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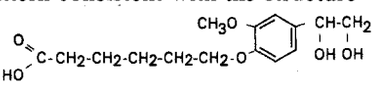
Specific and Sensitive Radioimmunoassay for 3-Methoxy-4-hydroxyphenylethyleneglycol (MOPEG)

Abstract. *Antibodies that specifically bind the norepinephrine metabolite 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG) were produced in rabbits after injection of a derivative of MOPEG conjugated with bovine thyroglobulin. A sensitive radioimmunoassay was devised with this antiserum, in which as little as 0.5 nanogram of MOPEG can be accurately measured with a final antibody dilution of 1:180. The antibody appears to be specific for MOPEG, since tritiated MOPEG was not displaced from the antibodies by norepinephrine, epinephrine, dopamine, serotonin, or their major metabolites, including MOPEG-sulfate (333 nanograms each).*

The major terminal metabolite of norepinephrine in the central nervous system of the rat (*1*) and man (*2*) is 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG), and changes in the concentration of MOPEG and its sulfate conjugate (MOPEG-sulfate) faithfully reflect changes in the amount of norepinephrine released by nerve activity in the brain (*3-6*). On the basis of observations of this type (*3-6*), we wished to use changes in the regional concentrations of MOPEG in rat brain to gauge the effects of pharmacological agents on central noradrenergic neurotransmission. However, each of the methods of analysis used in the studies cited above presented problems. The measurement of MOPEG by either fluorometry (*3*, *4*) or gas-liquid chromatography (*5*, *6*) requires large amounts of tissue, so that the same anatomical region from several rat brains would have to be pooled for assay. The quantification of MOPEG by gas chromatography combined with mass spectrometry is extremely specific and sensitive (*1*, *2*), but the instrumentation is expensive and not widely available. We therefore undertook the development of a specific and sensitive radioimmunoassay for MOPEG.

Because MOPEG is a small molecule that occurs naturally in the body, it is not antigenic; furthermore, MOPEG has no free carboxyl or amino groups that can be used to couple it to a carrier protein. To obtain a derivative of MOPEG that has a free carboxyl group and an aliphatic bridge to position the 3-methoxyphenylethyleneglycol portion of MOPEG some distance from the surface of the carrier protein, we synthesized 3-methoxy-4-(5-carboxypentoxy)-phenylethyleneglycol by reacting MOPEG with 6-bromohexanoic acid (Fig. 1). The equivalent of 400 mg of free MOPEG as the piperazine salt was added to 4 ml of water, and the *p*H of the solution was adjusted to 6 with 1*N* H_2SO_4. Free MOPEG was extracted four times with 3 ml of water-saturated ethyl acetate. After evaporation of the pooled ethyl acetate fractions with a stream of nitrogen, 199 mg (49 percent) of the MOPEG was recovered. It is important that MOPEG be extracted from the aqueous solution of its piperazine salt at a *p*H of 5 or above; below *p*H 4, even for short exposures, a dimerization reaction occurs, and less free MOPEG is obtained from the extract. The 199 mg of MOPEG was dissolved in 1 ml of water, and the *p*H was adjusted to 10 with concentrated NaOH. In a separate test tube, an equimolar amount (210 mg) of 6-bromohexanoic acid (Aldrich) was added to 1 ml of water, and the *p*H was raised to 11 with concentrated NaOH. After the two solutions were mixed (final *p*H, 10.0), a stream of nitrogen was bubbled through the reaction mixture for 1 minute. The reaction vessel (an acid-washed 13-ml glass centrifuge tube) was stoppered, sealed with Parafilm, and placed in a 100°C oil bath. After 1 hour, the *p*H of the reaction mixture had dropped to 5.85. At this point, 214 mg of 6-bromohexanoic acid was added to the reaction mixture, and the *p*H was again adjusted to 11 with concentrated NaOH. After the reaction mixture was degassed with nitrogen, the reaction vessel was sealed and the mixture was again incubated for 1 hour at 100°C. As before, the *p*H decreased to 5.6 during this incubation. The *p*H was raised to 7.7 with concentrated NaOH, and the mixture was washed four times with 3 ml of chloroform. The *p*H of the reaction solution was then adjusted to 2.3 with 2*N* HCl, and the reaction product was extracted three times with 3 ml of water-saturated ethyl acetate. After evaporation of the ethyl acetate under a stream of nitrogen, 70 mg of a viscous oily material was recovered. Mass spectral analysis revealed the presence of a compound with a molecular weight of 298 and a fragmentation pattern consistent with the structure



