Biochemical Pharmacology, Vol. 24, pp. 1965–1969. Pergamon Press, 1975. Printed in Great Britain.

COVALENT BINDING OF *TRANS*-STILBENE TO RAT LIVER MICROSOMES*

EDWARD L. DOCKS† and GOPAL KRISHNA
Laboratory of Chemical Pharmacology, National Heart and Lung Institute, Bethesda, Md. 20014, U.S.A.

(*Received* 9 *December* 1974; *accepted* 21 *February* 1975)

Abstract—A number of estrogenic compounds have been shown to bind covalently to tissue macromolecules. Some of these agents cause impairment of liver function as measured by bromosulfophthalein clearance time. *Trans*-stilbene, a weak synthetic estrogen, which has been shown to be hydroxylated in several animal species, was investigated to determine if it covalently binds to tissue macromolecules. Liver damage was also evaluated histopathologically. *Trans*-stilbene binds covalently *in vivo* to various tissues of non-pretreated, phenobarbital-pretreated and 3-methylcholanthrene-pretreated rats. Pretreatment with 3-methylcholanthrene caused the greatest amounts of ^{14}C-*trans*-stilbene to covalently bind to plasma and liver proteins. Studies *in vitro* showed that ^{14}C-*trans*-stilbene became covalently bound to hepatic microsomes and that the covalent binding of ^{14}C-*trans*-stilbene to liver microsomes from non-pretreated, phenobarbital-pretreated and 3-methylcholanthrene-pretreated rats was linear with time for at least 20 min. Oxygen and NADPH were necessary for binding. Carbon monoxide inhibited covalent binding to microsomes from non-pretreated rats and, to a much lesser extent, to microsomes from 3-methylcholanthrene-pretreated rats. The effects of various pretreatments on the covalent binding *in vitro* paralleled those of the binding *in vivo*. Although *trans*-stilbene was seen to bind covalently *in vivo* and *in vitro*, no histopathological abnormalities of liver or kidney were observed. Pretreatment of rats with phenobarbital or 3-methylcholanthrene, before administration of *trans*-stilbene, also did not cause any tissue abnormalities in liver or kidney. Glutathione did not seem to act as a protective agent in this study because, even after complete depletion of this substance, *trans*-stilbene did not cause any significant liver or kidney histopathological changes.

It has been proposed by several investigators that the toxicities elicited by certain hepatotoxins, carcinogens and therapeutic agents might be mediated through the formation of chemically reactive metabolites that react with tissue macromolecules [1–8]. For example, previous work in this laboratory has shown that liver necrosis caused by halogenated benzenes is due to arene oxides [9] that alkylate tissue macromolecules [4–6]. In addition, acetaminophen causes liver necrosis presumably through the formation of a toxic metabolite [7, 8]. It has been reported that several estrogens covalently bind to various tissue macromolecules both *in vitro* and *in vivo* [10–14]. Some estrogens have also been reported to cause impairment of liver function, as evidenced by sulfobromophthalein (BSP) clearance times [15]. It seems possible that the covalent binding of estrogens to tissue macromolecules may result in the impairment of liver function.

Trans-stilbene is a weak synthetic estrogenic agent, whose activity is increased by hydroxylation of the benzene rings in the *para*-positions [16, 17]. It has been reported that *trans*-stilbene is hydroxylated in several animal species [18] possibly through an arene oxide intermediate. Since other arene oxide intermediates have been postulated to cause tissue damage [4–6, 9], and also since some estrogenic agents not only cause impairment of liver function [15] but also covalently bind to tissue macromolecules [10–14], this work was initiated to determine if *trans*-stilbene covalently binds to tissue macromolecules, and if so, does its reactive metabolite cause tissue damage.

METHODS

Male Sprague–Dawley rats (160–200 g) obtained from Hormone Assay Laboratories (Chicago, Ill.) were used in these experiments. Animals were allowed water and food (Purina Lab Chow) *ad lib.* Rats were pretreated with either phenobarbital (80 mg/kg, i.p.) 72, 48 and 24 hr prior to the experiment, or 3-methylcholanthrene (20 mg/kg, i.p.) 72, 60 and 48 hr prior to the experiment. Control rats were injected with the appropriate vehicle. In some experiments, diethyl maleate (0·6 ml/kg, i.p.) was given 30 min before *trans*-stilbene [19].

