Biochimica et Biophysica Acta, 327 (1973) 365–374

BBA 67075

RAT BRAIN PHENOLSULFOTRANSFERASE—PARTIAL PURIFICATION AND SOME PROPERTIES

ANDREW FOLDES AND JAMES L. MEEK

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington D. C. (U.S.A.)

(Received June 12th, 1973)

SUMMARY

A rapid, simple and sensitive radioassay for phenolsulfotransferase (EC 2.8.2.1) activity is described based on the transfer of $^{35}SO_4$ from phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) to a phenolic acceptor. The assay involves precipitation of unreacted ([^{35}S]PAPS) with $Ba(OH)_2$ and $ZnSO_4$, leaving the product aryl [^{35}S]-sulfate in solution. The assay is suitable for use with the phenolic catecholamine metabolites found in brain.

Phenolsulfotransferase has been purified 30-fold from rat brain. The substrate specificity of the partially purified enzyme toward endogenous phenols is investigated, and K_m values, pH optima and relative velocities are reported for various catecholamines and their acidic and neutral metabolites.

INTRODUCTION

Sulfate conjugation is an important pathway in the catabolism and excretion of catecholamines[1–3] as well as many other phenols[4]. The enzyme involved, phenolsulfotransferase (3′-phosphoadenylylsulfate: phenol sulfotransferase, EC 2.8.2.1), has been partially purified from rat and guinea pig liver[5,6]. Phenolsulfotransferase is also known to occur in brain, but has been less well characterized. Since a metabolite of norepinephrine, 3-methoxy-4-hydroxyphenylglycol (MOPEG) occurs in brain largely as a sulfate conjugate[7,8], a better understanding of sulfate conjugation in brain appears desirable. None of the currently available assays for phenolsulfotransferase[5,9,10] are adequate to study the kinetics of the brain enzyme with the biogenic amine metabolites as substrates. In this paper is reported a rapid sensitive radioassay for phenolsulfotransferase activity and its use in the partial purification and substrate specificity studies of the brain enzyme. A preliminary account of this work has been presented[11].

Abbreviations: PAPS, phosphoadenosine 5′-phosphosulfate; MOPEG, 3-methoxy-4-hydroxyphenylglycol.

EXPERIMENTAL

Materials

A solution of phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) (0.2–0.8 Ci/mmole) in 50 % ethanol was obtained from New England Nuclear Co., Boston, Mass. and stored at — 20 °C. For use, aliquots of this solution were diluted 1:10 with deionized water daily because of the instability of dilute aqueous solutions of PAPS. MOPEG and alumina gel Cγ were obtained from Sigma Chemical Co., St. Louis, Mo. MOPEG-sulfate (RO 4 6028) was a gift of Hoffman La Roche, Nutley, N. J.

Silica gel GF-coated thin-layer chromatography plates were obtained from Analtech, Newark, Del. DEAE-Sephadex A-50 and Sephadex G-200 were purchased from Pharmacia, Piscataway, N.J. and were swollen, defined (degassed in the case of the G-200) and equilibrated with the elution buffer before use.

Methods

Brains were obtained from between twenty and forty rats (Sprague–Dawley strain, Zivic Miller Labs., Allison Park, Pa.) weighing 160–400 g. The brains (50–100 g) were either frozen for short periods or homogenized immediately in 5 vol. of cold 10 mM phosphate buffer, pH 6.4, using a pre-cooled Waring blender. The homogenate was then centrifuged (30 000 × *g*, 20 min) at 4 °C and the inactive sediment discarded. The supernatant solution was centrifuged at 100 000 × *g* for 60 min at 4 °C. This supernatant solution was decanted and fractionated with $(NH_4)_2SO_4$. Initial experiments showed that the fractions precipitating between 20–40, 40–60 and 60–80 % saturation with respect to $(NH_4)_2SO_4$ contained comparable amounts of protein. However, the fraction precipitating between 40 and 60 % saturation had twice the specific activity of either of the other fractions. For further purification the "40–60 % fraction" was dissolved in approx. 5 ml of 10 mM phosphate buffer, pH 6.4, and dialyzed against a 200-fold excess of the same buffer for 3 h.

