Biochemical Pharmacology, Vol. 34, No. 1, pp. 45–49, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

SULFATION OF PEPTIDES AND SIMPLE PHENOLS BY RAT BRAIN PHENOLSULFOTRANSFERASE

INHIBITION BY DICHLORONITROPHENOL

OSVALDO GIORGI and JAMES L. MEEK*

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeth's Hospital, Washington, DC 20032, U.S.A.

(*Received* 2 *December* 1983; *accepted* 23 *April* 1984)

Abstract—Brain phenolsulfotransferase (PST) is involved in the sulfation of simple phenols like dopamine and of precursors of biologically active peptides like cholecystokinin octapeptide (CCK-8). Therefore, inhibition of brain PST would provide a new approach to studying the sulfation of CCK-8 and other sulfated compounds. Since 2,6-dichloro-4-nitrophenol (DCNP) produces a prolonged and selective inhibition of the sulfoconjugation of exogenous phenols by the liver, we decided to examine the applicability of DCNP to studies of sulfation of CCK-8 and other compounds by brain. DCNP was capable of completely inhibiting PST activity in rat brain homogenates incubated with *p*-nitrophenol, phenol or dopamine as substrates. The IC_{50} values for *p*-nitrophenol and dopamine were 12 and 14 μM respectively. The concentrations of DCNP in brain cortex and plasma were measured by high pressure liquid chromatography (HPLC) after a dose of 100 μmoles/kg, i.p. Peak concentrations of 380 μM in plasma and 25 μmoles/kg in brain were achieved 30 min after injection. Subsequently, DCNP concentrations decreased with half-lives of 8 and 6 hr in plasma and brain cortex, respectively. To establish if DCNP can inhibit CCK sulfation *in vivo*, rats were injected with 100 μmoles/kg, i.p., of the drug 30 min before injection of $^{35}SO_4^{2-}$ into the cerebral cortex and were killed 4.5 hr later. DCNP caused a 55% inhibition of [^{35}S]CCK-8-SO_4 formation as measured by HPLC. No change in the content of endogenous CCK-8-SO_4 was detectable, however, in the brain cortex of rats treated with DCNP for up to 4 days, indicating that the PST which remained active was capable of maintaining CCK-8 content at steady state.

Phenolsulfotransferase (EC 2.8.2.1, PST) catalyzes the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to a wide variety of acceptors including phenolic drugs, biogenic amines and their metabolites [1]. PST or a related enzyme is also probably responsible for the sulfation of tyrosine residues that occurs during the post-translational stages of processing of proteins into peptides. A few endogenous peptides are known to contain sulfate residues. Cholecystokinin octapeptide (CCK-8) requires a sulfate for biological activity; gastrin does not [2]. If PST could be inhibited in brain for prolonged periods, new approaches to studying processing and turnover of cholecystokinin and other peptides would be made possible. What is needed is a potent long-acting inhibitor of brain PST. Mulder and Scholtens [3] have reported that a prolonged and selective inhibition of the sulfoconjugation of phenolic compounds by liver is obtained after a single injection of 2,6-dichloro-4-nitrophenol (DCNP). This compound appears to be the best PST inhibitor available. We therefore decided to examine the applicability of DCNP to studies of CCK sulfation and turnover in rat brain. We investigated the pharmacokinetics of DCNP in brain and plasma, the ability of DCNP to inhibit the sulfation *in vitro* of several simple phenols by rat brain homogenates, and the ability of DCNP to block *in vivo* the formation of CCK-8-SO_4. We also examined nitrovinyldihydroxybenzene which was designed as a "suicide" inhibitor of PST [4]. Several forms of PST have been demonstrated in various tissues either by isolation [5, 6] or by thermostability differences with different substrates [7–9]. We chose to use simple phenols as substrates, since they are substrates for various PST forms, and neither the natural precursor of CCK nor artificial substrates for the enzyme which sulfate this peptide are known.

MATERIALS AND METHODS

[^{35}S]PAPS (2.8 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA); phenol, *p*-nitrophenol and dichloronitrophenol were purchased from the Aldrich Chemical Co. (Milwaukee, WI); and 2,3-dihydroxynitrovinylbenzene was a gift of J. L. Costa and K. L. Kirk [4]. CCK-8-SO_4 was a gift of M. Ondetti, Squibb Institute. $Na_2{}^{35}SO_4$ (carrier free) was purchased from ICN (Irvine, CA). Dopamine HCl and pargyline were obtained from the Sigma Chemical Co., St. Louis, MO).

