Biochemical Pharmacology, Vol. 22, pp. 2319–2327. Pergamon Press, 1973. Printed in Great Britain.

EFFECT OF TRITON X-100 ON THE CONJUGATION OF TETRAHYDROCORTISONE, *IN VITRO**

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(*Received* 7 *February* 1972; *accepted* 2 *March* 1973)

Abstract—The detergent, Triton X-100, increased the conjugation of estrone, estradiol and tetrahydrocortisone (THE) by uridine diphosphate glucuronyl transferase (UDPGT) in rat liver microsomes; the maximal increase of the conjugation of these substrates was measured when the concentration of Triton in the incubation mixtures was 0·05 per cent. However, the magnitude of the increase and the effect seen with varying Triton concentration were substrate dependent, which is consistent with the hypothesis that multiple forms of UDPGT may be present in hepatic microsomes. The effects of various compounds which had previously been shown to either increase or decrease the conjugation of THE in non-activated enzyme preparations were re-examined in Triton-activated preparations. Compounds such as β-diethylaminoethyldiphenylpropylacetate (SKF-525A) and 7-hydroxychlorpromazine which inhibited conjugation in non-activated preparations also inhibited conjugation in Triton-activated preparations. Alternatively, the demethylated metabolites of chlorpromazine, which increased activity in non-treated preparations, decreased activity slightly in preparations maximally stimulated by Triton. Bisubstrate kinetic analysis of the THE conjugation of UDPGT also revealed differences between the properties of the non-treated and Triton-activated enzyme preparations. Triton activation caused an increase in the V_{max} of the reaction in the forward direction while having an insignificant effect on the dissociation constant for THE.

THE ACTIVITY of the microsomal enzyme, uridine diphosphate glucuronyl transferase (UDPGT), can be increased *in vitro* by a variety of means including treatment with the non-ionic detergent Triton X-100.[1–3] This action of Triton has been postulated to be the consequence of a dispersion of the membranous material surrounding the enzyme which exposes additional active sites.[2]

Since activation by Triton could provide an effective means for enhancing the sensitivity of assay procedures for UDPGT, it was of interest to determine to what extent the detergent alters the properties of the enzyme. We had previously reported that treatment with Triton increased the maximal velocity (V_{max}) for the conjugation of the steroid tetrahydrocortisone (THE) without significantly changing the apparent Michaelis constant (K_m).[4] These studies, as with most kinetic analysis carried out with UDPGT, were done by classical Michaelis–Menten treatment of the enzyme

* Supported by USPHS Grants MH-11468, GM-15431 and U. of M. Grant 457-0350 4909-02.

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reaction. Specifically, excess uridine diphosphoglucuronic acid (UDPGA) was added to the enzyme system and the bisubstrate reaction treated as though it were a unisubstrate enzyme reaction. Recent bisubstrate analysis of this enzyme reaction utilizing *p*-nitrophenol as the aglycone substrate has indicated that, for many published kinetic analyses, saturating concentrations of UDPGA are not realized.[5] Therefore, re-evaluation of our previous findings on the effect of Triton on V_{max} and K_m for THE were undertaken.

Examination of several other factors was also carried out. It had been shown that the measured increase in activity of UDPGT caused by treatment with Triton differs markedly and this difference was dependent on the aglycone substrate used in the assay procedure.[2] The aglycones used in this study of UDPGT included a number of exogenous phenolic substrates and bilirubin. We investigated this substrate-dependent activity change utilizing several steroids which are endogenous substrates for the enzyme system along with *p*-nitrophenol. In addition we had previously demonstrated that a variety of compounds could either decrease or increase the conjugation of THE by UDPGT, *in vitro*.[3] The behavior of several of these compounds was reinvestigated with the Triton-activated preparations to determine if they would retain or lose their properties which were demonstrated in non-activated microsomes.

METHODS

Male Sprague–Dawley rats (Holtzman Co., Madison, Wis.) weighing 200–250 g were used in these experiments. The animals were allowed access to food and water at all times.

