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A STUDY OF NOREPINEPHRINE METABOLISM IN RAT BRAIN USING A DOUBLE LABEL TECHNIQUE*

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Abstract—Using a double-label procedure, the incorporation of endogenously-derived $^{35}SO_4$ into phenylethylene glycol sulfates (PGS) was estimated. When [^{3}H]norepinephrine and [^{35}S]cysteine were injected concomitantly into the brain, about 30–80 percent of the tritium and about 4 percent of the ^{35}S retained in the brain 1 hr later were in PGS. In B_6-deficient rats, the proportion of ^{35}S was increased as was the $^{35}S/^3H$ ratio. Probenecid caused a significant increase in the amount of PGS found in the brain, but a minimal enrichment of ^{35}S was observed, suggesting that there was little or no effect on sulfotransferases. Cysteine in high concentrations inhibits the incorporation of tritium into PGS.

Several lines of evidence have demonstrated that a major route of catecholamine metabolism occurs via sulfate conjugation [1–4]. Sulfation reactions are particularly important in brain, because the formation of phenylglycolsulfate (PGS) esters constitutes a major pathway of norepinephrine catabolism in most species, including man [5]. In rats, NE is metabolized primarily to 3,4-dihydroxyphenylethyleneglycol sulfate (DHPG-SO_4) and its 3-methoxy derivative (MHPG-SO_4) [1, 6]. The reaction is catalyzed in brain and other tissues by sulfotransferases capable of utilizing ethanolic and glycolic catecholamine metabolites as substrates, as well as other phenolic substances [4, 7].

In several of the studies published to date, $Na_2\,^{35}SO_4$ has been used to study sulfation reactions *in vitro* and *in vivo* [6, 7]. Under these conditions, activation of sulfate ions must occur, followed by transfer to acceptor molecules, as described by Eccleston and Ritchie [7].

Brain tissue contains a very active transsulfuration pathway, which is mediated by the formation of cystathionine. The end product is cysteine. Degradative processes for the latter are obscure, but are believed to proceed to sulfate ions via alanine sulfinic acid [8]. The intermediate, cystathionine, occurs in large quantities in primate brain, and accumulates in the brain of other mammals if a pyridoxine deficiency occurs.

The interaction and interdependency between catecholamines and sulfur in brain should be of greater than passing significance. In this paper, we describe experiments in which some of the possibilities were investigated and evaluated.

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METHODS AND MATERIALS

L-[^{35}S]cysteine hydrochloride (sp. act. 100 mCi/m-mole) was obtained from Amersham–Searle. L-[^{35}S]-methionine and L-[^{3}H]norepinephrine with sp. act. of 211 and 3.42 Ci/m-mole respectively, were purchased from New England Nuclear, as was Aquasol counting cocktail. Radioactivity measurements were made on a Beckman Model LS-230 liquid scintillation counter equipped with an Olivetti computer. Glusulase, containing 46,472 units/ml as sulfatase, was bought from ENDO Laboratories. Probenecid (*p*-(dipropylsulfanoyl)-benzoic acid) was a Sigma product. Pyridoxine-deficient and pyridoxine-complete test diets were obtained from Nutritional Biochemicals, as was desoxypyridoxine phosphate. The composition of the diets are listed in the manufacturer's brochures. 4-Hydroxy-3-methoxyphenylethyleneglycol sulfate (MHPG-SO_4) was generously supplied by Hoffman-LaRoche. An Aminco-Bowman spectrofluorometer was used to measure fluorescence.

Rats (Harlan, SD, males) weighing between 150 and 200 g were used in these studies. Except for those used in dietary experiments, they were kept for about 1 week (four animals per cage) prior to the experiment in quarters equipped for a daily light–dark cycle (12:12 hr). They received Purina rat chow and tap water *ad lib.* Intracerebral injections were done using procedures described by Farris and Griffith [9]. Probenecid (200 mg/kg) was administered i.p. as a 1% solution in phosphate buffer, pH 7.4, 1 hr prior to the i.c. injections. Controls were injected with equal vol. of saline. The rats were decapitated 1 hr after the final injection (from 9–10 a.m.) and the brains were subjected to procedures described by Meek and Neff [10] for the isolation of MHPG-SO_4. Recoveries of added MHPG-SO_4 were 82 ± 8 percent. Under the conditions of our experiments, the procedure does not distinguish between DHPG-SO_4 and MHPG-SO_4. However, since these two compounds account for practically all of the NE metabolized, the procedure was used to isolate the ^{35}S-labeled conjugate fraction. Under these conditions, it was necessary to determine

