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PRODUCTION OF ANTIBODIES TO CATECHOL-*O*-METHYLTRANSFERASE (EC 2.1.1.6) OF RAT LIVER

MARCEL ASSICOT and CLAUDE BOHUON

Laboratoire de Biologie Clinique et Expérimentale, Institut Gustave Roussy, 94 Villejuif, France

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Abstract—A potent and specific antibody to the catechol-*O*-methyltransferase (EC 2.1.1.6) has been produced in rabbits by injecting the purified enzyme from rat liver. This antibody inhibited the catechol-*O*-methyltransferase from various organs of the rat and from liver of some mammalian species. The specificity of the antibody was demonstrated by the lack of inhibition of several other *O*-methylating enzymes. The usefulness of this antibody for pharmacological research is emphasized.

SEVERAL enzymes are responsible for the various *O*-methylations, *in vivo*. Successively a soluble catechol-*O*-methyltransferase,[1] an hydroxyindol-*O*-methyltransferase[2] (EC 2.1.1.4), an indophenol-*O*-methyltransferase,[3] a microsomal catechol-*O*-methyltransferase[4] and recently a phenol-*O*-methyltransferase[5] have been described. One of these *O*-methylating enzymes, soluble catechol-*O*-methyltransferase has been obtained in highly purified form permitting its use as an antigen. We report the production of antibody to catechol-*O*-methyltransferase of rat liver and first results for interactions of this antibody with catechol-*O*-methyltransferase from various species and with other *O*-methyltransferases.

METHODS

Preparation of enzymes

Catechol-*O*-methyltransferase (COMT) to be used as antigen was purified from soluble supernatant fraction of rat liver. Details of purification procedure will be described in a subsequent publication by M. Assicot and C. Bohuon. The specific activity of preparations was tested before each injection and their homogeneity controlled with polyacrylamide gel electrophoresis as described by Davis.[6] The source of COMT from various tissues was the soluble supernatant fraction obtained by mixing tissues with 4 vol. isotonic KCl and centrifuging at 105,000 *g* for 30 min.

The microsomal fraction of rat liver was obtained by centrifuging homogenates in KCl at 9000 *g* for 10 min. The supernatant fraction was centrifuged at 105,000 *g* for 60 min. The microsomes were washed in KCl, resedimented (four times) and re-suspended in isotonic KCl before utilisation. Hydroxy-indole-*O*-methyltransferase from bovine pineal gland was purified by the method of Axelrod and Weissbach[7] modified by Baldessarini and Kopin.[8]

Enzyme assays

COMT (soluble and microsomal) activity was determined by measuring the amount

of metanephrine formed from epinephrine and methyl ^{14}C-S-adenosyl-methionine, 10 μmoles of potassium phosphate buffer (pH 7·8 for soluble COMT, pH 7 for microsomal COMT), 1 μmole of magnesium chloride, 0·15 μmole of epinephrine, 6 mμmoles of ^{14}C-S-AMe (Sp. Act. 30 mc/mM) and enzyme preparation, in a final volume of 150 μl. After an incubation at 37° for 3 min, the reaction is stopped by adding 0·5 ml of 0·5 M borate buffer pH 10. The formed metanephrine was extracted into 5 ml of ethylacetate. After shaking and centrifuging, 2 ml portions of the organic layer were used for the determination of radio-activity in 10 ml of scintillation fluid (4 g PPO; 0·1 g POPOP; toluene q.s.p. 1000, 400 ml ethanol).

The microsomal phenol-*O*-methyltransferase activity was determined as described by Axelrod *et al.*[5] by measuring the radio-activity of anisole formed from phenol and ^{14}C-S-AMe.

Hydroxy-indole-*O*-methyltransferase activity was estimated by determination of melatonin formed from *N*-acetyl serotonin and ^{14}C-S-AMe by a method close to that described by Axelrod.[7] The mixture containing enzyme preparation (700 μg of proteins), 70 μmoles of sodium phosphate tampon pH 6·8, 0·2 μmole of *N*-acetyl serotonin, 3 mμmoles of unlabelled S-AMe, 0·36 mμmole of ^{14}C-S-AMe (Sp. Act. 55 mc/mM), in a final volume of 1 ml was incubated at 37° for 30 min. After incubation, 2 ml of 1 N NaOH was added to the mixture and the samples shaken 10 min with 8 ml of chloroform for extraction of melatonin. The organic layer was washed with 1 ml of NaOH (three times) and 5 ml of the extract was transferred in counting vials, evaporated under nitrogen and the residue redissolved in 10 ml of scintillating fluid (naphthalen 125 g; PPO 12 g; POPOP 0·30 g; dioxane q.s.p. 1000).

