COO-1602-31

A SIMPLE MICRORADIOMETRIC TECHNIQUE FOR THE RAPID MEASUREMENT OF MONOAMINE OXIDASE ACTIVITY IN RAT TESTICULAR MINCED AND TEASED-TUBULAR PREPARATIONS[1]

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DISCLAIMER

Footnotes

1. This research was sponsored by Utah State University Research Project U-300, and U.S. Atomic Energy Commission Grant No. AT(11-1)-1602.

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Running Head: Monoamine Oxidase Activity

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Monoamine oxidase (MAO, Monoamine: O_2 oxidoreductase (deaminating) EC. 1.4.3.4) activity has been demonstrated in many vertebrate and invertebrate tissues (1,2,3,4). Several quantitative methods have been developed to determine the activity of this enzyme in various tissue extracts (5, 6, 7) using different substrates. Onesubstrate commonly used in MAO determinations is 5-hydroxytryptamine (5-HT) where 5-HT (5, 6) is converted into 5-hydroxy-indole acetic acid (5-HIAA).

Different investigations have shown (2, 8, 9) that MAO is present in the male sex organs and that small amounts of endogenous 5-HT (60-100 nanograms/ gram of tissue) are present in rat testes (10, 11). Large doses of 5-HT, when injected into adult male rats, caused deleterious effects in their gonads that were evidenced by a reduction in testicular weight and altered spermato-genesis (12, 13, 14, 15).

The following investigations were undertaken in order to develop a simple, rapid method for the quantitative assay of MAO activity in various testicular preparations so that the enzyme activity could be related to physiological and pathological states of the testis.

METHODS

Testicular tissue was obtained from adult male rats (Holtzman strain--12 weeks of age) that were maintained under controlled conditions in our small animal colony. The animals were given Purina lab chow and water ad libitum.

After decapitating the animals, the testes were quickly removed, chilled, decapsulated, and portions of one testis from each animal were finely minced with razor blades on a teflon block. Aliquots of the minced tissue preparations were weighed and incubated as described below. Teased-tubular preparations were obtained from decapsulated testes as described elsewhere (17). The substrate used for the MAO assay was 5-HT-2-^{14}C binoxolate (18 mCi/mMole New England Nuclear Corp.) or 5-HT-1-^{14}C creatinine sulfate (45 mCi/mMole Schwarz/Mann).

First Procedure:

In the first assay procedure tested, 0.05 μCi (0.9μM) aliquots of 5-HT-^{14}C in 100 μl were added to 50-ml Erlenmeyer flasks. Then 120 mg of either teased-tubules or minced tissue were transferred with 3 ml of phosphate buffer (NaCl, 127 mM; KCl, 5mM; mg SO_4, 26mM; K_2HPO_4, 69.8 mM; $NaH_2PO_4H_2O$, 12.5 mM; pH 7.4) to the flask. The mixture was then incubated for 1 hr. The resulting 5-HIAA-^{14}C was extracted from the incubation mixture by a modified procedure previously outlined for pineal culture extracts (18) as indicated below. After the incubation was terminated with 2 ml of 2 N HCl, the contents of the flasks were centrifuged at 240 x g for 5 min. Three ml of each supernatant solution were transferred to clean, 15-ml, glass-stoppered centrifuge tubes containing 6 ml of recently distilled diethyl ether. After shaking the tubes for 5 minutes, the two phases were allowed to separate. The ether layers were subsequently transferred to clean, glass-stoppered centrifuge tubes. Each sample was re-extracted with a 2-ml aliquot of ether.

Two ml of 0.1 M phosphate buffer (pH 8.0) were added to the pooled ether aliquots for each sample (the pH was not allowed to drop below 7.9 at this stage). The tubes were shaken for 5 minutes and the phases were allowed to separate, the ether layers were removed and discarded, and the aqueous phases were washed twice with 4-ml aliquots of ether. After discarding the ether layers, 1 ml of 1 N HCl was added to each sample, followed by 6 ml of ether.

