The aqueous layer which showed a spot of D-glucose at *Rf* 0.15 on a paper chromatogram (*n*-butanol: AcOH: H_2O=4: 1: 5) was concentrated and filtered through cation ion exchange resin. The filtrate was then concentrated to dryness to afford a substance, which gave D-glucose phenylosazone of mp 210.5—213° with phenylhydrazine (mixed fusion).

Methylation of IV——IV was methylated with diazomethane for 5 days to afford colorless needles (V) of mp 145—147°. *Rf*: 0.33 (TLC, acetone: ether: *n*-hexane=2: 5: 5). $[\alpha]_D^{24}=-15.0$ (c=1.00, $C_2H_2Cl_4$). NMR (d_6-acetone): 2.39—2.98 (octet, 2H, J_{AB}=16.5 Hz, J_{AX}=8.7 Hz, J_{BX}=5.7 Hz, C_4–H_2), 3.71 (s, 3H), 3.78 (s, 3H), 3.80 (s, 6H) (OMe×4), 4.00 (m, 1H, C_3–H), 4.16 (br, 1H, OH), 4.64 (d, 1H, J=8.8 Hz, C_2–H), 6.03, 6.12 (each d, 1H, J=2 Hz, C_6–H, C_8–H), 6.96—6.99 (m, 3H, C_2'–H, C_5'–H, C_6'–H). MS: 346 (M^+, 64), 180 (79), 167 (100), 166 (7), 165 (26), 151 (41), 137 (20). *Anal.* Calcd. for $C_{19}H_{22}O_6$: C, 65.88; H, 6.40. Found: C, 65.99; H, 6.47. V was proved to be identical with 5,7,3',4'-tetramethyl-(+)-catechin[9] of mp 145—147°, ($[\alpha]_D^{16}=-13.1°$ (c=1.07, $C_2H_2Cl_4$); $[\alpha]_D^{16}=+7.3°$ (c=1.01, dimethylsulfoxide)), obtained by the methylation of (+)-catechin from gambir, by the mixed fusion, IR and TLC.

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Studies on Radioimmunoassay for 2,5-Dimethoxy-4-methylamphetamine

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A sensitive and specific method of radioimmunoassay for 2,5-dimethoxy-4-methylamphetamine (DOM) was developed, using anti-DOM antiserum obtained by immunizing guinea pig with DOM-glutaraldehyde-HSA conjugate and ^{125}I-N-succinyl-DOM-tyrosine methylester (^{125}I-DOM) as a labeled hapten.

DOM, ^{125}I-DOM and antibody came up to equilibrium over 15 hours at 4° of the incubation time in the radioimmunoassay system at pH 7.4 in phosphate buffer. Bound ^{125}I-DOM was precipitated with satd.$(NH_4)_2SO_4$ and the radioactivity of the bound labeled hapten was determined by γ-counting.

The displacement curve was linear when the percentage binding of ^{125}I-DOM was plotted against logarithmic increase of unlabeled DOM from 1 to 100 ng.

The antiserum showed less affinity for various phenylisopropylamine derivatives and biogenic amines and there was no interfering substance in a normal serum.

Keywords——radioimmunoassay; DOM; 2,5-dimethoxy-4-methylamphetamine; N-succinyl-DOM-tyrosine methylester; ^{125}I-N-succinyl-DOM-TME; hallucinogen; DOM-HSA

2,5-Dimethoxy-4-methylamphetamine (DOM) is a considerably potent hallucinogen which reveals the effect, lasting for about eight hours, with only a few mg.

The development of analytical method more sensitive than the conventional one of DOM was desired for pharmacokinetic studies.

Radioimmunoassay (RIA) technique using ^{3}H-labeled DOM has been reported[2)] but was not sensitive enough to be satisfactory for the determination of DOM in biological fluids.

In the present paper, we wish to report a modified, more sensitive RIA method using ^{125}I-labeled hapten synthesized according to the route as shown in Chart 1.

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1) Location: *1-18-1, Kamiyoga, Setagaya-ku, Tokyo, 158, Japan.*

2) Y. Kido, K. Nagamatsu, and C. Ishizeki, *Yakugaku Zasshi*, **94**, 1290 (1974).