whether contaminating ^{35}S-containing materials were present in the isolated product. The thin-layer chromatography system used by Meek and Neff [6] proved inadequate for this purpose because of acid hydrolysis and salt interference. The problem was solved by using a double-label technique in which the ^{3}H and ^{35}S content was monitored throughout as follows: brain homogenates from rats which had been injected with [^{3}H]NE and [^{35}S]cysteine were treated by procedures described by Meek and Neff [10]. In this procedure, endogenously-formed inorganic sulfate is removed with barium hydroxide during the deproteinization step. After chromatography, the conjugate fraction is eluted, divided into two portions, adjusted to pH 6.0 with buffer containing EDTA, and hydrolyzed for 24 hr with and without glusulase. In our modification, the hydrolysis mixtures were treated with barium hydroxide again to remove inorganic sulfate [10] and were then rechromatogrammed. About 10 per cent of the enzyme-treated conjugate fraction and all of the radioactivity in the control fraction was retained by the second column, indicating that at least 90 per cent of the ^{35}S isolated from the first column was esterified as conjugate and had been hydrolyzed. This was consistent with the 96–100 per cent recovery of tritium in the eluate from the second column.

In some experiments, pyridoxine-deficient rats were used. Procedures for preparing and treating the diets have been previously described [11]. Young rats, weighing about 90–100 g were divided into groups of six. One group received a pyridoxine-deficient diet supplemented with desoxypyridoxine phosphate (10 mg/kg of diet). The control group received the same diet except that pyridoxine had been added and the desoxypyridoxine phosphate omitted. After about 4 weeks, animals which had received the B_6-deficient diet weighed 185 ± 8 g *versus* 274 ± 6 g for the controls. Acrodynia of the feet and tail was apparent in the B_6-deficient group and the coat had lost its lustre. Animals from both groups received i.c. injections and their brains were subjected to analyses as described above. In these experiments, the efficiency of the isolation procedures was monitored which required the pooling of two brains for each analysis. Other experimental details are presented in the legends to the tables.

RESULTS

Expressed as percent total dose, the data presented in Table 1 show that the retention and incorporation of ^{3}H is relatively unaffected by B_6-deficiency. As expected, retention and utilization of ^{35}S administered in the form of cysteine may be altered by the nutritional inadequacy. For instance, the percent retention of the total injected dose is reduced from 0.41 in the controls to 0.24 in the deficiency. However, the fraction incorporated into glycols is increased. This is consistent with an expected decrease in the metabolic turnover of brain sulfate. When the data in Table 1 are examined in terms of the percent of brain radioactivity in the conjugate fraction, they show, firstly, that MHPG-SO_4 is indeed a major metabolite of NE in rat brain. In the controls, an amazing 82 percent of the retained tritium was present as sulfate ester. Secondly, these data suggest that in the B_6-deficient animals, the incorporation of NE into phenylglycols is drastically reduced whereas the opposite occurs for ^{35}S. This is reflected in the $^{35}S/^{3}H$ ratio for the conjugates (Table 1).

Table 1. Effects of vitamin B_6 on retention and incorporation of radiolabel into phenylglycol sulfates by brain after intracerebral injection of [^{3}H]NE and [^{35}S]cysteine

Diet	^{3}H	^{35}S
	Percent	
	A. Retained	
B_6-Deficient	1.24 ± 0.16	0.24 ± 0.09
Complete	1.12 ± 0.47	0.41 ± 0.19
	B. PGS	
B_6-Deficient	0.73 ± 0.13	0.021 ± 0.004
Complete	0.92 ± 0.31	0.013 ± 0.002

Each rat received 40 μl of an aqueous solution of [^{35}S]cysteine (62.8 μCi, sp. act., 100 mCi/m-mole) and [^{3}H]NE (9.6 μCi, sp. act., 3.42 Ci/m-mole) injected into the cisterna magna. Each value is the mean of 3 samples ($n = 3$) each of which was prepared by pooling two brains prior to homogenization. All values are expressed as percent total dose.

