PREPARATION OF TRITIUM-LABELLED meta-AMINOLEVAMISOLE OF HIGH SPECIFIC RADIOACTIVITY BY CATALYTIC DEHALOGENATION

James A. Lewis[1,3] and Ian Paterson[2]
Departments of Biological Sciences and Chemistry
Columbia University, New York, NY 10027

SUMMARY

We describe the synthesis of tritiated meta-aminolevamisole (MAL) of high specific radioactivity. Unlabelled MAL was iodinated with iodine monochloride in aqueous hydrochloric acid. The major reaction product, L[-]2,3,5,6-tetrahydro-6-(5-amino-2-iodophenyl)imidazo [2,1-b]thiazole, was catalytically dehalogenated with carrier-free tritium gas in methanolic potassium hydroxide over 10% palladium-carbon. After extraction and chromatography, a radioactive species ([^{3}H]-MAL) was identified with the same chromatographic mobility as unlabelled MAL. [^{3}H]-MAL possesses the same high biological activity in contracting nematode muscle as authentic unlabelled MAL, co-chromatographs with unlabelled MAL in three different TLC systems and is about 90% radiochemically pure. By bioassay and competitive inhibition studies, the specific radioactivity of [^{3}H]-MAL appears greater than 15 Ci/mmole and is probably close to 29 Ci/mmole. [^{3}H]-MAL was shown to be useful for detecting a high affinity, saturable binding activity in extracts of the nematode Caenorhabditis elegans.

Key Words: meta-aminolevamisole, tritium-labeling, anthelmintic, levamisole, catalytic deiodination

INTRODUCTION

Levamisole is a drug neurotoxic to nematodes (1). It causes rapid contraction of nematode muscles followed by spastic paralysis and at high doses, death. It is the l-isomer and more active form of d,l-tetramisole and bears structural and pharmacological resemblances to the nematocides nicotine and pyrantel (2,3,4). The stereospecificity of levamisole, its low effective concentration, and the effects of substitution on the phenyl ring suggest that

1. Present address: Dept. of Biological Sciences,Univ. of Missouri,Columbia,MO
2. Present address: Dept. of Chemistry,Univ. of Cambridge,Cambridge,England
3. To whom reprint requests should be addressed.

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levamisole acts by specifically binding to a receptor protein.

Uncoordinated mutants of the nematode Caenorhabditis elegans can be isolated that are immune to the neurotoxic effects of levamisole (5,6). We have shown that such mutants possess genetic and pharmacological properties consistent with acetylcholine receptor deficiency although our hypothesis does not rigorously rule out other possibilities (4). To better define the levamisole receptor and its possible deficiency in levamisole-resistant mutants, a biochemical assay for the receptor was needed. The compounds used to assay vertebrate cholinergic receptors, α-bungarotoxin, benzyltrimethylammonium, and 3-quinuclidinyl benzilate, do not have significant pharmacological potency on nematodes (4). Meta-aminolevamisole, L[-]2,3,5,6-tetrahydro-6-(5-aminophenyl)-imidazo [2,1-b]thiazole, (MAL, I in Figure 1), however, is an extremely potent nematode muscle agonist and its aryl amino moiety would facilitate the preparation of both an iodinated precursor and subsequent introduction of tritium by catalytic dehalogenation (7).

In this work we describe the synthesis of tritiated meta-aminolevamisole of high specific radioactivity ([^{3}H]-MAL). Our basic method is shown in Figure 1.