The dialyzed aliquot was then assayed for protein and enzyme activity and treated with alumina Cγ (0.5 g dry weight/g protein). The suspension was stirred for 30 min at 0 °C and centrifuged (30 000 × *g*, 10 min). The supernatant solution was discarded and the enzyme eluted from the gel by stirring with 0.2 M phosphate buffer, pH 6.4. The supernatant solution, after centrifugation was adjusted to pH 7.2, dialyzed against 2 l of 10 mM Tris buffer, pH 7.2, containing 100 mM NaCl and applied to a 20 cm × 1.5 cm column of DEAE-Sephadex anion-exchange resin equilibrated with the pH 7.2 buffer. Fractions of 5 ml were collected at an initial flow rate of approx. 20 ml/h. Nonadsorbed proteins were eluted with the above buffer, and then the adsorbed proteins were eluted by a linear 0.1–1.0 M NaCl gradient in 10 mM Tris buffer, pH 7.2, of 200 ml total volume.

The eluted fractions were assayed for protein and enzyme activity and the active fractions concentrated by positive pressure dialysis on a Diaflo ultrafilter (Amicon Corp., Lexington, Mass.) using a 10 000 mol. wt exclusion membrane.

Attempts to further purify the column eluate by chromatography on a 60 cm × 1.5 cm column of Sephadex G-200 proved unsuccessful as essentially all of the applied protein was eluted as a single heterogeneous peak containing all of the applied enzyme activity. Rechromatography of the DEAE-Sephadex eluate at a higher pH (8.0) on DEAE-Sephadex also did not result in further purification.

The enzyme assay was based on the conversion of phenol to phenyl[^{35}S]sulfate or of MOPEG to MOPEG[^{35}S]sulfate. In each case, [^{35}S]PAPS served as the sulfate donor. The 1.0-ml incubation mixture routinely consisted of 10 mM phosphate buffer, pH 6.4; 50 μM phenolic substrate, 0.4–4.0 μM [^{35}S]PAPS, (0.1–1.0 μCi) and sufficient enzyme to transfer 5–20 % of the $^{35}SO_4$ to the phenol. The incubation at 37 °C was started by the addition of enzyme and stopped exactly 10 min later by the addition of 0.2 ml of 0.1 M barium acetate to precipitate the protein and the phosphate buffer. Then 0.2 ml of 0.1 M $Ba(OH)_2$ was added to each tube, followed by 0.2 ml of 0.1 M $ZnSO_4$. The precipitate was removed by low speed centrifugation and the barium *plus* zinc step was repeated. This treatment precipitates proteins and nucleotides[12] including the unconverted [^{35}S]PAPS. In control experiments, at least 99.5 % of the radioactive PAPS added in the absence of enzyme was removed by two such precipitations. Blanks were assayed as above, omitting only the phenolic substrate. The supernatant (2.2 ml) after the second precipitation was transferred to scintillation vials for measurement of radioactivity. For assay of dopamine, norepinephrine, and serotonin, the phosphate buffer was replaced by 0.1 M glycine–NaOH buffer, pH 9.0, containing 1 mM mercaptoethanol to protect the substrates from oxidation.

With MOPEG as the phenolic substrate, the identity of the reaction product was studied by thin-layer chromatography. The assay was performed as above (20 % conversion of PAPS to MOPEG-sulfate) with the omission of the barium acetate step. The supernatant solution was lyophilized and the residue, redissolved in methanol, was applied to a silica gel GF plate. More than 90% of the radioactivity migrated similarly to authentic MOPEG-sulfate in two solvent systems. The R_f values were 0.43 in butanol–acetic acid–water (12:3:5, by vol.) and 0.76 in isopropanol–ammonia–water (10:1:2, by vol.).

