11-18-82

005778

OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

TO: Henry Jacoby, PM 21
Registration Division (TS-767C)

THRU: Christine F. Chaisson Ph.D.
Toxicology Branch (TS-769)

SUBJECT: Metalaxyl, 100-607/2500. Submission of Additional Data: Mouse oncogenesis, mutagencity studies and 90 day smoke inhalation study.

Request

The following studies were received by the Agency with a letters dated June 10, 1982 and August 10, 1982 submitted by Dr. Gene Holt, Senior Regulatory Specialist, Government Affair of the Ciba-Geigy Corporation. No reference was made to a specific registration request.

Conclusion.

1) The mouse oncogenicity study review can not be completed because of missing data. The evaluation of the study will be completed when the missing data is supplied.

2) Two of the mutagenicity studies were unacceptable and the others can be acceptable if information requested by Dr. John Chen (Memo to David Ritter, dated (October 25, 1982) of this Branch can be supplied.

3) The results of the 90 day subchronic inhalation study suggested that the animals were not exposed to sufficient quantities of tobacco smoke necessary to simulate smoke exposure in humans. This information was transmitted by phone to Dr. Ada Kung of the Company in regards to this submission and a submitted protocol for smoke inhalation study with Prime Plus.

The review of the individual studies is attatched.

SBG 11/18/82

Stanley B. Gross, Toxicologist
Toxicology Branch
Hazard Evaluation Division (TS-769C)

TS-769C:SGross:CM#2:Rm 824:557-1511: 11/18/82

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2. CGA 48 988 : Oncogenicity in Dietary Administration to Mice for Two Years.

The study carried out by Life Science Research, England, for Ciba-Geigy. Report 80/CIA008/442, June 17, 1981.

Groups of mice of the ICI Alderley Park strain (Swiss strain origin) were subjected to diets containing technical metalaxy at levels of 50, 250 and 1250 ppm for two years. The animals were observed daily for clinical signs while growth and body weights, food consumption, and observations for the occurance of tumor masses were carried out weekly. Based on the information provided in the report, no differences were detected between the control animals and any of the animals on the treatement regimens.

The experiment was well executed and well documented except for the summary tabulations of the pathological findings--Tables 12 to 15, parts A to C inclusive. These tables compare separately the pathological findings for the controls vs group #4 (1250 ppm) for weeks 0 to 52, weeks 53 to 104, and over 104 weeks; and similar data for groups #2 (50 ppm) and #3 (250 ppm) for the different time periods. All of these table list the number of animals examined, however, they do not provide information on the number of individual tissues examined such that one could determine the percentage of tissues that had pathological lesions. The only indicated the number of animals examined. Nor is it possible to readily extract this information from elsewhere in the report-- the tables on individual animal findings also did not contain this information.

Dr. Louis Kasza, the Branch's pathologist, advises that reviewers cannot assume that the number of animals examined (as shown in the summary tables) represents the number of organs examined. He suggests that the data be tabulated in a manner similar to Tables I and II attached. Table I (Summary Incidence Table) includes the tabulation of numbers of organs examined along with the pathological conditions. Including the data from all of the treatment groups in one table, allows easier comparisons between the various groups. This table indicates the type of death (scheduled sacrifice vs moribund sacrifices and found dead animals) and allows the comparison of the total numbers of tissues to be compared to the number of animals which were originally in each of the test groups. Table II (Histopathology Incidence Table) groups the animals by sex and treatment group with similar pathology and provides a cross reference by number to the individual animal. This table also allows one to evaluate the severity of the pathological changes which might be different for the different treatment groups.

Also missing from the report is information on historical controls for the tumor incidence by tissue for this strain of animal. This information should include recent control data for this strain for the laboratory-- Life Science Reserch-- and

SUMMARY INCIDENCE TABLE

Environmental Protection Agency

Female Mice

	Group I			Group II			Group III			Group IV		
	Scheduled Sacrifice	Moribund Sacrifice & Deaths	Total	Scheduled Sacrifice	Moribund Sacrifice & Deaths	Total	Scheduled Sacrifice	Moribund Sacrifice & Deaths	Total	Scheduled Sacrifice	Moribund Sacrifice & Deaths	Total
LIVER (NO. EXAMINED)	(22)	(52)	(74)	(34)	(42)	(76)	(24)	(52)	(76)	(22)	(53)	(75
Hepatocellular Carcinoma		4	4	2	1	3	2	1	3	1	1	2
Hepatocellular Adenoma*	2/2	1/1	3/3	1/1	4/3	5/4	30/12	24/11	54/23	54/17	21/12	7...
Malignant Lymphoma	3	9	12	2	7	9	1	7	8		6	6
Granulocytic Leukemia		1	1								1	1
Angiosarcoma								2	2		1	1
Carcinoma, Metastatic											1	1
Sarcoma, Metastatic		1	1		1	1						
Reticulum Cell Sarcoma				1		1		1	1			
Hepatocholangiocarcinoma												
Multifocal Hepatocellular Degeneration		1	1				1	2	3		3	3
Basophilic Foci				1		1				1	2	
Mononuclear Cell Infiltration	6	9	15	9	1	10	9	2	11	8	8	16
Foci of Mononuclear Cells				1		1					1	1
Angiectasis	1		1	2	1	3		1	1	2	3	5
Focus of Cellular Change				3		3				1		1
Multifocal Hepatitis	16	10	26	23	4	27	13	7	20	14	9	23
Multifocal Necrosis	2	6	8	3	8	11	1	6	7	2	6	8