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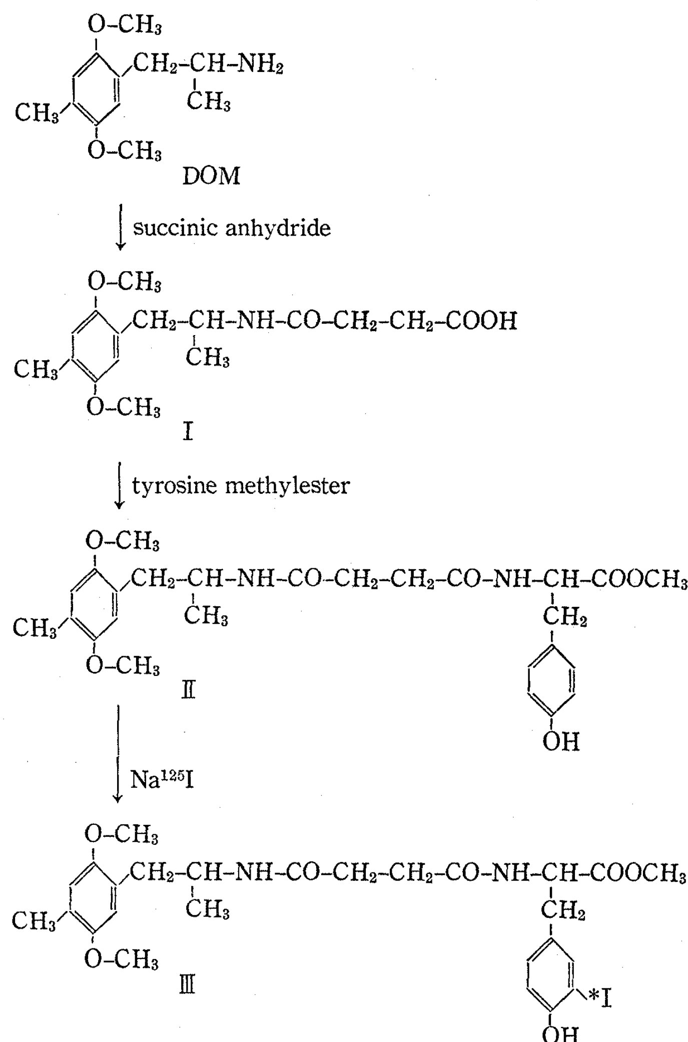

Chart 1

Experimental

Material——The chemicals used in this study were of the reagent grade from commercial sources and used without any purification.

Amphetamine derivatives were given to us from Dainabot Radioisotope Laboratories, Ltd.

Preparation of Antigen——To a solution of 20 mg of HSA and 6 mg of DOM–HCl in 2 ml of 0.1 M phosphate buffer (pH 7.0) was added dropwise 1.0 ml of 2×10^{-3} M aqueous glutaraldehyde. After 2 hr, 0.1 ml of 1 M lysine was added to consume unreacted glutaraldehyde. Then the reaction mixture was dialyzed against 0.15 M NaCl for 48 hr, and the solution inside sack was made to 2 ml with water to give the antigen solution.

Preparation of Antiserum——A solution of the antigen was emulsified with an equal volume of complete Freund's adjuvant. Five guinea pigs were immunized by subcutaneous injection of the emulsion every other week 5 times.[2] Blood was collected 7 days after last injection and the serum was examined for antibodies to DOM. The serum of adequate titer (30% binding of ^{125}I-DOM at 1 : 100 dilution) was used to the following experiments.

N-Succinyl-DOM (I)——To 10 ml of the chloroform solution of succinic anhydride (500 mg) and DOM (500 mg) was added pyridine (0.5 ml) and the mixture was refluxed for 4 hr. The reaction mixture was poured into 100 ml of cold 5% $NaHCO_3$ solution and stirred for 1 hr. The aqueous layer was washed once with 10 ml of chloroform. The solid product produced by neutralizing with 1 N HCl was isolated by filtration, washed with cold water and recrystallized from EtOH to give 500 mg of I, mp 126—127°. IR ν_{max}^{KBr} cm^{-1}: 1700 (C=O). Mass Spectrum (MS) *m/e*: 309 (M^+), $C_{16}H_{23}NO_5$.