Recently it was reported that probenecid, which increases steady state levels of MHPG-SO_4 [12, 13], stimulates liver sulfotransferases *in vitro* [14, 15]. This observation led to speculation that part of the probenecid-induced increase in brain MHPG-SO_4 could be due to increased enzyme activity as opposed to alterations in acid transport systems. The double-label technique was used to study these possibilities.

In a preliminary experiment using a large dose of [^{3}H]NE (2.4 μmole) injected i.c. in the absence of cysteine, we confirmed that probenecid causes an increased retention of tritium (72 percent greater than controls) and a comparable increase in tritiated conjugates [13], expressed as percent total dose. Expressed a percent radioactivity in the brain, there was little or no difference in the amount found in the glycol sulfates of control (45%) versus drug-treated animals (42%). Preliminary observations suggested that the concomitant injection of cysteine *per se* effected the incorporation of tritiated NE. Experiments to test this hypothesis were initiated and the

Table 2. Effects of probenecid on retention and incorporation of radiolabel into PGS by brain after intracerebral injection of [^{3}H]norepinephrine and [^{35}S]cysteine

Drug	^{3}H	^{35}S
	Percent	
	A. Retained	
None	1.32 ± 0.18 (4)	0.37 ± 0.04 (4)
Probenecid	1.64 ± 0.21 (6)	0.44 ± 0.03 (6)*
	B. PGS	
None	0.63 ± 0.10 (4)	0.029 ± 0.002 (4)
Probenecid	0.43 ± 0.10 (6)	0.034 ± 0.002 (6)*

Each rat received 40 μl of an aqueous solution of [^{35}S]cysteine (66.6 μCi, sp. act., 27.8 mCi/m-mole) and [^{3}H]NE (7.5 μCi, sp. act., 27.7 Ci/m-mole). Values are expressed as means ± S.E. and represent percent total dose. Sample number is contained in parentheses.

* Significantly different from controls, $P < 0.05$.

Table 3. Effects of cysteine and probenecid on the incorporation of [^{3}H]NE into PGS

	No Probenecid			Probenecid		
	^{3}H	^{35}S	^{35}S/^{3}H	^{3}H	^{35}S	^{35}S/^{3}H
μmole	Percent			Percent		
0	56	—	—	44	—	—
0.83	51	—	—	43	—	—
2.40*	48*	7.6*	0.04*	26*	7.8*	0.10*

Conditions have been described in the legend to Table 2 except that unlabeled cysteine was injected. $n = 5$ for each analysis.

* Calculated from data in Table 2.

results are shown in Tables 2 and 3. These experiments differ only in that the cysteine was either labeled (Table 2) or unlabeled (Table 3) and for that reason differed in concentration. The data show that injection of large doses of cysteine concomitantly with probenecid reduced the effects of the latter, both in the retention of tritium by brain, which is now only 25 percent greater than controls (Table 2), and upon the percent of tritiated NE incorporated into PGS (Table 2). Injection of probenecid caused a small increase in the percent incorporation of brain ^{35}S into PGS (Table 2).

DISCUSSION

Sulfur injected into rat brain in the form of [^{35}S]cysteine can be incorporated into phenolic sulfate esters. As mentioned above, alanine-3-sulfinic acid serves as a precursor for inorganic sulfate, but it also can be oxidized to organic sulfate such as taurine and isoethionic acid [8]. Although taurine is found in practically all areas of the brain [16] and has received considerable attention as a possible neurotransmitter [17], there is no evidence that it is a substrate for sulfotransferases. In squid nerve, large amounts of isoethionic acid are formed from cysteine, but not by way of taurine [18]. In the absence of evidence to the contrary, the most likely source of the sulfate in the glycol esters is 3′-phosphoadenylylsulfate.

