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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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JUN 6 1984

OFFICE OF PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Chlorpropham (CIPC)/Bud-Nip on tobacco, EPA#748-233. Data submitted in response to conditional registration. Caswell 510A.

TO: Robert Taylor, PM 25
Registration Division (TS- 697C)

FROM: Stanley Gross, Toxicologist
Toxicology Branch (TS-769C) SG 9/27/83

THRU: Christine Chaisson, Section Head ~~9/27/83~~
William Burnam, Chief
Toxicology Branch (TS-769C) CFChaisson 6/5/84

Note: This is copy of original review. WB 6/6/84

These data were submitted with a letter of April 5, 1983 from John F. Flanagan, Supervisor of Pestisticide Registration, of PPG Industries, Inc. Barberton, Ohio. The submission is in response to the requirements for additional studies specified when Bud-Nip was conditionally registered for use on tobacco (EPA Form 8570-6 (Rev. 5-76) dated July 14, 1982 sent to PPG by Thomas Adamczyk of Registration Division). The submitted studies included:

1) Ames mutagenicity test using metabolite I.
2) Ames mutagenicity test using metabolite II.
3) Primary eye irritation study using PPG-575 1EC.
4) Four hour acute inhalation study using PPG-575 1EC.
5) Preliminary smoke inhalation study.
6) 21 Day smoke inhalation study.

The Company (Dr. James Barter, toxicologist, and Flanagan) met with OPP representatives (C. Sandusky, J. Dziuban and S. Gross) on 9/8/82 to discuss the toxicity testing requirments for Bud Nip on tobacco. We indicated at that time that OPP has had little direct experience in the testing of pesticides on tobacco but that we recently required 21 day smoke inhalation studies using cigarette tobacco spiked with pesticide residues. We referred the PPG to Roger Jenkins of Oak Ridge National Laboratories. Jenkins had experience in nose only smoke inhalation

testing systems. We also required mutagenicity testing of the key metabolites in tobacco. Dr. Barter found that Dr. Carol Henry of Microbiological Associates also had experience with nose only smoke inhalation systems with rodent and together, they provided the submitted inhalation study.

Recommendations.

1) The Ames mutagenicity studies using metabolites I and II, the eye irritation study and the 4 hour inhalation study are acceptable as submitted.

2) The 21 day smoke inhalation study showed adverse effects due to the CIPC/metabolites added to the tobacco which ~~does~~ does not seem to provide an adequate margin of safety from the expected use of tobacco treated with Bud-nip.

3) Since it is possible that the present smoke inhalation study represented too much exposure to cigarette smoke and not enough time to evaluate the effects of the pesticide residues, it is recommended that the protocol be re-evaluated relative to some of the questions discussed below.

Discussion on Smoke Inhalation Testing Requirements.

The focus here is on cigarette tobacco and not tobacco used in cigars, pipes or for chewing. Although there are almost ~~a~~ 100 registered pesticides (about 1800 formulations) manufactured by at least 24 companies, the Agency as well as most has had very little direct experience evaluating toxicity studies for assessing possible hazards due to pesticides applied to tobacco. Over the years, different requirements have been advanced which may provide some confusion to registrants. These requirements and a summary of some of the problems raised by the present protocol are presented here.

Requirements in the Past.

In 1973, G. Whitmore, in a memo to C. Williams referring to a S-75 Statement, indicated that the cancer aspects of pesticides on tobacco will not be evaluated, however, the pyrolytic products should be evaluated at 0, 1x and 5x the residue levels seen in tobacco, tested at 1 hr/day, 5 days/week for 3 weeks. The July 25, 1975 proposed Guidelines required of subchronic inhalation testing on the technical formulation when residues exceeded 0.1%. A smoke inhalation study on

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Prowl applied to tobacco used 30 ml puffs of smoke sent into a 72 liter chamber but was turned down by J. Doherty (10/12/79) for inconsistancies in the results. C. Frick (8/14/79) required 90 day studies using technical Previcur because of high tobacco residues of 707 ppm.

In the 1978 proposed Guidelines (Article 163.83 of the 1978 Proposed Guidelines (FR 43: 37336 to 37403, August 22)), life-time oncogenicity studies using the technical material in two species were required for pesticides applied to tobacco. The same requirements were stated when Subpart F was published as revise in the October 1982 NTIS publication of the Guidelines which are referenced in Article 158.135 of the Proposed Data Requirements (FR 47: 53192-221, November 24, 1982). The Toxicology Branch however, has not required long-term smoke inhalation. It would be difficult and expensive to demonstrate the increased risk of cancer beyond that which has already been associated with cigarette smoking.

The 1978 proposed requirement for the chemistry subpart of the Guidelines (article 163.64-1, FR 43: 29724, July 10, 1978) set a cut-off limit of 0.1 ppm for testing requirements. Residues in tobacco or smoke exceeding 0.1 ppm were to be identified chemically and were to be tested using the 90 day subchronic inhalation studies. The implication here was to waive these requirements pesticides below 0.1 ppm. A possible rationale for this cut-off limit might be as follows: if a 60 kg smoker smoked 60 cigarettes a day (considerably more than the 40 cigarettes per day used for the analyses below), this would represent 1 ug/kg/day if <u>all</u> of the pesticide residue in the average cigarette was inhaled and absorbed. This would be a thousand fold less than a 1 mg/kg/day life-time feeding study assuming complete absorption of the pesticide in the feed. Most pesticide food tolerances are set on NOELs much above the 1 mg/kg/day.

<u>Recent Testing Requirements</u>.

