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UDP GLUCURONYLTRANSFERASE AND PHENOLSULFOTRANSFERASE FROM RAT LIVER *IN VIVO* AND *IN VITRO*—III

THE EFFECT OF PHENOLPHTHALEIN AND ITS SULFATE AND GLUCURONIDE CONJUGATE ON CONJUGATION AND BILIARY EXCRETION OF HARMOL*

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Abstract—Harmol (7-hydroxy-1-methyl-9H-pyrido-(3,4b) indol) is excreted in the rat in bile and urine after intravenous (i.v.) injection, in the form of harmol-sulfate and harmol-glucuronide. The effect of phenolphthalein, phenolphthalein-β-D-glucuronide and phenolphthalein-sulfate on both harmol conjugations *in vitro* and *in vivo* has been studied. Phenolphthalein and phenolphthalein-glucuronide inhibited strongly glucuronidation of harmol *in vitro* by a Triton X-100 activated post-nuclear supernatant. Phenolphthalein-sulfate was less inhibitory. Sulfation of harmol was only inhibited at higher concentrations of phenolphthalein-glucuronide and phenolphthalein-sulfate. When phenolphthalein and its conjugates were injected simultaneously with harmol, a decrease of biliary excretion of harmol-sulfate and harmol-glucuronide was found and a great increase in urinary excretion of these conjugates. The total amount of harmol-sulfate synthesized *in vivo* increased and the amount of harmol-glucuronide decreased. These results can be explained by (1) inhibition of glucuronidation of harmol by the phenolphthalein derivatives, and (2) an inhibition of biliary excretion of harmol-sulfate and harmol-glucuronide by the phenolphthalein derivatives, probably due to competition of harmol and phenolphthalein conjugates for the same transport-carrier from liver cell to bile. This carrier should transport both sulfate and glucuronide conjugates.

Several endogenous substances or xenobiotics containing phenolic hydroxylgroups in the molecular structure can be both glucuronidated and sulfated in rat liver *in vivo* by UDP glucuronyltransferase (E.C. 2.4.1.17) and phenolsulfotransferase (E.C. 2.8.2.1) respectively. Previously we reported that in the rat harmol (7-hydroxy-1-methyl-9H-pyrido-(3,4b) indol) is excreted in bile and urine after i.v. injection in the form of its sulfate and glucuronide conjugates [1, 2]. *In vivo*, therefore, UDP glucuronyltransferase and phenolsulfotransferase must be competing for the same substrate harmol. Thus, when one of the conjugating enzymes is inhibited the ratio of sulfate to glucuronide conjugate formed, will change.

Phenolphthalein is a substrate of presumably only UDP glucuronyltransferase. Therefore it might inhibit only glucuronidation of harmol by substrate competition. If that occurs *in vivo* the degree of sulfation of harmol might be enhanced. We have investigated the effects of phenolphthalein on harmol conjugation *in vitro* with a post-nuclear rat liver supernatant as enzyme source of both UDP glucuronyltransferase and phenolsulfotransferase. *In vivo* we have injected phenolphthalein simultaneously with harmol and observed the changes in excretory pattern of the harmol conjugates.

Phenolphthalein is converted *in vivo* to phenolphthalein-glucuronide and effects observed *in vivo* with phenolphthalein might be due to effects of phenolphthalein-glucuronide. Therefore phenolphthalein-glucuronide (and phenolphthalein-sulfate) was included in this study because it might influence conjugation of harmol by product inhibition, and might compete for biliary and urinary excretion of the harmol conjugates. The results show that phenolphthalein and its derivatives inhibited *in vitro* predominantly harmol glucuronidation and, less, sulfation. *In vivo* a slight shift is found in the presence of the phenolphthalein derivatives towards sulfation of harmol.

MATERIALS AND METHODS

Chemicals. Phenolphthalein was obtained from Merck (Darmstadt, Germany); harmol HCl from Fluka (Buchs, Switzerland); Triton X-100, ATP (disodium salt) and phenolphthalein-β-D-glucuronide (sodium salt) from Sigma (St. Louis, U.S.A.);

* Parts I and II of this series are refs. 1 and 2.

phenolphthalein-sulfate (tri-potassium salt) from British Drug House (Poole, England); UDP glucuronate (disodium salt) from Boehringer (Mannheim, Germany) and saccharo-1,4-lactone from Calbiochem (San Diego, U.S.A.).