Covalent binding in vitro. Male rats were killed by decapitation. Their livers were immediately removed, washed in 0·9% saline, and homogenized with 3 vol. of 1·15% KCl–0·02 M Tris–HCl, pH 7·4 (Tris–KCl). The homogenate was centrifuged at 9000 *g* for 15 min and the supernatant fluid separated and centrifuged for 1 hr at 105,000 *g*. The microsomes were resuspended in 30 ml Tris–KCl buffer and recentrifuged at 105,000 *g* for 1 hr. The washing of microsomes with Tris–KCl buffer helped to remove most of the hemoglobin. The resulting pellet was resuspended in a volume of the Tris–KCl buffer equivalent

* Presented in part at the 1973 Federation of American Societies for Experimental Biology Meeting, Atlantic City, NJ.

† Recipient of a Postdoctoral Fellowship from the National Institute of General Medical Sciences and a Hoffmann-La Roche Fellowship. Present address: U.S. Borax Research, Anaheim, Calif.

to the original liver weight. The suspension was then adjusted to contain 5 mg protein/ml.

The covalent binding *in vitro* of ^{14}C-*trans*-stilbene to protein was determined as described previously [11]. The incubation mixture consisted of ^{14}C-*trans*-stilbene, rat liver microsomes and an NADPH-generating system. The final incubation mixture (1 ml) consisted of rat liver microsomes (1 mg protein), 0·108 mM NADH, 2 mM nicotinamide, 0·2 mM NADP, 2 mM glucose 6-phosphate, and 0·4 units/ml of glucose 6-phosphate dehydrogenase. The mixture was preincubated with shaking for 2 min at 37°, and then 10 μl of a 10 mM solution of ^{14}C-*trans*-stilbene (1 μCi) in dimethylformamide was added to the incubation mixture. The mixture was then incubated at 37° for varying amounts of time, and then 1 ml of 10% trichloroacetic acid was added to stop the enzymatic reaction and to precipitate the protein. The sample was centrifuged, and the resulting pellet was resuspended in 5 ml methanol–ether (3 : 1), heated at 60° for 10 min and then centrifuged again. This process was repeated 10 times. After aspiration of the final wash, which contained no radiolabel, the protein was dissolved in 1 ml of 1 N sodium hydroxide. The covalently bound radioactivity was determined by counting an aliquot of the above basic solution as described for the covalent binding experiments *in vivo*. Protein was determined by the method of Lowry *et al.* [22]. Student's *t*-test was used to evaluate the significance of differences between treated animals and the corresponding controls.

In addition to the covalent binding *in vitro* of *trans*-stilbene, the relationship of this binding to the metabolism of *trans*-stilbene was also studied. For the metabolism studies, 10 μl of a 10 mM solution of ^{14}C-*trans*-stilbene (1 μCi) in dimethylformamide was incubated with microsomes from control or 3-methylcholanthrene-pretreated rats with an NADPH-generating system as described above. After 10 min, the reaction was terminated by extracting with 3 ml heptane containing 5% isoamyl alcohol.

Ten μl of the organic phase was applied to a thin-layer chromatography plate (Silica gel F-254, layer thickness 0·25 mm). The plate was developed for a distance of 10 cm with benzene in a previously equilibrated chamber. The spot corresponding to *trans*-stilbene was visualized with the aid of a short wavelength u.v. light. The entire plate was then divided into 1-cm sections, starting 0·5 cm below the origin, and the Silica gel from each section was scrapped into scintillation vials and the radioactivity was determined. An authentic pure sample of *trans*-stilbene had an R_f value of 0·60. According to Sinsheimer and Smith [21], hydroxylation of *trans*-stilbene results in products having R_f values between 0·00 and 0·34 in this solvent system. Therefore, radioactivity corresponding to an R_f value of 0·60 was considered to be derived from unmetabolized *trans*-stilbene and the radioactivity corresponding to R_f values between 0·00 and 0·20 was considered to be derived from metabolites of *trans*-stilbene. No other radioactivity was observed on the plate except for the two areas cited above.