*Number of neoplasms/number of animals with neoplasms.

Environmental Protection Agency

Female Mice

Group IV - Sacrificed

ANIMAL NUMBER	800	807	815	817	818	819	822	824	828	831	833	834	836	844	845	856	857	860	862	863	865	556					
LIVER																											
Hepatocellular Carcinoma																						P					
Hepatocellular Adenoma (No. Present)	1	6	2	5	1	4	5		4		1	5		2	7	1		1	1		4	4					
Malignant Lymphoma																											
Granulocytic Leukemia																											
Angiosarcoma																											
Carcinoma, Metastatic																											
Sarcoma, Metastatic																											
Reticulum Cell Sarcoma																											
Hepatocholangiocarcinoma																											
Multifocal Hepatocellular Degeneration																											
Basophilic Foci			2																								
Mononuclear Cell Infiltration	1					2					3		1	1		2	1			1							
Foci of Mononuclear Cells																											
Angiectasis						2								3													
Focus of Cellular Change															3												
Multifocal Hepatitis	2	2	4		3	3	3	1		3		2	2	2			3		2			4					
Multifocal Necrosis												2										2					

Key: P = Present, N = No Section, A = Autolysis, X = Not Remarkable
1 = Minimal, 2 = Slight, 3 = Moderate, 4 = Moderately Severe/High
5 = Severe/High, I = Incomplete Section

control data provided in the literature.

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The evalution of this study can be completed when the Registrant provides the requested information.

2. Test for Non-Disjunction in Saccharomyces cerevisiae D 61 with CGA 48 988. (Test for Mutagenic Properties in Yeast).

Study carried out by Ciba-Geigy Limited, Basle, Switzerland. # 79/1891, April 10, 1980.

Organisms of S. cervisiae, strain D 61 were incubated with technical CGA-48-988 (?% purity) at concentrations of 40, 200 and 1000 ug/ml suspended in DMSO to determine possible non-dysjunction in yeast cells. The test involves counting the number of monosomic colonies that develop resistance to cyclohexamide as a result of exposure to the test substance. Monosomic conversion is detectable by the number of white colonies which would otherwise grow as red colonies. 4-nitroquinoline-N-oxide was used as a positive control.

The results of the test are shown in Table 1 (taken from page 835 of the submission). The increase in monosomic colonies compared to the positive controls were not striking nor dose dependent compared to the positive controls. This test could be improved by increasing the concentration of the test substance to produce more toxicity similar to that achieved by the positive controls. Metabolic activation was not tested nor did the report include data on the variability of the results. According to Dr. Irving Mauer, geneticist in this Branch, the D 61 strain of this organism is not typically used for this test.

Discussion. See Evaluation of Report 2 by Dr. Chen attached to the end of this Reviewer's comments. The study needs additional information to be acceptable.

3. Saccharomyces cerevisiae D7/Mammalian-Microsome Mutagenicity Test in Vitro with CGA-48-988 (Test for Mutagenicity Properties in Yeast Cells).

This study was carried out by Ciba-Geigy, Report Number 822561, January 18, 1982.

Organisms of the S. cerevisiae D 7 strain were incubated with technical CGA-48-988 (%AI?) suspended in DMSO at concentrations ranging from 400 to 10000 ug/ml to test for mitotic cross over (shown by the development of pink to red colonies); mitotic gene conversion (expressed as loss of tryptophane requirement for growth); and reverse mutation (as shown by the loss of the requirement of isoleucine for growth). 4-nitroquinoline-N-oxide was used as a positive control without activation and cyclophosphamide monohydrate was used as a positive control with S-9 rat liver microsomal activation.

METALAXYL

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____ Description of the product manufacturing process.

____ Description of quality control procedures.

____ Identity of the source of product ingredients.

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____ A draft product label.