Immunisation of the rabbits

Purified COMT of rat liver in 0·2 M potassium phosphate buffer pH 7·5 was emulsified with an equal volume of complete Freund's adjuvant (Difco). 1 ml of this preparation (200 μg of enzyme) was injected into the foot pads of albino rabbits. Following the primary injection of the antigen, two subsequent booster injections were given at two week intervals. Animals were bled by cardiac puncture or ear wein. The antiserum was stored at 4°.

RESULTS

Purity of the antigen

The specific activity of COMT preparations used as antigen was 3·3 $\pm$ 0·2 μmole of metanephrine formed per milligram of protein per 3 min. The enzyme was purified approximately 450-fold as compared to the supernatant soluble fraction. The enzyme dissolved is quite unstable and its activity decreases very quickly at this degree of purification. This lack of activity did not modify the antigenecity. Polyacrylamide gel electrophoresis of 200 μg of the purified preparation carried out at 20° with 7·5 polyacrylamide under a constant current of 5 mA per tube at pH 8·5 for 45 min yielded one protein band (Fig. 1).

Antibodies production in the rabbit. Table 1 summarizes special features of antibodies production in the rabbit. The amount of antibody in the serum samples is estimated by measuring the antiserum inhibitory effect on the purified enzyme activity.

Antibody production was not apparent 8 days after the primary injection of antigen, but after 2 weeks the rate of the inhibition by the antiserum of rabbit No. 1 was 22

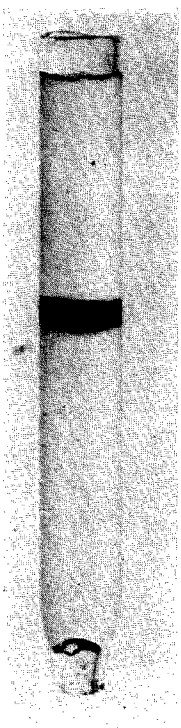

FIG. 1. Electrophoretic analysis of purified catechol-*O*-methyltransferase on polyacrylamide gel.

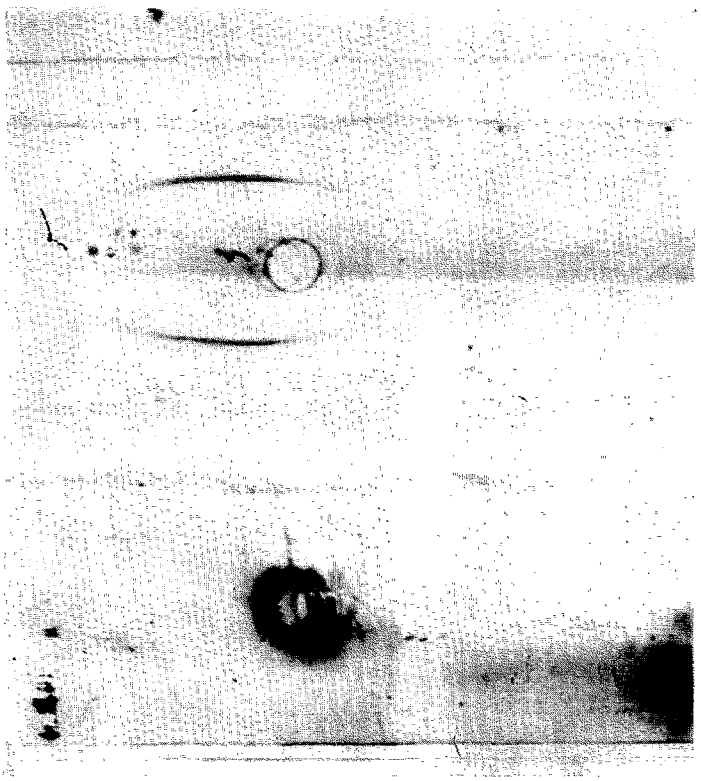

FIG. 2. Agar immuno-electrophoresis of antibodies to catechol-*O*-methyltransferase from rat liver. Rabbit antiserum (at the top) and normal serum were put in the central wells. Purified catechol-*O*-methyltransferase was placed in the longitudinal troughs in the plate.