Studies in vivo. Animals were given various doses (200–800 mg/kg) of *trans*-stilbene, i.p., in sesame oil such that a 200-g rat received 1 ml of solution. The oil solutions were heated slightly to dissolve the solid *trans*-stilbene. The animals were killed by decapitation at various times after injection of the *trans*-stilbene. Paraffin sections of the liver and kidney were prepared and stained with periodic acid-Schiff reagent and hematoxylin or with hematoxylin and eosin [22].

Covalent binding in vivo. *Trans*-stilbene (1,2-^{14}C; sp. act. 1·64 mCi/m-mole, Mallinkrodt, > 99·0 per cent pure) diluted with nonlabeled *trans*-stilbene was given, i.p., to male rats in sesame oil at a dose of 400 mg/kg (125 μCi/kg). Some rats were pretreated with phenobarbital or 3-methylcholanthrene. Rats were killed, by decapitation, at various times, and blood was collected in tubes containing heparin, centrifuged and serum transferred to a clean tube and diluted with 3 vol. of water. Tissues were homogenized in 3 vol. of water. An aliquot (1 ml) of the homogenate or diluted serum was added to 10 ml of 10% trichloroacetic acid in order to precipitate the protein. The sample was then centrifuged, and the resulting pellet resuspended in 5 ml methanol–ether (3 : 1), heated at 60° for 10 min and then centrifuged. The washing procedure with the methanol–ether was repeated 10 times to remove radioactivity that was not covalently bound to protein. After aspiration of the final wash, the protein was dissolved in 2 ml of 1 N sodium hydroxide. The covalently bound radioactivity was determined by counting an aliquot of the above basic solution in 15 ml scintillation phosphor consisting of 0·4% BBOT [2,5-bis(5-*tert*-butylbenzoxazoyl)thiophene], 0·8% naphthalene and 40% methylcellosolve in toluene [23]. Protein concentration was determined by the method of Lowry, *et al.* [26].

RESULTS

Covalent binding in vitro. ^{14}C-*trans*-stilbene (0·1 M) was incubated for varying lengths of time with washed rat liver microsomes in the presence of an NADPH-generating system. The data in Fig. 1 indicate that covalent binding to rat liver microsomal protein was linear for at least 20 min. The rate of covalent binding of *trans*-stilbene to liver microsomal protein was not altered by pretreating the rats with phenobarbital but was markedly increased by pretreating the rats with 3-methylcholanthrene.

The covalent binding of ^{14}C-*trans*-stilbene to rat liver microsomes is apparently mediated by a microsomal mixed-function oxidase, because it requires an NADPH-generating system and oxygen (Table 1).

Moreover, the covalent binding is inhibited by SKF 525-A (β-diethylamino-ethyl diphenylpropylacetate, 0·5 mM) and glutathione (1 mM) (Table 1). However, the role of cytochrome P-450 in the formation of the reactive metabolite is uncertain. As shown in Table 1, an atmosphere at 80% carbon monoxide and 20% oxygen decreased the covalent binding of *trans*-stilbene by more than 97 per cent when microsomes from control rats were used in the incubation mixture. In contrast, this atmosphere inhibited the covalent binding by only 60 per cent when rat liver microsomes from phenobarbital-pretreated rats were used and 27 per cent when 3-methylcholanthrene-pretreated rats were used.