PST assay. PST was assayed as described by Foldes and Meek [10] with modifications by Anderson and Weinshilboum [11]. Male Sprague–Dawley rats (Zivic Miller, PA, 150–180 g) were decapitated and their brains removed, weighed and homogenized in 40 vol. of 10 mM sodium phosphate buffer, pH 6.4, containing 0.25% bovine serum albumin (BSA). After centrifugation (16,000 *g*, 10 min), PST activity was assayed in a 50-μl aliquot of the supernatant

* To whom correspondence should be addressed.

fraction (approximately 30 μg protein). The homogenates were incubated for 20 min at 37° in a mixture containing 4 μM [^{35}S]PAPS, a phenol, and 10 mM sodium phosphate buffer, pH 6.4, in a final volume of 250 μl. Whenever dopamine was used as substrate, 1 mM pargyline was also included in the incubation mixture. In blank tubes sodium phosphate was substituted for the phenol. The reaction was stopped by adding 200 μl of 0.1 M barium acetate and transferring the tube to ice-cold water. Unreacted [^{35}S] PAPS was removed by precipitation with 200 μl of 0.1 M $Ba(OH)_2$ and 200 μl of 0.1 M $ZnSO_4$. After centrifugation (600 *g*, 10 min), the sequential addition of barium acetate, $Ba(OH)_2$ and $ZnSO_4$ and centrifugation were repeated. Radioactivity in a 1.1-ml aliquot of the final supernatant fraction was measured by scintillation counting. PST activity was linear with time for at least 60 min and with protein content up to 200 μg/tube.

To analyze the effects of DCNP and nitrovinyldihydroxybenzene, homogenates were preincubated for 15 min at 37° in the presence of different concentrations of the tested inhibitor with saturating concentrations of substrate. Inhibitors were added as aqueous solutions. The reaction was started by adding [^{35}S]PAPS.

DCNP Pharmacokinetics. Rats were anesthetized with ether at intervals after i.p. injection of DCNP in corn oil (100 μmoles/kg, 1 ml/kg), and 2–3 ml of blood was collected from the abdominal aorta into heparinized plastic tubes. Brains were then removed, and a piece of frontal cortex (area 2, approximately 100 mg) was dissected for assay. Blood was centrifuged (600 *g*, 10 min), and a 100-μl aliquot of plasma was diluted in 10 vol. of 90% methanol to precipate proteins. After centrifugation (8000 *g*, 4 min), the methanol supernatant fraction was evaporated to dryness in a vacuum centrifuge. Cerebral cortex was homogenized in 1 ml of 90% methanol, using an ultrasonic cell disrupter (Kontes, Inc.). After centrifugation at 8000 *g* for 4 min, the supernatant fractions were evaporated in a vacuum centrifuge. Plasma and brain samples were dissolved in 300 or 150 μl of mobile phase respectively. Recovery of DCNP for analysis by HPLC was 80% for both cortex and plasma. The HPLC system consisted of a Milton–Roy minipump, a Rheodyne 7010 injection valve with a 50 μl loop, a Bio-Rad ODS-10 reverse phase column (25 × 0.4 cm) and an Altex–Hitachi variable wavelength spectrophotometer. The mobile phase (60% methanol/50 mM sodium phosphate buffer, pH 6.2) was pumped at a rate of 1 ml/min. Absorbance was measured at 420 nm, 0.02 to 0.05 absorbance units full scale.

Incorporation of $^{35}SO_4^{2-}$ into CCK-8-SO_4. For isolation of [^{35}S]CCK-8-SO_4 by reverse phase HPLC, a method was used similar to that previously described [12] except that the sample cleanup was simplified: samples of cerebral cortex were homogenized in 1 ml of 90% methanol containing approximately 1 μg CCK-8-SO_4 and centrifuged. The entire supernatant fraction was injected onto a 3 cm × 4.6 mm AX300 anion exchange guard column used as a sample "loop" in the HPLC system. The guard column was washed with 0.4 ml of 0.5% trifluoroacetic acid to remove methanol and non-ionic impurities. For chromatography, the "loop" was inserted into the stream and a gradient started from 20% acetonitrile/0.2% trifluoroacetic acid to 70% acetonitrile at 5%/min. Four minutes after starting the gradient when CCK-8-SO_4 but not $^{35}SO_4^{2-}$ was eluted onto the reverse phase column, the sample "loop" was returned to the "load" position. The fraction containing the CCK-8-SO_4 (7.4 to 8.1 min) (and the fractions (blanks) immediately before and after the CCK-8-SO_4 peak were collected for scintillation counting.