After the tubes were shaken for 5 minutes, the ether layers were transferred to conical test tubes containing 100 µl of carrier 5-HIAA. After initially evaporating the ether with a stream of dry nitrogen gas, the tubes were rinsed three times with ether to concentrate the radioactivity in the tips of the tubes. The residue in each tube was redissolved in a small amount of ether and quantitatively transferred to thin-layer chromatofilm strips 3 cm wide (silica gel--Eastman Kodak Co.). The chromatographic strips were subsequently developed in a chloroform:methanol:acetic acid system (65:30:5). A 2 pi, Geiger-Müller, electronic Auto scanner (Nuclear Chicago Actigraph III) was used to locate the 5-HIAA-^{14}C. Carrier 5-HIAA was located on the strips by using an ultraviolet light visual scanner (254 mµ). Those portions of the strips containing the radioactivity and carrier 5-HIAA were cut out and placed in liquid scintillation counting vials. After the addition of a standard scintillation cocktail, the vials were placed in a liquid scintillation counter (Packard Instr. Co.) and the contents of each vial were counted for ten minutes. The counting efficiency was 88% and

external standardization was used to correct for quenching. Three 10-minute counts were recorded for each sample. The recovery of the 5-HIAA-^{14}C, as determined by recovery of authentic 5-HIAA-^{14}C (New England Nuclear Corp.) added to samples, was approximately 30%.

Several investigations were undertaken to define the limitations of this assay. Testes from adult male rats were removed, decapsulated, and finely minced with razor blades on a teflon block as described above. Five samples (120 mg each) were homogenized in 3 ml of phosphate buffer and were transferred to Erlenmeyer flasks as described above. Five samples were prepared by taking a portion of the decapsulated testis (120 mg each) and lightly teasing it apart with teasing needles to increase the surface area. Five teased-tubule samples (120 mg each) were also prepared as described elsewhere (17). Five aliquots of teased-tubules (120 mg each) were homogenized in 3 ml of buffer, and were transferred to Erlenmeyer flasks. These samples were then assayed for MAO activity as described above.

Irradiated testicular tissue preparations were obtained from male rats (Holtzman strain) that had been irradiated when 12 weeks of age with 250 R of whole-body x-irradiation (250 kvp, 1 mm Al and 0.5 mm Cu Filter-Westinghouse Quadrocondex). Aliquots (120 mg each) of teased-tubular and homogenized tissue were obtained from 5 control and 5 irradiated animals 42 days after treatment. These samples were then assayed for MAO activity as described above.

The effects of various substrate concentrations on this MAO assay were determined for homogenized and teased-tubular preparations by using five aliquots each of the two tissue preparations. The substrate concentrations ranged from 0.05 μc to 2.0 μc (0.1 to 37.0 μMolar). The samples were then assayed using the procedure outlined above.

Second Procedure:

For the second series of investigations, we derived an assay for determining MAO activity in testicular preparations from procedures reported by other workers testing nerve tissue (16) and pineal preparations for MAO activity (19). Ten μl of 5-HT-^{14}C (0.5 μc, 0.65 mMolar) were transferred to conical microtubes (8 mm I.D. x 7 cm length) that were held on ice prior to incubation. Forty μl of 0.1 M phosphate buffer (pH 7.4) were added to each tube. One gram of decapsulated testicular tissue was homogenized in 5 ml of de-ionized distilled ice-cold water. Ten μl of the homogenate (2 mg of testicular tissue) were transferred to each tube, and the contents carefully mixed. After incubating the samples for 30 minutes at 38^{o}, the reaction was terminated by adding 10 μl of 3N HCl. Two hundred μl of analytical grade, distilled ethyl acetate were added to each tube and the contents of the tubes were mixed (Vortex Jr. mixer). Following a brief centrifugation, 100 μl of the ethyl acetate layers were transferred to clean tubes containing 75 μl of 0.3 N HCl and mixed to remove the last traces of the radioactive substrate. The tubes were shaken, centrifuged, and 50 μl of the ethyl acetate layers were chromatographed on thin-layer strips as

described above. Carrier 5-HIAA and 5-HT, 20 μg each, were streaked on the origin of each strip just prior to developing the strips as indicated above.

Radioactivity and carrier 5-HT and 5-HIAA were located on the strips as described above and sections of the strips containing radioactivity corresponding with carrier 5-HIAA were cut out and counted as described above. Three 1-min. counts were made for each sample. 5-HIAA-^{14}C was added to non-incubated samples to determine relative recovery, which was approximately 90%.

The effects of different substrate concentrations on MAO activity were ascertained by using homogenized testicular tissue (2 mg) (prepared as described above). Samples were incubated in duplicate with substrate concentrations of 0.19, 0.39, 0.58, 0.78, and 0.97 mMolar (0.2 through 1.0 μc).