Tetrahydrocortisone 1,2-^{3}H (20 Ci/m-mole, radiopurity > 97 per cent), estrone 4-^{14}C (45·2 mCi/m-mole, radiopurity > 99 per cent) and 17-β-estradiol-^{14}C (52 mCi/m-mole, radiopurity > 99 per cent) were obtained from the New England Nuclear Corp.; tetrahydrocortisone, estrone, 17-β-estradiol and uridine diphosphoglucuronic acid from the Sigma Chemical Company and Triton X-100 from the Rohm & Haas Co. The ^{14}C-estrone and ^{14}C-estradiol were used without further purification; ^{3}H-tetrahydrocortisone was repurified when necessary by a procedure previously described.[3]

Enzyme assay. Washed liver microsomes were prepared by differential centrifugation.[3] Each incubation mixture contained microsomes from the pooled livers of two rats. Reaction mixtures were incubated in 15-ml glass-stopped centrifuge tubes at 37° under air. The reactions were terminated by the addition of toluene or a toluene–isoamylalcohol mixture followed by vigorous shaking to both extract the unreacted substrate and inactivate UDPGT. Incubation mixtures for the Triton activation studies and substrate effect studies consisted of microsomes (1·0 ml obtained from 125–250 mg of liver), 1·0 ml of 0·2 M Tris buffer (pH 7·6 for THE, pH 8·4 for estrone and estradiol), containing UDPGA (9 μmoles), and 0·1 ml of a propylene glycol solution of the steroid. Buffer pH was measured at 25°. The buffer pH values used in the incubations were those found to give optimal activity for the respective substrates. Drugs were added in solution in Tris buffer to bring the final volume to 3 ml.

For the kinetic studies, the volumes of the microsomes, buffer and propylene glycol solution of steroid were halved. Variable amounts of THE and UDPGA were added to the tubes with the final volume being constant at 1·5 ml. The concentration

of microsomes in both the non-activated and activated enzyme preparations was approximately 2 mg of protein/ml of incubate. Protein concentration was measured by a modified biuret procedure using bovine serum albumin (Sigma) as a standard.[6]

Measurement of conjugation. The rate of conjugation of THE was measured by estimation of the formation of its glucuronide, as previously described.[3] The rates of conjugation of estrone and estradiol were measured by a modification of the procedure used for THE. Unconjugated estrone and estradiol were removed from the incubation mixtures by two extractions with 10 ml of toluene. The tubes were shaken for 40 min, centrifuged, and 8-ml aliquots of the organic phase were transferred to scintillation vials containing 3 ml of ethanol, 5 ml of toluene, and 2 ml of phosphor [50 g of 2,5-diphenyloxazole (PPO) and 0·625 g of 1,4-bis-[2-(5-phenyloxazolyl)] benzene (POPOP)/liter of toluene]. After each extraction, the aqueous phase was frozen in an acetone-dry ice bath and the remaining organic phase removed by aspiration. After removal of the unconjugated estrone or estradiol, the aqueous phase was acidified with 0·5 ml of 2 N HCl and the respective glucuronide extracted into 5 ml of water-saturated *n*-butanol. After being shaken for 20 min, the tubes were centrifuged and 3-ml aliquots of the organic phase were transferred to scintillation vials containing 3 ml of ethanol, 10 ml of toluene and 2 ml of phosphor.

Radioactivity was measured by liquid scintillation counting and all data were corrected for counting efficiency by external standardization and for the small amount of radioactivity (< 0·5 per cent) extracted into butanol from the non-incubated controls. Using this procedure, 99·5 $\pm$ 0·5 per cent of the radioactivity added to incubation mixtures was consistently recovered as estrone, estradiol or their respective glucuronides. Appropriate non-incubated blanks were used in all determinations. Concentrations of Triton X-100 of less than 0·1% had no apparent effect on the extraction of the steroids or their conjugates.

Measurement of p-*nitrophenol conjugation.* The disappearance of *p*-nitrophenol was measured by a modification of the method of Isselbacher.[7]

Analysis of kinetic data. Initial velocities were determined using a 10-min incubation time for the non-stimulated preparations and a 5-min incubation time for the Triton-activated preparations. It was determined that the reaction was linear for these times and protein concentration range utilized. A modified Lineweaver–Burk equation was used in plotting the reciprocals of velocity and substrate in order to determine the dissociation constants for the enzyme reaction.[8] All data were fitted by the method of least squares.