Little unreacted MOPEG was detected, but unreacted 6-bromohexanoic acid (and possibly 6-hydroxyhexanoic acid) was present. No further attempt was made to purify the 3-methoxy-4-(5-carboxypentoxy)phenylethyleneglycol or to corroborate the proposed structure. This derivative of MOPEG was coupled to bovine thyroglobulin with a water-soluble carbodiimide. The above reaction product (5 mg) was dissolved in 200 μl of water, and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma) was dissolved in 200 μl of water. To each of these solutions was added 150 μl of 0.1*M* $NaHCO_3$. The two solutions were then mixed together, and 25 mg of bovine thyroglobulin (Sigma), dissolved in 300 μl of water, was added to the mixture. The mixture was stirred constantly for 24 hours at room temperature and then dialyzed for 2 days (at 4°C) against 4 liters of 0.1*M* phosphate buffer, *p*H 7.5, containing 0.1*M* NaCl. After 3.8 ml of fresh dialysis buffer was added to the dialyzed mixture, the protein-hapten complex was stored in 300-μl portions at −20°C.

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The ^{3}H-labeled MOPEG to be used in the radioimmunoassay was synthesized enzymatically by incubating 1 μg (in 60 μl of water) of 3,4-dihydroxyphenylethyleneglycol (DOPEG) (Sigma) with 4.4 μg (100 μl) of ^{3}H-labeled *S*-adenosylmethionine (specific activity, 64 Ci/mmole; New England Nuclear) in a solution containing 200 μl of rat liver catechol-*O*-methyltransferase [prepared by the technique described in (*7*)], 40 μl of 25 m*M* $MgCl_2$, and 100 μl of 2*M* tris-HCl buffer, *p*H 8.6, for 3 hours at 37°C, with gentle shaking. The reaction was stopped by adding 20 μl of 0.3*M* EDTA, *p*H 7.3, and the ^{3}H-MOPEG was extracted three times with 3 ml of water-saturated ethyl acetate. After evaporation of the ethyl acetate under a stream of nitrogen, the ^{3}H-MOPEG was stored in methanol at 4°C. When the purity of this ^{3}H-MOPEG was assessed by thin-layer chromatography (cellulose MN-300 developed with a mixture of isopropanol, ammonium hydroxide, and water at a ratio of 16:1:3), the enzymatically prepared ^{3}H-MOPEG appeared as a single peak with a retardation factor (R_f = 0.73) identical to that of authentic ^{14}C-MOPEG (Research Products International).

Six New Zealand White rabbits were injected at 15 to 20 intradermal sites with this protein conjugate (0.78 mg protein per rabbit emulsified in 2 ml of Freund's complete adjuvant) at 6-week intervals. The antibody titer against MOPEG was determined by measuring the amount of ^{3}H-MOPEG bound by serial dilutions of rabbit serum obtained 7 days after the last booster injection of immunogen. Antibody titer against MOPEG had developed in rabbits 5 and 6 by the third injection of immunogen. One week after the fifth injection, these two rabbits were exsanguinated. At this time, the dilution of antibody that bound 50 percent of the ^{3}H-MOPEG was 1:60 for antibody 5 and 1:180 for antibody 6. Standard curves, prepared by using known amounts of unlabeled MOPEG to displace ^{3}H-MOPEG from these antibodies, showed that as little as 0.5 ng of MOPEG could be measured with antibody 6 (Fig. 1). At a concentration of 2.0 ng of MOPEG per assay tube, the intra- and interassay coefficients of variation were 6.7 and 10.9 percent, respectively. Free and antibody-bound ^{3}H-MOPEG were separated by the addition of saturated ammonium sulfate followed by centrifugation. These antibodies were shown to specifically bind MOPEG, since as much as 1000 ng of MOPEG-sulfate, norepinephrine, epinephrine, dopamine, serotonin, normetanephrine, metanephrine, 3-methoxy-4-hydroxymandelic acid, 3,4-dihydroxymandelic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, and 5-hydroxy-3-indoleacetic acid did not displace ^{3}H-MOPEG from these antibodies. High concentrations (1000 ng) of DOPEG displaced 16 percent of the bound ^{3}H-MOPEG, but concentrations in the physiologic range (< 100 ng) exhibited no cross-reactivity.

