (20.8 mCi/m-mole) and (1′,2,3′,4,5,6′,7′,8-^{14}C) bilirubin (16.9 mCi/m-mole) were obtained from Radiochemical Center, Amersham.

Male Sprague–Dawley rats (200–250 g) were fed *ad lib.* a standard diet containing 20 per cent protein (Altromin, Lage-Lippe, Germany). Animals were treated either with galactosamine-HCl (400 mg/kg, i.p., for 3 hr [5]), with orotic acid (1 g/kg, i.p., for 2 hr [5]) or with insulin (4 I.U./kg, i.p., for 2 hr [6]). When the pretreated livers were perfused, 5 mM galactosamine-HCl or 5 mM orotic acid, or 4 I.U. insulin, respectively, were added to 70 ml perfusion medium at the start of perfusion. Carbon tetrachloride was dissolved in olive oil (1:1, v/v) and was given orally at 1,3 and 5 ml CCl_4/kg. Animals were sacrificed after 24 hr [7].

Previously described methods were used for the determination of 1-naphthol glucuronidation and sulfate ester formation in the isolated perfused rat liver, for estimating UDP-glucuronic acid levels in liver tissue and for the preparation of liver microsomes [2]. Bilirubin glucuronidation in perfused liver and in fistula rats was estimated from the rate of appearance of [^{14}C]bilirubin conjugates in bile and the analysis of labeled bilirubin conjugate according to Heirwegh *et al.* [8] as described [3]. With this method bilirubin glucuronidation can only be roughly estimated for the following reason: under the conditions of diazotization tetrapyrroles are split into two dipyrroles. Pure bilirubin monoglucuronide would therefore yield azodipyrrole and azodipyrrole glucuronide in a ratio of 1:1 [9]. The ratio of azodipyrrole glucuronide plus nonglucuronide polar azodipyrrole to azodipyrrole was found to be about 7:3 in fistula bile [Table 2] indicating that a high proportion of bilirubin was conjugated at both propionic acid groups. The proportion of bilirubin mono- and diconjugates with glucuronic acid and other groups is unknown, thus the proportion of glucuronic conjugates cannot be predicted accurately. However, it can be clearly shown whether glucuronic conjugates are decreased or increased after treatment in comparison with control bile.

*Previous papers in the series are references [2] and [3].

† Present address: Kowa company, Ltd., Higashimurayama, Tokyo, Japan.

‡ Present address: Al Azhar University, Kairo, Egypt.

Assay of UDP-glucuronyltransferase. Assays were performed at 37° in a total volume of 1 ml containing 100 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ as well as different acceptor substrates as indicated. The enzyme reaction was started by the addition of 3 mM UDP-glucuronic acid.

In controls UDP-glucuronic acid was omitted. (a) 1-naphthol glucuronidation: 0.5 mM 1-naphthol dissolved in 0.25% (v/v) dimethylsulfoxide was incubated with 0.04 μCi 1-[1-^{14}C]naphthol and 1 mg microsomal protein of 0.2 ml liver homogenate (1:5, w/v). After 0.5 to 2 min incubation the reaction was stopped by addition of 0.4 M trichloroacetic acid-0.6 M glycine buffer, pH 2.2. Following centrifugation at 3000 *g* for 5 min the supernatant was extracted with 8 ml chloroform to remove unreacted naphthol. The radioactivity of the aqueous phase was determined in Brays scintillation fluid [10]. Zero-time blanks were subtracted. The rate of naphthol glucuronidation was calculated from the total radioactivity of the original naphthol solution which corresponded with 500 nmole 1-naphthol. The identity of the glucuronide in the aqueous phase was verified by t.l.c. on cellulose plates with ethanol-1 M ammonium acetate (9:1, v/v) as the solvent system and by the detection of 1-naphthol after hydrolysis with β-glucuronidase. (b) Bilirubin glucuronidation was determined according to Van Roy and Heirwegh [11] as previously described [12] except that 0.05 mM bilirubin was used.

Protein was determined according to Lowry *et al.* [13] using bovine serum albumin as the protein standard.