RESULTS

Confirmation of glucuronide formation. The formation of the glucuronides of estrone and estradiol by microsomal preparations was confirmed in a manner similar to that previously described for THE.[3] When UDPGA was omitted from incubation mixtures, over 99 per cent of the estrone or estradiol was recoverable with two toluene extractions. Moreover, the UDPGA-dependent formation of polar, butanol-extractable metabolites of estrone and estradiol was prevented when preparations were incubated at 4° or contained microsomes which had been heated for 1 min in a boiling water bath. When reaction mixtures which had been incubated for 15 min were inactivated by heating and reincubated for 24 hr with 3000 units of β-glucuronidase at pH 5·0, no butanol-extractable metabolites of either substrate were measured (Table 1). If

TABLE 1. CONFIRMATION OF GLUCURONIDE FORMATION*

Reincubation conditions	Butanol-extractable radioactive metabolite (nmoles)
Estrone	
No additions	9·56 (9·15–9·79)
Plus β-glucuronidase (3000 units)	0 (0)
Plus β-glucuronidase and saccharo-1,4-lactone (10^{-3} M)	6·19 (6·12–6·26)
Estradiol	
No additions	14·01 (13·84–14·27)
Plus β-glucuronidase	0·13 (0·10–0·17)
Plus β-glucuronidase and saccharo-1,4-lactone	12·37 (12·15–12·60)

* Butanol-extractable metabolites were formed by incubating 100 nmoles for 15 min as described in Methods. The reaction was terminated by placing tubes in a boiling water bath for 1 min. Acetate buffer (0·1 M, pH 5·0) and glacial acetic acid were added to adjust incubate to pH 5·0. The tubes were reincubated for 18 hr at 37° with the appropriate additions. Values are the average of four incubation mixtures. The range is in parentheses.

saccharo-1,4-lactone, a specific inhibitor of β-glucuronidase,[9] was added during the reincubation, considerable amounts of butanol-extractable metabolites were recovered. In addition, thin-layer chromatographic analysis of the butanol-extractable phase was made before and after hydrolysis with β-glucuronidase. This showed that the steroid which was not present in the free form before incubation with the enzyme appeared after an incubation treatment. All these findings were consistent with the view that the butanol-extractable radioactivity formed from the ^{14}C-estrone and ^{14}C-estradiol was indeed their glucuronides.

Stimulation of the conjugation of steroids and p*-nitrophenol (PNP) by Triton X-100.* It had been shown that Triton X-100 could increase the conjugation of a number of substrates by UDPGT, including THE and *p*-nitrophenol.[1–3] Moreover, it was of interest that the maximal increase of the conjugation of a variety of compounds occurred when the concentration of the detergent in incubation mixture was approximately 0·05 per cent.[2] Although there were marked differences between the extent of the increases of their conjugation, the maximal increase of the conjugation of estrone, estradiol, THE and PNP occurred in the presence of 0·05% Triton in our enzyme preparation (Fig. 1). Additional differences in the activation profiles of these compounds were also apparent. For example, with THE and PNP the activity of Triton-treated microsomes remained greater than control preparations even at concentrations of Triton as high as 0·5% which strikingly clarify the incubation mixture. The results obtained with PNP were consistent with those of Winsnes[2] but differ with those of Lueders and Kuff[1] who observed no conjugation in the presence of 0·3 to 0·5% Triton. When the substrate was estrone or estradiol, there was indeed lower than normal activity at these higher concentrations of Triton. Moreover, with estradiol a small but significant ($P < 0·001$, $N = 4$) inhibition of conjugation was observed at 0·01 per cent. This inhibition of conjugation at low concentrations of Triton resembled that previously described by Winsnes[2] using *o*-aminophenol as the aglycone substrate.

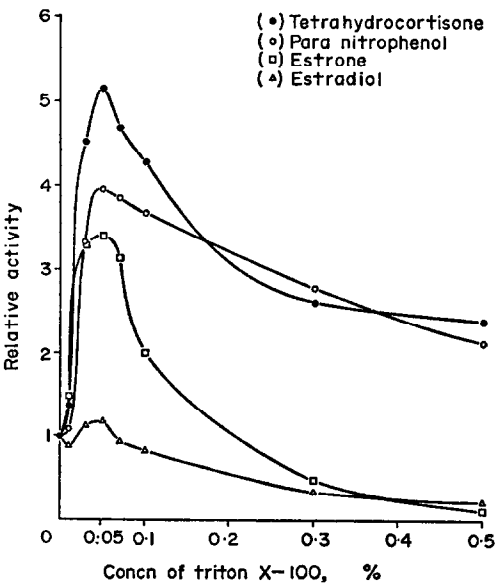

FIG. 1. Effects of Triton X-100 on conjugation. Incubation conditions are described under Methods. Relative activity of 1 = 0·35 nmole of THE, 4·83 μmoles of *p*-nitrophenol, 0·19 nmole of estrone, 3·88 nmoles of estradiol metabolized/mg of microsomal protein. Each point is the average value obtained from four incubation mixtures.