N-Succinyl-DOM-Tyrosine Methylester (DOM-TME) (II)——I (309 mg, 1 mmol) and tyrosine methylester (195 mg, 1 mmol) were dissolved in 10 ml of chloroform. N,N′-Dicyclohexyl carbodiimide (206 mg, 1 mmol) in 2 ml of chloroform was added dropwise to the above solution under stirring, and the stirring was continued further for 18 hr at room temperature.

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The precipitate produced in the reaction mixture was filtered off and the filtrate was extracted with 100 ml of chloroform. The extract was washed with 1 N HCl, with 1 M $NaHCO_3$ and with H_2O, successively. The chloroform layer was dried over anhydrous Na_2SO_4 and evaporated to dryness leaving a crystalline product which was recrystallized from EtOH to afford colorless DOM–TME (280 mg), mp 209°. MS *m/e*: 486 (M^+), $C_{26}H_{34}N_2O_7$.

^{125}I-N-Succinyl-DOM-TME (^{125}I-DOM) (III)——To the mixed solution of 20 μl of the DOM–TME in MeOH (20 μg/ml) and 80 μl of 0.5 M phosphate buffer (pH 7.4) was added carrier free ^{125}I (as Na^{125}I in 0.1 M NaOH, 2 mCi). Two min later exactly, 20 μl of a freshly prepared, aqueous solution of Chloramine-T (7 mg/ml) was added to the mixture. Then 2 min later, 20 μl of a freshly prepared, aqueous solution of sodium bisulfite (7 mg/ml) was added. After additional 2 min, 0.5 ml of dimethylformamide and 0.3 ml of H_2O were added and then the product was separated from the resulting solution through the column of AG 1×8 (100—200 mesh, chloride form, 5×40 mm) by eluting with MeOH. III was readily obtained in crystalline form by evaporating MeOH.

The purity of the product was determined as 98% by TLC ($CHCl_3$: EtOH=2: 1). The specific activity and the radiochemical yield were 2.5 Ci/mg and 54%, respectively.

Standard Procedure for RIA——To 10 μl of ^{125}I-DOM in 0.5 M phosphate buffer (approximately 4000 cpm) were added 0.1 ml of the diluted antiserum (1: 100) with phosphate buffer, varying volume from 10 to 100 μl of the working solution of DOM or 10 μl of the sample fluid and sufficient volume of phosphate buffer (pH 7.4) to make the total volume 0.4 ml. The resulting mixture was mixed well by use of Thermomixer and incubated overnight at 4°. Free and bound ^{125}I-DOM were separated by centrifugation (5000 rpm at 4° for 15 min) after the addition of 0.4 ml of satd.$(NH_4)_2SO_4$ to the incubation mixture. The separated precipitates (bound-^{125}I-DOM) were dissolved in 0.4 ml of phosphate buffer (pH 7.4) and precipitated again by adding 0.4 ml of satd.$(NH_4)_2SO_4$. After decantation of supernatant, the wall of the centrifuge tube was carefully cleaned down by filter paper. The radioactivity of precipitate in the tube was determined by Aloka Auto Well Gamma System JDC-751 (Aloka Co., Ltd.).

Results and Discussion

Condition of RIA

Optimum incubation condition on pH and the kind of buffer solution susceptible of enough antigen-antibody reaction was examined using phosphate, borate and Tris buffer solutions of various pH (Fig. 1). Phosphate buffer of pH 7.4 gave satisfactory results.

As the results of the experiment changing the incubation time, free and bound ^{125}I-DOM came up to equilibrium in the incubation time of over 15 hours at 4° or 6 hours at 37° (Fig. 2).

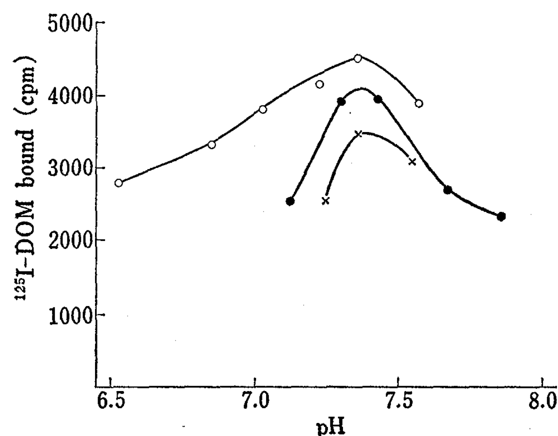

Fig. 1. Binding of ^{125}I-DOM by Antiserum at Various pH in Different Buffer Solution

—○—, phosphate buffer, —●—, borate buffer, —×—, Tris buffer.