In order to determine K_m and V_{max} values for the

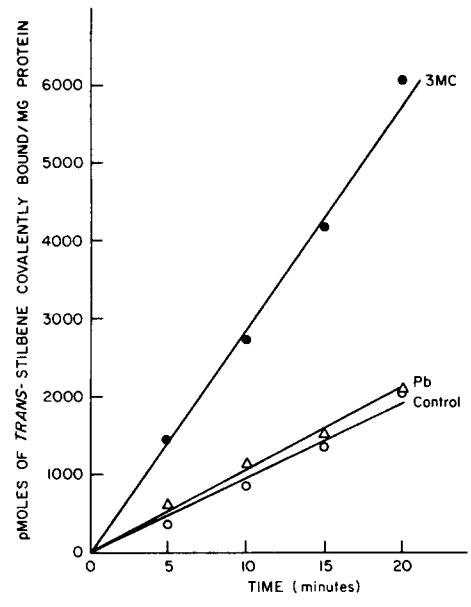

Fig. 1. Covalent binding *in vivo* of ^{14}C-*trans*-stilbene in rat liver microsomal protein as a function of time. Key: (○) non-pretreated rats; (△) phenobarbital-pretreated rats (80 mg/kg, i.p., for 3 days); (●) 3-methylcholanthrene-pretreated rats (20 mg/kg, i.p., 72, 60 and 48 hr prior to use). Rat liver microsomes were incubated for various lengths of time with an NADPH-generating system and ^{14}C-*trans*-stilbene (0·1 mM).

covalent binding of ^{14}C-*trans*-stilbene to rat liver microsomal protein, reciprocal covalent binding velocities were plotted against reciprocal substrate concentrations and the kinetic parameters of the Michaelis–Menten equation were calculated according to Lineweaver and Burk [24]. The results of these calculations are shown in Table 2. Both the K_m and V_{max} values are significantly different when using microsomes from 3-methylcholanthrene-pretreated rats compared to control rats. Pretreatment of the rats with 3-methylcholanthrene greatly increased the rate of covalent binding, as evidenced by the greater than 6-fold increase of V_{max} over the values observed when microsomes from control rats were used.

Figure 2 shows the total metabolism *in vitro* of ^{14}C-*trans*-stilbene (0·1 mM) by rat liver microsomes. The rate of metabolism of *trans*-stilbene was linear for 10 min. However, the rate of metabolism of *trans*-stilbene by liver microsomes was increased 10-fold by 3-methylcholanthrene pretreatment of the animals.

Covalent binding in vivo. After the administration of ^{14}C-*trans*-stilbene (400 mg/kg, 125 μCi/kg, i.p.) to rats, the amount of covalently bound radiolabeled material was determined after 24 hr. The data in Table 3 show that only less than 150 pmoles radiolabeled *trans*-stilbene was covalently bound mg of protein to any tissues from non-pretreated rats or from phenobarbital or 3-methylcholanthrene-pretreated rats. Liver contained the greater amount of covalently bound material. Rats which were pretreated with 3-methylcholanthrene showed the highest amount of covalently bound material in the liver after 24 hr. The liver of phenobarbital-pretreated rats showed the least amount of covalently bound material after 24 hr. A qualitatively similar result was observed in the case of covalently bound material to plasma with the exception that the quantities were two to three times less than in the liver. Only small amounts of radiolabeled material were bound to heart. Fat had similar quantities of covalently bound radioactivity to those found in liver for non-pretreated, phenobarbital- and 3-methylcholanthrene-pretreated rats. For all tissues examined, the amount of covalently bound material decreased by 10 per cent at 48 and 72 hr (not shown).

Studies in vivo. *Trans*-stilbene when administered to non-pretreated male rats at doses of 100, 200, 400 or 800 mg/kg did not cause any liver or kidney histopathological abnormalities within 48 hr. Furthermore, induction of the mixed-function oxidase system, by administration of phenobarbital or 3-methylcholanthrene prior to the administration of *trans*-stilbene at a dose of 200 mg/kg, also did not cause any liver or kidney histopathological abnormalities within 48 hr. Even when rats were pretreated with diethyl maleate to deplete the tissues of reduced glutathione (GSH) [5, 6], no damage was seen in liver and kidney after the administration of *trans*-stilbene, at doses of 200 or 400 mg/kg.