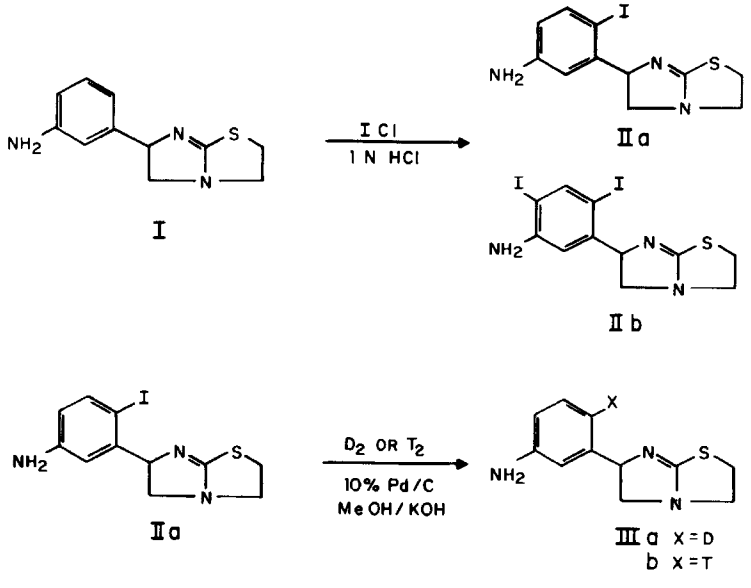

Figure 1

EXPERIMENTAL

Chemicals. Meta-aminolevamisole was a gift from American Cyanamid. Palladium-carbon was purchased from Aldrich Chemical Co., deuterium oxide (99.8%) and 35% deuterium chloride in deuterium oxide (99%) from Sigma Chemical Co. The solvents used were ACS grade and were obtained from Fisher Scientific.

Thin layer chromatography. Reactions and column chromatograms were monitored for the most part by use of Gelman ITLC type SG plates developed in acetone/methanol 10:0.4. Meta-aminolevamisole compounds were less strongly absorbed to this medium than to standard silica gel G plates, which required much more polar, slowly developing solvents for reasonable separations.

Iodination of MAL with iodine monochloride (ICL). We followed the iodination method described by Wallingford and Krueger (12). MAL (657 mg, 3.0 mmole) was dissolved in 3.45 ml of water and 0.6 ml of concentrated hydrochloric acid. ICl (36 mg, 3.3 mmole) was added to 2.44 ml of water and 0.53 ml of concentrated hydrochloric acid cooled on ice. The chilled ICl suspension was then stirred into the MAL solution at room temperature. An immediate yellow precipitate formed. Stirring was continued for 1 1/2 hours at room temperature. The pH of the solution was then raised to around 11 with concentrated ammonium hydroxide, the precipitate dispersed with several ml of methanol and then extracted with 50 ml of chloroform. The combined aqueous and organic layers were then filtered through Celite to remove insoluble material. The aqueous layer was washed twice with chloroform and the pooled chloroform layers washed with 20 ml of 5% sodium sulfite, dried over magnesium sulfate, and evaporated to dryness, yielding 976 mg of material.

Purification of L[-]2,3,5,6-tetrahydro-6-(5-amino-2-iodophenyl)imidazo [2,1-b]thiazole (IIa). The crude iodination reaction product was dissolved in methylene chloride and half the material was applied to a 1.8 by 34 cm Florisil column (32 gm). The column was eluted with the series of solvents shown in Table 1 and the indicated weights of material collected. The reaction products

consisted of a front-running minor species and a less rapidly eluted major product. The major product identified by its NMR and mass spectra characteristics was IIa (Figure 1; Table 2, column 3) and the more rapidly eluted minor product L[-]2,3,5,6-tetrahydro-6-(5-amino-2,4-diiodophenyl)imidazo [2,1-b]-thiazole (IIb, Figure 1; Table 2, column 2). The identity of the monoiodinated product was further confirmed by the ability to catalytically dehalogenate it back to material with the chromatographic mobility, biological activity, and NMR spectra of MAL.

Table 1. Chromatography of iodinated MAL compounds on Florisil.

Fractions	Weight of material, mg	Composition
3,4,5,6	93	I_2MAL
7,8	77	~1/4 I_2MAL, ~3/4 IMAL
9 to 34	269	IMAL

Crude material (484 mg) from the reaction of MAL with ICl was applied to a Florisil column as described in Experimental. The column was eluted with the following solvents collected in 50 ml fractions as indicated: CH_2Cl_2/EtAc 1:3 (#1-22); EtAc (#23,24); EtAc/MeOH 100:1 (#25,26); EtAc/MeOH 19:1 (#27-32); EtAc/MeOH 4:1 (#33,34). The yield obtained was 326 mg of IMAL (compound IIa) and 112 mg of I_2MAL (compound IIb), representing 63 and 16 mole percent of the starting material, respectively.