Early last year, members of the Branch met with another registrant at which time it was ~~it was~~ decided that the Branch would concern itself with testing for possible pharmacological effects of the pesticide and its metabolites which cause adverse effects beyond those expected from the smoke of the cigarette itself and also to examine the oncogencity potential as might be revealed in an Aimes mutagenicity test. A smoke inhalation protocol used by Hazleton Laboratories was approved which involved the smoking of 18 cigarettes/day for 21 days using the smoke from cigarettes spiked with several times (thousands) the amount of residues expected on the cigarettes. This protocoal called for a 1000 fold dilution of the cigarette smoke and proved to be an inadequate simulation of smoke exposure for a variety of reasons. I then contacted a number of additional inhalation

scientists with experience with rodent smoke inhalation studies. On the basis of their commments we proposed the use of the nose only exposures using 350 ml chambers and 10% smoke dilution, similar to the methods used by Dr. Henry and her group at Microbiological Associates.

More recently, the cut-off limit of 0.1 ppm was applied in the case of chlorpyrifos (April 29, 1983 memo from G. Burin to Jay Ellenberger). Using the 60 cigarettes/day basis and 100% absorption, the cigarette tobacco exposure to the pesticide amounted to 0.3% of the ADI.

Human Inhalation Exposures to Smoke Products

The inhalation route of exposure is different from the usual exposures to pesticides because absorption from the lung is usually very rapid, often equivilent to IV injections. Absorption through the skin is generally very slow by comparison. Absorption through the intestinal tract is generally less complete, slower and leads to the liver, which often transforms the pesticide to less toxic metabolites which are more readily excreted from the body. Because absorption through the lung is rapid without the benefit of liver biotransformation, critical organs in the body may be exposed to higher and more damaging levels of pesticides than seen by exposures to similar external dosage levels on a per kg level through other routes.

From some estimates, heavy smokers smoke 40 cigarettes per day. If the average puff from a cigarette is 35 ml and this is taken into an average tidal volume equal to 500 ml, the cigarette smoke of human smoker is diluted 35/500 = 14.5% smoke concentration. Assuming 100% transfer to the lungs and cigarettes containing 1 ppm (1 ug/1gm cigarette), a smoker would receive 40ug/ 60 kg = 0.667 ug/kg/day over the many years he/she might be smoking for each 1 ppm of residue in tobacco.

The 100% transfer (deposition) assumption may be extreme. Dr. Wm Coate from Hazleton Laboratories in one of his communications indicated the following which allows for lower estimates of dosage: 40% of the smoke is contained in the mainstream; 65% of the smoke gets through the cigarette filter; 90% of the tobacco is treated with the pesticide; 80% of the cigarette is smoked (leaving a butt length of 20 mm); therefore the smoker received (1 x 0.4 x 0.8 x 0.9 x 0.65 = 0.187 ug/cigarette) x 40 cigarettes/60 kg = 0.125 ug/kg/day from cigarettes containing 1 ppm residues. Further, Dr. Roger Jenkins of Oak Ridge National Laboratores, indicated that deposition of smoke in humans varies from 20% to 80%.

Exposures in the CIPC Rodent Smoke Inhalation Study

In the exposure system used in the 21 day CIPC smoke inhalation study, 12 mice are exposed to the smoke from 9 cigarettes/day for 21 days. A 35 ml puff is diluted to 350 ml producing a 10% dilution, however, the mice rebreath the same smoke for 30 second (respiratory rates estimated at 109/ minute, range of 97 to 123-- 1974 Biological Data Book) from the smoke in the 350 ml chamber. Assuming 1 ppm pesticide in a cigarette (for comparison with the human analysis above) and all of the pesticide from the 9 cigarette exposure is transferred to the lungs of the 12 mice, the 9 ug/(12 x 0.020kg mouse) would represent 37.5 ug/kg/day exposure to pesticide or 56.22 times human exposure (37.5 ug/kg/day divided by 0.667 ug/kg/day). According to Dr. Domingo Aviado (communication of 7/18/83), the rat would be a better model than the mouse for simulating smoke inhalation effects.

Similar loss factors (loss to side stream, filters, etc.) described for humans by Dr. Coate would apply also to the cigarettes used in rodent smoke inhalation study. However, in the case of the human, the exhaled smoke is lost to the air surrounding the human. In the case of the animal inhalation system, the animal rebreaths his exhaled air and the exhaled air of the other animals. Therefore rodents have a better chance of absorbing considerably more of the smoke products containing the pesticide residues than in the case of the human. These comparisons may help to explain why the rats had COHb levels of 30 to 50% compared to heavy smokers which have COHb of 5 to 10%, and suggests the animal exposures to the cigarette smoke in this system may be excessive for the purposes of evaluating the effects of the pesticide.

Questions Concerning the CIPC Smoke Study.

The results from the 21 day smoke exposure study of tobacco treated with CIPC/ metabolites indicated statistically significant effects which may or may not be biologically significant because morphological changes relatable to CIPC were not seen in any organ. Several concerns arise in considering this study:

a) As indicated above, the animals may have been exposed to an excessive amount of cigarette smoke and proportionally more pesticide residues. Their blood COHb levels were quite high (30 to 50%) compared to that expected in a heavy smoker (5-10%). The animals did not seem to tolerate their smoke exposures-they struggled, became lethargic, etc. A revision of the exposure system may be necessary to better approximate human exposures.