DISCUSSION

The covalent binding of ^{14}C-*trans*-stilbene was approximately the same when using microsomes from either non-pretreated rats or phenobarbital-pretreated rats. However, if rat liver microsomes from 3-methylcholanthrene-pretreated rats were used, the covalent

Table 1. Effect of various inhibitors of the covalent binding of ^{14}C-*trans*-stilbene to liver microsomes*

Inhibitors	Covalent binding of *trans*-stilbene to microsomes (pmoles/mg protein/10 min)		
	Control	Phenobarbital	3-MC
None	1339 ± 292	1431 ± 194	2767 ± 555
SKF 525-A (0·5 mM)	488 ± 253	0	918 ± 248
GSH (1 mM)	225 ± 74	664 ± 238	1228 ± 145
N_2 (100%)	0	170 ± 113	1560 ± 711
CO–O_2 (8 : 2)	36 ± 35	561 ± 133	2005 ± 491
Minus NADPH-NADH and NADPH-generating system	149 ± 23	108 ± 35	105 ± 43

* Rat liver microsomes from control, phenobarbital- and 3-methylcholanthrene-pretreated rats were incubated with 0·1 mM ^{14}C-*trans*-stilbene as described in Methods.

Table 2. Apparent K_m and V_{max} for covalent binding of *trans*-stilbene to rat liver microsomes*

Pretreatment	K_m (μM)	V_{max} (pmoles/mg/min)
None	50	100
Phenobarbital	21	139
3-Methylcholanthrene	17	667

* Rat liver microsomes were prepared and incubated with ^{14}C-*trans*-stilbene and an NADPH-generating system as described in Methods. Values are the mean of at least three rats.

binding increased markedly (Fig. 1). The covalent binding of *trans*-stilbene to liver microsomes requires an NADPH-generating system and is markedly decreased under an atmosphere of nitrogen. Thus, covalent binding of *trans*-stilbene does not occur directly, but apparently is dependent on the conversion of *trans*-stilbene to an active metabolite.

Moreover, the addition of SKF 525–A to the microsomal incubation mixture decreased the covalent binding to the microsomal protein. Covalent binding to microsomes from control rats is decreased when the microsomes are incubated in an atmosphere of CO–O_2 (8 : 2). When microsomes from 3-methylcholanthrene-pretreated rats were used, the covalent binding of *trans*-stilbene also decreased but to a much smaller extent than when control microsomes were used. The reason for this is not clear.

GSH at a concentration of 1 mM also inhibited covalent binding (Table 1). This result infers that the active metabolite, which covalently binds to microsomal protein, is an arylating agent. GSH is a nucleophilic compound capable of reacting with electrophilic-alkylating agents. One function of GSH *in vivo* may be to protect the liver from attack by active metabolites of various compounds. In the case of bromobenzene, it has been found that GSH protects the liver by reacting with a toxic active metabolite of bromobenzene [23]. GSH caused a decrease in the covalent binding *in vitro* of *trans*-stilbene to rat liver microsomal protein. However, if GSH acted as a protective agent in the liver, depleting GSH *in vivo*, using diethyl maleate, might lead to liver necrosis after *trans*-stilbene was administered. However, depletion of GSH before administration of *trans*-stilbene (200–400 mg/kg) did not cause any tissue abnormalities, as observed by histopathological examination. It has also been observed that *trans*-stilbene does not cause a decrease in GSH levels in the rat liver even after administration of very large amounts, up to 1 g/kg.

Metabolism of *trans*-stilbene is directly related to covalent binding. In Fig. 2 are data which demonstrate that metabolism is greatly increased when ^{14}C-*trans*-stilbene and an NADPH-generating system are incubated with rat liver microsomes obtained from animals pretreated with 3-methylcholanthrene. As mentioned previously, covalent binding *in vivo* of ^{14}C-*trans*-stilbene also markedly increased. Thus, the increase in covalent binding appears to be dependent on the metabolism of *trans*-stilbene. The metabolic products in this study have not been identified, but they are probably hydroxylated stilbenes as reported previously [18, 21].