Catalytic dehalogenation of IIa.

a. Dehalogenation using hydrogen gas. Compound IIa (40 mg, 0.12 mmole) was dissolved in 8 ml of methanol, 0.2 M in potassium hydroxide. To this material in a 25 ml round bottom flask, 81 mg of 10% palladium-carbon was added. The flask was attached to a T tube apparatus fitted on one end with a balloon of hydrogen and connected on the other to a water aspirator. The reaction mixture was frozen with a dry ice/ethanol bath and the reaction flask evacuated and flushed four times with hydrogen gas. The reaction mixture was warmed and stirred for 2 hours at room temperature. The reaction was terminated by freezing the

Table 2. Properties of MAL compounds.

	I_2MAL	IMAL	DMAL	MAL
R_f	0.83	0.73	0.34	0.34
^{1}H NMR peaks, ppm downfield				
aromatic	7.9s(0.7) 6.9s(0.7)	7.6-7.4d(0.8) 6.9-6.7d(0.8) 6.4-6.2dd(1)	7.2-6.9m(1.2) 6.7-6.4m(1.7)	7.3-7.0m(1.6) 6.9-6.5m(2.6)
benzilic	5.6-5.2m(1)	5.7-5.4m(1)	5.4-5.0m(1)	5.6-5.3m(1)
other	4.4-2.7m(7.6)	4.3-2.7m(8.4)	4.2-2.7m(10.7)	4.4-2.9m(10)
Mass spectra, major peaks,m/e				
	472,471 346,345 222,220	346,220	-	-

R_f values given for chromatography on Gelman ITLC type SG plates in acetone/methanol 10:0.4. NMR spectra were obtained in deuterated chloroform using tetramethylsilane as a standard except DMAL was done in deuterated acetonitrile. Integrated peak values are normalized to the distinctive benzilic hydrogen. s, singlet; m, multiple; d, doublet; dd, doublet of doublets. Mass spectra were obtained by chemical ionization. I_2MAL = compound IIb; IMAL = compound IIa; DMAL = compound IIIa.

reaction mixture and then evacuating and flushing the flask three times with air. Thin layer chromatography revealed essentially complete conversion of starting material to a product with an R_f of MAL. The reaction mixture was filtered through Celite and 0.5 ml of concentrated hydrochloric acid was added to the filtrate along with 15 ml of distilled water and 15 ml of chloroform. The yellowish color which formed was extracted into the chloroform layer. After removal of this layer, an additional 30 ml of chloroform was added and the solution made alkaline with 1 ml of concentrated ammonium hydroxide. The alkaline chloroform extract was dried over magnesium sulfate and evaporated to dryness. The product, redissolved in methylene chloride, was applied to a

5 cm high Florisil column packed in a Pasteur pipette and then eluted with 5 aliquots of ethyl acetate/methanol 5:1 and 6 aliquots of methanol, each 3.8 ml. Elutions 2 through 5 with ethyl acetate/methanol were pooled and evaporated, yielding 16 mg of a slightly yellow material (63% yield). TLC and NMR showed the product was mostly MAL with some tarry contaminant. Bioassay of the ability of the material to contract cut C. elegans showed the material to have 70-80% of the potency of authentic MAL (Table 3). The iodinated starting material at 2,000 times the concentration had no biological activity. Reaction of MAL with ethyl acetate, especially during rotary evaporation, appeared to be the source of the tarry contaminant. The contamination caused by the use of ethyl acetate was markedly greater in the purification of MAL than in the purification of the iodinated MAL compounds. Besides avoiding the use of ethyl acetate, it may also be more desirable to substitute methylene chloride for extraction and sodium carbonate for pH adjustment in our purification scheme.

Table 3. Biological activity of MAL compounds.