Enzyme preparation. Male Wistar rats (200–300 g) who had free access to food and water were killed by decapitation. The liver was removed into ice-cold 0·15 M KCl and a 20% homogenate was made with a Potter–Elvehjem homogenizer with Teflon pestle. The postnuclear supernatant was prepared by centrifuging the homogenate during 15 min at 600 *g* at 0–4°. This supernatant was made 0·25% (v/v) in respect to Triton X-100 [5]. Thereafter it was diluted two- or three-fold with 0·15 M KCl and used as enzyme preparation, containing both microsomal UDP glucuronyltransferase and cytosolic phenolsulfotransferase.

PAPS generating system. 3′ phosphoadenosine 5′ phosphosulfate (PAPS), the donor substrate of the sulfate group in the sulfation reaction, was generated in a preincubation according to the method of Van Kempen and Jansen [3]. After termination of PAPS generation by boiling, the resulting PAPS solution contained about 150–200 μM PAPS [2, 4], along with 50 mM K_2SO_4, 2·2 mM $MgCl_2$ and 4·4 mM ATP. Excess sulfate in this solution (which was inhibitory towards glucuronidation) was removed by the addition of 0·53 ml of a 1 M $BaCl_2$ solution to 10 ml of the PAPS solution, immediately after boiling. The $BaSO_4$ precipitate and the coagulated protein were spun off and the supernatant was the PAPS solution.

Simultaneous measurement of UDP glucuronyltransferase and phenolsulfotransferase with harmol as substrate. The medium in which both glucuronidation and sulfation were measured had the composition: 500 μl of the PAPS solution; 100 μl of the substrate solution (1·5 mM harmol in water); 200 μl of a 7·5 mM UDP glucuronate solution in 0·67 M Tris–HCl buffer, pH 7·4 (prepared by mixing 0·67 M Tris with 0·67 N HCl to pH 7·4), which contained 20 mM $MgCl_2$; 100 μl water and 100 μl of the enzyme preparation. The incubation was for 15 min at 37° after which the tubes were cooled in icewater and protein was coagulated by putting the tubes in a boiling waterbath for 1 min. After centrifugation 30 μl of the clear supernatant was applied to a thin-layer chromatography (t.l.c.) plate (silicagel 60 F 254, Merck, Darmstadt, Germany). Separation and elution from the plate of the glucuronide and sulfate conjugate were as described before [1]. Characteristics of this assay have been submitted elsewhere. (G. J. Mulder, *Analyt. Biochem.* in press).

Measurement of UDP glucuronyltransferase with phenolphthalein as substrate. This was performed as described before, in Tris–HCl buffer, pH 7·4 [5].

Determination of harmol conjugates. This method was described before [1].

Bile cannulation experiments. Male rats were used in the experiments, weighing 280–350 g (Wistar, TNO, Zeist, The Netherlands). Bile cannulation and collection of urine from the bladder at the end of the experiment were performed as described before under pentobarbital (Nembutal®) anesthesia [1, 6]. Injections were in the vena jugularis externa in a volume of 0·25 ml/100 g body wt. The kidneys were not ligated in the present experiments.

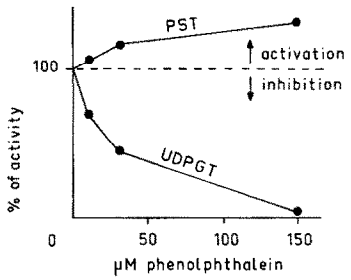

Fig. 1. Effect of phenolphthalein on UDP glucuronyltransferase (UDPGT) and phenolsulfotransferase (PST) with harmol as substrate. Phenolphthalein was added to the incubation dissolved in ethanol such that the final concentration in the incubation was 2·5% (v/v). This concentration of ethanol did not affect UDP glucuronyltransferase and phenolsulfotransferase activity. Activity without phenolphthalein present is 100%.

RESULTS

Inhibition of harmol conjugation in vitro *by phenolphthalein and its conjugates.* Phenolphthalein inhibited glucuronidation of harmol *in vitro* with the post-nuclear rat liver supernatant as enzyme preparate. Figure 1 shows that harmol glucuronidation was already inhibited for about 30 per cent at 10 μM phenolphthalein. At 150 μM it was nearly 100 per cent inhibited. Harmol sulfation, on the other hand, was stimulated by phenolphthalein up to 25 per cent at 150 μM.