This second assay system was checked for linearity with respect to incubation time (15, 30, 45, and 60 mins) and amount of tissue (1 through 10 mg of homogenized tissue). All assays were made in duplicate. Various amounts of testicular tissue (1 through 10 mg homogenized tissue) were assayed in duplicate for MAO activity as outlined above with a 30-minute incubation time.

The precision of the procedure was determined by taking five aliquots of homogenized tissue (2 mg) and five aliquots of homogenized teased-tubules (2 mg) from the testes of one animal and assaying for MAO activity. To ascertain the degree of variability in MAO activity among animals of the same age group, one aliquot of homogenized tissue and one of homogenized teased-tubules were obtained from each of six rats and were assayed as described above.

RESULTS

First Procedure:

When the MAO activity of teased-tubular preparations from control and irradiated rats were compared with results from homogenized tissue preparations (Table 1), significant differences were observed among the teased-tubular, but not the homogenized preparations. To ascertain if the type of tissue preparation was responsible for this difference in results, homogenized testicular tissue, lightly teased testicular tissue, teased-tubules, and homogenized teased-tubules were assayed for MAO activity (Table 1). A significant decrease in MAO activity was observed when the testicular tissue was homogenized. No decrease in activity was observed, however, when teased-tubular preparations were homogenized. The data indicate an inhibitor of MAO activity in the interstitial cells but not in the seminiferous tubules. Moreover, when teased-tubular and minced preparations were incubated with increased amounts of 5-HT-^{14}C (Fig. 1), a marked increase in activity was seen in the minced testicular preparations above a substrate concentration of 9.3 μM, indicative of a competition at low substrate levels with an endogenous substance presumed to be 5-HT. No such increase was noted for the teased-tubular preparations.

Second Procedure:

Incubation with a smaller incubation volume, less tissue, and various amounts of 5-HT-^{14}C, resulted in a maximum MAO activity at a substrate concentration of approximately 0.75 mM (Fig. 2). When homogenized testicular tissue was incubated for various time intervals (Fig. 3) the rate of 5-HIAA-^{14}C formation was linear up to 45 min.

When whole testicular tissue was homogenized and incubated with 5-HT-^{14}C and compared with homogenized teased-tubules from the same animal (Table 2), the whole testicular preparations had more activity than the teased-tubular preparations. Incubation of whole testicular tissue preparations and teased-tubular preparations from six additional animals (Table 2) demonstrated no significant differences between the two preparations. Good precision with low standard error of mean values was observed. Increasing aliquots of homogenized testicular tissue (Fig. 4) and a 30 min. incubation time gave a linear relationship with from 1 to 10 mg of testicular tissue.

DISCUSSION

The data show that the first procedure described in this paper (substrate concentration of 0.9 μM) was acceptable for use with teased-tubular preparations, but not for homogenized or minced preparations. Good results were obtained with the second procedure (substrate concentration of 0.75 mM) when measuring the MAO activity of homogenized whole testicular tissue. Endogenous 5-HT from the interstitial tissue appears to interfere with the first procedure more than the second because of the low substrate concentration. Other inhibitors, however, may also be present, and at low substrate concentrations these might effectively interfere with determinations of MAO activity, in testicular preparations containing interstitial cells.

The second procedure described in this paper indicated (Table 2) that MAO activity of homogenized testicular tissue is equal to or higher than the activity in the seminiferous tubules, which agrees with findings of other workers (9). The assay was linear with incubation times up to 45 minutes and with an optimal substrate concentration of 0.75 mM. The assay was also linear with from 1 through 10 mg of tissue. The second method is more rapid and less expensive than the first procedure, and is equally useful for whole tissue and teased-tubular preparations. This procedure could be used with testicular biopsies where only small amounts of tissue (2 mg) are available for study.

SUMMARY

Two radiometric ways of measuring monoamine oxidase activity in testicular tissue preparations are described. Both methods measure the rate of conversion of 5-HT-^{14}C into 5-HIAA-^{14}C. The limitations and advantages of each method are discussed.