Measurement of endogenous CCK-8-SO_4 content. Cerebral cortex (area 2, approximately 100 mg) was removed and homogenized in methanol. After centrifugation (16,000 *g*, 10 min), the supernatant fraction was passed through a DEAE-Sephadex A-25 0.6 × 5 cm column. Unsulfated CCK was eluted with 3 ml of 0.05 M HCl and CCK-8-SO_4 was subsequently eluted with 3 ml of 0.1 M HCl. The eluate was adjusted to pH 7.4 with 0.1 M Tris base, and the amount of CCK-8-SO_4 present in the eluate was measured by radioimmunoassay. The antibody was a gift of M. Iadarola, NIH.

RESULTS

The activity of PST in whole rat brain homogenates was measured with various concentrations of *p*-nitrophenol, phenol and dopamine. Substrate inhibition was observed with high concentrations of *p*-nitrophenol and phenol, but not with dopamine up to 4 mM (Fig. 1). The V_{max} values for these three substrates were nearly identical while the affinities differed widely: *p*-nitrophenol (K_m = 0.31 μM), phenol (K_m = 12 μM), and dopamine (K_m = 1300 μM) (Table 1). The sensitivity of PST to inhibition by DCNP and nitrovinyldihydroxybenzene is also shown in Table 1. Both compounds were capable of completely inhibiting PST activity regardless of the substrate used. Similarly, the IC_{50} values did not show significant variations when PST was assayed with saturating concentrations of *p*-nitrophenol, phenol or dopamine as substrates.

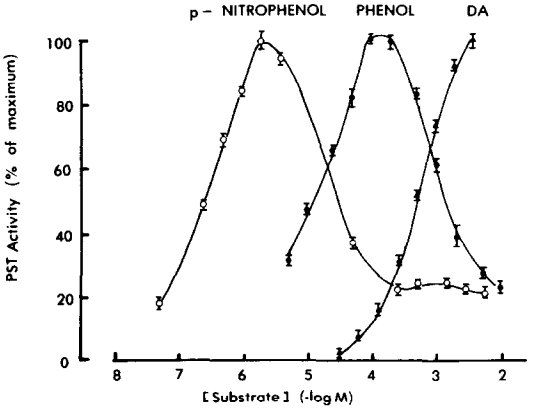

Fig. 1. PST activity in whole rat brain homogenates incubated with different concentrations of *p*-nitrophenol (○—○, phenol (●—●), or dopamine (▲—▲). Ordinate: PST activity as percent of maximum. Abscissa: molar concentration of substrate. Shown are the mean ± S.E.M. of triplicate determinations.

Table 1. Kinetic parameters of PST in rat brain: Inhibition by DCNP and nitrovinyldihydroxybenzene*

Substrate	V_{max} (pmoles/mg protein/min)	K_m (μM)	IC_{50} DCNP (μM)	IC_{50} Nitrovinyl-dihydroxybenzene (μM)
p-Nitrophenol (4 μM)	22	0.31	12	9
Phenol (50 μM)	19	12	NA†	6
Dopamine (4 mM)	22	1300	14	6

* V_{max} and K_m values were calculated from Eadie–Hofstee plots of data shown in Fig. 1. To analyze the inhibitory effects of DCNP and nitrovinyldihydroxybenzene, brain homogenates were incubated with the indicated (saturating) concentrations of substrate in the presence of different concentrations of the tested inhibitor. The IC_{50} values (i.e. the concentration of the drug required to decrease the control PST activity by 50%) were calculated by log-probit analysis.

† NA = not assayed.

PST activity was measured in homogenates pre-incubated with DCNP before and after dialysis to establish if the inhibition of PST induced by this agent is reversible or not. As shown in Table 2, the effect of DCNP was reversed completely by dialysis, indicating that the inhibition was reversible. After dialysis, a significant decrease in PST activity in control homogenates was also observed (Table 2). This loss of PST activity could be prevented by adding 0.25% BSA to the homogenates (not shown). Under these experimental conditions, however, PST inhibition by DCNP was not reversed by dialysis, since this agent binds to albumin [13]. Therefore, we did not use BSA in our subsequent dialysis experiments.