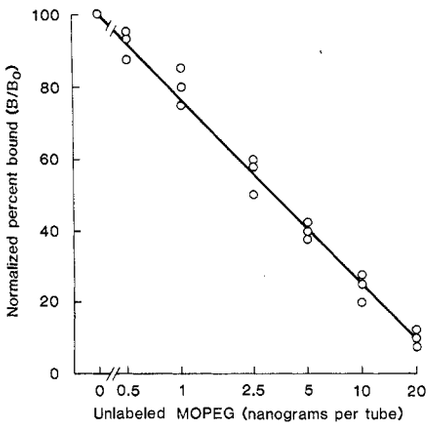

Fig. 1. Displacement of ^{3}H-labeled MOPEG from antibody 6 by unlabeled MOPEG. The final antibody dilution was 1:180, and the 0 percent binding (no unlabeled MOPEG added) was 40 percent.

This radioimmunoassay was used to measure the total concentration of MOPEG in seven discrete regions of the brain (*8*) in untreated (control) and phentolamine-treated (18.6 mg/kg, subcutaneously, for 3 hours) Sprague-Dawley rats (Simonsen Laboratories). Each tissue sample was homogenized in ten volumes of cold 0.2*M* $ZnSO_4$ followed by an equal volume of cold 0.2*M* $Ba(OH)_2$. After centrifugation at 30,000*g* for 20 minutes in a refrigerated centrifuge, the *p*H of the supernatant was adjusted to 7 with 0.2*M* H_2SO_4. Any additional precipitate was removed by centrifugation, and each sample was lyophilized. The residue was suspended in 965 μl of water, and 25 μl of water containing 100 units of Sigma H-1 sulfatase and 10 μl of 2*M* sodium acetate at *p*H 6 were added. Prior to use, the sulfatase was dialyzed to remove contaminating endogenous MOPEG. This amount of sulfatase was sufficient for the hydrolysis of 264 ng of MOPEG-sulfate during a 3-hour incubation, and the recovery of MOPEG-SO_4 added to brain was 96 ± 3 percent. The samples were assayed with antibody 6 at a final dilution of 1:180 (Table 1).

The accuracy of this radioimmunoassay is demonstrated by the comparable values for the regional concentrations of MOPEG in rat brain obtained by this means and by gas chromatography–mass spectrometry (*1*). Sprague-Dawley rats were used in both analyses, although they were obtained from different suppliers. The mean values determined by the two methods vary from −29 percent (cerebellum) to +14 percent (corpus striatum). As a further indication of the reliability of this radioimmunoassay, animals treated with phentolamine exhibited the expected increase (*9*) in total MOPEG content in all brain regions (Table 1). Thus, this specific and sensitive radioimmunoassay for MOPEG yields accurate values and can be used in neurochemical studies in rats.

The concentrations of free MOPEG and MOPEG-sulfate in rat brain are approximately 13 and 75 ng/g, respectively (*1*, *10*). Human cerebrospinal fluid contains a total MOPEG concentration of 6 to 20 ng/ml (*11*, *12*), of which 25 percent is MOPEG-sulfate (*11*). Free, sulfated,

Table 1. Comparison of the values obtained for total MOPEG concentration of the different brain regions (*8*) of the Sprague-Dawley rat as determined by gas chromatography–mass spectrometry (GC-MS) (*1*) and by radioimmunoassay, and values obtained after treatment with phentolamine as determined by radioimmunoassay. The predicted increase (*9*) in norepinephrine release elicited by phentolamine is shown, with percent increases given in parentheses. Values are means ± standard errors and are expressed as picomoles of MOPEG per gram of tissue weight (N = 6).

Brain region	MOPEG (pmole/g)		
	GC-MS base	Radioimmunoassay	
		Base	After phentolamine treatment
Pons-medulla	768 ± 50	686 ± 67	1089 ± 102* (+ 59 percent)
Hypothalamus	1246 ± 115	1106 ± 60	1485 ± 120† (+ 34 percent)
Corpus striatum	476 ± 66	543 ± 62	801 ± 100* (+ 48 percent)
Hippocampus	577 ± 36	564 ± 60	915 ± 38† (+ 62 percent)
Midbrain	715 ± 49	775 ± 59	1107 ± 87* (+ 43 percent)
Cortex	556 ± 53	610 ± 43	752 ± 36* (+ 23 percent)
Cerebellum	380 ± 21	269 ± 39	361 ± 26* (+ 34 percent)

*P < .05. †P < .01. Comparison by unpaired t-test of values for controls and phentolamine-treated rats, as measured by radioimmunoassay.