RESULTS AND DISCUSSION

1. Influence of UDP-glucuronic acid levels on 1-naphthol and bilirubin glucuronidation. The formation of UDP-glucuronic acid is regulated primarily at the level of UDP-glucose dehydrogenase [14]. Similar levels of UDP-glucuronic acid have been found *in vivo* and in the perfused rat liver [2]. The level in the perfused organ was not altered during prolonged glucuronidation indicating that the regeneration of the nucleotide could not be exhausted under our perfusion conditions. The level of UDP-glucuronic acid can be decreased by the administration of galactosamine which traps uridine nucleotides primarily as UDP-galactose [5]. The decrease of UDP-glucuronic acid could be reversed by the administration of orotic acid the precursor of uridine nucleotides. It was therefore of interest to learn whether a high dose of orotic acid may increase the nucleotide level above its normal value. Insulin treatment has been reported to increase the hepatic UDP-glucuronic acid content by an unknown mechanism [6].

As shown in Table 1, galactosamine treatment significantly decreased the level of UDP-glucuronic acid both *in vivo* and in the perfused liver. Treatment with orotic acid or insulin only slightly increased the nucleotide level. Carbon tetrachloride treatment will be discussed later. Under the conditions of treatment, listed in Table 1, 1-naphthol glucuronidation was determined. The formation of 1-naphthol glucuronide was decreased in galactosamine-treated livers (Fig. 1). The decrease in glucuronic acid conjugation was compensated by increased sulfate ester formation. Treatment with orotic acid or insulin (data not shown) slightly increased glucuronide formation. In contrast to 1-naphthol glucuronidation bilirubin glucuronidation was not significantly altered by galactosamine-treatment (Fig. 2). This was demonstrated in perfused liver as well as in fistula rats (data not shown) no matter whether a trace dose of [^{14}C]bilirubin or a saturating concentration of bilirubin was used. Similarly treatment with orotic acid or insulin did not alter bilirubin conjugation. In order to exclude the possibility that an altered glucuronide conjugation was compensated by other conjugation pathways, bilirubin conjugates in bile were analysed with the method of Heirwegh *et al.* [8]. Non-glucuronide conjugates are shown collectively as discussed previously [3]. No significant alterations of bile pigment composition were found after treatment with either galactosamine, orotic acid or insulin (Table 2).

Table 1. Hepatic levels of UDP-glucuronic acid after various treatments of rats

Treatment	UDP-glucuronic acid levels *In vivo* (μmole/g liver)	In perfused liver (μmole/g liver)
Control	0.30 ± 0.03	0.29 ± 0.07*
Galactosamine (400 mg/kg i.p., 3 hr)	0.17 ± 0.02	0.18*
Orotic acid (1 g/kg i.p., 2 hr)	0.38 ± 0.03	
Insulin (4 I.U./kg i.p., 2 hr)	0.38 ± 0.04	
CCl_4 (1 ml/kg, *per os* 24 hr)	0.35 ± 0.05	
CCl_4 (5 ml/kg, *per os* 24 hr)	0.27 ± 0.05	

* data taken from reference [2]

UDP-glucuronic acid was determined in liver tissue, excised under ether anesthesia, as described in Methods. The mean ± S.D. of 4 experiments is shown.

The differential effect of galactosamine on 1-naphthol and bilirubin glucuronidation in intact liver was interesting since both reactions are dependent upon the concentration of UDP-glucuronic acid when assayed in non-activated microsomes (Fig. 3). When kinetic properties were studied in the presence of the allosteric activator, UDP-*N*-acetylglucosamine, 1-naphthol glucuronidation remained dependent upon UDP-glucuronic acid: bilirubin glucuronidation, however, tended to be independent on the endogenous substrate in the physiological concentration range. The latter finding confirms observations by Vessey *et al.* in guinea pig microsomes [15]. The fact that bilirubin glucuronidation becomes practically independent of the UDP-glucuronic acid concentration in the UDP-*N*-acetylglucosamine-activated form may explain why galactosamine treatment has no effect on bilirubin glucuronidation in the intact liver.