Before attempting *in vivo* inhibition of PST with DCNP, we examined its fate and tissue distribution following administration to rats. The concentrations of this drug in brain cortex and plasma were measured by HPLC at different times after injection at a dose of 100 μmoles/kg, i.p. As shown in Fig. 2, DCNP reached a peak concentration of 380 ± 24 μM in plasma and 25 ± 5 μmoles/kg wet weight in brain cortex 30 min after injection and disappeared with a half-life of 8 and 6 hr in plasma and brain cortex respectively. This dose of DCNP, which should cause substantial but not complete inhibition of PST in brain, had no obvious behavioral effects. Larger doses could not be used to increase PST inhibition in brain because of toxicity. DCNP must therefore be injected repeatedly in order to achieve a prolonged inhibition of brain PST activity. The latter condition must be satisfied to induce a significant decrease in CCK-8-SO_4 content in the brain since the half-life of this compound is about 17 hr [12].

To establish if DCNP can inhibit the synthesis of CCK-8-SO_4 *in vivo*, rats were injected with 100 μmoles/kg, i.p., of the drug 30 min before the intracortical administration of 50 μCi $^{35}SO_4^{2-}$ (38 pmoles) into area 2 of the cerebral cortex. Animals were killed 4.5 hr after injection of the labeled tracer. At this time, free $^{35}SO_4^{2-}$ had declined to 1% of the starting content while [^{35}S]CCK-8-SO_4 content was near its maximum, and the bulk of the radioactivity present in brain was probably incorporated into membrane components such as sulfatides and mucopolysaccharides with very long half-lives [12]. DCNP caused a 55% decrease in formation of labeled CCK-8-SO_4 (Table 3). The incomplete inhibition is not surprising in view of the concentration of inhibitor likely to be in brain during the 4-hr period (20–25 μmoles/kg) and the K_i seen for simple phenols (12 μM). A similar inhibition of incorporation of $^{35}SO_4^{2-}$ into other tissue components was also observed.

We attempted to change the endogenous content of CCK-8-SO_4 by repeated injection of the maximum tolerated dose of DCNP. Rats were injected with DCNP (100 μmoles/kg, i.p.) followed by doses of 50 μmoles/kg, i.p., at 8-hr intervals for 2 or 4 days.

Table 2. DCNP-induced inhibition of PST: Reversal by dialysis*

Substrate	PST activity (pmoles/mg protein/min) Before dialysis Control	Before dialysis DCNP	After dialysis Control	After dialysis DCNP
Dopamine (300 μM)	5.07 ± 0.13	0.25 ± 0.25	2.98 ± 0.10†	3.09 ± 0.15‡
Phenol (100 μM)	22.5 ± 0.08	3.20 ± 0.03	13.2 ± 0.32†	15.1 ± 0.11‡
p-Nitrophenol (4 μM)	22.1 ± 0.30	3.93 ± 0.39	15.1 ± 0.24†	16.1 ± 0.60‡

* Homogenates were incubated at 37° for 5 min in the absence or in the presence of 100 μM DCNP. Aliquots of the control and DCNP-treated homogenates were taken and assayed for PST activity before and after 24-hr dialysis in 4 liters of 10 mM sodium phosphate buffer, pH 6.4, containing 1 mM dithiothreitol. Shown are the mean ± S.E.M. of triplicate determinations.

† $P < 0.001$ vs non-dialyzed control group (two-tailed Student's *t*-test).

‡ $P < 0.001$ vs non-dialyzed DCNP-treated group.

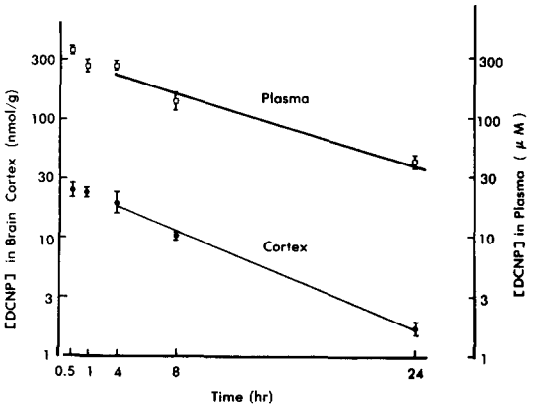

Fig. 2. DCNP concentrations in plasma and brain cortex of rats at different times after injection. Rats received 100 μmoles/kg, i.p., of DCNP (dissolved in corn oil, 1 ml/kg) and were killed at intervals. Ordinates: DCNP content expressed as nmoles/g wet weight in brain cortex (●—●) and as μM concentration in plasma (□—□). Abscissa: time after DCNP injection (hr). Shown are the mean ± S.E.M. of four rats for each time point. Half-life times for DCNP in plasma (8 hr) and in brain cortex (6 hr) were calculated by linear regression analysis.