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TABLE 1

Comparison of MAO activity in homogenized, teased tissue, and teased-tubular preparations (using the first procedure)

TISSUE	ACTIVITY (Counts/10 Minutes)	P VALUES
Radiation Experiment	(per Animal basis)	
Teased-Tubules		
Control	244,158,074 ± 26,455,022	$P < 0.01$
Irradiated	176,563,332 ± 15,857,019	
Homogenized Tissue		
Control	20,073,480 ± 2,966,935	
Irradiated	17,915,460 ± 3,498,971	$P > 0.50$
Various Tissue Preparations	(per 100 mg tissue)	
Homogenized Tissue	5,639 ± 834	$P < 0.001$
Lightly Teased Tissue*	34,473 ± 1,388	$P < 0.001$
Teased Tubules	67,019 ± 4,729	$P > 0.50$
Homogenized-Tubules	60,866 ± 10,351	

* Whole testicular tissue was lightly teased apart with forcepts to increase surface area.

TABLE 2

Comparison of Homogenized Tissue and Homogenized Teased-Tubules (using the second procedure)

TISSUE	ACTIVITY (Counts/Minute)		P VALUE
Homogenized Whole Tissue	70,805 ± 2,808[1]		
		*	P < 0.001
Homogenized Teased-Tubules	50,046 ± 587		
Homogenized Whole Tissue	54,826 ± 1,855		
		**	P > 0.50
Homogenized Teased-Tubules	55,374 ± 1,571		

[1] Standard Error of Mean

* Tissue from the same animal

** Tissue from six animals of the same age

Legends for Figures

Fig. 1. The effect of substrate concentration on rat testicular MAO activity using minced (dotted lines) and teased-tubular (solid line) preparations and the methods outlined in the first procedure (CPM = counts per minute).

Fig. 2. The effect of substrate concentration on rat testicular MAO activity using homogenized tissue and the mthods outlined in the second procedure (CPM = counts per minute).

Fig. 3. The effect of incubation time on rat testicular MAO activity using homogenized tissue and the methods outlined in the second procedure (CPM = counts per minute).

Fig. 4. The effect of enzyme concentration (amount of homogenized tissue) on rat testicular MAO activity using the methods outlined in the second procedure (CPM = counts per minute).