The results suggest that UDP-*N*-acetylglucosamine is an important intracellular effector of the enzyme. The differential effects of the allosteric effector on 1-naphthol and bilirubin glucuronidation are in

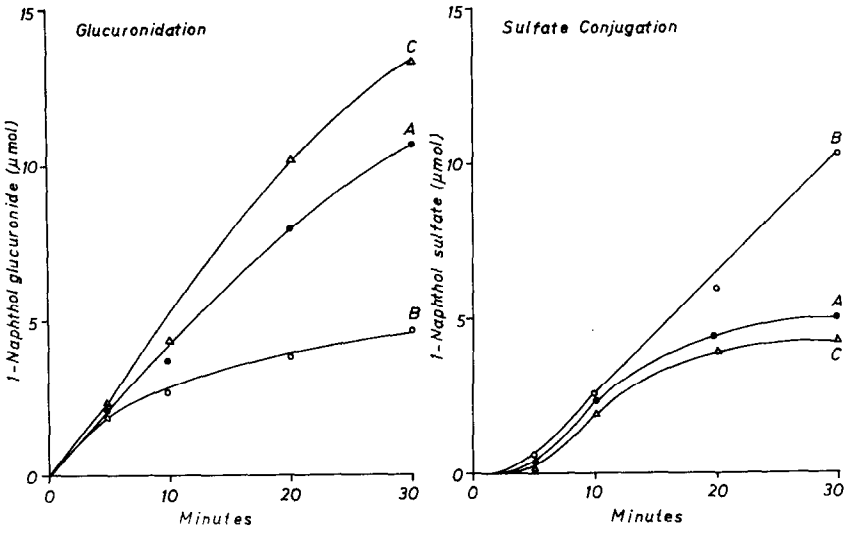

Fig. 1. Effects of treatment with galactosamine and orotic acid on the excretion of 1-naphthol conjugates into the perfusion medium of the perfused rat liver. (A) Control; (B) galactosamine treatment (400 mg/kg, i.p., 4 hr); (C) treatment with orotic acid (1 g/kg, i.p., 3 hr). 1-Naphthol conjugates were determined as described in Methods. The mean of 4 experiments is shown.

favour of the hypothesis that these two substrates are glucuronidated by different forms of UDP-glucuronyltransferase. There is accumulating evidence for a multiplicity of this enzyme [1, 12, 16–19].

2. Influence of carbon tetrachloride treatment on glucuronide formation in the intact liver. Carbon tetrachloride is known as a hepatotoxic agent [20]. During its metabolism a reactive radical is formed which causes lipid peroxidation in the microsomal membranes. Since the activity of microsomal UDP-glucuronyltransferase is constrained by the phospholipids of the membrane [21] it was of interest to investigate the effect of CCl_4 treatment on 1-naphthol and bilirubin glucuronidation in the intact liver. Conditions of treatment were used which have been demonstrated to cause stimulation of UDP-glucuronyltransferase activity in liver microsomes [7]. As shown in Fig. 4, a strong and dose dependent decrease of 1-naphthol glucuronidation in perfused liver of CCl_4 treated rats was found. At high doses sulfate ester formation was also decreased but to a lesser extent. The low glucuronide formation was not due to a decreased level of UDP-glucuronic acid (Table 1).

The effect of CCl_4 treatment in the intact liver is in contrast to the stimulated enzyme activity in liver microsomes. Therefore properties of the microsomal enzyme were studied in more detail. Liver microsomes from CCl_4 treated rats were compared with untreated controls (Table 3). UDP-glucuronyltransferase activity, without additions to the assay, was higher than in controls. When the enzyme was fully activated by the addition of 0.05% (w/v) Triton X-100 initial glucuronidation rates were lower than in controls. These findings confirm the results of Aitio [7] who concluded that the enzyme is activated by CCl_4-treatment. Interestingly the enzyme could not be activated by UDP-*N*-acetylglucosamine in the CCl_4-treated group. It has been shown that UDP is an effective product inhibitor in the absence of UDP-*N*-acetylglucosamine. In its presence the K_i for UDP

Table 2. Analysis of bilirubin conjugates in rat fistula bile after various treatments