Trans-stilbene covalently binds to tissues *in vivo* (Table 3). Less ^{14}C-*trans*-stilbene was bound covalently to plasma and liver in rats pretreated with phenobarbital than was bound to those same tissues in non-pretreated rats. Pretreatment with 3-methylcholanthrene increased the amount of radiolabeled

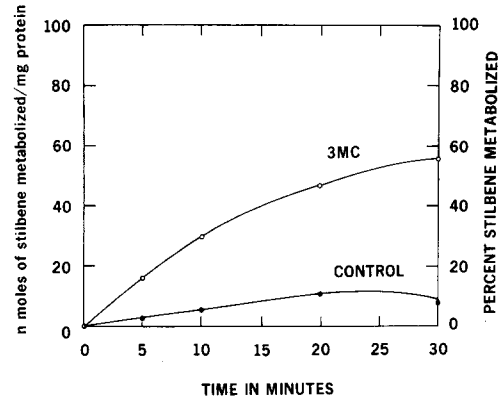

Fig. 2. Percent of *trans*-stilbene metabolized *in vitro* using rat liver microsomes. Either 1 mg protein/ml of control microsomes or microsomes from 3-methylcholanthrene-pretreated rats were prepared and incubated with ^{14}C-*trans*-stilbene (0·1 mM) and an NADPH-generating system as described in Methods. Incubation mixtures were extracted and analyzed by thin-layer chromatography as described in Methods. Each point represents three different determinations. All standard errors were less than 5 per cent.

Table 3. Covalent binding *in vivo* of ^{14}C-*trans*-stilbene to various tissues 24 hr after administration*

Tissue	pmoles *trans*-stilbene covalently bound/mg protein ± S.E.M. Non-pretreated	Phenobarbital pretreated	3-Methylcholanthrene pretreated
Plasma	41·6 ± 3·5	27·6 ± 1·3	63·7 ± 1·8
Heart	14·6 ± 1·4	7·1 ± 0·2	8·6 ± 0·7
Lung	43·1 ± 9·3	25·0 ± 0·4	24·8 ± 8·6
Liver	102·0 ± 10·3	58·2 ± 4·4	139·0 ± 6·9
Kidney	81·9 ± 24·5	60·8 ± 9·5	68·9 ± 6·3
Fat	109 ± 33·3	49·1 ± 8·4	51·7 ± 11·3

* ^{14}C-*trans*-stilbene (400 mg/kg) was injected, i.p., into groups (three rats each) of control, phenobarbital- or 3-MC-pretreated rats. Animals were killed 24 hr after stilbene administration, various tissues were removed and covalently bound stilbene was determined as described in the text.

material covalently bound to liver and plasma. Therefore, it appears that 3-methylcholanthrene increases the metabolism *in vivo* of *trans*-stilbene, thereby causing a greater amount of an active metabolite to become covalently bound to various tissues. The results *in vivo* closely parallel the results *in vitro* discussed above, and it appears that in both cases the covalent binding is markedly increased with 3-methylcholanthrene pretreatment.

Covalent binding to microsomal protein, *in vitro* as well as *in vivo*, has been used as a measure of the amount of an active metabolite formed from various compounds [3–8]. In several cases, it has been shown that by either inducing or inhibiting drug metabolism, thereby causing changes in the amount of the active metabolite formed and concomitantly changes in the amount of compound covalently bound to tissue macromolecules, compounds can become more or less hepatotoxic. For example, the relationship of metabolism to toxicity has been examined for bromobenzene [4–8] and acetaminophen [8]. In the present study with *trans*-stilbene, although 3-methylcholanthrene pretreatment caused greater amounts of an active metabolite to bind covalently both *in vitro* and *in vivo*, no tissue damage was observed, as measured by histopathological evaluation. There are several reasons why a compound may bind covalently but not produce tissue damage. First, not enough of the active metabolite may be formed at the doses which were used. By inducing the drug-metabolizing enzyme system with either phenobarbital or 3-methylcholanthrene, more of the active metabolite can be formed. For example, 3-methylcholanthrene pretreatment increases the covalent binding of *trans*-stilbene. Even after this treatment, however, *trans*-stilbene did not cause tissue damage, presumably because less than 150 pmoles *trans*-stilbene is bound/mg of liver protein. This amount may not be enough to produce necrosis since, as we have shown earlier, bromobenzene does not cause liver necrosis until 1 nmole bromobenzene metabolite is bound/mg of liver protein [6]. It is also possible that the animal may possess a system or compound which detoxifies any active metabolite formed before it can react with a target tissue and cause damage. As mentioned previously, GSH is probably not responsible for this protective effect in the case of *trans*-stilbene. Furthermore, the active metabolite which is formed may covalently bind to tissue macromolecules, but not cause tissue damage because the metabolite is bound at an inactive position of the tissue.