TABLE 1. PRODUCTION OF ANTIBODIES TO CATECHOL-*O*-METHYLTRANSFERASE OF RAT LIVER IN THE RABBITS

Rabbit No. 1			Rabbit No. 2			Rabbit No. 3		
Day	Exp.	Inhibition %	Day	Exp.	Inhibition (%)	Day	Exp.	Inhibition (%)
0	puncture (1st Inj.)	0	0	puncture (1st Inj.)	0	0	puncture (1st Inj.)	0
			8	puncture	0			
			10	(2nd Inj.)				
14	puncture	22				14	(2nd Inj.)	
			24	puncture	91			
28	puncture (2nd Inj.)	10				28	puncture (3rd Inj.)	91
						30	puncture	100 (20 μl)
33	puncture	38						
			37	puncture (3rd Inj.)	87			
			50	puncture	100 (20 μl)	50	puncture	98 (20 μl)

80 μl of rabbit antiserum (20 μl when specified) were pre-incubated with 0·2 μg of purified COMT, 20 μl of 0·5 M potassium phosphate buffer pH 7·6 and 10 μl of 0·1 M $MgCl_2$. After 15 min 0·15 μmole of epinephrine and 6 mμmoles of ^{14}C-S-AMe were added. The reaction was carried on as described in text. For every determination, a control test was made by incubating 80 μl (or 20 μl) of normal rabbit serum.

per cent. Then it decreased to 10 per cent on day 28 without giving a subsequent booster injection. A second injection on day 10 to a rabbit No. 2 and on day 14 to rabbit No. 3 raised the rate of inhibition up to 91 per cent, 14 days later. The intensity of this secondary response remained approximately constant, but a third injection given to rabbit No. 3 and to rabbit No. 2 respectively 2 and 4 weeks after the second injection resulted in a 5-fold increase in antibody level.

Precipitin analysis was carried out by adding increasing amounts of COMT to a constant amount of rabbit antiserum and evidence was obtained for antigen–antibody reaction. Use of a suitable pool of rabbit antisera will permit an accurate study of the mechanism and general characteristics of the antigen–antibody reaction.

An agar immuno-electrophoretic analysis of the antiserum is shown in Fig. 2 (Hyland agar gel IEP system). After the antiserum electrophoresis was performed at 20° under a current of 15 mA for 70 min, the rat liver COMT was placed in the longitudinal trough in the plate and the immuno-diffusion carried out for 24 hr. An incurved and well limited line of precipitation was obtained.

Cross reactivity and antibody specificity

The production of antibody to COMT of rat liver being demonstrated, interaction of this antibody with COMT from several tissues in various species was investigated. Reaction of antibody with other biologic *O*-methylating enzymes also was tested. All studies were made by measuring the antiserum inhibition of enzymatic activities. A few cross reactivity observed by enzymatic methods were confirmed by immuno-electrophoretic precipitation.

Cross reactivity of the antibody with preparations of COMT from liver were

TABLE 2. INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE ACTIVITY BY ANTIBODY IN VARIOUS SPECIES

Species	Inhibition (%)
Rat	94
Guinea pig	47
Cat	55
Cow	0
Man	66
Rabbit	0

5 μl of soluble supernatant fraction from rat and cow liver and 50 μl from other species were pre-incubated at 37° with 20 μl of the antiserum, 20 μl of 0·5 M potassium phosphate buffer pH 7·6, and 10 μl of 0·1 M $MgCl_2$. Reaction was continued by adding ^{14}C-S-AMe and epinephrine as described in "Methods". The percentage of inhibition is the COMT activity observed with the antiserum compared to the enzyme activity obtained when 20 μl of normal rabbit serum was added to incubation mixture.

TABLE 3. INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE ACTIVITY FROM VARIOUS TISSUES BY ANTIBODY

Species	Tissue	Inhibition (%)
Rat	liver	98
	spleen	98
	lung	97
	brain	42
	heart muscle	98
Man	liver	68
	neuroblastoma	59
	pheochromocytoma	73

50 μl (5 μl for rat liver) of soluble supernatant fraction from various tissues were pre-incubated at 37° for 15 min with 20 μl of antiserum, 20 μl of 0·5 M potassium phosphate buffer pH 7·6 and 10 μl 0·1 M $MgCl_2$. The reaction was started by adding epinephrine and ^{14}C-S-AMe as described in "Methods". Control test were performed concurrently by pre-incubating every enzyme with 20 μl of normal rabbit serum.

observed in three species, guinea pig, cat and man (Table 2). The rates of inhibitions were respectively 47, 55 and 66 per cent. On the contrary, no inhibition of COMT from rabbit and cow liver could be detected even when the amount of antiserum was much increased in the reaction mixture.