A smoke inhalation protocol submitted to the Agency in 1981, used a Mark IV HRC rodent smoke machine in which the exposures were to 6% smoke on a projected nominal concentration basis (Huntingdon Research Center, Huntingdon, England). Since, this organization seems to have extensive experience with smoke inhalation studies, a 6% smoke may be an improvement over the 10% level used in the present study.

b) The changes in of the relative weights of so many organs (heart, kidney, brain, spleen); the effects on blood phosphorus and the increase in reticulocytes suggest the possibility of definite effects due to the CIPC/metabolites. Abnormalities of the spleen had been noted in previously submitted dog study and the increase in reticulocytes raises the question increased RBC fragility and/or hypersplenism which need to be evaluated.

c) The lack of morphological changes within the short 21 day experiment is not suprizing and points to the need to extend the study. It was hoped that a negative finding in the 21 day exposure study could act as a screen to avoid more costly long-term smoke exposure studies. The results of this experiment indicates longer exposures are necessary, perhaps extending to a 6 month study to better simulate the long-term smoking exposure pattern of the smoking public.

d) The animals in this study did not seem to tolerate well their smoke exposures. This is in contrast to human smokers, and dogs and monkeys exposed smoke through face masks who (in my observations) seemed to enjoy their smoke exposures. It seems likely that the rodents used in this study may need to be acclimated to the smoke before they are given their experimental exposures. The Huntingdon Center protocol (cited above) utilizes such an acclimatization period during which the smoke concentrations are increased over a 7 day period.

e) Since margins of safety for subchronic tests are usually based on 1000 and 2000 fold safety factors, the recommended spiking levels of 1x, 2x and 5x or the 50x used in the CIPC smoke study should be increased.

Conclusion.

The above considerations indicates the need to reduce the smoke exposure of the test animal, possibly change the species to the rat, increase the amount of residue used to spike the cigarettes (especially more that the recommended 5 x's), provide a period for acclimatization and extend the number of exposure period to 3 or 6 months to allow more time for morphological and other abnormalities to develop if they are to do so.

PPG has been extremely helpful in cooperating with the Agency to meet the recommended objectives of this test. We will appreciate their further support in this effort and would hope that they can get some of the other companies who will need to meet these requirement to help finance the research necessary to develop an acceptable protocol.

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5) Acute Toxicity of Cigarette Smoke After Inhalation in Mice.

Study carried out for PPG Industries by Microbiological Associates, Bethesda MD. Study number I-1727.001 completed on 1/27/83. This was a range finding study to help establish smoking regimens which were to be used in the main smoke inhalation study discussed below.

Methods. Mice (B6C3F1/CrlBL strain) were exposed in nose only smoke inhalation systems, 3 animals/sex/exposure regimen. The experimental design was as follows:

Experimental Design

Group[a]	%Smoke[b]	Exposure[c] time (min)	Cigarettes	Total no.of puffs
1T	30%	40.5	9	81
2T	20%	40.5	9	81
3T	10%	40.5	9	81
4T	20%	54	12	108
5T	10%	54	12	108
6	0%	NA	NA	NA
7R	10%	40.5	9	81
8R	10%	54	12	108

[a] T= CIPC treated cigarettes; R= Kentuck reference cigarette 2A1 (no CIPC).
[b] 10% smoke using one cigarette, 20% smoke using 2 cigarettes and 30% smoke using 3 cigarettes in the Walton machine.
[c] Smoke exposure time = 30 sec/per puff x 9 puffs/ cigarette x 9 or 12 cigarettes. Total experimental time approx. 18 min/cig or 2.7 hour for 9 cigs. and 3.6 hours for 12 cig.

Group 6 was a sham or air treated group of rats. Groups 7R and 8R were exposed to the smoke of cigarettes referred to as PPG-441-2525 made from Kentucky 21A reference tobacco with no added CIPC or metabolites. Groups 1T, 2T, 3T, 4T and 5T were exposed to cigarette smoke from " high dose" test cigarettes designated as PPG 441-2528. These cigarettes were made from the Kentuck reference 2R1 tobacco which had been spiked with CIPC to a mean concentration of 51 ppm (SD=17ppm) and homogenity expressed as %SD = 33%. The following mean values were obtained from 5 cigarettes of each type:

	RTD[a] (mm water)	Weight (g)	TPM/Cigarette[b]) (mg)
Reference	60.6 ± 5.5[c]	1.09 ± 0.04	32.2 ± 5.3
Test-High Dose	76.4 ± 8.5	1.17 ± 0.04	32.4 ± 4.7

[a] RTD = Resistance to draw
[b] TPM = Total particulate matter collected on a Cambridge filter after 9 puffs/cigarette.
c Standard deviation.

The cigarette smoke was generated using Walton Horizontal Smoking Machines (Walton, Process and Instruments, Inc. Brooklyn, NY). which blew 35 ml puffs of air through 1, 2 or 3 cigarettes at one time over a 2 second period of time. The smoke thus formed was fed into a 350 ml chamber such that the smoke was diluted to 10, 20 or 30% smoke atmosphere depending on whether, 1, 2 or 3 cigarettes were used. The animals placed inside of stainless steel tubes (obtained from Process and Instruments Corportation, Brooklyn, NY) equiped with a neck-slot with a restraining spring. A chin rest ensured that the nose of the animal was aligned with the conical shaped opening of the exposure nodule. The nose of the animal protruded into the 350 ml chamber through a dental rubber dam diaphragm which formed a seal to prevent body exposure. With this arrangement, one to 12 mice could be exposed to smoke at once.

The Walton machine under standard conditions generates in two seconds a 35 ml puff volume, one puff every minute. The smoke is held in the chamber for 28 seconds, then the chamber is purged for 30 seconds with fresh air and the cycle repeated for a total of 9 puffs at which time the cigarette butt is approximately 23 mm. The chambers are purged for approximately 9 minutes between cigarettes while the spent cigarettes are removed and the next cigarette is lighted and set in place.