Effect of various substrates on the conjugation of THE in stimulated and non-stimulated microsomes. We had previously demonstrated that a number of substances could alter the conjugation of THE by UDPGT in non-activated microsomes.[3] Compounds which contained hydroxyl groups and would thus make them potential substrates for UDPGT were found to inhibit THE conjugation. Similarly, the potent inhibitor of various microsomal oxidative pathways of metabolism, β-diethylaminoethyl-diphenylpropylacetate (SKF-525A), was found to be a potent inhibitor of THE conjugation. In contrast to the hydroxylated chlorpromazine substrates used in the study, the structurally related demethylated compounds possessing no functional groups capable of conjugation were found to actually increase the conjugation of THE.

In order to assess the effects that Triton activation has on enzyme behavior, these previous experiments were repeated with a selected number of previously studied substrates. The same microsomes were used as the source of enzyme with the only difference being the presence of absence or 0·05% Triton. The results of these studies are found in Table 2. It can be seen that all the compounds which inhibited THE conjugation in the non-activated preparation also inhibited the Triton-treated preparation. For those compounds which increased activity of the non-treated enzyme, it was found that in the Triton-activated preparation their behavior was that of slight inhibition at best. The behavior exhibited by these demethylated chlorpromazine (CPZ) metabolites in the Triton-activated microsomes was similar to that of nortriptyline, the demethylated metabolite of amitriptyline.[3] In that previous study, it was postulated that the nortriptyline was behaving as a detergent due to its appreciable surface active properties. This same mechanism could also be similarly postulated for the demethylated metabolites of chlorpromazine.

TABLE 2. EFFECTS OF VARIOUS COMPOUNDS ON THE CONJUGATION OF THE*

Compound added†	THE conjugation relative activity‡	
	With Triton	Without Triton
Control	100	100
SKF-525A	11	22
7-OH CPZ	36	18
7-OH Nor_1 CPZ	48	34
7-OH Nor_2 CPZ	47	60
Nor_1 CPZ	82	180
Nor_2 CPZ	90	214
p-OH amphetamine	47	48
p-Cl amphetamine	99	84
p-OH norephedrine	82	73

* Each value is the average of at least four determinations.

† Concentration of compounds added 5×10^{-4} M. Substrate concentration $1{\cdot}3 \times 10^{-5}$ M.

‡ Relative activity of 100 = 3·3 nmoles of THE glucuronide formed in 10 min without Triton and 11·5 nmoles formed in 5 min with Triton (0·05%).

Bisubstrate kinetic analysis of UDPGT. We had previously reported that Triton activation does not appear to significantly alter the apparent K_m for the conjugation of THE but markedly increases its V_{max}.[4] It was assumed that the UDPGA concentration used in these previous experiments (3×10^{-3} M) was high enough so that the apparent K_m measured for THE would be independent of UDPGA concentration. "Saturating" concentrations of UDPGA were required since UDPGT catalyzes a bisubstrate reaction.

The determination of an apparent K_m value for the aglycone substrate of UDPGT in the presence of "saturating" concentrations of UDPGA as done by a number of investigators including ourselves has several severe limitations. The first of these is that the concentration of UDPGA used in most kinetic experiments, although seemingly in excess, is not "saturating" as pointed out by the recent studies of Vessey and Zakim.[5] More important, however, is the fact that the K_m value determined by classical Lineweaver–Burk analysis of the data does not have the same meaning as that for a true unisubstrate reaction which is classically described by Michaelis–Menten kinetics. Depending on the true mechanism of this bisubstrate reaction, the constants determined by classical plotting procedures can take on different meanings.[10]

The bisubstrate kinetic analysis and determination of the kinetic constants for the conjugation of THE by both the non-treated and Triton-activated enzyme preparations were done according to procedures described by Florini and Vestling.[11] The results of those experiments are found in Figs. 2 and 3. When THE was variable the substrate and UDPGA the fixed substrate, a series of plots which intersected to the left of the $1/v$ axis resulted. This pattern of intersecting plots occurred with either enzyme preparation. The intersection pattern was indicative of sequential rather than a ping-pong type of mechanism but the data were insufficient to determine the order in which the two substrates combine with the enzyme.[10] The common intersection point to the left of the $1/v$ axis described a dissociation constant for THE but the