and glucuronidated MOPEG are found in about equal concentrations of 6 to 10 ng/ml in human plasma (*13*), whereas the sulfate and glucuronide conjugates of MOPEG predominate in equal concentrations of 0.5 to 0.7 μg/ml in human urine (*14*). Rat urine, like human urine, contains little free MOPEG, but the concentration of the sulfate conjugate (3 μg/ml) is about six times that of the glucuronide conjugate (0.5 μg/ml) (*14*). This radioimmunoassay is thus sufficiently sensitive to quantify MOPEG in tissue, cerebrospinal fluid, plasma, and urine and can therefore be used in many areas of biomedical research. For example, several groups of investigators (*15*) have suggested that the urinary levels of MOPEG prior to treatment are predictive of the therapeutic response to tricyclic antidepressants in some depressed patients. A recent prospective study supports this hypothesis (*16*). Only preliminary studies of cerebrospinal (*17*) and urinary levels of MOPEG in hypertension (*18*) and myocardial infarction (*19*) have been conducted, and the diagnostic and therapeutic implications of changes in the plasma concentration of MOPEG in these and other diseases have yet to be determined (*20*).

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Two Classes of Single-Stranded Regions in DNA from Sea Urchin Embryos

Abstract. *Native DNA from sea urchin embryos contains single-stranded regions (gaps) of up to 3000 nucleotides. The longer gaps (more than 1400 nucleotides) are nonrandomly distributed and are rich in histone gene sequences, other moderately repetitive sequences, and polypyrimidines. The shorter gaps are associated with DNA replication. A method for isolation of the two classes of single-stranded DNA pieces is reported.*

Isolated native eukaryotic DNA of diverse origins contains single-stranded regions (gaps) interspersed throughout the double helical DNA (*1-13*). Some of these single-stranded regions have been associated with DNA replication sites that were active in vivo at the time that the DNA was isolated. Other single-stranded regions may not be associated with replication and may be at nonrandom positions on the genome (*1*, *4-6*). We now report some experiments that distinguish and characterize two classes of gaps in sea urchin embryo DNA; one is rich in replication forks or regions, and the other is rich in histone gene DNA sequences, with the gaps therefore nonrandomly distributed.

Under conditions in which single-stranded DNA is not cut, we have used the double-stranded DNA specificity of certain prokaryotic restriction endonucleases to generate a set of DNA pieces that include those containing the naturally occurring gaps. In some experiments, DNA from the morula-stage embryos of the sea urchin *Strongylocentrotus purpuratus* was isolated and cut into specific pieces with Hind III endonuclease. These pieces were separated by column chromatography with benzoylated naphtholated diethylaminoethyl cellulose (BND) into double-stranded pieces without gaps (eluted early from the column and shown in Fig. 1 as peak I) and pieces containing internal single-stranded regions of 20 nucleotides or more (*4*, *14*). These single-stranded pieces of DNA were selectively retained on the column and then eluted by a caffeine gradient according to the length of the internal single strand for each DNA piece; the gaps ranged from 20 to 3000 bases per DNA piece, as eluted by a 0 to 1 percent caffeine gradient. The BND columns had been calibrated according to the total length of the single-stranded regions per DNA piece eluted at different concentrations of caffeine; the length of the double-stranded portions of any piece containing gaps was shown not to influence this calibration (see legend to Fig. 1). Figure 1 shows that the single-stranded pieces elute as two major peaks; one peak at about 0.15 percent caffeine (peak II) contains short gaps of about 500 nucleotides (the total length of single-stranded regions per DNA piece), and the other peak at about 0.5 percent

Table 1. DNA replication positions relative to gap length in DNA pieces. ^{3}H-labeled DNA from morula-stage sea urchin embryos was isolated, treated with restriction enzymes, and fractionated as described in the legend to Fig. 1, except that the embryos were exposed to [^{3}H]thymidine (1.0 μCi per milliliter of seawater medium; 20 Ci/mmole) for 3 minutes before the DNA isolation. Fractionation of the restriction pieces yielded those without gaps (< 20 bases per gap; fractions 3 to 7, in the vicinity of peak I), those with short gaps (20 to 500 bases per gap; fractions 9 to 12, in the vicinity of peak II), and those with long gaps (1400 to 3000 bases per gap; fractions 14 to 27, in the vicinity of peak III). Incorporation of ^{3}H was determined by scintillation counting. Unfractionated DNA (10^4 count/min; 100 percent) was treated with restriction enzymes and applied to a BND column. Means ± range for three experiments are shown.

DNA pieces	Percent of ^{3}H in DNA pieces labeled in vivo
Short gaps	69 ± 5
Long gaps	12 ± 3
Without gaps	13 ± 3
Unfractionated	100

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