The animals were observed for toxicity during the exposures and for 24 hours after. They were weighed 24 hours before exposure, just after exposure, and before sacrifice. Retroorbital blood samples were obtained at the end of the exposure and were analyzyed for carboxyhemoglobin using an IL-CO-Oximeter (Instrument Laboratories). Urine from animals in Groups 3T and 7R was collected for 24 hours immediately after the exposures and the urine was shipped frozen to PPG for analyses of CIPC metabolites.

Results. Some of the results are summarized in Table 6 (attached) taken from the submitted report. None of the sham treated animals or the animals exposed to 10% smoke died. Mortalities occurred in animals receiving 20 and 30% smoke. The animals which died experienced struggling, labored breathing, gasping and convulsions. All of the treated animals were lethargic, unable to walk, were uncoordinated, cold just after their exposures. Most of the animals exposed 10% smoke were normally active 24 hours after their exposures. The sham treated animals experienced no adverse effects.

The sham treated animals had mean COHb of 1.9 % while the smoke treated animals had COHb which ranged from 40 to 73% in proportion to the concentrations of smoke and the duration of the exposures. The urinary excretion from the control animals (Group 6) and the high test group (7R) contained the following:

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Table 6

SELECTED SUMARY OF ACUTE TOXICITY DATA

taken from Laboratory report

roup	Treatment/ Number of Exposures	Total Number of Puffs	Survival Male	Survival Female	Percent Survival	Mean Percent COHb
1T	30%/9	43.8 ± 26.0[a]	1/3	0/3	16.7	72.9
2T	20%/9	61.5 ± 24.4[b]	2/3	1/3	50.0	65.5±4.5[d]
3T	10%/9	81.0 ± 0.0[c]	3/3	3/3	100.0	46.0±7.9[d]
4T	20%/12	22.5 ± 10.7[b]	0/3	0/3	0.0	-
5T	10%/12	108.0 ± 0.0[c]	3/3	3/3	100.0	44.3±2.6[d]
6	Sham	-	3/3	3/3	100.0	1.9±0.3
7R	10%/9	81.0 ± 0.0[c]	3/3	3/3	100.0	40.4±5.7[d]
8R	10%/12	108.0 ± 0.0[c]	3/3	3/3	100.0	63.6±8.5[d]

tandard deviation.

roup 2T significantly different ($p \leq 0.05$, Studentized Range Test) rom 4T.

ice received scheduled total number of puffs.

roups 2T, 3T, 5T, 7R and 8R significantly different ($p < 0.05$, Studentized nge Test) from Group 6, sham-exposed control.

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	Wt. Urine (g)	CIPC-II metabolyte (ug)
Control	17.5	ND (<0.3)
Test	13.8	1.02

Conclusion. Well executed study from which the investigators chose to use the 10% smoke concentrations for the main study.

6. Study of the Toxicity of CIPC Derived Residues in Tobacco Smoke Administered to Mice for 21 Days.

Study performed for PPG Industries by Microbiological Associates, Bethesda, MD, Report No. I-1727.005, March 28, 1983. EPA accession no. 249884.

Methods. Mice (B6C3F1/CrlBL) were exposed in a nose only smoke inhalation system, 10 animals/sex/exposure regimen, 7 days/week for 21 days. The exposure regimens were as follows:

Exposure Group		CIPC (ppm)	Metabolite I[a] (ppm)	Metabolite II (ppm)
1	(Sham)	Air only	0	0
2	Reference Cig.	0	0	0
3	Low	35	2	12
4	Mid	113	6	26
5	High	452	21	104

[a] Calculated on the basis of the percentage of metabolite II analyzed relative to the nominal treatment level.

Group 1 animals were placed in exposure chambers and handled in ways similar to the smoke exposed animals only they were exposed to air rather than to cigarette smoke. Group 2 was exposed to cigarette smoke made from Kentucky reference tobacco 2A1 without any spike of CIPC/metabolites. Groups 3, 4 and 5 were exposed to cigarettes made from the reference tobacco which was treated by PPG with nominal concentrations of CIPC/metabolites as follows before being made into cigarettes:

Treatment Levels on Tobacco (nominal concentrations, ppm)

Group	Spike[a]	CIPC[b]	Metabolite I[c]	Metabolite II[d]
2	Reference	0	0	0
3	5x	60	3	15
4	15x	180	9	45
5	50x	600	30	150

[a] Levels of CIPC or metabolites relative to that expected on on normally treated cigarette tobacco.
[b] Isopropyl 3-chlorocarbanilate.
[c] Isopropyl 2-hydroxy-3-chlorocarbanilate.
[d] Isopropyl 4-hydroxy-3-chlorocarbanilate.

The CIPC/metabolite treatments were applied to shredded cigarette Kentucky reference 2A1 tobacco at the Barberton Technical Center, PPG Industries. The treated tobacco was shipped to the Tobacco and Health Research Institute, University of Ketucky, where the cigarettes were manufactured. Each cigarette weighed approximately 1.1 gm, had resistance-to-draw (RTD) values ranging from 63 to 74 mm water. The RTD's were measured using a Filtrona pressure drop tester (Cigarette Components (UK) Ltd., Wembley, England). Total particulate matter (TPM) collected on Cambridge filters 9 puffs from a cigarette averaged 30 to 34 mg for the different groups of test cigarettes.