Compound tested	Concentration tested, μm	Fraction of worms contracted	Elapsed time, minutes
IMAL	200	0/5	90
MAL from dehalogenation with hydrogen	0.1	7/10	33
MAL control	0.1	10/11	36
[^{3}H]-MAL from dehalogenation with tritium	0.1*	14/15	25
MAL control	0.1	14/15	27
MAL control	0.2	14/15	18
MAL control	0.3	14/15	12

MAL compounds were tested for biological activity as described in Experimental and compared to authentic MAL tested at the same time. IMAL = compound IIa.
*Concentration if carrier-free.

b. Dehalogenation using deuterium. The dehalogenation was performed similarly to that described above using hydrogen gas. To obtain deuterium gas, 11.5 ml of deuterium oxide 5% in deuterium chloride was boiled in the presence of 8 gm of zinc and the evolved gas collected in a balloon. Compound IIa (42mg, 0.12 mmole) was dehalogenated over 10% palladium-carbon as described above but after 2 hours reaction, TLC showed only about 50% conversion to the expected MAL reaction product, possibly due to the limited amount of deuterium generated and its dilution with air. The reaction was worked up as described for dehalogenation with hydrogen except that during Florisil chromatography, the column was eluted with six aliquots of ethyl acetate/methanol 3:1 and six aliquots of methanol, each 3.8 ml. Fractions 2 through 6 of the first eluent and fractions 1 and 2 of the second were combined and evaporated, yielding 10 mg of material (38% yield). The product was again tinged with yellow but co-migrated on TLC plates with authentic MAL and had the NMR spectra of material that was substantially compound IIIa (Table 2, column 4).

c. Dehalogenation with tritium gas. This dehalogenation was performed by New England Nuclear Custom Labeling Service according to our instructions. Compound IIa (34.5 mg, 0.10 mmole) was dissolved in 7 ml of 0.2 M potassium hydroxide in methanol and added to a reaction vessel with 70 mg of 10% palladium-carbon. The reaction mixture was stirred vigorously for 2 hours at room temperature in the presence of one atmosphere of tritium gas (25 Curies) while protected from light. The reaction was terminated by adding 0.5 ml of concentrated hydrochloric acid and taking the mixture to dryness, a difference from previous reaction protocols that was necessitated by the large amount of tritium used. Labile tritium was removed by the repeated addition and evaporation of methanol. The reaction mixture was then dissolved in methanol, filtered to remove the catalyst, evaporated to dryness, and dissolved in 20 ml of 0.01 N HCl and then diluted with 0.01 N HCl for shipment in aliquots containing less than 10 mCi/ml. A total of 637 mCi (22% of theoretical incorporation) was

received from New England Nuclear and stored quick-frozen in liquid nitrogen.

Purification of [^{3}H]-MAL. Forty nine mCi of the tritiated reaction material received from New England Nuclear in 9.6 ml of 0.01 N HCl was extracted with 10 ml of methylene chloride. The aqueous layer was then made alkaline (pH~12) with 0.24 gm of sodium carbonate and extracted with two 20 ml portions of methylene chloride and then one 10 ml portion. The combined methylene chloride fractions were dried over magnesium sulfate and filtered through Celite. The filtrate was concentrated to a few ml volume and applied to a 5 cm high column of Florisil in a Pasteur pipette. The column was eluted with solvents as described in Figure 2, avoiding the use of ethyl acetate, and the eluates assayed for radioactivity and analyzed by thin layer chromatography. Fractions 5 to 15 were pooled, filtered through cotton to remove Florisil particles, evaporated to dryness, redissolved in 4 ml of 0.01 N HCl and stored quick-frozen in liquid nitrogen. About 90% of the radioactivity was shown to co-migrate with unlabelled MAL in three different

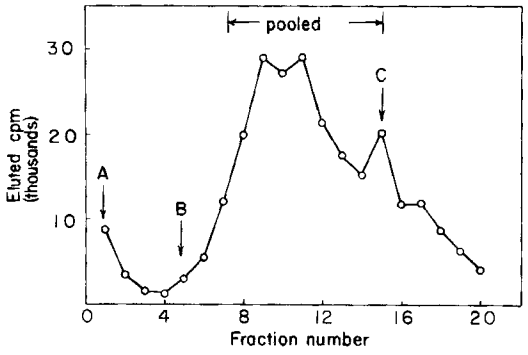

Figure 2. Tritiated material (3 x 10^{10} cpm) received from New England Nuclear was processed as described in Experimental. Essentially all radioactivity was extractable from alkaline solution with methylene chloride. A good part of the material was lost due to absorption to magnesium sulfate used as a drying agent (1.9 x 10^{10} cpm). The remainder was applied to a 5 cm Florisil column in a Pasteur pipette and eluted with the following mixtures of methylene chloride/methanol applied as indicated by the arrows: A, 10:1 mix; B, 2:1 mix; C, 1:2 mix. Fractions of 3.8 ml were collected and 10 µl of a 1:100 dilution of each fraction counted. Recovery of applied material was complete. The indicated fractions were pooled as [^{3}H]-MAL.