Treatment	Azodipyrrole	Azodipyrrole glucuronide	Non-glucuronide azodipyrrole conjugates	Excretion of bilirubin conjugates 3 hr after labeling
	(% of radioactivity)			(% of dose)
Control	31	53	16	95
Galactosamine–HCl (400 mg/kg, i.p., 4 hr)	37	53	10	95
Orotic acid (1 g/kg, i.p., 3 hr)	34	59	7	92
Insulin (4 I.U./kg, i.p., 3 hr)	36	46	17	90
CCl_4 (3 ml/kg, *per os*, 24 hr)	32	34	28	92
CCl_4 (5 ml/kg, *per os*, 24 hr)	29	28	34	92

Bilirubin conjugates were analyzed according to Heirwegh *et al.* [8] 3 hr after intravenous injection of 0.1 μCi [^{14}C]bilirubin. The mean of 3 experiments is shown.

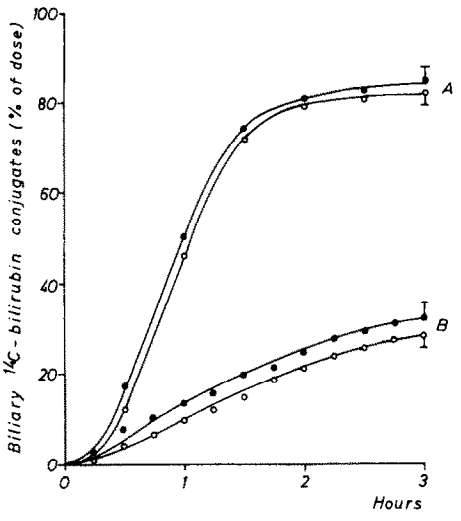

Fig. 2. Effect of galactosamine treatment on the biliary excretion of bilirubin conjugates in the perfused rat liver. (●) Control, (○) galactosamine treatment (400 mg/kg, i.p., 4 hr). (A) 0.1 μCi [^{14}C]bilirubin (=3.5 μg) was added to the perfusion medium at zero time. (B) 25 mg bilirubin was added together with [^{14}C]bilirubin. Bile was collected every quarter hour and counted for radioactivity. The mean ± S.D. of 4 experiments is shown.

is markedly increased [22]. This is also seen in Table 3 in the control group. However in the CCl_4-treated group UDP was an effective inhibitor even in the presence of UDP-*N*-acetylglucosamine. These results suggest that the allosteric effector has lost its regulatory properties in microsomes from CCl_4-treated rats. It is very likely that in these microsomes the constraint of UDP-glucuronyltransferase is released, and that the release of the constraint is associated with the loss of regulatory properties of UDP-*N*-acetylglucosamine.

It has been demonstrated previously that the release of the constraint of UDP-glucuronyltransferase leads to a number of other alterations, e.g., to inhibition of the enzyme by other UDP-sugars [21]. This inhibition is probably caused by competition between UDP-glucuronic acid and other UDP-sugars for a common binding site. In the constrained form the enzyme is specific for UDP-glucuronic acid. Lack of activation by UDP-*N*-acetylglucosamine as well as increased inhibition by UDP and possibly by UDP-sugars may be dominant factors leading to the profound decrease of 1-naphthol glucuronidation in the intact liver after treatment with CCl_4.

The total amount of bilirubin conjugates excreted in livers from CCl_4-treated animals was not significantly different from that of controls described in Fig. 2. However, when bilirubin conjugates in bile were

Table 3. Effect of CCl_4-treatment on microsomal UDP-glucuronyltransferase activity

Additions to assay	UDP-glucuronyltransferase (nmole/min/mg protein) Control	CCl_4-treated
None	3.1 ± 0.6	10.1 ± 2.5
None + UDP	1.8 ± 0.4	2.9 ± 1.1
UDP-*N*-acetylglucosamine	10.7 ± 1.3	11.4 ± 1.6
UDP-*N*-acetylglucosamine + UDP	9.3 ± 1.5	4.5 ± 1.9
Triton X-100	44.5 ± 6.0	32.0 ± 9.2
Triton X-100 + UDP	25.6 ± 4.5	22.7 ± 4.2

Rats were treated with 5 ml/kg CCl_4 *per os*. Liver microsomes were prepared after 24 hr and assayed for UDP-glucuronyltransferase activity as described in Methods. The concentration of the nucleotides was 3 mM, that of Triton X-100 0.05% (w/v). The mean ± S.D. of 4 experiments is shown.