The exposure methods used by Microbiological Associates have been described in two reports from the open literature:

1) Guerin, M.R., Stokely, J.R., Higgins, C.E., Moneyhun, J.H. and Holmberg, R.W. Inhalation Bioassay Chemistry--Walton Horizontal Smoking Machine for Inhalation Exposure of Rodents to Cigarette Smoke. J. Natl. Canc. Inst., 63, 441-448, 1979.

2) Henry, C.J., Whitmire, C.E., Lopez, A., Dansie, D.R., Avery, M.D., Caton, J.E., Stokely, J.R., Homberg, R.W., Guerin, M.R. and Kouri, R.E. The Dosimetry and Distribution of Whole Cigarette Smoke Particulates in Inbred Strains of Mice: Comparison of a Large Smoke-Exposure Machine (SEM) with a Small-Capacity Smoke Exposure Machine (Walton). In: Pulmonary Toxicology of Respirable Particles, C.L. Sanders, F.T. Cross, G.E. Dagle, and J.A. Mahaffey (Eds.), Technical Information Center, U.S. Department of Energy, NTIS. 177-192, 1980.

The cigarette smoke was generated using a Walton Horizontal Smoking Machines (Walton, Process and Instruments, Inc. Brooklyn, NY) which produced 35 ml (nominal) puffs of cigarette smoke which was fed into a 350 ml chamber where it was diluted with clean air to a concentration of 10% smoke. The animals were placed inside of stainless steel tubes (Process and Instruments Corporation, Brooklyn, NY) equiped with a neck-slot with a restraining spring. A chin rest ensured that the nose of the animal was aligned with the conical shaped opening of the exposure module. The nose of the animal protruded into the

the 350 ml chamber through a dental rubber dam diaphragm which formed a seal to prevent body exposure.

The Walton machine generated the 35 ml puff of smoke in 2 seconds, one puff per minute. The smoke was held in the chamber for 28 seconds, then the chamber purged for 30 seconds with fresh air and the cycle repeated for a total of 9 puffs at the end of which time the cigarete butt was approximately 23 mm. The chambers were purged for 9 minutes between cigarettes while the spent cigarettes were removed and the next cigarette lit and set in place.

This regimen resulted in the following exposures for all smoke-exposed groups:

10% (v/v) smoke concentration
30 second smoke/30 second air/minute
9 puffs/exposure
9 exposures/day
81 puffs/day
9 minutes rest between exposures
40.5 minutes total time/day exposed to smoke
153 approx. minutes/day exposure period
189 total exposures or 1701 total puffs in 21 days.

The animals were observed any adverse effects during each exposure and at least one additional time each day. They were weighed 24 hours prior to the first exposure and weekly thereafter. Food consumption was determined after 7, 14 and 21 days on test. Four animals per test group were bled from the retro-orbital sinus 3-5 minutes after their exposures on days 7, 14 and 21 and the blood analyzed from carboxyhemogobin (COHb). Urine was collected from 6 females/ group in metabolism cages for approximately 19-20 hours after exposure on days 8, 15 and 20. The urine samples were stored frozen and shipped to PPG for analyses of CIPC and metabolites. Livers, kidneys and lungs of 2 animals/sex/ group were obtained 6 hours after the final exposure for the purpose of analyzing for aryl hydrocarbon hydroxylase (AHH) levels. Because of buffer problems, the AAH determinations were not reliable (however, the investigators include the results obtained in Appendix D.

Necropsies were performed at the end of the study. The weights of the lungs, heart, liver, spleen, kidneys and brain were obtain. The following organs were examined grossly and placed in fixative: spinal cord (thoracic), heart, brain, thigh muscle (L), eyes, sciatic nerve (L), Pituitary, trachea, larynx, esophagus, adrenals (R&L), thyroids (R&L, with parathyroids), lungs and bronchi, kidneys, liver, spleen, thymus, any gross lesions, salivary gland, mandibular lymph node, stomach, small intestine, pancreas, large intestine, turbinates, skin, urinary bladder, ovaries, uterus, mammary gland, prostrate, testes, mesenteric lymph nodes, sternum (bone marrow), Zymbal's

gland. All tissues were processed and examined microscopically in the HD (group 5) and in the reference group (group 2). In the other groups (Low, Mid and Sham), only the respiratory tissues, liver, heart, kidneys, spleen and gross lesions were examined histologically.

The hematological tests performed were: segmented neutrophil counts, monocyte counts, platelet cell counts, red blood cell counts, hematocrit value, lyphocyte counts, eosinophil counts, reticulocyte counts, white blood cell counts, and hemoglobin concentration.

Blood/serum was analyzed for the following: glucose, blood urea nitrogen, albumen, globulin, albumin/globulin ratio, creatinine, cholesterol, triglycerides, uric acid, calcium, phosphorus, total protein, potassium, alkaline phosphatase, lactic dehydrogenase and sodium.

Results.

The mice exposed to air only showed no signs of adverse reactions. The mice exposed to smoke from the reference cigarettes or the spiked cigarettes exhibited considerable stress during their exposures, struggling in the holders and exhibiting shallow breathing. The smoked-exposed animals were lethargic, uncoordinated and breathing shallow immediately after exposure but recouperated in time.

Mortalities. One female (of 13 animals) died on the first day in the low dose group and was replaced immediately. One male of 12 males from the mid dose group died on day 11 of pneumonia and one male of 12 from the high dose died on day 10. Many of the organs of the latter animals were autolyzed.