At the end of the treatment the rats were killed, and CCK-8-SO_4 in cerebral cortex was separated from any free CCK-8 and measured by radioimmunoassay. As shown in Table 4, there was no effect of this treatment on CCK-8-SO_4 content.

Table 4. CCK-8-SO_4 content in the cerebral cortex of rats treated with DCNP*

Treatment	CCK-8-SO_4 (pmoles/g wet wt)
Control	278 ± 56
DCNP (48 hr)	260 ± 19
DCNP (96 hr)	225 ± 17

* Rats received an initial injection of 100 μmoles/kg, i.p., of DCNP, followed at 8-hr intervals by doses of 50 μmoles/kg, i.p., for 2 or 4 days. Control rats received the equivalent volume of solvent (corn oil, 1 ml/kg, i.p.). At the end of the treatment, animals were decapitated, and the cerebral cortex was removed, weighed and homogenized in 1 ml of 90% methanol. Sulfated CCK-8 was separated from free CCK-8 by ion exchange chromatography and measured by radioimmunoassay. Shown are the mean ± S.E.M. of five animals in each experimental group.

DISCUSSION

The ability of tissues to sulfate a wide variety of phenols is due to a number of enzymes, collectively called phenol sulfotransferase. Four such enzymes have been purified to homogeneity from rat liver [6]; more may occur in that tissue with different substrate specificity. Differences in enzyme type may occur between tissues: rat liver enzyme preparations were reported to have a rapid-equilibrium random mechanism [14] while a rat brain enzyme had an ordered sequential mechanism [15]. Evidence exists for PST heterogeneity in human platelets and brain based on thermostability of activity with various substrates and selective inhibition [7–9].

While the liver PST is known to be important in detoxification of phenols, the role of the enzymes in other tissues is less clear. Some form of PST is likely to be involved in sulfation of tyrosine residues in proteins and peptides. In brain, simple phenols (e.g. dopamine and 4-hydroxy-3-methoxyphenylglycol), proteins and peptides (e.g. CCK-8-SO_4) can be sulfated. A good, preferably irreversible, inhibitor of PST would be useful to study the role of such sulfation processes. DCNP, was shown to be a long-lasting peripheral inhibitor of sulfation, but not glucuronidation [3]. Pentachlorophenol has a similar effect, although shorter in duration. These compounds are probably dead-end inhibitors but, as shown here, are reversible. Although these compounds are chemically similar to well known inhibitors of oxidative phosphorylation (e.g. dinitrophenol), the specificity of inhibition of conjugation would suggest that the drug effects are not mostly due to an inhibition of energy metabolism. Nitrovinyldihydroxybenzene, although designed as a "suicide" inhibitor [4], also turned out to be revers-

Table 3. Effect of DCNP on the incorporation of $^{35}SO_4^{2-}$ into CCK-8-SO_4*

	Radioactivity (cpm/mg protein) Homogenate	Pellet	CCK-8-SO_4
Control	194,740 ± 36,930	148,520 ± 22,240	119 ± 19
Treated	72,060 ± 16,800†	59,320 ± 14,200‡	53 ± 14†

* Rats received a dose of DCNP (100 μmoles/kg, i.p., 1 ml/kg) or the equivalent volume of the solvent. Thirty minutes later animals were injected into area 2 of the cerebral cortex with 50 μCi of $^{35}SO_4^{2-}$ in a volume of 0.1 μl. Four and one-half hours after injection of the tracer, the rats were killed and the injected areas were removed and homogenized in 1 ml of 90% methanol. Radioactivity was measured in an aliquot of the homogenate. After centrifugation (8000 *g*, 4 min) CCK-8-SO_4 present in the supernatant fraction was separated by HPLC and the cpm in the eluate fraction corresponding to CCK-8-SO_4 were measured. The pellet was resuspended in 1 ml of 0.5 M NaOH, and the protein content and radioactivity were measured. Shown are the mean ± S.E.M. of five animals in each experimental group.

† $P < 0.025$ vs corresponding control group (two-tailed Student's *t*-test).

‡ $P < 0.01$ vs corresponding control group.

ible. Thus, its inhibitory effect on PST activity was reversed completely by dilution of the homogenate (data not shown).

