

EASTMAN

Eastman Chemical Company
P. O. Box 511
Kingsport, Tennessee 37662-5054
Telephone: 1-800-EASTMAN Ext. 5208

TOXICITY SUMMARY

for

EASTMAN[®] TXIB FORMULATION ADDITIVE

(2,2,4-TRIMETHYL-1,3-PENTANEDIOL DIISOBUTYRATE, CAS NO. 6846-50-0)

Prepared by:
Product Safety & Health
Eastman Chemical Company
Kingsport, Tennessee 37660

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TXIB Toxicity Summary Overview

Study	Method	Result
Acute Oral Toxicity	LD ₅₀ , OECD 425, rat	>2,000 mg/kg (highest dose tested)
Acute Oral Toxicity	LD ₅₀ , other, rat	>3,200 mg/kg (highest dose tested)
Acute Oral Toxicity	LD ₅₀ , other, mouse	>6,400 mg/kg (highest dose tested)
Acute Dermal Toxicity	LD ₅₀ , OECD 402, rabbit	>2,000 mg/kg (highest dose tested)
Acute Dermal Toxicity	LD ₅₀ , other, 24 hr occlusive patch, guinea pig	>20,000 mg/kg (highest dose tested)
Acute Inhalation Toxicity	LC ₅₀ , rat	>5,300 mg/m ³ (highest concentration tested)
Skin Irritation	Primary Dermal Irritation/Corrosion, OECD 404, rabbits	No evidence of irritation was observed
Skin Irritation	24-hr occlusive wrap; guinea pig	Slight
Skin Irritation	24-hr occlusive patch three 9 applications; human, 1.0%	No evidence of irritation was observed
Eye Irritation	OECD 405; GLP, rabbit	Slight
Skin Sensitization	Other; guinea pig, 1.0%	No sensitization was observed
Skin Sensitization	HRIPT; human, 1.0%	No sensitization was observed
Repeat Dose Approx. 44 days	OECD 422; GLP, rat, gavage, 30, 150, and 750 mg/kg	
Repeat Dose - 90 days	Other; dog 0.1, 0.35 and 1.0% in diet	NOAEL 1%
Repeat Dose - 90 days	US FDA Redbook; GLP, rats, 30, 150, and 750 mg/kg in the diet	NOAEL 150 mg/kg (males), 750 mg/kg (females)
Repeat Dose - 100 days	Other; rat, 0.1 and 1.0% in diet	NOAEL 1.0% (approx. 772 mg/kg in males and 858 mg/kg in females)
Repeat Dose – Special	Other; rat, 0.1 and 1.0% in diet for various durations up to 100 days	Increases in liver weight and induction of liver enzyme activities reversed after 47 day removal of TXIB diet.
Reproductive and Developmental Toxicity	OECD 422; GLP, rat, 30, 150, and 750 mg/kg	NOEL 750 mg/kg
Reproductive and Developmental Toxicity	OECD 421; GLP, rat, 1.5, 4.5, or 15.0 ppm (91, 276, 905 mg/kg in males and 120, 359, 1135 mg/kg in females)	NOAEL 4.5 ppm (276 mg/kg in males and 359 mg/kg in females.) NOEL for teratogenicity 15.0 ppm (approx 1000 mg/kg)

Genotoxicity	Mutagenicity OECD 471; GLP, Ames OECD 476; GLP, HGPRT in CHO cells	Negative Negative
	Chromosomal Aberration; OECD 473, GLP, CHO cells	Negative
Adsorption, Distribution, Metabolism, and Elimination	Other, rats, oral gavage	TXIB was rapidly adsorbed, metabolized, and excreted

TXIB Toxicity Summary

Acute Toxicity Data

Acute Oral Toxicity

Study 1

Oral LD₅₀: >2,000 mg/kg; rat (highest dose tested)

Method: OECD 425 with GLP assurances. Rats (N=5 females) were dosed with 2,000 mg/kg. After the first rat showed no toxicity, 4 more were administered test material.

Discussion and Interpretation: The test article was of very low toxicity. No deaths were noted at this high dose with all animals surviving the scheduled 14-day observation period. Clinical signs consisted of a wet anogenital area on Day 0 and 1. All animals were normal from Day 2 through 14 gaining weight in a normal manner. Necropsy results were normal.

Reference: Acute Oral Toxicity – Up and Down procedure; MB Research Laboratories; Project number MB 07-16050.01; Report Date: November 14, 2007.

Study 2

Oral LD₅₀: > 3,200 mg/kg; rat (highest dose tested)
> 6,400 mg/kg; mouse (highest dose tested)

Method: Other; Rats (N=2/dose) were dosed with test article at doses of 800, 1600, and 3200 mg/kg. Mice (N=2/dose) were exposed to 400, 800, 1600, 3200, and 6400 mg/kg.

Discussion and Interpretation: The test article was of very low toxicity. No deaths were noted at this high dose with all animals surviving the scheduled 14-day observation period. Clinical signs in rats consisted of moderate weakness and vasodilation. All mice were noted as normal.