Body weights (Table 10 from the report, attached) showed that the animal weights were evenly distributed at the start of the experiment. The body weights of all animals including the sham groups dropped slightly by day 7 of the experiment but increased slightly during the following 2 weeks. There were no statistically significant differences between groups although there was a weak tendency for the sham groups to be slightly heavier than the smoke-treated groups.

Food consumption in the smoke treated animals decreased (Table 13 from the report) compared to the sham groups, however there were no statistically significant differences in any of the test groups. Although all of the animals showed an increase in food consumption from week 1 to week 3, the increase in food consumption of the high dose animals were 1/2 (males) and 1/4 (females) as much as the increases in the other test groups.

Table 10 (From report)

SUMMARY

BODY WEIGHTS OF MICE

Mean Weight (g)/Group

Group/ Cigarette Type	Days on Test: 24 hrs Prior	Day 7	Day 14	Day 21
		Males		
1 - Sham	23.5	22.5	23.0	23.4
2 - Reference	23.4	22.5	22.2	22.6
3 - Low Test	23.9	22.6	22.5	23.0
4 - Mid Test	23.9	22.4	22.4	22.9
5 - High Test	23.7	22.6	22.5	22.6
		Females		
1- Sham	18.4	17.9	18.1	18.9
2 - Reference	18.4	17.7	17.5	18.1
3 - Low Test	18.9	18.0	18.0	18.4
4 - Mid Test	18.6	17.9	17.9	18.5
5 - High Test	18.6	17.8	17.7	18.0

Table 13 (from report)

SUMMARY OF FOOD CONSUMPTION

Group/Cigarette	% Change Relative to Sham-Exposed Control (Group 1)[a] Weeks on Test 1	2	3	% Change from 1 to 3 Weeks[b]
Males				
1 - Sham	NA[c]	NA	NA	+9
2 - Reference	-20	-22	-21	+7
3 - Low Test	-17	-22	-21	+3
4 - Mid Test	-14	-19	-16	+7
5 - High Test	-17	-19	-21	+3
Females				
1 - Sham	NA	NA	NA	+21
2 - Reference	-17	-22	-20	+17
3 - Low Test	-14	-19	-17	+16
4 - Mid Test	-10	-22	-17	+12
5 - High Test	-7	-16	-20	+4

[a] The mean value from 2 cages/group was used to determine the percent change from the sham-exposed control.

[b] The mean value from 2 cages/group was used to determine the percent change from Week 1 to Week 3.

[c] Not applicable. All group changes are relative to the sham-control group.

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Carboxyhemoglobin analyses indicated that the smoke treated animals received significant exposures to carbon monoxide while the sham treated animals did not:

	COHb (%)		
Group	Day 7	Day 14	Day 21
1 - Sham	2.1	1.8	2.1
2 - Ref.	46.9	49.1	30.1
3 - Low	47.6	36.8	41.4
4 - Mid	53.6	43.7	42.7
5 - High	42.5	35.8	47.7

COHb was approximately 2% in the sham animals and ranged from 30 to 50% in the smoked animals. There were no differences between the different smoke exposed animals nor was there a tendency for COHb to increase as the study progressed.

Organ Weights based on absolute and relative weights showed a consistant statistically significant increase in the weight of the lungs of the smoked animals compared to that of the sham treated animals. The relative weights of the heart (males and females) and kidneys (males only) were statistically decreased in the high test groups. The relative weights of the brains of the males and females of the high test group tended to increase, achieving statistical significance only in the males. The relative weights of the spleen to increased in a linear faction from the sham group up through the dosage groups, however the increase did not achieve statistical significance. Except for the lung, there were no morphologicl changes in the organs from any of the test groups which might suggest developing problems in these organs.

Hematological effects. The only consistant change in hematological parameters was a consistant decrease in the reticulocytotic count of males and females as one proceeded from the sham groups down through the smoke groups (Tables 28 and 29 taken from the report). There were no morphological abnormalities of the bone marrow seen and no other hemopoietic changes were observed-- including the WBC's, RBC's, Ht or Hb. Bilirubin was not measured.

Blood chemistry. There were statistically significant differences for phosphurs, triglycerides, lactic dehydrogenase and creatinine. Phosphorus in the CIPC treated smoke groups were decreased from the sham and reference groups in both sexes. Triglycerides of the high dose groups were decreased compared to the other groups and achieved statistical significance only in the males-- the LD group females (45 mg/dl) had even lower levels than that of the HD females (47 mg/dl). The changes

Table 26 (from report)

SUMMARY OF ORGAN-TO-BODY WEIGHT RATIO BY GROUP
MALES

Organ Weight (g)/Body Weight (kg)[a]

Group/Cigarette Type		Lung	Heart	Liver	Spleen	Kidneys	Brain
1/Sham		8.253	5.041	50.538	2.850	15.370	17.736
	±S.D.	1.155	0.259	2.798	0.289	1.354	0.810
2/Reference		10.777[b]	4.706	46.969	2.919	13.163[b]	18.778
	±S.D.	0.670	0.262	3.709	0.363	0.662	0.915
3/Low		11.326[b]	4.575	46.072	2.967	13.571[b]	18.537
	±S.D.	0.694	0.290	4.250	0.466	0.126	0.900
4/Mid		11.292[b]	4.966	47.167	3.179	13.736[b]	18.934
	±S.D.	0.630	0.297	4.096	0.453	0.797	1.205
5/High		11.128[b]	5.227[c,b]	46.227	3.333	14.705[c]	20.255[b,c,d,e]
	±S.D.	0.857	0.681	3.552	0.705	1.316	1.100

[a] Body weight immediately after exposure on Day 21 was used to calculate organ weight/body weight ratios.