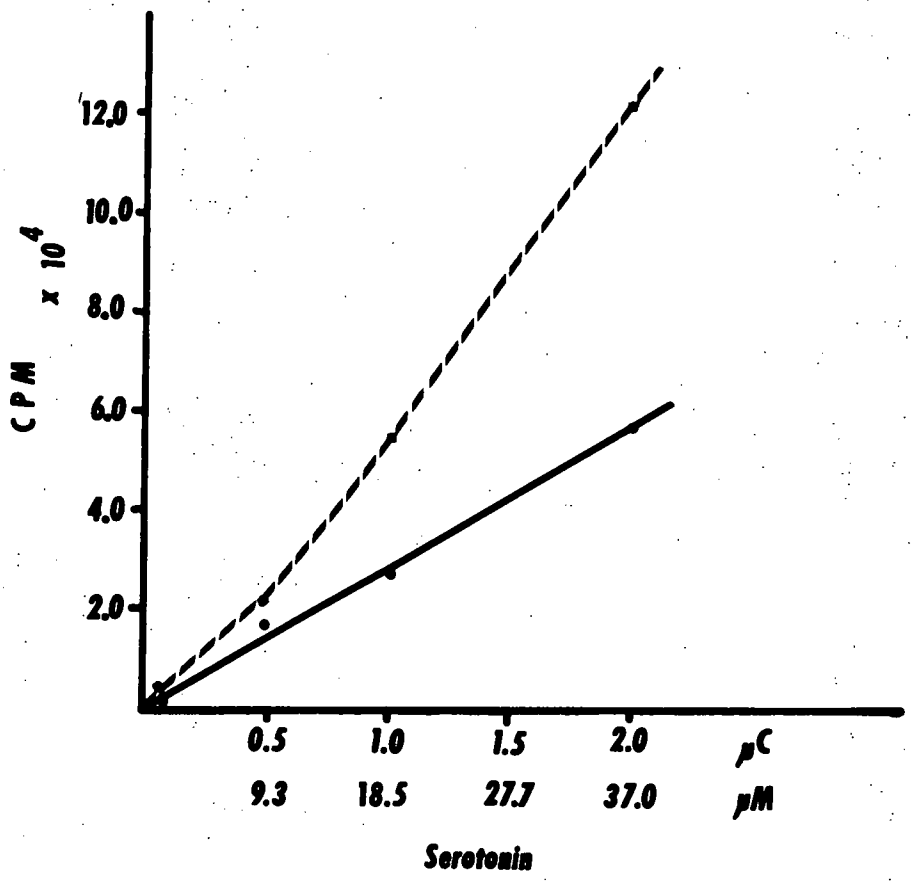

Fig. 1
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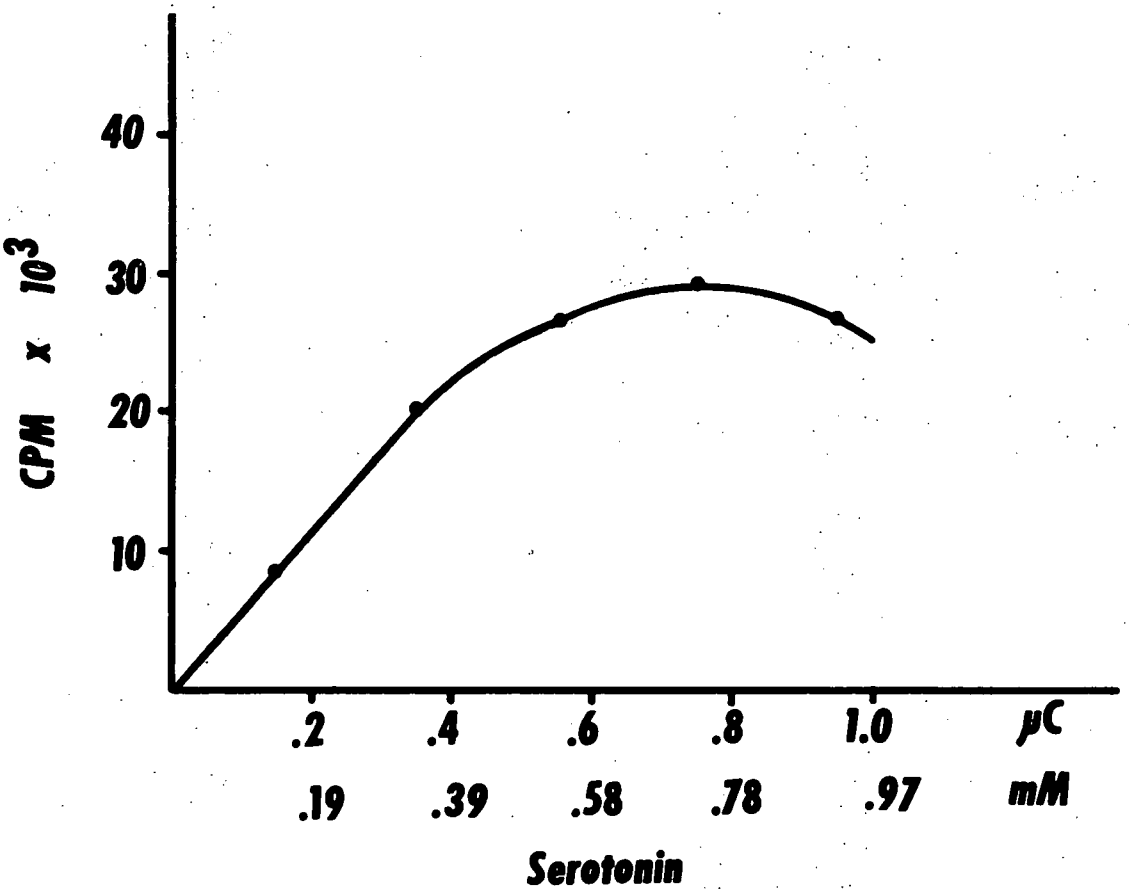