Reference: Kodak Toxicity Report (acute toxicity data); Report Date: 9-20-61

Acute Inhalation Toxicity

Inhalation LC₅₀: >5,300 mg/m³; rat (highest concentration tested)

Method: Other; Rats (N=3/dose) were exposed to concentrations test article at either 120 or 5,300 mg/m³ for 6 hours (10 or 453 ppm). This LC₅₀ concentration is approximately 100X greater than saturated vapor concentration. The studies were conducted in 1961.

Discussion and Interpretation: The test article was of very low toxicity. No deaths were noted at either concentration with the only clinical sign noted to be "pink extremities" at the highest concentration.

Reference: Kodak Toxicity Report (acute toxicity data); Report Date: 9-20-61

Acute Dermal Toxicity

LD₅₀: >2,000 ml/kg; rabbit (highest dose tested)

Method: OECD 402 with GLP assurances. New Zealand white rabbits (N=5/sex) were exposed to 2,000 mg/kg body weight with material held in place for 24 hours. Animals were monitored for 14 days

Discussion and Interpretation: The test article was of very low toxicity. No deaths were noted with all animals surviving the scheduled 14-day observation period. Clinical signs consisted of occasional diarrhea, few feces, and soiling of the anogenital area. All animals were normal by Day14 gaining weight in a normal manner. Necropsy results revealed abnormalities of the spleen, thymus, and pancreas.

Reference: Acute Dermal Toxicity/LD₅₀ in Rabbits; MB Research Laboratories, Project number MB 07-16050.02; Report Date: November 14, 2007.

Acute Dermal Irritation and Toxicity

Study 1

Dermal LD₅₀: >20,000 ml/kg; guinea pig (highest dose tested)

Dermal Irritation: Slight

Method: Other; Three guinea pigs were exposed to 5 - 20 ml/kg TXIB under an occlusive wrap for 24 hours.

Discussion and Interpretation: The test material induced slight irritation and was of very low toxicity following dermal exposure. Animals exhibited slight to moderate edema and erythema with some desquamation noted at 7 and 14 days. No deaths were noted although 2 of 3 animals lost weight. No other clinical signs were noted.

Reference: Kodak Toxicity Report (acute toxicity data); Report Date: 9-20-61

Study 2

Result: Negative; humans

Method: The study involved 200 volunteers using a Modified Draize Procedure and involved the application of a discontinuous series of nine repetitive applications over a three-week induction period of a 1.0% solution in acetone using a semi-occluded patch. Induction was followed by an approximate two-week rest period with a challenge consisting of a single application of test material to naïve skin

Discussion and Interpretation: Under the conditions of this study, TXIB was found to be nonirritating and did not induce any evidence of sensitization in human volunteers. Responses during induction consisted of isolated instances of slight to mild redness. Responses during challenge consisted of three instances of slight redness.

Reference: Hill Top Research, Inc. "Repeated Insult Patch Test in Humans (Modified Draize Procedure). 2001.

Acute Dermal Irritation

Study 1

Dermal Irritation: None

Method: OECD 404 with GLP assurances. Three rabbits were exposed to 0.5 ml of test material for 4 hours under an occlusive wrap. Reactions were scored at 24, 48, and 72 hours.

Discussion and Interpretation: The test material induced no evidence of irritation based on an absence of edema and erythema. All animals gained weight and no other clinical signs were noted.

Reference: Primary Dermal Irritation/Corrosion in Rabbits; MB Research Laboratories, Project number MB 07-16050.03; Report Date: November 21, 2007.

Study 2

Dermal Irritation: Slight

Method: Other; Three animals were exposed for 24 hours to 5 - 20 ml/kg under an occlusive wrap.

Discussion and Interpretation: The test material induced slight irritation and was of very low toxicity following dermal exposure. Animals exhibited slight to moderate edema and erythema with some desquamation noted at 7 and 14 days. No deaths were noted although 2 of 3 animals lost weight. No other clinical signs were noted.

Reference: Kodak Toxicity Report (acute toxicity data); Report Date: 9-20-61

Acute Ocular Irritation

Result: Slightly irritating; rabbit

Method: OECD 405; the study followed GLP assurances and was conducted in 1990. Six rabbits were used with 3 having their eyes washed and 3 being unwashed after having been instilled with 0.1 ml of test material into one eye.

Discussion and Interpretation: The test material induced slight to no irritation. Only 1 animal in the unwashed group showed conjunctival redness (score of 1) after 1 hour that cleared by 24 hours. All the animals whose eyes were washed showed a conjunctival redness (score of 1) at one hour and again were all normal by 24 hours.

Reference: 2,2,4-trimethyl-1,3-pentanediol diisobutyrate Synonym: Texanol isobutyrate (TXIB) Acute eye irritation study in the rabbit, Eastman Kodak Company, August 2, 1990

Dermal Sensitization Potential

Study 1

Result: Negative; guinea pig

Method: Other; Three guinea pigs were exposed to 1% test material in an organic solvent.