[b] Significantly different from Group 1/sham-exposed control ($p \leq 0.05$, Studentized Range Test).

[c] Significantly different from Group 2/Reference ($p \leq 0.05$, Studentized Range Test).

[d] Significantly different from Group 3/Low ($p \leq 0.05$, Studentized Range Test).

[e] Significantly different from Group 4/Mid ($p \leq 0.05$, Studentized Range Test).

Table 27 (from report)

SUMMARY OF ORGAN-TO-BODY WEIGHT RATIO BY GROUP
FEMALES

Organ Weight (g)/Body Weight (kg)[a]

Group/Cigarette Type		Lung	Heart	Liver	Spleen	Kidney[b]	Brain
1/Sham		9.084	5.387	49.344	3.671	13.316	23.580
	±S.D.	0.849	0.472	4.326	0.560	0.440	0.699
2/Reference		12.213[c]	4.808	45.724	3.742	12.524	24.390
	±S.D.	0.747	0.276	4.192	0.621	0.552	1.339
3/Low		12.498[c]	4.917	45.787	3.630	12.685	24.302
	±S.D.	1.052	0.252	2.496	0.252	0.655	1.290
4/Mid		12.201[c]	5.128	44.955	3.882	12.667	24.595
	±S.D.	1.197	0.587	2.291	0.598	0.752	1.428
5/High		12.852[c]	5.602[d]	45.402	4.166	13.393	25.203
	±S.D.	0.584	0.965	4.110	0.951	0.945	1.029

[a] Body weight immediately after exposure on Day 21 was used to calculate organ weight/body weight ratios.

[b] ANOVA, $p \leq 0.05$, Studentized Range Test, No significant differences between groups.

[c] Significantly different from Group 1/Sham-exposed control ($p \leq 0.05$, Studentized Range Test).

[d] Significantly different from Group 2/Reference ($p \leq 0.05$), Studentized Range Test).

TABLE 28 (from report)

FINAL HEMATOLOGY DATA FOR MALES

TOXICITY OF CIPC-DERIVED RESIDUES IN TOBACCO SMOKE IN B6C3F1/CRL BL MICE
TEST ARTICLE: CIPC RESIDUES
SUMMARY

GROUP		WBC (THOUS/CU MM)	RBC (MIL./CU.MM.)	HB (G./DL.)	HCT (%)	RETIC. COUNT[a] (%)
SHAM	MEAN	7.0111	8.5989	16.6667	42.1222	3.0889
	S.D.	0.9080	0.4073	0.5362	2.4570	0.4372
	N	9	9	9	9	9
REF	MEAN	7.6778	8.1233	16.4444	39.0222	2.1556
	S.D.	1.4158	0.7787	1.1392	3.8271	0.7002
	N	9	9	9	9	9
LOW CIPC	MEAN	7.3000	8.4875	16.7778	41.4375	2.5444
	S.D.	2.3780	0.6061	0.4024	2.0653	0.9632
	N	9	8	9	8	9
MID CIPC	MEAN	8.3400	8.2270	16.5800	39.5700	2.2500
	S.D.	0.7011	0.5821	0.6250	2.6683	0.6381
	N	10	10	10	10	10
HIGH CIPC	MEAN	7.0778	8.1563	16.4000	39.5125	1.9778
	S.D.	1.2863	0.3633	0.8352	2.3363	0.8167
	N	9	8	9	8	9

[a]ANOVA, $P \leq 0.05$, Scheffe's Test, no significant differences between groups (Appendix B).

TABLE 29 (from report)

FINAL HEMATOLOGY DATA FOR FEMALES

TOXICITY OF CIPC-DERIVED RESIDUES IN TOBACCO SMOKE IN B6C3F1/CRL BL MICE
TEST ARTICLE: CIPC RESIDUES
SUMMARY

GROUP		WBC (THOUS/CU MM)	RBC[a] (MIL./CU.MM.)	HB (G./DL.)	HCT[a] (%)	RETIC. COUNT[a] (%)
SHAM	MEAN	6.0333	7.7733	15.9333	38.2444	3.0222
	S.D.	0.7794	0.5616	0.9526	2.6940	0.5142
	N	9	9	9	9	9
REF	MEAN	6.9000	7.9950	16.6000	39.4000	2.6700
	S.D.	1.5875	0.8499	0.9165	3.7160	1.2561
	N	10	10	10	10	10
LOW CIPC	MEAN	5.7444	8.6800	16.9889	42.7125	2.1889
	S.D.	1.2167	0.2197	0.3296	0.9833	0.7524
	N	9	8	9	8	9
MID CIPC	MEAN	6.5300	8.4270	16.6300	41.3300	2.1100
	S.D.	1.3107	0.6408	0.7987	3.4049	0.5587
	N	10	10	10	10	10
HIGH CIPC	MEAN	6.2333	8.4288	16.7333	41.8000	1.8000
	S.D.	0.8902	0.6049	0.8660	3.1159	0.9798
	N	9	8	9	8	9

[a] ANOVA, $p \leq 0.05$; Scheffe's Test, no difference between groups (Appendix B).

in creatine and LDH were not relatable to the exposures to the smoke or to the CIPC treatmentk.

Pathological morphology. The only histopathological findings which were relatable to the smoke inhalation exposures were the peribronchial lyphocytosis, the infiltration of the alveoli with macrophages and golden brown pigment seen in almost the all of the lungs of the smoke treated animals while 8 M and 7 F of the 10 animals each of the sham treated groups had normal lungs. Few other morphological abnormalities were found and none of these could be related to the experimental regimens.