____ The product confidential statement of formula.

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The results are shown in Tables 1 to 7 taken from pp 845 through 848 of the submission. The initial tests involving concentrations of metalaxyl at 400 to 10,000 ug/ml were repeated using concentrations of 2000 to 8000 ug/ml because the 10000 ug/ml concentration produced excessive toxicity. Statistical analyses indicated that none of the exposures to metalaxy produced results which were statistically significantly different from those of the vehicle controls. The positive controls produced the significant effects as expected.

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Discussion. See the attached Report 3 by Dr. Chen. This study may be acceptable when information requested by Dr. Chen is supplied.

4. Mutagenicity Test on Saccharomyces cerevisiae MP-1 In Vitro with CGA-48-988 (Test for Mutagenic Properties in Yeast Cells).

Study carried out by Ciba-Geigy, #79/1346, April 10, 1980.

MP-1 strain of S. cerevisiae was tested with technical CGA-48-988 (?% AI) (solvent used?) without activation to determine possible intergenic recombinants (mitotic cross-over) recognizable by their adenine deficiency; possible intragenic recombinants (mitotic gene conversions) determined by tryptophane prototrophy and forward mutation determined by their resistance to cycloheximide. 4-nitroquinoline-N-oxide was used as a positive control.

The results are shown in Table 1 taken from page 858 of the submission. The results are based on 10 plates/treatment however the variability of the results were not reported. The data were subjected to chi square analyses which showed no significant changes in the metalaxyl treatments from the solvent controls. The positive controls which showed significant increases for intergenic and intragenic recombinants but not for the forward mutation (cyloheximide-resistance data from the Table).

Discussion See Report 4 by Dr. Chen. Not acceptable.

5. Nucleus Anomaly Test in Somatic Interphase Nuclei. CGA-48-988. Chinese Hamster. (Test for Mutagenic Effects on Bone Marrow Cells).

Study carried out by Ciba-Geigy, No. 78-3007, June 18, 1979.

Chinese hamsters (3 per sex per treatment group) were administered orally technical CGA-48-988 (%AI?) in 0.7%

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carboxy methyl cellulose daily for two days at levels of 595, 1190 and 2380 mg/kg. Twenty-four hours after the second treatment, the animals were sacrificed and the marrow of both femurs was removed by aspiration and fixed and stained on microscopic slides and evaluated for nuclear anomalies including a) single Jolly bodies, b) fragments of of nuclei in erythrocytes, c) micronuclei in erythroblasts, d) micronuclei in leucopoietic cells, and e) polyploid cells. Cyclophosphamide was administered at 128 mg/kg as a positive control and 0.7% CMC as a solvent control. The results are shown in Table 1 (taken from page 865 of the data submission). The percents of the anomalies (based on examining 1000 bone marrow cells within the treatment groups were essentally the same as those of the vehicle controls while the cylcophosphamide produced the expected increase in anomalies.

Note-- The oral LD50=669 mg/kg for the rat based on previous submissions. This report does not indicate any observed toxicity in the hamsters at the highest doses of 2380 mg/kg. It might also be noted that this test is usually carried out using the mouse instead of hamster (according to Dr. Irvin Mauer, of this Branch).

Discussion See Report 5 by Dr. Chen. Study not acceptable.

6. L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenicity Test. CGA-48-988. (In vitro test for mutagenic Properties of Chemical Substances in Mamalian Cells).

Study carried out by Ciba-Geigy. No 811258, February 1, 1982.

Technical CGA-48-988 (%AI?) at concentrations ranging from 0.0625 to 1.0 mg/ml in DMSO was used with mouse lymphoma cells of the L5178Y/TK(+/-) strain (source unspecified) to test for possible forward point mutation demonstrated by the ability of the cells which are insensitive to 5-bromodeoxyuridine. Cells from the stock culture were cleansed of spontaneous TK(-/-) mutants by incubating for 24 hours with a mixture of thymidine, hypoxanthine, methotrexate and glycine. A preliminary test was carried out with and without the addition of S-9 liver fraction to determine the levels of CGA-48-988 which produce the appropriate amount of toxicity-- a 10% reduction in cell population compared to the negative or solvent (DMSO) control. Ethyl methylene sulfonate (0.5 ul/ml) was used as a positive control without activation and dimentyl nitrosamine (0.5 ul/ml) was used as a positive control with activiation.

Discussion. See Report 5 by Dr. Chen. Study may be acceptable with the submission of additional information per Dr. Chen's comments.

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7. Autoradiographic DNA Repair Test on Rat Hepatocytes. CGA-49-988 (In Vitro Test for DNA-D aging Properties).