Fig. 2
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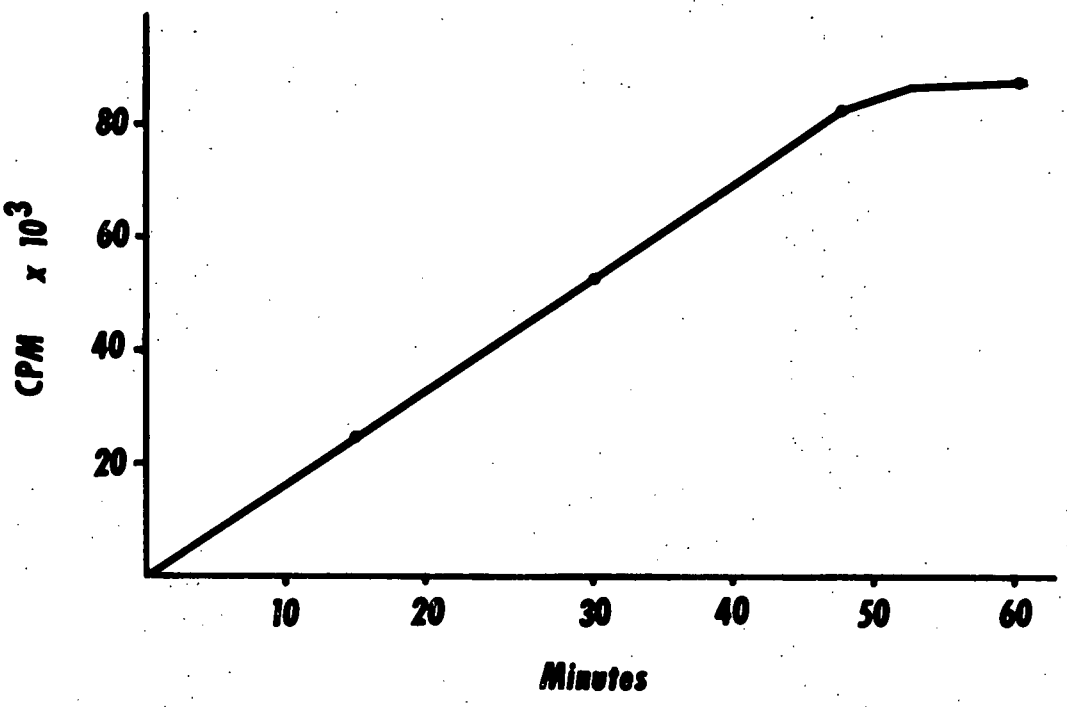

Fig. 3
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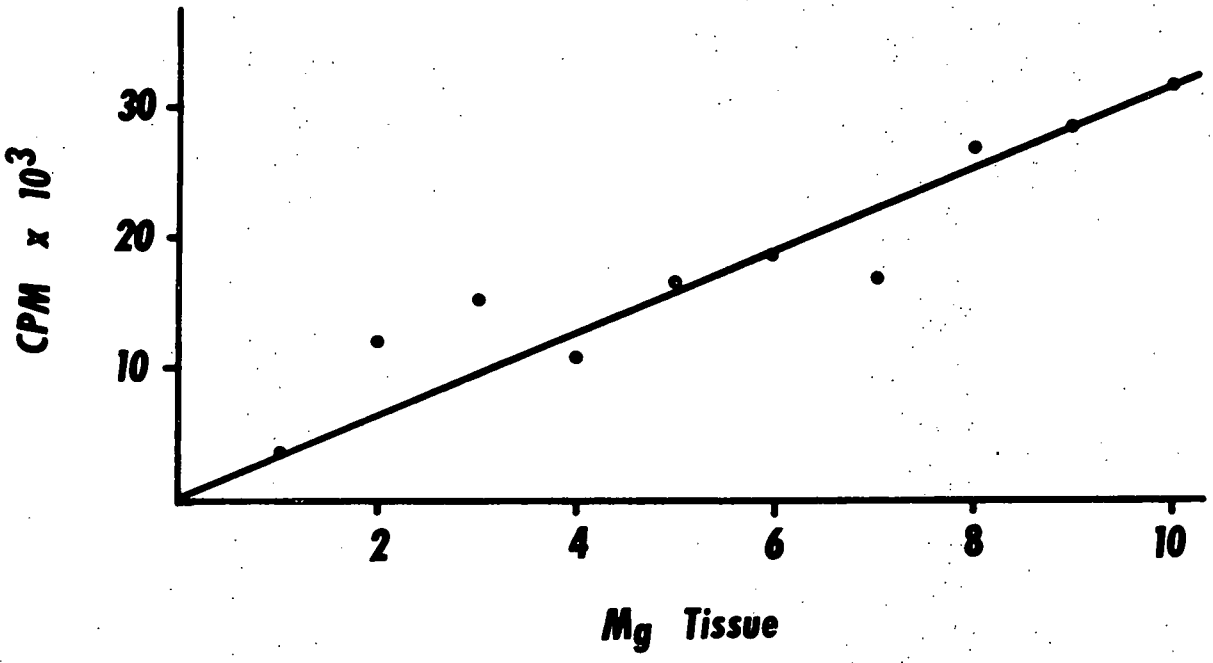

Fig. 4
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