Phenolphthalein-glucuronide also inhibited harmol glucuronidation strongly (Fig. 2). Up until 0·10 mM it did not affect phenolsulfotransferase but at higher concentration also this enzyme activity was inhibited.

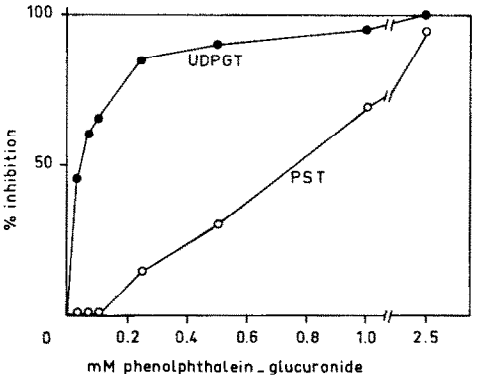

Fig. 2. Inhibition of phenolsulfotransferase (PST) and UDP glucuronyltransferase (UDPGT) by phenolphthalein-glucuronide.

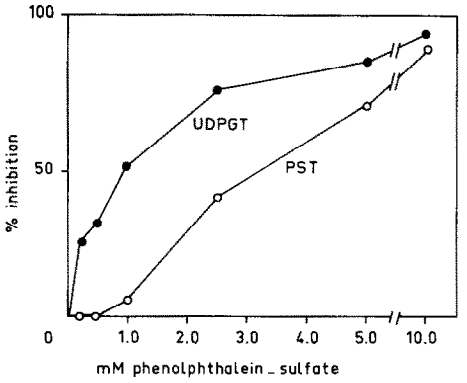

Fig. 3. Inhibition of phenolsulfotransferase (PST) and UDP glucuronyltransferase (UDPGT) by phenolphthalein-sulfate.

Thus, at 0·25 mM phenolphthalein-glucuronide, phenolsulfotransferase activity was inhibited to 15 per cent and UDP glucuronyltransferase to 85 per cent. Phenolphthalein-sulfate was less inhibitory. At 0·25 mM it inhibited UDP glucuronyltransferase to 25 per cent and phenolsulfotransferase not at all (Fig. 3). Only from 1·0 mM onward phenolsulfotransferase was clearly inhibited. The possibility that inhibition by phenolphthalein-glucuronide and phenolphthalein-sulfate were due to liberation of free phenolphthalein by β-glucuronidase and arylsulfatase respectively during the incubation was excluded, because phenolphthalein-sulfate was not at all hydrolyzed (at pH 7·4) during the incubation, whereas phenolphthalein-glucuronide was hydrolyzed to a limited extent. Thus, during the incubation with 30 μM phenolphthalein-glucuronide as inhibitor, less than 1 μM free phenolphthalein was present at the end of the incubation. The inhibition by the conjugates thus cannot be explained by the resulting low level of free phenolphthalein and, therefore, these inhibitions are due to the compounds themselves.

Inhibition of phenolphthalein glucuronidation in vitro *by harmol.* Phenolphthalein glucuronidation *in vitro* (at 150 μM phenolphthalein) was inhibited about 3 per cent at 0·15 mM harmol and 20 per cent at 1·5 mM harmol; when 50 μM phenolphthalein was used as substrate concentration, resulting in about 2 times lower velocity of phenolphthalein conversion compared with substrate concentration 150 μM, the same degree of inhibition by harmol was found. This suggests non-competitive inhibition.

Effect of phenolphthalein, phenolphthalein-glucuronide and phenolphthalein-sulfate on biliary and urinary excretion of harmol conjugates. When harmol is injected intravenously (i.v.) in rats, it is eliminated in bile and urine as harmol-sulfate and harmol-glucuronide [1, 2]. Phenolphthalein, phenolphthalein-glucuronide and phenolphthalein-sulfate caused a reduction of biliary excretion of harmol, both of harmol-sulfate and harmol-glucuronide (Fig. 4). Phenolphthalein-glucuronide inhibited most strongly, phenolphthalein and phenolphthalein-sulfate were about equally inhibitory. In Fig. 4a only the results for the first period of an hour after injection of harmol are given. During the 2nd hr of bile collection the amounts of harmol-sulfate and harmol-glucuronide in bile were still lower than in the control group.

The three phenolphthalein derivatives caused a very great increase of the urinary excretion of harmol-sulfate. There were similar increases in the amount of harmol-glucuronide excreted in urine (Fig. 4b).