In animals receiving nutritionally complete diets, the incorporation of ^{35}S into sulfate esters amounted to about 0.01–0.03 percent of the total injected dose. However, it is also possible that the amount incorporated is relative to the total dose of cysteine (Tables 1 and 3). In B_6-deficient animals, the percent incorporation was increased probably as a result of decreased metabolic turnover. The formation of NE and its metabolites are subject to marked changes during B_6-deficiencies because dopa decarboxylase activities are decreased [19]. Sulfate metabolism is also altered, but no efforts have been made to relate these changes to NE catabolism. In the current study, which was originally designed to test the effects of a B_6-deficiency on incorporation of ^{35}S from [^{35}S]methionine, the primary sites of B_6 action were by-passed. Except for its important role in transport processes, which is currently under investigation in our laboratory, vitamin B_6 has little or no direct effect on NE and cysteine catabolism.

The MHPG-SO_4 fraction isolated 1 hr after a dose of [^{3}H]NE contained approximately one half of the brain tritium. With the exception of the value reported in Table 1 for controls (82%), this percentage was relatively constant throughout these experiments. The one exceptionally high value is not easily explained. In any case, the data in Table 1 should be cautiously interpreted because they represent pooled samples from a relatively low number of subjects ($n = 6$). Even so, the percent of brain ^{35}S found in the MHPG-SO_4 fraction 1 hr after injection of [^{35}S]cysteine was significantly increased in the B_6-deficient animals. The ^{35}S/^{3}H ratio in the MHPG-SO_4 fraction was therefore greater in these animals. Since the retention of ^{35}S by the brain was either unaffected or decreased in these rats, the enrichment of ^{35}S in the MHPG-SO_4 fraction was not due to increased availability of the label. As indicated earlier, the increase was probably due to a slower metabolic turnover of brain sulfur.

With probenecid, the double-label data clearly showed an enrichment of sulfur in the MHPG-SO_4 fraction as compared to tritium (Tables 2 and 3). Although this was consistent with the observation that probenecid stimulated sulfotransferase *in vitro*, the data were equivocal in that the expected percent increase in retention and incorporation of tritium did not occur. In other words, the increased ^{35}S/^{3}H ratio (Table 3) was due to a decrease in ^{3}H, combined with a slight increase in ^{35}S. The data in Table 3 suggest that the presence of high concentrations of cysteine diminishes or obliterates the animal's response to probenecid.

Attempts to interpret these data are subject to the limitation inherent in the experimental design. For instance, high concentrations of cysteine injected i.c. should, and did, invoke behavioral changes in the experimental subjects. Animals receiving 2.4 μmole of cysteine appeared to be narcotized and, in some, convulsions occurred. At lower concentration (0.83 μmole) no such responses were observed. One of the major problems then is the unavailability of a [^{35}S]cysteine preparation of high and constant sp. act. Experiments using L-[^{35}S]methionine (sp. act. > 100 Ci/m-mole) were ambiguous because of a very low incorporation of isotope into MHPG-SO_4 (Brown and Zawad, unpublished observations). Another limitation is the obvious need to measure any changes in the sp. act. of [^{3}H]PGS and [^{35}S]PGS.

The cysteine-induced decrease in the probenecid reaction could be due to either decreased formation of PGS or to increased amounts of endogenous precursors. In other words, high concentrations of intracerebral cysteine could lead to sulfation of NE or dopamine thereby lowering the concentration of PGS. Since probenecid affects the excretion of organic acids, its affect could be partly or completely prevented. On the other hand, high concentrations of cysteine could induce mobilization of NE. This would dilute the tritium and lead to an increase in the ^{35}S to ^{3}H ratio. Another interesting possibility was suggested recently by Tudball and Griffiths [20]. They showed that a dramatic decrease in dopamine and NE occurred in the brains of rabbits treated with methionine and homocysteine (injected i.p.). They

suggest that the primary change is associated with transport processes. Our data do not distinguish among these possibilities, but experiments to do so are technically feasible and are currently under way.

The data presented here clearly show that probenecid does affect the concentration of brain conjugates, *in vivo*, as suggested by Van Kempen *et al.* [15], but not necessarily by stimulating sulfotransferases. The most definitive conclusion is that PGS concentrations are not a valid reflection of catecholamine metabolism in the presence of various types of drugs.

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