Study performed by Ciba-Geigy, No. 811259, January 19, 1982. 005778

An initial toxicity assay was carried out by culturing hepatocytes taken from male rat (strain not specified) with levels of metalaxyl ranging from 1 ug/ml to 2000 ug/ml and determining viability of the cells by vital staining. Using cells from a second rat and the concentrations metalaxyl shown in Table 1 (taken from page 885 of the submission), the DNA assay was carried out. 4-nitroquinoline-N-oxide and dimethyl nitrosame were used as positive controls. After being cultured overnight, the cells were incubated with tritiated thymidine and incubated for 5 hours. The cells were then stained with haematoxylin-eosin and allowed to develop autoradiagraphs over a 6 day period. From the results shown in the table, the radiactivity from metalaxyl treated cells was comparable to the solvent and negative controls while the positive controls showed the expected increased incorporation of the thymidine.

Discussion. See comments by Dr. Chen, Report 7. Study acceptable if additional information can be supplied.

8. Subchronic Inhalation Study in Rats. CGA-48988 Treated Cigarettes. Final Report.

Study performed for Ciba-Geigy by Hazleton Laboratories America, No. 483-202, January 18, 1982.

Exposure Methods. Four groups of Fisher 344 rats (10/sex/group) were exposed to the smoke of cigarettes (Class A, "standardized") made with tobacco spiked with CGA-48-988 as follows:

Group	1	Control	0 ppm
Group	2	Low dose	130 ppm
Group	3	Mid dose	3900 ppm
Group	4	High Dose	13,000 ppm

The animals were exposed for approximately four hours per day, 5 days per week for 13 weeks. The exposure method involved the use of an ADL/Mark II (A.D. Little, Inc.) piston-type of smoking machines which delivered 35-ml (nominal), two-second puffs of cigarette smoke at 60 second intervals into 38 liter cylindrical chambers containing the test animals.

The 35 ml puff of smoke was theoretically distributed within the chamber by turbulance (with the aid of air at 2 L/min flow according to Dr. Wm. Coate of Hazleton Laboratories, verbal communication) and remained in the chamber in a static condition for 20 seconds. The chamber was then purged with compressed air for 36 seconds at a rate of 50 L/min theoretically reducing the concentration of the smoke in the chamber by 75% (T_{75} for

clearance). This cycle was continued 6-9 times until the butt length of the cigarette was approximately 20 mm for each cigarette. The chambers were purged continously for 6 minutes between cigarettes. The animals were exposed to the smoke of 18 cigarettes was delivered in this manner during each 252-minute exposure.

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Particulate and vapor phase smoke samples were obtained weekly. Sampling was carried out during the 20 second static phase after each puff. Particulate samples were caught on Gelman 25 mm thick glass fibers and weighed under standardized drying conditions. These were collected from the second puff to the next to the last puff and the particulate materials used to determine TPM (Total Particulate Matter). Smoke residues were also determined from the particulate material caught on these filters using a gas chromatography method supplied by the Company. Chamber CO concentrations were measure after the first and last puff using samples which were passed through the Gelman fiber filters. The Co was analyzed using a Bechman Medical Co. Infrared CO Analyzer.

Prior to the initiation of the study, five groups of two animals/sex/group were exposed for a single four hour period to air only, non-spiked cigarettes and to cigarettes spiked with CGA-48-988 at concentration levels used in the study in order to characterize the plasma nicotine levels expected in 90 day study.

The exposure animals were observed daily and weighed weekly. At the end of the study the animals were sacrificed and the usual clininal laboratory and pathological studies carried out.

Results. There were no compound related effects. Except

TABLE
Summarizes means for the chamber TPM, CGA-48-988 residues, CO and ranges for plasma nicotine.

Group/CGA Spike (ppm)	TPM (ug/L)	Chamber CGA (ug/L)	Chamber CO (ppm)	Plasma Nicotine (Pretest only) (ug/L)
Air (pretest only)		-	-	0.13-6.23
1/ Unspiked	25	0	41	3.88-18.12
2/ 130	51	0	43	6.83-20.11
3/ 3900	51.5	1	49	2.60-12.88
4/ 13000	60.4	5	48	6.70-19.77

for a few animals which developed eye irritation there were no signs of toxicity. There were no differences in body weights, food comsumption, hematology, blood chemistry, organ weights, or pathological findings which could be related to the test material. The above TABLE lists means for the chamber TPM, CGA-48-988 residues and the ranges for the pretest plasma nicotine levels. The CO measurements are based on samples taken at the end last puff of the cigarette-- no CO was detectable after the first puff. Rather than the means, the ranges of plasma nicotine are presented in order to show the variability of the determinations and the overlapping values between groups.

Discussion. The following discussion will attempt to supply a rationale for rejecting the findings of this study and will point out short-comings in the protocol used. Because the Toxicology Branch has had little experience with this type of inhalation study and because this Reviewer recently approved a similar smoking inhalation protocol (on theoretical grounds without the benefit of the data from the present study), this discussion will provide some detail in order to anticipate some questions the Company may raise.