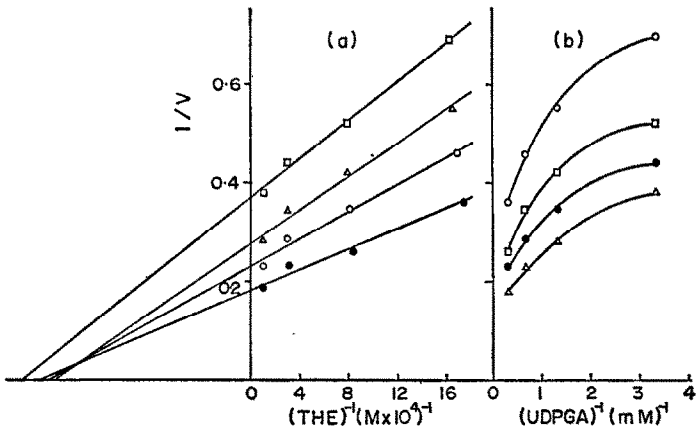

FIG. 2. Typical double reciprocal plot of initial rate of THE glucuronide formation in non-activated preparations. Plot a represents varying concentrations of THE at different fixed concentrations of UDPGA, and plot b represents varying concentrations of UDPGA at different fixed concentrations of THE. Velocity is expressed as nmoles of THE glucuronide formed in a 10-min incubation period per incubate. Each point is the average value obtained from two determinations. The fixed concentrations of UDPGA in plot a were 0·3 mM (□), 0·75 mM (△), 1·5 mM (○) and 3 mM (●). The fixed concentrations of THE in plot b were $6{\cdot}67 \times 10^{-6}$ M (○), $1{\cdot}33 \times 10^{-5}$ M (□), $3{\cdot}33 \times 10^{-5}$ M (●) and 1×10^{-4} M (△).

reaction this constant described is not definable until the reaction mechanism is known.[10] The value for this dissociation constant for THE (which will be termed K_{THE}) was $0{\cdot}75 \times 10^{-5}$ M for the non-treated enzyme and $1{\cdot}1 \times 10^{-5}$ M for the Triton-activated enzyme. These values were similar in magnitude to our previously published apparent K_m values for THE.[3]

Secondary plots of the intercepts vs the reciprocals of the fixed substrate also yield additional information.[11] The intercept on the $1/S$ axis gives another kinetic constant which will be termed K'_{THE}. The V_{max} values were 0·22 and 2·0 nmoles/min/mg of

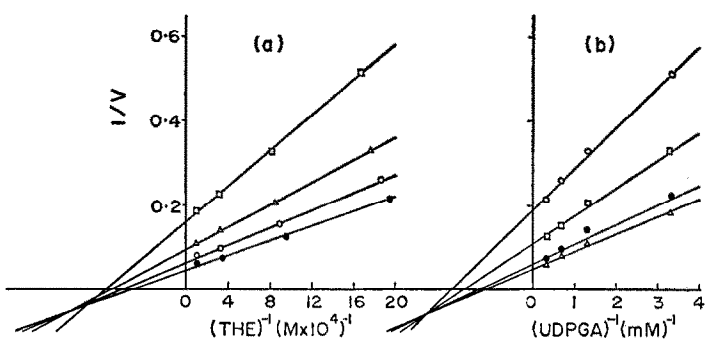

FIG. 3. Typical double reciprocal plot of initial rate of THE glucuronide formation in Triton-activated preparations. Plot a represents varying concentrations of THE at different fixed concentrations of UDPGA, and plot b represents varying concentrations of UDPGA at different fixed concentrations of THE. Velocity is expressed as nmoles of THE glucuronide formed in a 5-min incubation period per incubate. Each point is the average value obtained from two determinations. The concentrations and appropriate symbols for THE and UDPGA concentrations are the same as in Fig. 1.

protein for the non-treated and activated preparations respectively. The K' values were 5×10^{-4} M and 1×10^{-3} M for the non-treated and Triton-activated preparations.

The interpretation of the experiments where UDPGA was the variable substrate and THE the fixed substrate proved more difficult. The non-treated enzyme gave non-linear plots making determination of a dissociation constant for UDPGA impossible. This non-linear behavior was similar to that reported by Winsnes[2] for non-treated mouse-liver enzyme preparations. Linear behavior of the reciprocal plots was seen for the Triton-activated preparation. The plots again intersected to the left of the $1/v$ axis, indicative of sequential mechanism. The value of the dissociation constant determined from the common intersection point of the reciprocal plots and which will be termed K_{UDPGA} was approximately 4×10^{-4} M. A secondary plot of the intercepts vs 1/THE concentration gave a value of $1{\cdot}7 \times 10^{-5}$ M for K'_{UDPGA}.