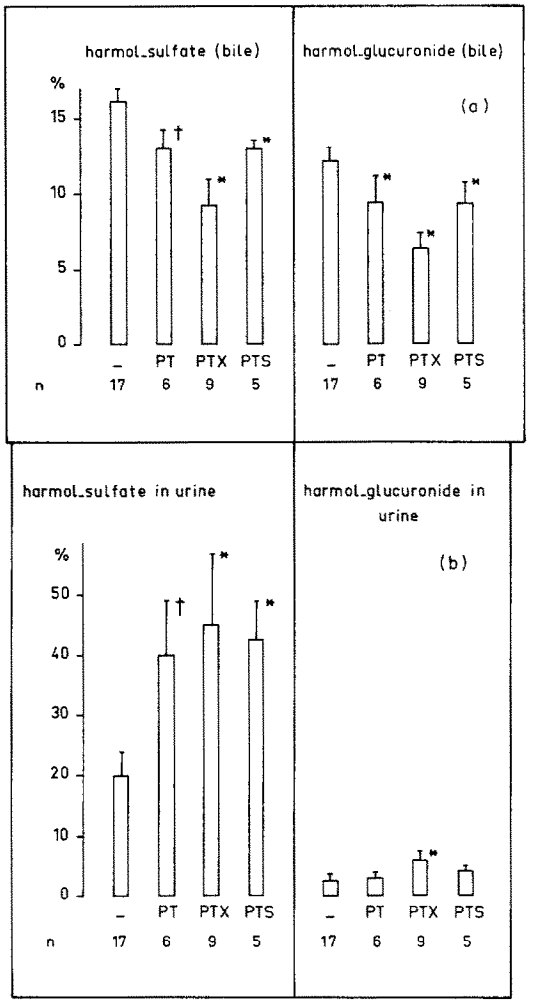

Fig. 4. Effect of phenolphthalein (PT), phenolphthaleinglucuronide (PTX) and phenolphthalein-sulfate (PTS) on biliary (4a) and urinary (4b) excretion of harmol-sulfate and harmol-glucuronide after i.v. injection of harmol. Harmol was given to rats (20 μmoles/kg), alone or in combination with either PT, PTX or PTS (77·5 μmoles/kg). The percentage of the dose excreted as harmol-sulfate or harmol-glucuronide during 1 hr after injection in bile and during 2 hr in urine is given ± S.E.M. n is the number of animals used.* Significantly different from the control group (Wilcoxin; $P < 0.05$); † the same at $P < 0.10$.

Table 1. Effect of phenolphthalein, phenolphthalein-glucuronide and phenolphthalein-sulfate on glucuronidation and sulfation of harmol

Group	n	Harmol-sulfate (% of dose)	Harmol-glucuronide (% of dose)	Harmol-sulfate/ Harmol-glucuronide
Control	17	44·4 ± 3·5	17·6 ± 0·9	2·58 ± 0·21
Phenolphthalein-group	6	58·3 ± 9·1	14·5 ± 1·9*	3·97 ± 0·41*
Phenolphthalein-glucuronide group	9	59·5 ± 9·5†	15·5 ± 0·8	3·95 ± 0·70†
Phenolphthalein-sulfate group	5	61·8 ± 5·4*	15·8 ± 1·0	3·99 ± 0·47*

Harmol (20 μmoles/kg) was injected i.v., and bile and urine were collected during 2 hr thereafter. The total amount of harmol-sulfate and harmol-glucuronide in bile and urine was determined and the percentages of the dose excreted as sulfate and glucuronide conjugates ± S.E.M. are given in the first part of the table. The third column gives the ratio of sulfate to glucuronide conjugate excreted in bile and urine. n is the number of animals used.

* Significantly different ($P < 0·05$) from the control group; † the same for $P < 0·10$.

Effect of phenolphthalein, phenolphthalein-glucuronide and phenolphthalein-sulfate on conjugation of harmol by UDP glucuronyltransferase and phenolsulfotransferase in vivo. The three phenolphthalein derivatives caused an increase in the total amount of harmol-sulfate excreted in bile and urine during 2 hr; these increases are statistically significant to various degrees as is evident from Table 1. The corresponding total amount of harmol-glucuronide was decreased. When for each rat the ratio of the total amount of harmol-sulfate to the total amount of harmol-glucuronide was computed, we found that the phenolphthalein derivatives caused a significant increase of this ratio. Thus, harmol conjugation was shifted to sulfation by these compounds.