TLC systems: 1) Gelman ITLC plates type SG eluted with acetone/methanol 10:0.4 (R_f 0.28); 2) Gelman ITLC plates type SG eluted with methylene chloride/methanol 10:0.7 (R_f 0.26); and 3) Gelman ITLC plates type SAF eluted with methanol/water/ammonium hydroxide 10:1:0.4 (R_f 0.56).

Bioassay of MAL activity. MAL compounds were dissolved in an artificial perienteric salts solutions and tested for their ability to cause muscle contraction as described by Lewis, Wu, Levine, and Berg (4). The time required for a test solution to contract cut wild type C. elegans was compared to the time required for an authentic MAL standard.

Assay of specific [^{3}H]-MAL binding activity in total extracts of C. elegans. The specific [^{3}H]-MAL binding activity present in extracts of the nematode C. elegans and the ability of authentic unlabelled MAL to inhibit this binding activity were determined by Lewis and Fleming (8) and specific examples of this work are shown in this paper to substantiate the radiochemical characteristics and the biological activity of the [^{3}H]-MAL synthesized.

RESULTS AND DISCUSSION

Synthesis of L[-]2,3,5,6-tetrahydro-6-(5-amino-2-iodophenyl)imidazo [2,1-b]-thiazole. Our results show that it is easy to convert MAL to an iodinated precursor which then can be tritiated to high specific radioactivity while maintaining biological activity. The only unsatisfactory aspect of our purification scheme is the use of ethyl acetate as a solvent in column chromatography. It was originally chosen because it afforded a gradual, well-controlled elution of the iodinated MAL compounds and MAL from Florisil columns. However, ethyl acetate appears to react with MAL compounds, especially during rotary evaporation. The use of mixtures of methylene chloride and methanol for column chromatography is only a passable substitution as MAL compounds are then eluted much more rapidly and in a much less controllable way from Florisil.

In our reaction scheme, a moderate amount of the diiodinated product IIb is formed. This might be used to synthesize tritiated MAL of even higher specific radioactivity. For our use of [^{3}H]-MAL in receptor binding assays, however, the principal limitation is the background of nonspecific binding, which would only rise accordingly with ligand of higher specific radioactivity. If it was desirable to synthesize the diiodinated MAL compound in larger quantity, one would probably want to conduct the iodination reaction in an organic solution such as acetic acid as the limited solubility of the monoiodinated product in aqueous solution would probably hinder further reaction.

Catalytic dehalogenation of L[-]2,3,5,6-tetrahydro-6-(5-amino-2-iodophenyl)-imidazo [2,1-b]thiazole. The feasibility of dehalogenation over 10% palladium-carbon in a hydrogen atmosphere was investigated for several solvent/base combinations. Of the methods tried, reaction in methanolic potassium hydroxide appeared to convert almost all the iodinated starting material to MAL after only 2 hours of reaction at room temperature. Reaction of the iodinated MAL compound in dioxane 0.14 M in triethylamine or dimethylformamide 1.4 M in triethylamine for 16 hours or more produced only a poor conversion to MAL and a tarry by-product.

Catalytic dehalogenation of the monoiodinated MAL compound with deuterium gas in methanolic potassium hydroxide solution showed the reaction could proceed without substantial incorporation of protons from the solvent. The deuterium reaction provides confirmation of our indirect calculation of a high specific radioactivity for the tritiation reaction. Both the dehalogenation with deuterium and that with hydrogen are likely to have yielded MAL of much higher purity if we had avoided the use of ethyl acetate in purifying the reaction products over Florisil columns.