The active metabolite which covalently binds is probably an arene oxide. Many arene oxides have been postulated to be responsible for tissue damage [9]. Since *trans*-stilbene is hydroxylated in several animal species [18], it is likely that an arene oxide is formed which could then lead to hydroxylated or polyhydroxylated products.

In summary, although *trans*-stilbene is a weak estrogenic agent [16, 17] and some estrogenic agents have been shown to cause liver impairment [15], it now appears that *trans*-stilbene does not cause any liver damage. In addition, some estrogenic agents have been shown to bind covalently to tissue macromolecules [10–14]. *Trans*-stilbene also binds covalently, the binding being related to metabolism, but this binding does not lead to hepatotoxicity. If further investigation shows that *trans*-stilbene does not cause tissue damage, it may be possible to design a synthetic estrogen which possesses estrogenic activity but lacks the adverse toxic effects. Further studies on the mechanism of covalent binding of *trans*-stilbene to tissue macromolecules, as well as the amino acid(s) to which this compound is covalently bound, may help us to understand, in greater detail, the relationship between covalent binding and toxicity.

REFERENCES

1. J. D. Judah, A. E. M. McLean and E. K. Mclean, *Am. J. Med.* **49,** 609 (1970).
2. J. H. Weisburger and E. K. Weisburger, *Pharmac. Rev.* **25,** 1 (1973).
3. B. B. Brodie, W. D. Reid, A. K. Cho, G. Sipes, G. Krishna and J. R. Gillette, *Proc. natn. Acad. Sci. U.S.A.* **68,** 160 (1971).
4. W. D. Reid, B. Christie, G. Krishna, J. R. Mitchell and B. B. Brodie, *Pharmacology* **6,** 41 (1971).
5. W. D. Reid, B. Christie, M. Eichelbaum and G. Krishna, *Expl. molec. Path.* **15,** 363 (1971).
6. W. D. Reid and G. Krishna, *Expl. molec. Path.* **18,** 80 (1973).
7. N. Zampaglione, D. J. Jollow, J. R. Mitchell, B. Stripp, M. Hamrick and J. R. Gillette, *J. Pharmac. exp. Ther.* **187,** 211 (1973).
8. J. R. Mitchell, D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187,** 185 (1973).
9. J. W. Daly, D. M. Jerina and B. Witkop, *Experientia* **28,** 1129 (1972).
10. P. H. Jellinck, J. Lewis and F. Boston, *Steroids* **10,** 329 (1967).
11. G. Krishna, G. U. Corsini, J. R. Gillette and B. B. Brodie, *Toxic. appl. Pharmac.* **19,** 361 (1972).
12. G. Corsini, G. Krishna, B. B. Brodie and J. R. Gillette, *Fith Int. Cong. on Pharmacology*, p. 14, Abstr. No. 277 (1972).
13. H. M. Bolt and H. Remmer, *Xenobiotica* **2,** 489 (1972).
14. H. Kappus, H. M. Bolt and H. Remmer, *Steroids* **22,** 203 (1973).
15. T. F. Gallagher, Jr., M. N. Mueller and A. Kappas, *Medicine, Baltimore* **45,** 471 (1966).
16. E. C. Dobbs and W. Lawson, *Nature, Lond.* **139,** 626 (1937).
17. S. W. Stroud, *J. Endocr.* **2,** 55 (1940).
18. J. E. Sinsheimer and R. V. Smith, *Biochem. J.* **111,** 35 (1969).
19. E. Boyland and L. F. Chasseaud, *Biochem. Pharmac.* **19,** 1526 (1970).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
21. J. E. Sinsheimer and R. V. Smith, *J. pharm. Sci.* **56,** 1280 (1967).
22. L. G. Luna, *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*, 3rd Edn, p. 159. McGraw-Hill, New York (1968).
23. I. G. Sipes, B. Stripps, G. Krishna, H. M. Maling and J. R. Gillette, *Proc. Soc. exp. Biol. Med.* **142,** 237 (1973).
24. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).