Biliary and urinary excretion of the phenolphthalein derivatives; effect of harmol. Phenolphthalein and its sulfate and glucuronide conjugate are readily excreted after i.v. injection in bile as shown in Table 2. Phenolphthalein-glucuronide and phenolphthalein-sulfate are excreted to 95 per cent in bile in 2 hr; only 2–4 per cent of the dose is found in urine after that time. These results are in agreement with those of Hirom *et al.* [7]. Phenolphthalein is converted in rat liver to phenolphthalein-glucuronide [8] which is subsequently excreted in bile. Its excretion rate is somewhat slower than that of both conjugates, injected i.v. themselves (Table 2). The presence of harmol did not influence any of these figures to a statistically significant extent; thus harmol did not affect glucuronidation of phenolphthalein *in vivo* nor biliary elimination of phenolphthalein-glucuronide (formed from phenolphthalein or injected as such) and phenolphthalein-sulfate.

DISCUSSION

The results show that harmol glucuronidation *in vitro* is equally strongly inhibited by phenolphthalein (presumably through substrate competition) and by phenolphthalein-glucuronide (presumably by product inhibition). Phenolphthalein-sulfate inhibited less strongly. Sulfation of harmol was less sensitive to the phenolphthalein conjugates and was not affected by phenolphthalein itself. The inhibitory potency of phenolphthalein-glucuronide is exceptionally high compared with that of several other glucuronides [9, 10]; of a series of glucuronides investigated with several substrates of UDP glucuronyltransferase, phenolphthalein-glucuronide was in all cases the strongest inhibitor [11].

This inhibition of glucuronidation of harmol found *in vitro*, and the relative lack of effect on sulfation, prompted us to study the effect of phenolphthalein and its derivatives *in vivo*. Table 1 shows that the ratio of

Table 2. Biliary and urinary excretion of phenolphthalein, phenolphthalein-glucuronide and phenolphthalein-sulfate

Compound	n	Bile		Urine	Total recovery
		1st hr	2nd hr		
Phenolphthalein	6	54·8 ± 6·0	13·0 ± 1·2	5·7 ± 2·2	73·4 ± 5·9
Phenolphthalein-glucuronide	4	73·4 ± 5·7	18·3 ± 2·1	1·5 ± 0·8	94·1 ± 6·6
Phenolphthalein-sulfate	4	80·4 ± 2·6	10·0 ± 0·6	3·7 ± 2·1	94·1 ± 2·5

The dose of these compounds was 77·5 μmoles/kg i.v. in the external jugular vein. Bile was collected during two fractions of an hour. Urine was collected after the end of these 2 hr. The results given are expressed as percentage of the dose excreted per period ± S.E.M.

harmol-sulfate to harmol-glucuronide synthesized *in vivo* can indeed be influenced: the concomitant administration of phenolphthalein and its derivatives caused an increase of sulfation relative to glucuronidation.

Phenolphthalein-sulfate and phenolphthalein-glucuronide are rapidly excreted in bile in the rat after i.v. injection and they do not appear to a considerable extent in urine, in agreement with the findings of Hirom *et al.* [7]. Phenolphthalein is excreted in bile as phenolphthalein-glucuronide [6, 8] and effects of phenolphthalein *in vivo* may be due to phenolphthalein itself or to phenolphthalein-glucuronide formed from it.

Phenolphthalein and its derivatives inhibited biliary elimination of both harmol-sulfate and harmol-glucuronide (Fig. 4). This was not due to a reduced synthesis (and thus supply) of these conjugates (Table 1). Therefore the most likely explanation is competition of phenolphthalein-sulfate and phenolphthalein-glucuronide for transport sites (carriers) which transport harmol-sulfate and harmol-glucuronide (and also the phenolphthalein derivatives) from liver cell into bile. A similar effect of phenolphthalein-glucuronide on the biliary excretion of some other glucuronides has been found previously [6]. The lack of effect of harmol on the biliary excretion of phenolphthalein-sulfate and phenolphthalein-glucuronide can only be explained, with the carrier model, if it is assumed that the affinity of the carriers for the harmol conjugates is much less than for the phenolphthalein conjugates (see also ref. 6). The results suggest that both phenolphthalein conjugates and both harmol conjugates may bind to the same binding site of the proposed carriers in parallel with the findings of Powell *et al.* [12]. Thus we did not find evidence for separate carriers for transport of sulfate and glucuronide conjugates as suggested by Smith *et al.* [13, 14]. They, however, have drawn their conclusions from experiments in which, on a molar base, very different doses of morphine and its glucuronide or sulfate conjugate were administered to rats and cats *in vivo*.