Synthesis of [^{3}H]-MAL. Catalytic dehalogenation of the monoiodinated MAL compound in the presence of tritium produced a large amount of radioactive product. Because the reaction workup leaves the product in the presence of a

large amount of inorganic salt, direct determination of specific radioactivity was not easily possible. The yield of tritiated material (22% of theoretical) is lower than we expected and might be attributable to our decision to have the reaction mixture acidified prior to the filtration step for removal of the catalyst. We thought the acidified compound would stand up to the series of evaporations required to remove labile tritium better than the material in alkaline solution but some of the protonated product may have been absorbed to the catalyst.

From the reaction mixture, [^{3}H]-MAL can be purified that is about 90% radiochemically pure as judged by TLC in three solvent systems. Several improvements are possible. A higher yield is obtainable in drying the methylene chloride extract by more sparing use of magnesium sulfate. By washing the Florisil column with several more fractions of methylene chloride/methanol 10:1 before switching to a 2:1 mixture of these solvents and then by taking only the peak most fractions of radioactivity, [^{3}H]-MAL can be obtained that gives a four times lower nonspecific background in [^{3}H]-MAL receptor binding assays. Perhaps by using starting material (IIa) purified without the use of ethyl acetate even higher quality [^{3}H]-MAL may be obtainable.

Biological activity and specific radioactivity of [^{3}H]-MAL. Our purified [^{3}H]-MAL has the appropriate high biological activity expected of a nearly radiochemically pure product maximally substituted with one tritium atom (Table 3). [^{3}H]-MAL made up to 0.1 μm concentration on the assumption of maximum specific radioactivity (29 Ci/mmole) has the same biological activity as an 0.1 μm solution of unlabelled MAL. If [^{3}H]-MAL had a lower specific radioactivity, it should contract cut worms proportionally faster, i.e. if [^{3}H]-MAL were really 15 Ci/mmole, it should contract worms at the rate that an 0.2 μm solution of authentic MAL does.

The specific radioactivity of [^{3}H]-MAL was calculated by comparing the affinity of [^{3}H]-MAL to the competitive inhibiting power of unlabelled MAL in a

specific [^{3}H]-MAL receptor binding assay using nematode extract (Figures 3 and 4). The authenticating details of this assay will be described by Lewis and Fleming (8). Unlabelled MAL has an IC_{50} value of 49 ± 11 nm for inhibiting the specific [^{3}H]-MAL binding observed at a concentration of 69,200 dpm of [^{3}H]-MAL per 100 µl. By assuming the chemical potency of [^{3}H]-MAL to be the same as that for unlabelled MAL (reasonable based on the bioassay results) and by using the parameters K_a and n derived from the BMDP3R fit to a Hill equation for several data sets such as the one shown in Figure 3, one can calculate I, the effective concentration of unlabelled MAL, in units of [^{3}H]-MAL dpm that would be needed to reduce the observed binding to 50% of its value when no unlabelled MAL is present. Expressing these conditions in terms of a Hill equation

$$B_{max}K_aL^n/(1 + K_aL^n + K_aI^n) = 1/2\ B_{max}K_aL^n/(1 + K_aL^n)$$

which reduces to

$$I = 10^{\log[1/K_a + L^n]/n}$$

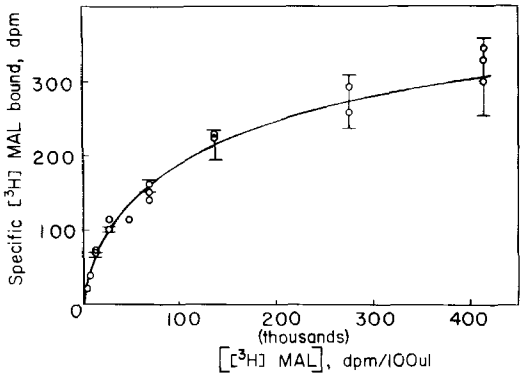

Figure 3. Specific binding of [^{3}H]-MAL to nematode tissue as a function of [^{3}H]-MAL concentration. Five times frozen and thawed extract made from predominantly first stage larvae, 1 mg extract protein per determination, was incubated on ice from 70 to 240 minutes with the indicated amounts of [^{3}H]-MAL in a 100 µl reaction volume as described by Lewis and Fleming (8). The specific [^{3}H]-MAL binding observed represents the mean difference between quadruplicate determinations of total and nonspecific [^{3}H]-MAL binding. The standard error of the difference is shown by the error bars. Nonspecific binding was linear with increasing [^{3}H]-MAL concentration and was 1416 dpm for the maximum [^{3}H]-MAL concentration assayed (415,431 dpm per 100 µl). The fitted curve for a Hill equation was obtained through weighted nonlinear regression using BMDP3R (14). The Hill parameters are Bmax = 500 ± 157 dpm, Ka = 2.5 ± 1.1 x 10^{-4} dpm/100 µl, and n = 0.68 ± 0.09 for the data as plotted.