DISCUSSION

The ability to stimulate UDPGT, *in vitro*, is not an exclusive property of Triton X-100, but is shared by other detergents such as digitonin[2] and sodium desoxycholate,[12] the enzyme phospholipase,[13] the chelating agent EDTA[14] the nucleotide UDP-*N*-acetyl-glucosamine,[2,15] as well as preincubation[1,2,16] and ultrasonication.[17]

The stimulation of conjugation by detergents such as Triton X-100 is in sharp contrast to their action on the multicomponent mixed function oxidases in hepatic microsomes. For example, previous studies from this laboratory[18] have shown that concentrations of Triton which maximally increase UDPGT activity abolish the metabolism of both hexobarbital and aminopyrine. This ability of Triton to enhance conjugation and impair oxidative metabolism can both simplify assay procedures for UDPGT and increase their sensitivity particularly with substrates such as steroids.

Since the activity of UDPGT, *in vitro*, varies with the substrate and the manner of activation, it is difficult to speculate concerning the assay conditions which most accurately reflect the activity of the enzyme, *in vivo*. The results of the present studies emphasize the need for caution in the use of both non-treated and Triton-activated preparations. For example, we found that the concentration of Triton which causes maximum increases in UDPGT activity was the same for a variety of substrates but that the extent of activation was highly substrate dependent. Whether this substrate dependency was due to a multiplicity of UDPGT forms remains unknown, but is a possibility. Moreover, depending on whether a non-treated or Triton-activated enzyme preparation was used, the response of UDPGT to possible inhibitors and stimulators could be altered.

It was found that compounds which inhibit the conjugation of THE do so whether or not microsomes have been activated by Triton. However, the response to the primary and secondary amine analogs of chlorpromazine was quite different in the activated and non-treated preparations. We had previously shown that nortriptyline, the demethylated metabolite of the antidepressant amitriptyline, increases UDPGT activity by a mechanism markedly similar to that of Triton.[3] Both nortriptyline and the chlorpromazine metabolites could act as detergents because they possess considerable surface activity as a result of their molecular structure.[19]

The results from the bisubstrate kinetic analysis were of greatest interest. The true mechanism of the reaction was not determined as this requires a number of additional

product inhibition studies.[10] However, certain conclusions could be made. One was that Triton activation does increase the V_{max} of the reaction in the forward direction. Additionally, Triton treatment does not appear to significantly alter the dissociation constant for THE. It should be noted that nothing was said about an apparent K_m value. Both ourselves and others have reported values for an apparent K_m value for a variety of substrates conjugated by UDPGT. It should be realized that often these reported values are second substrate dependent,[10] and more importantly the designation of the constant derived from simple Michaelis–Menten treatment of this bisubstrate reaction has little if any meaning. The determination of the true mechanism for a variety of substrates using the same enzyme preparation should lead to a better understanding of these enzyme-catalyzed reactions and may finally decide the question of whether UDPGT exists in multiple forms.

Finally, several explanations for the non-linear behavior of the 1/UDPGA plots with the non-treated enzyme could be proposed. High concentrations of UDPGA might itself cause an activation of UDPGT such as that seen with several of the phenolic substrates of the enzyme.[2,5] Since most activation procedures for UDPGT are not additive, this behavior would not be expected with an enzyme already activated with Triton. Alternatively, there might exist a saturable non-specific binding site for UDPGA in the microsomes which would serve to remove UDPGA from the active site. These sites would then be altered or abolished by treatment with Triton. Similarly, it is known that other enzymes are present in the microsomes which can destroy UDPGA.[14] A reduction in UDPGA concentration by an alternate enzymatic pathway would similarly give non-linearity to the 1/UDPGA plots. It may also account for the non-intersection behavior of the $1/v$ vs 1/THE plot at the lowest UDPGA concentration.

Acknowledgements—We thank Dr. Glenn E. Ullyot, Smith, Kline & French Laboratories, for *p*-hydroxyamphetamine, *p*-hydroxynorephedrine, chlorpromazine and SKF-525A; and Dr. A. A. Manian, National Institute of Mental Health, for chlorpromazine metabolites. The authors are indebted to Miss Martha Austerman for her valuable technical assistance.

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