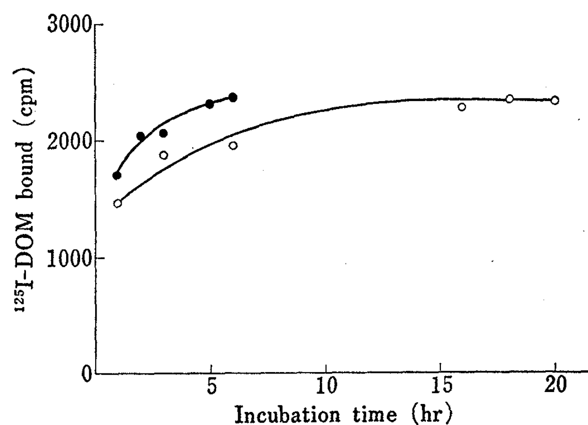

Fig. 2. Effect of the Incubation Time on the Binding of ^{125}I-DOM to Antiserum

—○—, 4° —●—, 37°.

Sensitivity of RIA

The addition of stepwise increasingly greater amounts of unlabeled DOM to the mixture of fixed amount of ^{125}I-DOM and the antiserum resulted in competitive inhibition with labeled DOM for binding to antiserum and the addition of as little as 10 pg of DOM resulted in 15%

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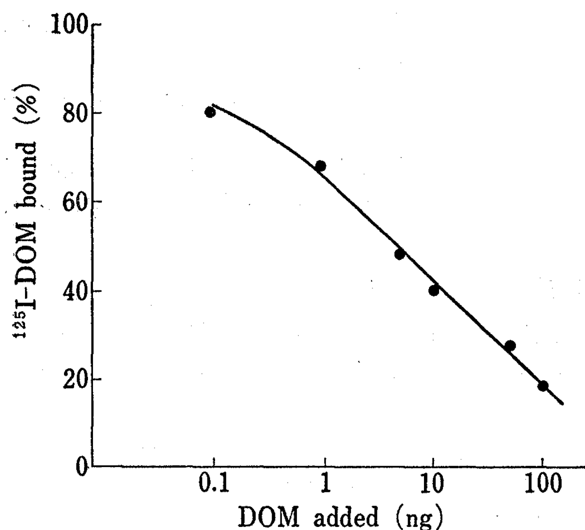

Fig. 3. Standard Curve for DOM

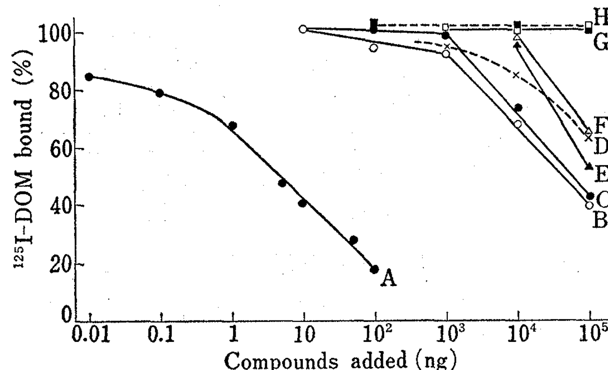

Fig. 4. Inhibition of Binding in the ^{125}I-DOM-Antiserum Reaction by Various Compounds

A: DOM, B: 2,5-dimethoxyamphetamine, C: 2-methoxy-4-methylamphetamine, D: mescaline, E: 2-methoxyamphetamine, F: 5-methoxyamphetamine, G: methamphetamine, H: 2,5-dimethoxy-4-methylbenzoic acid.

inhibition of binding of ^{125}I-DOM. A linear standard curve was observed by the addition of 1 to 100 ng of DOM (Fig. 3).

Specificity of Antibody

The specificity of the antibody towards phenylisopropylamine derivatives can be observed in Fig. 4.

Phenylisopropylamine derivatives monosubstituted on the benzene ring were poor inhibitors of immune reaction. For example, 2-methoxy- and 5-methoxyamphetamine caused 45% and 35% inhibition on the formation of antigen-antibody complex even at 100 μg level, respectively.