The purpose of the smoke inhalation study is to attempt to evaluate the potential pharmacological effects of pesticide residues in cigarettes made from tobacco treated with the pesticide. This study is intended to simulate exposures to humans by exposing animals to smoke from cigarettes treated at levels of pesticide corresponding to those expected in cigarettes and at levels considerably higher (5 to 20 times) in an effort to demonstrate that there will be little chance that the use of the pesticide on the tabacco will cause toxicity in excess of that inherent in the use of the tobacco without the pesticide.

A protocl similar to the one used here was recently approved by this Reviewer recently (Memo of June 3, 1982 from Gross to Taylor, Prime Plus (CGA-41065). Protocols for Mutagenicity and Smoke Inhalation Test. 100-EUP-72. Caswell 794C.) The approval of the protocol was based on discussions with Dr. Coate of Hazleton Laboratories, Drs. Gene Holt and Ada Kung of Ciba-Geigy and on the calculations that the animals would theoretically be breathing more smoke in 4 hours of exposure than would man smoking 40 cigarettes a day (heavy smoker) over a period of 8 to 12 hours even though the smoke the animals would receive would be highly diluted. This was based on the following type of calculations:

Assuming 1 ppm of pesticide residues in cigarette tobacco provides 1 ug/cigarette of pesticide residues;

35 ml/puff, 8 puffs/cigarette and 100% transfer of the residue to the body;

For Man: A 60 kg man of 60 kg smoking 40 cigarettes/day would received 40 ug/60 kg or 0.7 ug/kg/day.

For the Rat: a 35 ml puff into 38000 ml chamber is diluted 38000/35= 1086 times producing 1 ug/ 1086 ml of air for each cigarette. The rat breathing smoke 20 seconds our of each minute at an estimated 150 ml/min tidal volume and breathing smoke from 18 cigarettes/ 4 hour run.

Thus (1 ug/ 1086 ml air) x (200 ml/min x 1 min/puff x8 puffs) x (18 cig/ 4 hr) = 6.67 ug/day or 22.22 ug/kg/day for 0.3 kg rat.

Rat/Man: The rat was calculated to receive 31.7 times that of the man on a ug/kg/day basis.

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However, on a concentration basis:

35 ml smoke into 500 ml tidal volume for man is 500/35= 14.3 fold dilution of smoke while for the rat, 35 ml puff into a 38000 ml chamber was a 1086 fold dilution. The smoke received by the rat was 76 times more dilute than that of man.

(Note: In some of the calculations used by Dr. Coate he included in his calculations the following:
40% of the smoke is contained in the mainstream (therefore 60% of the smoke containing any pesticide would be lost to the side stream)
65% of the smoke gets through a filter (35% remains in the filter or unsmoked cigarette.
90% of the cigarette is treated with pesticide(basis?)
80% of the cigarette is smoked (smoked until the butt is 20 mm long)
Based on this only about 19% of the tobacco of a cigarette ends up being inhaled. Not all of this is deposited in the pulmonary tract or oral cavity (%?). The source of this information was not cited.)

The above considerations formed the bases of the approval of the Prime plus smoke inhalation protocol requested by Ciba-Geigy.

When the present study was received, it raised a number of questions which were posed to a number of investigators who have experience with smoke inhalation studies in animals. The Reviewer was also able to review data from a second smoke inhalation study carried out by Hazleton Laboratories in which the cigarette smoke was administered to lungs of dogs through tracheostomy openings. These analyses and discussions lead to the conclusion that the present study does not provide a sufficient exposure to cigarette smoke to assess the possible pharmacological effects sought:

1) Lack of Behavior Responses. Based on the report and conversations with Dr. Coate, the animals when smoke is introduced into the chambers showed no behavior responses at all. Animals in other smoking studies (like humans) have to develop a tolerance to smoking and this apparently is achieved by gradually increasing the smoke exposure before starting the testing periods.

2) Chamber CO Levels Cigarette smoke contains from 2.7 to 6% CO (p 21 of "The Health Consequences of Smoking. A Report of the Surgeon General: 1972". DHEW Pub. no (HSM) 72-7516) which corresponds to 27,000 and 60,000 ppm of CO. According to the report, no chamber CO levels were uniformly detectable after the first puff. This suggests the lack of good distribution of smoke within the chamber. The between puff

purges clears a theoretical 75% from the chamber and allows the CO to accumulate to about 50 ppm at the end of 6-8 puff/cigarette which indicates that the concentration of CO increased to the theoretical 1000th dilution level by the end of the cigarette exposure rather than presenting smoke at a uniform during the exposure cycles.