When MOPEG was omitted from the reaction, only one-tenth as much radioactivity was found in the supernatant after precipitation of PAPS. Part of this radioactivity remained at the origin (probably[^{35}S]PAPS and/or $^{35}SO_4$). Inorganic sulfate (if formed by PAPS sulfohydrolase[13] would be precipitated and thus would not interfere with the enzyme assay). A second part of the radioactivity migrated with R_f values of 0.18 and 0.52 in the acidic and basic solvents, respectively, (probably adenosine 5′-phosphosulfate which could be formed from PAPS by a 3′nucleotidase). Preliminary experiments showed that, unlike PAPS, more than 87% of authentic adenosine 5′-phosphosulfate was not precipitated by our procedure. If formed, adenosine 5′-phosphosulfate would thus remain in the supernatant to contribute to the substrate blank. However, from the low blank values obtained, it appears that breakdown of PAPS by 3′-nucleotidase, if the enzyme is present at all, is quantitatively negligible compared to the transfer of sulfate to phenolic acceptors.

When ATP was added to the complete reaction mixture in an attempt to inhibit PAPS breakdown[13], no species corresponding in mobility to authentic MOPEG-sulfate could be detected on the plates. The radioactive species present migrated similarly to the major species observed in the absence of phenolic substrate.

This assay method is applicable to all phenols, including steroids[12], whose sulfate esters do not precipitate in the presence of $Zn(OH)_2$ and $BaSO_4$. We examined the extent to which the sulfate esters formed from the biogenic amines and their metabolites were lost by adsorption during the assay. Labelled sulfate esters of norepinephrine, dopamine, MOPEG, dihydroxyphenylglycol, dihydroxyphenylacetic acid,

and homovanillic acid were prepared by injecting the phenols and $^{35}SO_4$ into rat brains, and purifying the resulting sulfate esters by electrophoresis[14]. The labelled esters were added to tubes containing phosphate buffer, then $ZnSO_4$ and $Ba(OH)_2$ were added as described above. After repeated centrifugation, the recovery of the esters of neutral and basic phenols in the supernatant was found to be 82–100%, while the recoveries of the sulfate esters of homovanillic acid, dihydroxyphenylacetic acid and vanillylmandelic acid were from 25–42%. The relative velocities in Table II were corrected for these recoveries.

Protein was routinely assayed according to the method of Lowry *et al.*[15] using dried bovine serum albumin as standard.

RESULTS

A 30-fold purification of the brain enzyme was obtained. Table I shows the recovery of protein and activity at each stage of a routine purification. On DEAE-Sephadex chromatography of the partially purified preparation, enzyme activity was associated with a single protein peak. The NaCl concentration at which this peak eluted was approx. 0.4 M at pH 7.2 (Fig. 1), 0.25 M at pH 6.0, and 0.5 M at pH 8.0. Neither of the latter pH values yielded better resolution or purification than pH 7.2.

TABLE I

PURIFICATION OF RAT BRAIN PHENOLSULFOTRANSFERASE

Phenolsulfotransferase activity was measured at pH 6.4 with phenol as substrate.

Purification stage	*Activity* ($cpm \cdot 10^{-6}/min$)	*Protein* (*mg*)	*Specific activity* ($cpm \cdot 10^{-3}/min$ *per mg*)	% *Recovery* *Activity*	*Protein*
Homogenate	15.3	11 730	1.3	100	100
30 000 × *g* Supernatant	9.9	2 066	4.8	65	18
100 000 × *g* Supernatant	8.9	1 430	6.2	58	12
40–60% $(NH_4)_2SO_4$	3.2	375	8.6	21	3
Post alumina	1.9	144	13.0	12	1.2
Post DEAE-Sephadex	1.4	40	35.0	9	0.3

Enzyme activity varied linearly with time of incubation at 37 °C in the range 0–20 min. Plots of activity against enzyme protein concentration also proved linear to 600 μg/ml. Hence, within these limits, Michaelis–Menten kinetics is applicable to the sulfate-transfer reaction.