Disubstituted derivatives of phenylisopropylamine, 2,5-dimethoxyamphetamine and 2-methoxy-4-methylamphetamine, were also poor inhibitors and demonstrated 62% and 61% inhibition at 100 μg.

So it was acknowledged that the antibody recognized the structure of phenyl moiety of DOM.

Mescaline, 3,4,5-trimethoxyphenetylamine, was a less potent inhibitor than the disubstituted amphetamines mentioned above, probably because it was slightly different from DOM in its side chain structure. Actually, 2,5-dimethoxy-4-methylbenzoic acid, which was

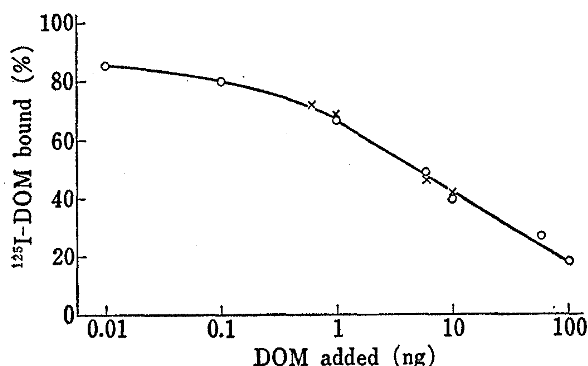

Fig. 5. Inhibition Curves of Antiserum-^{125}I-DOM Binding by Nonradioactive DOM in 0.02 M Phosphate Buffered Saline (pH 7.4) and in Guinea Pig Serum

—○—, buffered saline, —×—, guinea pig serum.

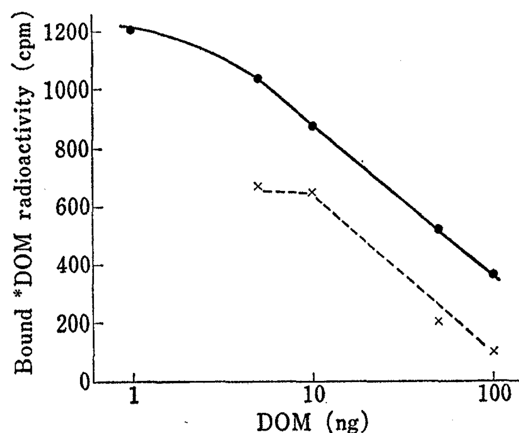

Fig. 6. Comparison of the Binding Efficiency of ^{125}I-DOM and ^{3}H-DOM to Antiserum

—●—, ^{125}I-DOM, —×—, ^{3}H-DOM.

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identical with DOM on the phenyl moiety, did not inhibit the antigen-antibody reaction. Therefore, the antibody should also recognize the side chain structure of DOM.

Ephedrine, methylamphetamine and the biogenic amines such as tryptamine, dopamine and norepinephrine failed to bind even at 100 μg level.

In further experiment, standard inhibition curves obtained by incubating in the buffered saline solution and in the guinea pig serum overlapped on a single line (Fig. 5). Namely, there was no interfering substance in the serum.

Comparison of the Two RIA Methods using ^{125}I-DOM and ^{3}H-DOM

Sensitivity of the RIA technique described in this paper was compared with that of the method using ^{3}H-labeled hapten in the most appropriate condition reported previously[2] (Fig. 6).

Due to the difference of the specific activities of ^{125}I-DOM (2.5 Ci/mg) and ^{3}H-DOM (0.1 mCi/mg), RIA technique with ^{125}I-DOM was more sensitive in scale of one order than ^{3}H-DOM RIA method.

^{125}I-DOM may be obtained easily by iodinating DOM–TME with carrier free Na ^{125}I by application of Greenwood's method[3] before use to afford the labeled compound having high specific radioactivity. Measurement of radioactivity of ^{125}I-labeled hapten is also more advantageous than that of ^{3}H-DOM in a viewpoint of counting efficiency.

^{125}I-DOM is more stable than ^{3}H-DOM. Namely, by allowing ^{3}H-DOM to stand for several months, its decomposition product was observed on the thin–layer chromatogram as a radioactive spot.[4] So, ^{3}H-DOM is greatly disadvantageous to store.

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3) F.C. Greenwood, W.M. Hunter, and J.S. Glover, *Biochem. J.*, **89**, 114 (1963).
4) Unpublished data.

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