Both DCNP and nitrovinyldihydroxybenzene inhibited sulfation of all three substrates tested. These data contrast with that of Rein *et al.* [7] in human brain and platelets where IC_{50} values for DCNP were several orders or magnitude lower for phenol than for dopamine. It may be that rat brain PST is not heterogeneous. Since tyrosine derivatives were suggested as model compounds for sulfation of proteins [6], it is interesting that *p*-nitrophenol inhibits sulfation of tyrosine methylester, but not the reverse [16]. A homogenous preparation of liver PST proved capable of sulfating peptides with an amino terminal tyrosine [6], but not with tyrosine at a position other than the terminus, which would be the normal situation in proteins. It may be that sulfation of endogenous proteins and peptides could be assayed under other conditions: K_m values, for example, for given substrates have been reported to vary 10- to 50-fold with pH [10] and up to 200-fold with oxidation state of the enzyme [17].

The pharmacokinetic data reported here show that inhibitory concentrations of DCNP can be achieved in the brain cortex after an i.p. dose of 100 μmoles/kg without any apparent toxic effect. The significant decrease in $^{35}SO_4^{2-}$ incorporation into CCK-8-SO_4 induced by DCNP pretreatment supports this contention. It should be pointed out that the "total" incorporation of $^{35}SO_4^{2-}$ into cortical tissue was dramatically reduced by DCNP which clearly indicates that the synthesis of other sulfated endogenous compounds (i.e. catecholamine metabolites, sulfolipids, etc.) is also affected by this treatment.

Since the concentration of DCNP in the brain decays with a half-life of 6 hr repeated injections are required to maintain inhibitory concentrations of this compound for a prolonged time. Even when injected repeatedly for up to 4 days, DCNP failed to induce a decrease in the brain content of CCK-8-SO_4. This finding is not in agreement with our experiment using incorporation of $^{35}SO_4$. We believe that the discrepancy is caused by the inability of our repeated DCNP injection regimen to completely and continuously inhibit PST. When higher doses of this inhibitor were used, severe toxic effects were observed. Development of a better pharmacological agent is therefore required in order to estimate the turnover rate of CCK-8-SO_4 by measuring the decrease in its brain content after PST inhibition. A compound is needed that will readily cross the blood–brain barrier after systemic injection and will completely block PST activity (preferably in an irreversible manner) without causing toxic effects.

Nevertheless, DCNP appears to be a potentially useful agent to study the sulfation and turnover rate of sulfated endogenous compounds *in vitro* and in peripheral tissues, where very high concentrations of this inhibitor can be achieved for long periods of time, apparently without producing toxic effects.

REFERENCES

1. G. J. Mulder, *Sulfation of Drugs and Related Compounds*. CRC Press, Boca Raton, FL (1981).
2. J. Rehfeld, *Am. J. Physiol.* **240**, G255 (1981).
3. G. J. Mulder and E. Scholtens, *Biochem. J.* **165**, 553 (1977).
4. J. L. Costa and K. L. Kirk, *Med. Hypotheses* **10**, 231 (1983).
5. R. D. Sekura and W. B. Jakoby, *Archs Biochem. Biophys.* **211**, 352 (1981).
6. R. D. Sekura, M. W. Duffel and W. B. Jakoby, *Meth. Enzym.* **77**, 197 (1981).
7. G. Rein, V. Glover and M. Sandler, *Biochem. Pharmac.* **31**, 1893 (1982).
8. P. R. Butler, R. J. Anderson and D. L. Venton, *J. Neurochem.* **41**, 630 (1983).
9. C. Reiter and R. M. Weinshilboum, *J. Pharmac. Exp. Ther.* **221**, 43 (1982).
10. A. Foldes and J. L. Meek, *Biochim. biophys. Acta* **327**, 365 (1973).
11. R. J. Anderson and R. Weinshilboum, *J. Lab. clin. Med.* **94**, 158 (1979).
12. J. L. Meek, M. J. Iadarola and O. Giorgi, *Brain Res.* **276**, 375 (1983).
13. H. Koster, I. Halsema, E. Scholtens, J. H. N. Meerman, K. Sandy Pang and G. J. Mulder, *Biochem. Pharmac.* **31**, 1919 (1982).
14. M. W. Duffel and W. B. Jakoby, *J. biol. Chem.* **256**, 11123 (1981).
15. E. J. M. Pennings, R. Vrielink and G. M. J. Van Kempen, *Biochem. J.* **173**, 299 (1978).
16. P. Mattlock and J. G. Jones, *Biochem. J.* **116**, 797 (1970).
17. D. J. Barford and J. G. Jones, *Biochem. J.* **123**, 427 (1971).