3) Low Plasma Nicotine Levels. Plasma nicotine levels as determined here are about 1/1000th that seen in the control dogs used in the study mentioned above. In addition, the pretest exposure studies had nicotine levels which were less than some of the air control exposures. Discussions with Drs. Paul Nettescheim of NIEHS and Walley Dalbey and Roger Jenkins of ONRL indicated that these levels are quite low and that nicotine determiniations are not good indicators of smoke exposure for two key reasons-- nicotine is rapidly metabolized and the analytical determinations are difficult to carry out accurately. It seems that carboxy hemoglobin (COHb) determinations are a more suitable measure of smoke inhalation because the COHb is more stable metabolically.

Suggested Changes. Based of these observations, this Reviewer contacted Dr. Ada Kung of Ciba-Geigy on 6/28/82 to indicate that the results of this study raised questions on the suitability of this type of protocol for smoke inhalation studies. I suggested that they consider increasing the number of cigarettes used with the present equipment or consider using chambers similar to those used at ORNL at Oak Ridge (W.L. Maddox et al., "A tobacco smoke inhalation device for rodents", Archives of Environmental Health pages 64-71, March/April, 1978) which use 350 ml nose only chambers which allow the animals to breath smoke diluted to 10%. I suggested that they should attempt to exposure the animals to the point that the COHb reaches 5-10% which corresponds to the levels experienced by heavy smokers (Table 4 of page 23 of "The Health Consequences of Smoking. A Report of the Surgeon General: 1972. DHEW Publication No. (HSM) 72-7516). According to Drs. Dalbey and Jenkins, rats can tolerate COHb levels of 45% and the 5-10% COHb would help to indicate that the animals were being exposed to an adequate amount of smoke for the purposes of these studies.

It might be helpful to get together with Dr. Jenkins and others with experience in this area. Based on my conversation with Dr. Jenkins, he has for several years served as a consultant in this area of research.

purges clears a theoretical 75% from the chamber and as reported, allows the CO to accumulate to approximately 50 ppm at the end of 6-8 puff/ cigarette which indicates that the concentration of CO increased to the theoretical 1000th dilution level by the end of the cigarette exposure rather than presenting smoke at a uniform exposure level during the exposure cycles.

3) Low Plasma Nicotine Levels. Plasma nicotine levels as determined here are about 1/1000th that seen in the control dogs used in the study mentioned above. In addition, the pretest exposure studies had nicotine levels which were less than some of the air control exposures. Discussions with Drs. Paul Nettescheim of NIEHS and Walley Dalbey and Roger Jenkins of ONRL indicated that these levels are quite low and that nicotine determiniations are not good indicators of smoke exposure for two key reasons-- nicotine is rapidly metabolized and the analytical determinations are difficult to carry out accurately. Carboxy hemoglobin (COHb) determinations are a more suitable measure of smoke inhalation because the COHb is more stable metabolically.

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It might be helpful to get together with Dr. Jenkins and others with experience in this area. Based on my conversation with Dr. Jenkins, he has for several years served as a consultant in this area of research.

ADDENDUM Additional data sent with a letter of August 10, 1982 has been received but does not seem to have any direct bearing on the problems with the study discussed above. Therefore, these additional data was not reviewed at this time.

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October 25, 1982

To: David Ritter, Acting Section Head
Review Section #1
Toxicology Branch/HED (TS-769)

From: John Chen, DVM
Review Section #1
Toxicology Branch/HED (TS-769)

Subj: Metalxyl Fungicide CGA 48 988 - Review and evaluation of Mutagenicity Studies for Registration

Review comments for the six submitted reports from CIBA-GEIGY Limited, Basel, England are attached.

Report 2: Test for Non-Disjunction on Saccharomuces cerevisiae D61 With CGA 48 988

Evaluation:

The assay used for the detection of non-disjunction in the yeast cells D61 as assessed by the presence of monosomic white colonies resistant to cycloheximide appears to follow the procedures developed by Parry and Zimmermann (1976). However, the evaluation of the clastogenic activity (produce changes in chromosome number) of the test agent, CGA 48 988 in the yeast cells D61 cannot be accomplished without supplemental information accompanying the report.

The following inadequacies in the reporting of this study were noted:

1. Source of the D61 strain of S. cerevisiae and the appropriate genotype and chromosome number in D61 must be identified.

2. The culturing techniques described in the report were inadequate. For obtaining the consistency of experimental results, yeast cell culture should be propagated to early stationary phase of growth from a single isolated colony.

3. No cytotoxicity study for the selection of three dose levels of the test agent was included. Ideally at least 4 exposure concentrations of the test agent should be selected from the cytotoxicity study.

4. No clear indication of the treatment conditions and the termination procedures were given. Since the genetic activity of yeast cells from the reaction of various mutagens are largely dependent on the pH treatment condition, the response of the test agent at pH 7.0 as well as around pH 4.5 should be included. The termination procedures by separating yeast cells from the test chemical immediately after treatment must be also presented.