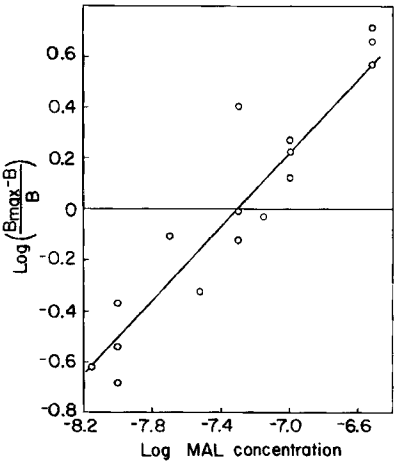

Figure 4. Hill plot of the displacement of specific [^{3}H]-MAL binding by unlabelled MAL. Five times frozen and thawed extract was incubated for 70 minutes on ice with 69,200 dpm of [^{3}H]-MAL and total binding was determined in the presence of the indicated concentrations of unlabelled MAL. Specific [^{3}H]-MAL binding was calculated by subtracting nonspecific binding measured in the presence of 10 μm unlabelled MAL only. B is the amount of specific [^{3}H]-MAL binding observed at a given drug concentration; Bmax, the specific [^{3}H]-MAL binding with no unlabelled MAL added to the total reaction mixture. The data were analyzed by weighted linear regression. The IC_{50} concentration of unlabelled MAL was calculated to be 49 ± 11 nm and the Hill coefficient, 0.73 ± 0.09.

L is the concentration of [^{3}H]-MAL in dpm units used in the competitive inhibition experiment. Substituting in values of K_a and n representing the best fit values from four independent direct binding experiments such as shown in Figure 4 provides four independent determinations of the potency of unlabelled MAL in equivalent [^{3}H]-MAL radioactive concentrations units with a mean value for I of 328,000 ± 23,000 (SEM) dpm per 100 ul. Correcting this value for 90% radiochemical purity and setting it equal to the IC_{50} value of unlabelled MAL in chemical concentration units yields a specific radioactivity for [^{3}H]-MAL of 27 Ci/mmole. Casting a simultaneous 95% confidence limit on the errors in the determinations of the equivalent radioactive and chemical concentrations sets a lower limit of 11 Ci/mmole on the specific radioactivity of [^{3}H]-MAL. From the agreement between the bioassay and the binding assay results, it appears that the specific radioactivity of [^{3}H]-MAL is probably greater than 15 Ci/mmole and may be close to the carrier-free value of 29 Ci/mmole. The dehalogenation reaction

with deuterium supports the conclusion of a high specific radioactivity. Carrier-free incorporation of tritium has also been reported under the same reaction conditions for iodotyrosine (13).

In a related paper, we will provide more detailed evidence that [^{3}H]-MAL detects the authentic levamisole receptor in our binding assay (8). Our evidence includes the demonstration that several mutants selected for their lack of response to levamisole are deficient in specific [^{3}H]-MAL binding activity. Altogether mutations in about 12 different genes confer resistance to the anthelmintic levamisole. The ability to synthesize [^{3}H]-MAL of high specific radioactivity will make it possible to determine which of these mutations directly affect levamisole receptor function. The availability of [^{3}H]-MAL thus will allow a combined genetic and biochemical analysis of receptor function that is not possible in most other receptor systems. The existence of [^{3}H]-MAL should also permit direct and rapid determination of structure-activity relationships for levamisole and related anthelmintics. There have been a number of investigations into the actions of levamisole on the vertebrate immune system (9-11). [^{3}H]-MAL might be of some use to researchers in this area, although the relative potency of levamisole compounds on the immune system appears to be far below its level of toxicity to nematodes.

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