Mg^{2+}, which have been reported to stimulate rat liver steroid sulfotransferase[5] had no activating effect on the partially purified brain phenolsulfotransferase preparation in the concentration range 10^{-6}–10^{-2} M. Mercaptoethanol, also reported to enhance the activity of liver steroid sulfotransferase, had no effect on brain phenolsulfotransferase when tested in the range 0.1–1.0 mM. High concentrations of ATP have been shown to inhibit the degradation of PAPS *in vitro*[13]. The effect of ATP was hence investigated on brain phenolsulfotransferase in order to determine whether ATP could be added to the incubation medium during assays of the crude enzyme fraction. We observed that even low levels of ATP inhibit phenolsulfotransferase (Fig. 2). Also, in the presence of 0.1 mM ATP, no MOPEG-sulfate formation could be demonstrated by thin-layer chromatography.

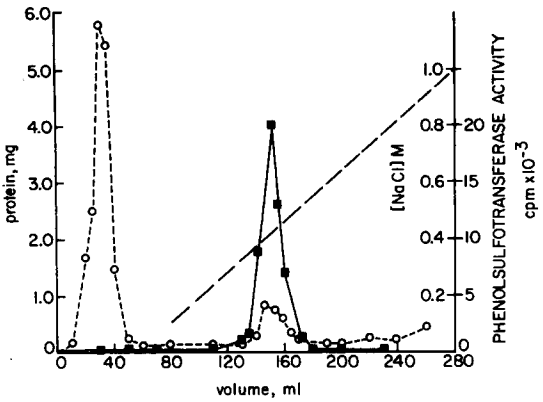

Fig. 1. Chromatography of phenolsulfotransferase on DEAE-Sephadex. A phenolsulfotransferase preparation was chromatographed on a 20 cm × 1.5 cm column of DEAE-Sephadex A-50 at pH 7.2. After washing off unadsorbed proteins with buffer, a linear 0.1–1.0 M NaCl gradient was applied after 80 ml. Enzyme activity (■—■) and protein (○---○) were assayed in duplicate in each 5-ml fraction.

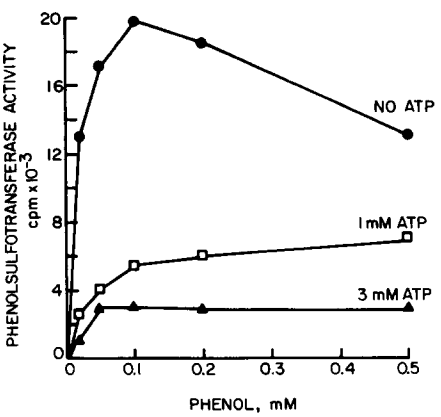

Fig. 2. Inhibition of phenolsulfotransferase by ATP. The effect of 1 and 3 mM ATP was examined on the activity of phenolsulfotransferase. Each point is the mean of 2 assays.

In addition to MOPEG, many other phenols related to the biogenic amines were substrates for the enzyme (Table II). The pH optima for phenol and the acidic and neutral amine metabolites ranged from 5.8 to 6.4 whereas the amines themselves had optimal activities at pH 9.0. The K_m values for the phenols ranged between 10 and 100 μM, with no obvious correlation between chemical structure and K_m. A typical K_m plot for phenol at pH 6.4 is shown in Fig. 3. Apparent substrate inhibition was seen for most of the compounds at concentrations exceeding approx. 5 times the K_m. Similar relative velocities were observed for the neutral compounds MOPEG and dihydroxyphenylglycol, and for the acidic metabolites, homovanillic acid, and dihydroxyphenylacetic acid, while for norepinephrine and dopamine lower velocities were obtained. Phenolsulfotransferase activity with serotonin was practically undetectable.

The K_m for PAPS at pH 6.4 was 0.30 μM. Routine assays were performed with

TABLE II

SUBSTRATE SPECIFICITY OF PARTIALLY PURIFIED RAT BRAIN PHENOLSULFOTRANSFERASE

The K_m values for the enzyme with the acidic and neutral phenols as substrates were determined at pH 6.4, while the K_m values for dopamine and norepinephrine were determined at pH 9.0. n.a., not assayed. The velocity values were corrected for recovery.