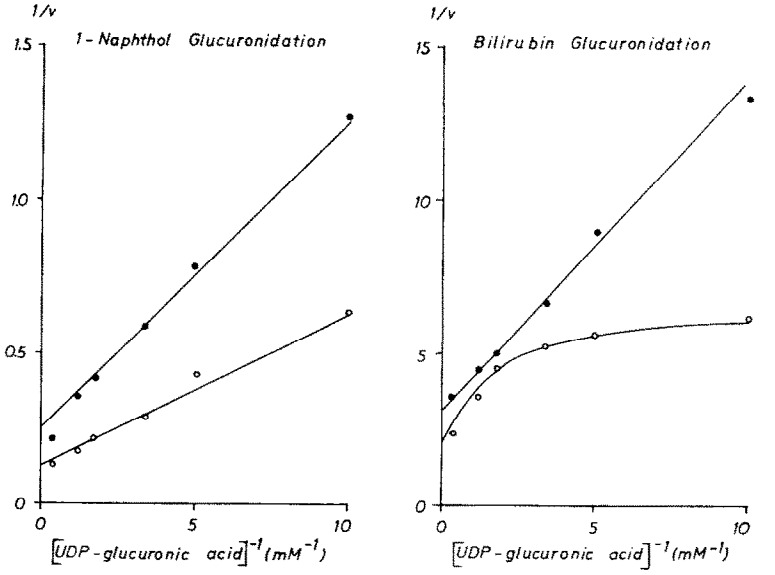

Fig. 3. Effect of UDP-*N*-acetylglucosamine on double reciprocal plots of initial rates of 1-naphthol and bilirubin glucuronidation. (●) Control; (○) addition of 3 mM UDP-*N*-acetylglucosamine to the assay. Liver microsomes were prepared and assayed for 1-naphthol and bilirubin glucuronidation as described in Methods. The mean of 3 experiments is shown.

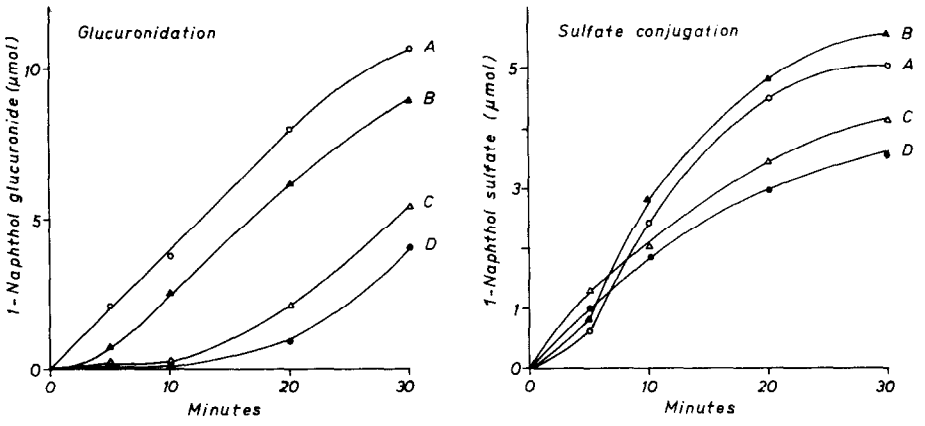

Fig. 4. Effects of CCl_4 treatment of 1-naphthol glucuronidation in the perfused rat liver. (A) Control; (B) CCl_4 treatment (1 ml/kg); (C) CCl_4 treatment (3 ml/kg); (D) CCl_4 treatment (5 ml/kg). Livers were hepatectomized and perfused after 24 hr. The mean of 3 experiments is shown.

analyzed with the method of Heirwegh *et al.* [8], bilirubin glucuronides were significantly decreased and concommittantly non-glucuronide conjugates were increased (Table 2). Bile pigment composition was altered similarly in postcholestatic bile which probably releases the constraint of UDP-glucuronyltransferase [3, 23]. Bilirubin glucuronidation was affected less severely than 1-naphthol glucuronidation in livers from CCl_4-treated rats, but the glucuronidation of both substrates was decreased.