5. The original data used for the summarized results in Table 1 for statistical analysis should be included. There is also no statistical analysis of data in the report.

Report 3: Saccharomyces cerevisiae D7/ Mammalian Microsome Muatgenicity Mutagenicity Test in Vitro With CGA 48 988

Evaluation:

The assay used for detecting the induction of mitotic crossing over, mitotic gene conversion, and reverse mutation of the test agent with and without the addition of metabolic activation from rat liver enzymes; appears to follow the procedures developed by Zimmermann (1975). However, the evaluation of the genetic activities of CGA 48 988 in the yeast cells D7 cannot be accomplished without supplemental information accompanying the report:

The following inadequacies in the reporting of this study were noted:

1. Source of the diploid strain D7 and the two haploid strains with the appropriate combination of alleles of which D7 was constructed must be identified..

2. the culturing techniques described in the report were inadequate. D7 should be propagated to early stationary phase of growth from a single isolated colony. Because of the unstable nature of the hetroallelic diploids, yeast cells should be determined the rate of spontaneous conversion prior to testing. Culture with a low content of convertant cells must be selected for the study.

3. No statements of justification were made to the selection of three dose levels of the test agent for the study. Ideally, at least 4 exposure concentrations of the test agent selected from cytotoxicity study should be used in the assay.

4. No clear indication of the treatment conditions and the termination procedures were given. Since the genetic activity of yeast cells from the reaction of various mutagens are largely dependent on the pH treatment condition, the response of the test agent at pH 7.0 as well as around pH 4.5 should be included. The termination procedures by spearating yeast cells from the test chemical immediately after treatment must be also presented.

5. The mitotic recombinants (the formation of cells with red and pink, white and pink, red and white sectored and other genetically altered colonies) formed from two different alleles of the gene locus ade 2 should be recorded. The number of convertants at the trp 5 locus on trytophan-free medium and the number of revertants from homoallelic reversion of the ilv1 gene on isoleucine-free medium should also be presented separately.

6. The original data used for the summarized results in Table 1, 2, & 3 for statistical analysis should be included. There is also no statistical analysis of data in the report.

Report 4: Mutagenicity Test on Sacchromyces cerevisiae MP-1 in Vitro With CGA 48 988

Evaluation:

The assay used for this study appears to be inadequate, not conducted according to the accepted procedures for yeast test, and hence, the results and their interpretations are unacceptable. The following inadequacies in performing the mutagenicity test on S. cerevisiae MP-1 were noted:

1. The results of this study derived from the value of only single experiment is unacceptable. At least duplicate experiments for each treatment study must be presented.

2. Since the cytotoxicity study of the test agent on MP-1 cells was not conducted, it is doubtful that the adequate exposure concentrations (upper or lower limits of the test agent) were selected for the study.

3. Because the correct response of the yeast cells MP-1 to 4-nitro-quinoline-N-oxide (5-15 ug/ml) was not indicated as expected in the forward mutation assay, it is questionable that the integrity of the yeast cell cultures was properly maintained in performing the assay.

4. Source of the multi-purpose MP-1 strain of S. cerevisiae and the appropriate combination of alleles and mating genotypes of which MP-1 was constructed were not identified.

5. The culturing techniques described in the report were inadequate. MP-1 should be propagated to early stationary phase of growth from a single isolated colony. Because of the unstable nature of the hetroacllelic diploids, yeast cells should be determined the rate of spontaneous conversion prior to testing. Culture with a low content of convertant cells must be selected for the study.

6. Since the genetic activity of yeast cells from the reaction of various mutagens are largely dependent on the pH treatment condition, the response of the test agent at PH 7.0 as well as around pH 4.5 should also be presented.

7. The original data used for the summerized results in Table 1 for analysis were not presented with the report. Results of the study should include the tests in the presence or absence of metabolic activation from rat liver enzymes.

Report 5: Nucleus Anomaly Test in Somatic Interphase Nuclei With CGA 48 988

Evaluation:

The assay procedure used for this study appears to be a modification of the mouse micronucleus test developed by Schmid (1975). Because the evaluation criteria in scoring the interphase nucleus anomalies from bone marrow cells and microsopic identification of various cell types of bone marrow were not clearly defined and established in the reporting of this study, and hence, the results and their interpretations are unacceptable. The following inadequacies in performing the assay were noted:

1. No established standard and criteria for scoring the interphase nuclei, fragments of nuclei in erythrocytes, micronuclei in erythroblasts, micronuclei in leucopoietic cells, single jolly bodies and polyploid cells were given. In bone marrow smears from mammals treated with chromosome-breaking agents, micronuclei can be found in numerous cell types provided that these cells have completed under the influence of the mutagen in one or a few mitosis. However, several critical disadvantages in scoring the nucleus anomaly from the various cell types of bone marrow should be considered: (a) Micronucleated cells are not as mitotically active as polychromatic erythrocytes; (b) The micronuclei in nucleated bone marrow cells such as erythroblasts, myelocytes, myeloblasts, and other leucopoietic cells are difficult to distinguish from normal nuclear lobes and projections under microscopic examination; (c) Artifacts can be formed by residues of stains and by basophilic granules of destroyed precursors of basophilic leucocytes.