Substrate	*pH optimum*	K_m *(μM)*	*Velocity (cpm/min per mg)*	
			pH 6.4	*pH 9.0*
Phenol	5.8	40	12 100	4 800
3-Methoxy-4-hydroxy-phenylglycol (MOPEG)	6.4	18	20 800	n.a.
3,4-Dihydroxyphenylglycol	6.0	17	22 800	n.a.
Homovanillic Acid	5.8	14	36 100	n.a.
Vanillylmandelic acid	6.2	100	30 800	n.a.
Dihydroxyphenylacetic acid	5.8	10	19 700	n.a.
Dopamine	9.0	20	920	4 000
Norepinephrine	9.0	100	n.a.	1 460
Serotonin	9.0*	n.a.	n.a.	< 200

* Only traces of activity were seen using serotonin as substrate.

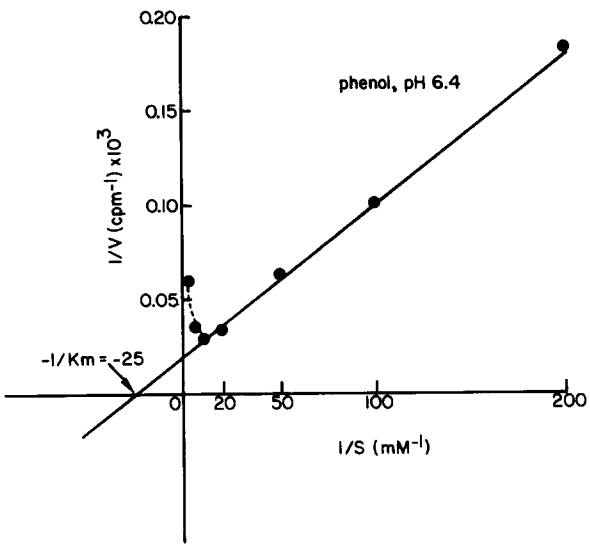

Fig. 3. Lineweaver–Burk plot for phenolsulfotransferase. The activity of the enzyme following DEAE-Sephadex chromatography was assayed at pH 6.4 using phenol as substrate. Each point represents the mean of 3 assays.

a suboptimal concentration of PAPS (0.4 μM) since unlabelled PAPS was not available and greater concentrations of [^{35}S]PAPS were too expensive. However, experiments performed with 4.0 μM PAPS gave the same K_m for phenol and the same ratio of velocities for phenol, MOPEG and homovanillic acid as did 0.4 μM PAPS. The variation of K_m with pH was investigated for the enzyme with phenol as substrate. The K_m was found to vary sharply with changes in pH, being 40 μM at pH 6.4, 6 μM at pH 7.2, and 80 μM at pH 5.6. A derived plot of $-\log_{10} K_m$ *vs* pH is shown in Fig. 4.

The crude enzyme prior to the $(NH_4)_2SO_4$ fractionation stage appeared stable

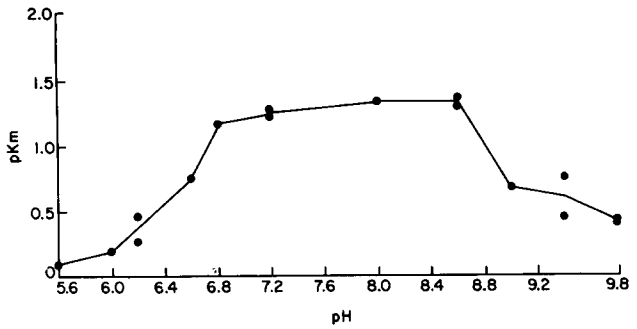

Fig. 4. The effect of pH on K_m of phenolsulfotransferase. K_m values were determined with phenol as substrate with the partially purified phenolsulfotransferase. The results are plotted according to the method of Dixon[19]. Each point represents a single graphical determination of K_m.pK_m = $-\log K_m$.

for at least 1 month at — 20 °C. The partially purified preparation was found to lose 20% of its activity in 7 days and 42% in 14 days when stored in small (200 μl) aliquots in glass test tubes at — 20 °C. When stored at 4 °C for 82 h, the partially purified enzyme preparation lost 60% of its activity at pH 6.0 and 80% of its activity at pH 8.0.