Treatment with galactosamine and CCl_4 have selective effects on glucuronide formation in the intact liver. Galactosamine treatment reduces the level of UDP-glucuronic acid without altering the constraint of the microsomal enzyme. This was judged from the activation by UDP-*N*-acetylglucosamine or Triton X-100 which was similar to controls. Carbon tetrachloride treatment probably releases the constraint of the enzyme but does not alter the hepatic level of UDP-glucuronic acid. The experiments with liver microsomes from CCl_4-treated rats demonstrate the importance of the constrained form of the enzyme for glucuronide formation in the intact liver. Information about the constraint may be conveniently obtained by testing the activation by UDP-*N*-acetylglucosamine.

Acknowledgements—This work was supported by the Deutsche Forschungsgemeinschaft. M.M.A. is grateful for a grant of the Alexander-von-Humboldt-Stiftung. The authors wish to thank Dr. H. Remmer for his interest and Mrs. B. Frank for skilful technical assistance.

REFERENCES

1. G. J. Dutton in *Glucuronic acid, Free and Combined.* (Ed. G. J. Dutton) pp. 185–299. Academic Press, New York (1966).
2. K. W. Bock and I. N. H. White, *Eur. J. Biochem.* **46,** 451 (1974).
3. M. M. Abou-El-Makarem and K. W. Bock, *Eur. J. Biochem.*, accepted for publication.
4. M. M. Abou-El-Makarem, G. Otani and K. W. Bock, *Biochem. Soc. Transact.*, in press.
5. D. O. R. Keppler, J. F. M. Rudigier, E. Bischoff and K. F. A. Decker, *Eur. J. Biochem.* **17,** 246 (1970).
6. B. Müller-Oerlinghausen, A. Hasselblatt and R. Johns, *Naunyn-Schmiedeberg's Arch. Pharmac. exp. Path.* **260,** 254 (1966).
7. A. Aitio, *FEBS Lett.* **42,** 46 (1974).
8. K. P. M. Heirwegh, G. P. Van Hees, P. Leroy, F. P. Van Roy and F. H. Jansen, *Biochem. J.* **120,** 877 (1970).
9. E. Halac, M. Dipiazza and P. Detwiler, *Biochim. biophys. Acta* **279,** 544 (1972).
10. G. A. Bray, *Annalyt. Biochem.* **1,** 279 (1960).
11. F. P. Van Roy and K. P. M. Heirwegh, *Biochem. J.* **107,** 507 (1968).
12. K. W. Bock, W. Fröhling, H. Remmer and B. Rexer, *Biochim. biophys. Acta* **327,** 46 (1973).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
14. E. Neufeld and C. Hall, *Biochem. biophys. Res. Commun.* **19,** 456 (1965).
15. D. A. Vessey, J. Goldenberg and D. Zakim, *Biochim. biophys. Acta* **309,** 75 (1973).
16. J. K. Isselbacher, F. M. Chrabas and C. R. Quinn, *J. biol. Chem.* **237,** 3033 (1962).
17. D. Zakim, J. Goldenberg and D. A. Vessey, *Biochim. biophys. Acta* **309,** 67 (1973).
18. M. M. Jacobson, W. Levin and A. H. Conney, *Biochem. Pharmac.* **24,** 655 (1975).
19. E. Del Villar, E. Sanchez, A. P. Autor and T. R. Tephly, *Molec. Pharmac.* **11,** 236 (1975).
20. R. O. Recknagel, *Pharmac. Rev.* **19,** 145 (1967).
21. D. Zakim, J. Goldenberg and D. A. Vessey, *Eur. J. Biochem.* **38,** 59 (1973).
22. D. Zakim and D. A. Vessey in *Membrane-bound enzymes* (Ed. A. Martonosi). Plenum Press, in press.
23. J. Fevery, B. Van Damme, R. Michiels, J. De Groote and K. P. M. Heirwegh, *J. clin. Invest.* **51,** 2482 (1972).