2. Cytotoxicity study for the selection of maximum tolerance dose of the test agent was not included.

3. The sample sizes (number of animals as well as cell count) were inadequate for the study. At least five females and five male animals per experimental and control group should be used in order to control the high variability in frequencies of micronuclei.

4. In the repeated treatment schedule, sampling time was inadequate for this study. Samples of bone marrow should be taken at least three times starting not earlier than 12 hrs after treatment with appropriate intervals following the first sample but not extending beyond 72 hrs.

5. The percentage of polychromatic erythrocytes, normochromatic erythrocytes and micronucleated cells per group was not presented.

6. Although mentioned in the procedure sheet, there is no statistical analysis of the data (X^2 - test).

Report 6: L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenicity Test With CGA 48 988

Evaluation:

The assay used to evaluate the mutagenic activity of the test agent in the L5178Y/TK$^{+/-}$ mouse lymphoma cell line with and without the addition of the metabolic activation from rat liver enzymes, appears to follow the procedures developed by Clive (1975 & 1979). However, the evaluation of the mutagenicity of CGA 48 988 cannot be accomplished without the following supplemental information accompanying the report:

1. The results of study(in the absence of metabolic activation from rat liver enzymes)derived from the value of only single experiment is unacceptable. At least duplicate experiments for each treatment study should be presented.

2. No cytotoxicity study for the selection of dose levels of the test agent was included. In order to cover a range adequate to define the mutagenic response of the test agent in the L5178Y mouse lymphoma cell line (use of a thymidine kinase (TK) heterozygote variant of this line to assay for resistance to bromodeoxyuridine), the highest concentration tested should produce a low level of survival; survival in the lowest concentration tested should approximate survival in the negative control.

3. The described procedures for cell growth, cell maintenance, and preparation of metabolic activation system were not clear and must be clarified:

   A. The L5178Y mouse lymphoma cells should be maintained in Fischer's mouse leukemia medium supplemented with L-glutamin, sodium pyruvate and horse serum (10% by volume). Cloning medium should consist of the proceeding growth medium with the addition of agar to a final concentration of 0.35% to achieve a semisolid state. Selection medium should be cloning medium containing 0.005% of bromcdeoxyuridine (BrdU).

   B. Checking for mycoplasma contamination and karyotype stability of the cell line prior to testing should be included.

   C. The final concentration of the necessary co-factors such as NADP (sodium salt) and isocitric acid, and other ingredients for the 4-hr treatment for metabolic activation should be identified.

4. The original data used for the summerized results in Table 1, 2, & 3 for statisrical analysis must be included in the report.

Report 7: Autoradiographic DNA Repair Test on Rat Hepatocytes With CGA 48 988

Evaluation:

The assay used to measure unscheduled DNA synthesis (UDS) from the uptake of ^{3}H-TdR into the DNA of primary rat liver cells, appears to follow the UDS method described by Williams (1977). However, the evaluation of the DNA-damaging effects of the test agent in the UDS assay using autoradiographic techniques cannot be accomplished without the following supplemental information accompanying the report:

1. The species, strain and age of test animals used for this study were not identified. The historical background incorporation rates of ^{3}H-TdR into untreated hepatocytes of the test animals should be recorded in the report.

2. The described procedures for the preparation of primary rat hepatocytes were not clear and must be clarified:

   A. The rat liver primary cells should be cultured in Williams' medium E (WME) supplemented with 10% fetal bovine serum, 2 mM L-glutamin, and 125 ug/ml gentamycin (This medium is referred to as complete WME; incomplete WME used for cell exposure contains no serum).

   B. Appropriate CO_2 concentration, temperature and humidity should be used in maintaining the cell cultures.

   C. The treated cells should be drained of medium, rinsed, fixed, dried, and attached to microslides. Slides must be dipped in autoradiographic emulsion, exposed at 4 C. for an appropriate length of time, developed and stained.

3. The net silver grains per nucleus were not given. UDS should be measured by the net nuclear grain count. The average number of grains in the cytoplasm (three nucleus-sized areas) must be substracted from the number of grains found over the cell nucleus to give the net nuclear grain count.

4. The original data used for the summerized results in Table 1 for statistical analysis should be presented with the report.