DISCUSSION

The method presented here for assaying the activity of phenolsulfotransferase is extremely simple, yet sensitive and useable with a variety of endogenous substrates. The fluorimetric phenolsulfotransferase assay[9] appears to be of similar sensitivity, but can only be used with fluorescent substrates such as methylumbelliferone which are not present in tissues. The methylene blue extraction technique[5] is less sensitive and can only be used with nonpolar substances such as steroids. The most commonly used assay[10] with *p*-nitrophenylsulfate as the substrate, has several drawbacks. This technique is based on the measurement of color formation as an active sulfate group is transferred from *p*-nitrophenylsulfate to PAPS and ultimately to acceptor phenols. This assay relies on two enzymic steps, and is kinetically too complex to allow ready interpretation of the observed apparent kinetic constants. For example, the observed pH optima probably represents both steps, not simply transfer of sulfate from PAPS to the final acceptor. Thus, using the *p*-nitrophenylsulfate technique, the pH optimum with phenol appeared to be 7.8, while in the present study and with liver phenolsulfotransferase[5] the pH optimum was 5.8. Further, the colorimetric assay is just sensitive enough to detect the enzyme in crude brain extracts and is not suitable for use during the enzyme purification. The enzyme showed marked activity with phenolic acids such as homovanillic acid as substrates, in contrast to previously published results[14,16]. The low pH required for optimum activity of the enzyme with these compounds may have inhibited the formation of PAPS from *p*-nitrophenylsulfate in the above reports.

In this study, the brain enzyme is obviously not completely pure, so the kinetic properties should be confirmed when further purification of the enzyme has been achieved. However, the ratio of activities towards phenol, dopamine, and MOPEG

remained constant throughout all of the purification stages in our study, suggesting that the sulfate conjugation of these three phenolic acceptors, and perhaps also of the other substrates is catalyzed by the same enzyme. This point cannot be conclusively proven until the brain enzyme is completely purified, but it appears that in brain, sulfate conjugation of simple phenols may be catalyzed by one enzyme, or a series of iso-enzymes. Using the partially purified brain phenolsulfotransferase preparation, the K_m for phenol was found to be independent of PAPS concentration, suggesting that the enzymic reaction is similar to that catalyzed by the enzyme from guinea pig liver, for which a rapid equilibrium random bi–bi reaction mechanism has been proposed[17]. Despite the finding that the K_m of the brain enzyme for phenol is much lower than that of the liver enzyme[5] the two enzymes appear to have similar properties. Although the activity of the partially purified enzyme for serotonin was barely detectable (Table II), serotonin-*O*-sulfate has been reported in brain, and brain "serotonin sulfotransferase" activity has been detected[18]. One possible reason for the low values obtained in our study with serotonin as substrate is that the barium/zinc precipitation technique used in this assay removes all barium-precipitable sulfates from the reaction mixture prior to counting. Thus, if serotonin-*O*-sulfate is completely precipitated by barium, no activity would be observed with serotonin using this assay. Unfortunately, authentic serotonin-*O*-sulfate was not available to study this point, and could not be synthesized by the method of Meek and Neff[14].

When — log K_m is plotted as a function of pH, the points of inflexion correspond to the pK values of the various species involved in the catalyzed reaction[19]. Upward inflexion points are characteristic of enzyme–substrate complex pK values, downward inflexion points are typical of the pK values of the free substrates or of charged groups within the active site and the slopes of the lines indicate the changes in charge on combination of the enzyme and substrate. For the sulfotransferase reaction with phenol as the substrate (Fig. 4), both inflexions are downward. These changes in K_m cannot be due to changes in the ionization of phenol, as the pK of phenol is 10.0. However, since the ionization constants of PAPS are not known, we cannot attribute the observed inflexion points to either PAPS or charged groups in the active site of the enzyme.