Metabolite II in Urine. From Table 8, attached (taken from page 27 of the Appendix D, Analysis Section of the report) indicates that the amount of metabolite II from pooled urine samples was dose related in the low, mid and high dosage groups of animals, however, the levels in the reference and low dose groups were generally too low to be detected.

Conclusions.

There are no Guidelines with which compare this directly. There no NOEL and the lowest dose tested was the LEL.

The study was well conceived, executed and reported and represents on the part of the Company and the Laboratory and excellent effort to develop a smoke inhalation study which could meet the objectives of the hazard assessment sought. The results, however, suggest that the smoking of the cigarettes spiked with CIPC/metabolites as tested in this regimen, produced adverse effects which raises questions on the need to modify and extend the exposure times:

1) The relative heart weights in the males and females were increased, achieving statistical significance in the high dose animals.

2) The relative kidney weights pregressively increased from the reference group through CIPC treated groups for both males and females achieving statistical significance in the HD males only.

3) The relative brain weights tended in increase in both sexes from the reference cigarettes through to the HD groups, achieving statistical significance only in the HD groups of both sexes.

4) The spleen of both sexes tended to increase in a linear fashion (not achieving statistical significance) from the sham groups, the reference cigarette groups through the CIPC tests groups and this may be coupled with 6) A statistically significant (linear) decrease in the reticulocyte counts in the corresponding animals.

7) There was a statically significant decrease in blood phosphate in the CIPC smoke groups compared to the reference cigarette groups of both sexes, and

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Table 8 (from report).

Analysis of Pooled Samples of Urine Collected from Six Mice[1] from the 21 Day Study after Exposure to Smoke from Cigarettes Spiked with CIPC

Group[3]	Day[2]	Sample No. PPC	Wt. Urine g	Metabolite II µg/ml	Metabolite II µg Excreted in Total Pooled Sample	Metabolite II µg Excreted per Mouse
Sham	8	5183	8.0	<0.04	<0.4	-
	15	5188	11.6	<0.04	<0.5	
	20	5193				
Refer-ence	8	5184	8.5	<0.04	<0.4	-
	15	5189	8.3	<0.04	<0.4	
	20	5194				
Low	8	5185	11.7	<0.04	<0.5	<0.1
	15	5190	12.5	<0.04	<0.5	<0.1
	20	5195	6.5	0.08	0.52	<0.1
Mid	8	5186	13.7	0.04	0.55	0.09
	15	5191	8.7	0.14	1.24	0.21
	20	5196	11.0	0.10	1.10	0.18
						Mean = 0.16
High	8	5187	12.3	0.23	2.83	0.47
	15	5192	8.3	0.41	3.44	0.57
	20	5197	6.4	0.65	4.16	0.69
						Mean = 0.58

1) Groups of six female mice held overnight in metabolism cages between daily smoking.
2) Day 8 - Urine collection was for 19 hours i.e. 2 hours after exposure until before next days exposure.
   Day 15 - Urine collection was for 21 hours i.e. immediately after exposure until before next days exposure.
   Day 20 - Urine collection was for 20 hours.
3) See Table 9 for nominal and measured concentrations of test materials in the cigarettes.

8) The food consumption increases over the three week period was 1/2 (males) to 1/4 (females) for the HD groups even though there were no obvious changes in body weight parameters.

Several questions are raised with the results of this study:

Margin of safety. PPG was told to spike the cigarettes at the 5, 15 and 50 x's the amount of CIPC and metabolites which were expected to be found in treated cigarettes. This was based on previous recommendations to other companies. Based on the analyses of the tobacco used, the spiking amounted to approximately 3, 10 and 40 x's the expected residues. Adverse effects were seen at the 40 times residue levels which does not allow for an adequate margin of safety. It might also be desirable to reevaluate the comparison of the animal exposure model to that of expected human exposures.

Extension of the Study. Although there were some organ weight, blood chemistry and hematological changes seen in the present study, there were no morphological changes seen except in the lung. A three week study is not likely to produce morphological changes when exposures to the pesticide are not high. Further, a three week smoke inhalation study is probably not an adequate exposure period to represent exposures for the smoking public which may represent years of exposure. Thus it is probably necessary that such a smoke inhalation study be extended at least to 6 months until the Agency aquires enough experience to determine if shorter exposures will suffice.

2) Lower COHb Levels. The smoke exposures resulting in lethargy following the exposures, by the high carboxyhemoglobin blood levels of 40 to 50% (compared to levels of 10 to 15% seen in heavy smokers, by the increased weights of the lungs which were found to contain pigmentation and lymphocytic infitration. It is possible that the exposure regimen used here represents an exposure which is more extreme than that expected by the inhalation of human. It may be desirable to reduce the smoke concentration to levels which will simulate blood COHb levels more consistant with humans.

3) Acclimatization. Humans who smoke develop a tolerance to the acute adverse effects. These animal exposures indicated that the animals were acutely stressed with the introduction of smoke to the air they breathed. It may be important for for the purpose of accessing the effects of the addition of the pesticide to the tobacco, to gradually acclimatize the animals to smoke starting with lower exposure rates and building up the exposure levels over a period of time. The initial

insult may set up a sequence of damaging effects which do not allow the animal to recover in order to develop the tolerance for smoking seen in humans.

An effort to further evaluate the type of studies that would be sufficient to assess the hazards of exposures to pesticides applied to tobacco need to be made by the Company (and other knowledgeable experts) in cooperation with the Agency in order to establish a basis for adequate testing procedures. On the other hand, the results from the present study may be sufficient to prevent the use of Bud-Nip on tobacco.