Previously we suggested that in the rat *in vivo* biliary excretion of harmol-sulfate may be rate limiting compared to the rate of sulfation, in the process of elimination of harmol [2]. The present results show that excretion of harmol-sulfate in urine is not rate limiting: if biliary excretion is inhibited the amount of harmol-sulfate normally excreted in bile can readily be eliminated in the same time in urine. Also when more harmol-sulfate is supplied due to increase of sulfation its elimination can be accommodated by increased urinary excretion (Fig. 4b). Thus harmol-sulfate seems to be eliminated in urine because it is excreted not quick enough in bile and, as a consequence, leaks back from liver into blood.

The present work suggests that the results found *in vitro* may also be relevant to the *in vivo* situation. However, at present it is not yet clear whether UDP glucuronyltransferase, as present *in vivo*, should be compared with (detergent-) activated, or with non-activated UDP glucuronyltransferase, in microsomes *in vitro*. The enzyme is constrained by the membrane environment [15] and *in vivo* this may be different from *in vitro*. Therefore, caution is required in the use of the effects, observed *in vitro* either with activated or non-activated microsomes, to explain *in vivo* findings.

It will be of interest to determine the character of the inhibitory effects of the phenolphthalein derivatives *in vitro*. Recently, Vessey and Zakim [16] reported that glucuronides activated UDP glucuronyltransferase, measured in non-activated microsomes from guinea-pig liver. The difference between their and our findings may be either a species difference (guinea-pig viz. rat) or, more likely, the use of non-activated (Vessey and Zakim) or Triton X-100 activated microsomes (present work). Moreover, at our concentration of UDP glucuronate (1·5 mM) we should not see activation any more, according to the data of Vessey and Zakim [16]. Further, the presumable noncompetitive inhibition of phenolphthalein glucuronidation by harmol requires further attention.

In conclusion: (1) phenolphthalein and its derivatives inhibit harmol glucuronidation and enhance sulfation of harmol *in vivo*; and (2) phenolphthalein and its derivatives inhibit biliary excretion of harmol-sulfate and harmol-glucuronide and thereby enhance urinary excretion of these conjugates.

REFERENCES

1. G. J. Mulder and A. H. Hagedoorn, *Biochem. Pharmac.*, **23**, 2101 (1974).
2. G. J. Mulder, K. Hayen-Keulemans and N. E. Sluiter, *Biochem. Pharmac.*, **24**, 103 (1975).
3. G. M. J. van Kempen and G. S. I. M. Jansen, *Analyt. Biochem.* **46**, 438 (1972).
4. G. S. I. M. Jansen and G. M. J. van Kempen, *Analyt. Biochem.* **51**, 324 (1973).
5. G. J. Mulder, *Biochem. J.* **125**, 9 (1971).
6. G. J. Mulder, *Biochem. Pharmac.* **22**, 1751 (1973).
7. P. C. Hirom, P. Millburn, R. L. Smith and R. T. Williams, *Biochem. J.* **129**, 1071 (1972).
8. P. Millburn, R. L. Smith and R. T. Williams, *Biochem. J.* **105**, 1275 (1967).
9. D. Zakim, G. Goldenberg and D. A. Vessey, *Biochim. Biophys. Acta* **309**, 67 (1973).
10. J. Marniemi and O. Hänninen, *Int. J. Biochem.* **4**, 95 (1973).
11. G. J. Mulder, *Biochem. Soc. Trans.*, in press (1975).
12. G. M. Powell, P. A. Gregory, A. K. Olavesen and J. G. Jones, *Biochem. Soc. Trans.* **1**, 1165 (1973).
13. D. S. Smith, R. E. Peterson and J. M. Fujimoto, *Biochem. Pharmac.* **22**, 485 (1973).
14. R. E. Peterson and J. M. Fujimoto, *J. Pharmac. exp. Ther.* **184**, 409 (1973).
15. D. Zakim, J. Goldenberg and D. A. Vessey, *Eur. J. Biochem.* **38**, 59 (1973).
16. D. A. Vessey and D. Zakim, *Biochem. J.* **139**, 243 (1974).