There has been some speculation concerning the role of the enzyme in brain[20], especially as the relative importance of sulfate conjugation of brain catecholamine metabolites appears to vary from species to species[8]. From the present results, it is evident that rat brain phenolsulfotransferase is capable of catalyzing the sulfate conjugation of a wide variety of phenolic metabolites. The low K_m values for most of these metabolites indicate that these compounds are potential substrates *in vivo* as well as *in vitro*. In addition to MOPEG, both dihydroxyphenylglycol and norepinephrine, shown here to be good substrates *in vitro*, have also been found to occur as sulfate conjugates *in vivo*[21,22].

It has been shown that MOPEG-sulfate but not unconjugated MOPEG, can be removed from the brain by a probenecid-sensitive active transport mechanism, which is specific for acidic metabolites[23]. Sulfate conjugation thus converts neutral phenolic metabolites into polar molecules capable of being actively transported out of the brain. Hence, brain phenolsulfotransferase may be an important part of the mechanism for the brain in the removal of potentially toxic metabolites and may also be involved in the detoxification of exogenous phenols which enter the brain.

REFERENCES

1 Richter, D. (1940) *J. Physiol. London* 98, 361–374
2 Axelrod, J., Kopin, I. J. and Mann, J. D. (1959) *Biochim. Biophys. Acta* 36, 576–577
3 Goodall, McC. and Alton, H. (1968) *Biochem. Pharmacol.* 17, 905–914
4 Williams, R. T. (1959) *Detoxification Mechanisms*, pp. 278–282, 2nd edn, Wiley and Sons, New York
5 Bannerjee, R. K. and Roy, A. B. (1966) *Mol. Pharmacol.* 2, 56–66
6 McEvoy, F. A. and Carroll, J. (1971) *Biochem. J.* 123, 901–906
7 Schanberg, S. M., Schildkraut, J., Breese, G. R. and Kopin, I. J. (1968) *Biochem. Pharmacol.* 17, 247–254
8 Schanberg, S. M., Breese, G. R., Schildkraut, J., Gordon, E. K. and Kopin, I. J. (1968) *Biochem. Pharmacol.* 17, 2006–2008
9 Van Kempen, G. M. J. and Jansen G. S. I. M. (1972) *Anal. Biochem.* 46, 438–442
10 Gregory, J. D. and Lipmann, F. (1957) *J. Biol. Chem.* 229, 1081–1090
11 Foldes, A. and Meek, J. L. (1973) *Fed. Proc.* 32, 797
12 Wengle, B. (1964) *Acta Chem. Scand.* 18, 65–76
13 Bailey-Wood, R., Dodgson, K. S. and Rose, F. A. (1970) *Biochim. Biophys. Acta* 220, 284–299
14 Meek, J. L. and Neff, N. H. (1973) *J. Neurochem.* 21, 1–9
15 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
16 Segal, H. L. and Mologne, L. A. (1959) *J. Biol. Chem.* 234, 909–911
17 Bannerjee, R. K. and Roy, A. B. (1968) *Biochim. Biophys. Acta* 151, 573–586
18 Hidaka, H., Nagatsu, T. and Yagi, K. (1969) *J. Neurochem.* 16, 783–785
19 Dixon, M. (1953) *Biochem. J.* 55, 161–170
20 Roy, A. B. (1971) in *Handbook of Experimental Pharmacology*, Vol. 28/2, pp. 545–548, Springer Verlag, New York
21 Häggendal, J. (1963) *Acta Physiol. Scand.* 59, 255–260
22 Sugden, R. F. and Eccleston, D. (1971) *J. Neurochem.* 18, 2461–2468
23 Meek J. L. and Neff, N. H. (1972) *J. Pharmacol. Exp. Ther.* 181, 457–462