In the same species, cross reactivity was also observed between antibody and COMT from various tissues. As shown in Table 3, antiserum inhibited COMT from several representative tissues of the rat. In all the organs studied, inhibition was about 100 per cent except in brain (42 per cent). Antiserum also had an appreciable inhibitory effect on the COMT activity from human pheochromocytoma and neuroblastoma (73 and 59 per cent).

Finally, the antiserum to COMT was not inhibitory of other *O*-methyltransferase enzymes, such as microsomal catechol-*O*-methyltransferase and phenol-*O*-methyltransferase from rat liver and hydroxy-indol-*O*-methyltransferase from cow pineal gland (Table 4).

TABLE 4. *O*-METHYLTRANSFERASES ACTIVITIES IN THE PRESENCE OF ANTISERUM TO CATECHOL-*O*-METHYLTRANSFERASE

Enzymes	Activity*	Inhibition (%)
COMT		
Normal serum	1·79	
Antiserum	0·14	94
Microsomal catechol-*O*-methyltransferase		
Normal serum	0·92	
Antiserum	0·94	0
Phenol-*O*-methyltransferase		
Normal serum	0·20	
Antiserum	0·21	0
Hydroxy-indol-*O*-methyltransferase		
Normal serum	0·64	
Antiserum	0·63	0

* For COMT and microsomal COMT, activity is mμmoles metanephrine formed for 3 min; for phenol-*O*-methyltransferase, activity is mμmoles anisole formed/30 min. For hydroxy-indol-*O*-methyltransferase activity is μg melatonin/30 min. For every experiment, enzyme examined was pre-incubated for 15 min with 80 μl of normal rabbit serum and 80 μl of antiserum. The enzymatic reaction was started by addition of S-AMe and substrate. Other conditions are described in "Methods".

DISCUSSION

During the progress of this work, we were in doubt on the ability of COMT to produce antibodies following injection. Molecular weight of COMT is about 24,000.* In fact, immunisation of rabbits with purified catechol-*O*-methyltransferase from rat liver yielded a potent antibody. Antigen–antibody interaction was demonstrated by specific precipitation and immuno-electrophoretic analysis. Moreover, antisera had a potent inhibitory effect on the COMT activity from rat liver. The produced antibody also exhibited cross reactivity with the enzyme from various sources. So, the same

* Unpublished results.

amount of antiserum inhibited at 100 per cent the COMT from liver, spleen, heart muscle and lung from rat, and brain only 42 per cent. It would be of interest to explain this decreased sensibility of cerebral COMT. This rate in brain is perhaps only due to tissue components brought about in the incubation medium by brain preparations.

Furthermore, cross reactivity was observed between the antibody and enzyme from several species, cow, pig, cat and man, but the antiserum has no inhibitory effect on cow enzyme. Failure to observe bovine COMT inhibition indicates that in this species, the enzyme is not similar to COMT from other tested species for antigenicity. Finally, it is interesting to note that the antibody not only inhibited man liver COMT, but also was a potent inhibitor of the enzyme from tumor tissues (pheochromocytoma and nuroblastoma). It will be of interest to investigate which enzyme from other various species can react with the antibody.

The specificity of the antibody was proved by the lack of inhibition on other *O*-methylating enzymes. The absence of inhibitory effect was noted with bovine pineal gland hydroxy-indol-*O*-methyltransferase and with microsomal phenol-*O*-methyltransferase from rat liver but also with the rat liver microsomal COMT. This enzyme which differs from soluble catechol-*O*-methyltransferase in its pH optimum and its response to cold stress, and benzpyrene[4] is proved to be a different protein with specific antigenic properties. Respective contribution of both these enzymes in the catabolism of catecholamines remains to be established. The ability of this antibody to inhibit exclusively soluble COMT activity point to its usefulness for solving this problem. Furthermore, *in vivo* studies are in project with purified antibodies. Our preliminary results obtained with the crude antiserum indicate an inhibition, *in vivo*.

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