Discussion and Interpretation: The results of the study indicate that the animals in this study had not been sensitized to the test material.

Reference: Kodak Toxicity Report (acute toxicity data); Report Date: 9-20-61

Study 2

Result: Negative; humans

Method: The study involved 200 volunteers using a Modified Draize Procedure and involved the application of a discontinuous series of nine repetitive applications over a three-week induction period of a 1.0% solution in acetone using a semi-occluded patch. Induction was followed by an approximate two-week rest period with a challenge consisting of a single application of test material to naïve skin

Discussion and Interpretation: Under the conditions of this study, TXIB was found to be nonirritating and did not induce any evidence of sensitization in human volunteers. Responses during induction consisted of isolated instances of slight to mild redness. Responses during challenge consisted of three instances of slight redness.

Reference: Hill Top Research, Inc. "Repeated Insult Patch Test in Humans (Modified Draize Procedure). 2001.

Genotoxicity Data

Mutagenicity Studies

Study 1

Result: Negative

Method: Ames assay +/- S9 microsomes using Salmonella and Escherichia coli bacterial strains following a protocol equivalent to OECD 471. The study was conducted under GLP assurances.

Discussion and Interpretation: The results indicate this test material did not induce mutations and should not be considered as a genotoxicant.

References: Mutagenicity test with EC 95-0205, TXIB in the Salmonella-Escherichia coli/mammalian-microsome reverse mutation assay with a confirmatory assay: September 12, 1995.

Study 2

Result: Negative

Method: The study followed OECD guideline 476 assessing HGPRT mutations in CHO cells +/- S9 microsomes. The study was conducted under GLP assurances.

Discussion and Interpretation: The results indicate this test material did not induce mutations and should not be considered as a genotoxicant.

References: Mutagenicity test on EC 95-0205 in the CHO/HGPRT forward mutation assay with duplicate cultures and a confirmatory assay: September 21, 1995.

Chromosomal Aberration Studies

Result: Negative

Method: The study followed OECD 473, in vitro chromosomal aberration assay in mammalian cell (Chinese hamster ovary cells) with and without S9 microsomes. The study was conducted under GLP assurances.

Discussion and Interpretation: The results of this study indicate this test material did not induce chromosomal aberrations and should not be considered as a genotoxicant.

Reference: Mutagenicity test on EC 95-0205, TXIB Measuring chromosomal aberrations in Chinese hamster ovary (CHO) cells: with a confirmatory assay with multiple harvests; October 25, 1995.

Repeated Exposure Studies

90-Day feeding study in rats

Result: The calculated mean compound consumption yielded actual dose levels of 30.28, 151.34, and 751.59 mg/kg for males and 30.84, 153.03, and 754.81 mg/kg in females. There were no test article induced deaths, ophthalmoscopic finding, clinical observations, or alterations in the functional observational battery findings. There were no differences seen in BW and significant reductions in food consumption were only noted on a sporadic basis in the high dose. There were no adverse test article-related effects seen on the hematology parameters or in the urinalysis. At 750 mg/kg the only clinical chemistry change deemed significant and to have occurred in both sexes was an increase in cholesterol on Days 15, 45, and 90 in males and on Days 45 and 90 in females. Males only at this dose exhibited an increase in total bilirubin (Day 90) and creatinine (Day 90). Other statistical changes at this dose, as well as occasional ones seen at 30 and 150 mg/kg, were either not dose related, consistent between sexes, or were still within control range and thus none were deemed to be of toxicological relevance. There were no macroscopic findings of relevance. Organ weight changes deemed to be a result of test article exposure at 750 mg/kg included male kidney weights and liver weight in both sexes. Histopathology of the kidneys in all treated males showed evidence of hyaline droplets do to an accumulation of alpha-2-u-globulin. In addition, it was noted that the incidence rate of chronic progressive nephropathy (CPN; a normal degenerative change) was increased, in males only, in animals receiving 750 mg/kg TXIB. The severity of the lesion however, was no different than controls and the histological appearance of all other organs was comparable to controls.

Method: US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, Subchronic study (13 weeks) with a functional observational battery assessment. Study utilized GPL assurances. Twenty Crl:CD(SD) rats/sex/dose were exposed to TXIB in their feed at varying concentrations to maintain a daily dose of approximately 30 150 and 750 mg TXIB/kg (diet concentrations were adjusted to maintain a constant dose level). Commensurate with a modern guideline study complete hematology and clinical chemistry profiles were conducted at Day 15, 45, and termination, as well as a robust number of tissues were harvested for analysis. Special emphasis was placed on kidney histopathology to assess for accumulation of alpha-2-u-globulin as well as the histopathology of the reproductive organs in males.

Discussion and Interpretation: TXIB was well-tolerated by the animals with a NOAEL of 150 mg/kg in males and 750 mg/kg in females. No NOEL level was established as alpha-2-u-globulin induced nephropathy was seen in males at all levels. There was no evidence of toxicity in the male reproductive organs at 750 mg/kg. Also the significance of the increased incidence of CPN is questionable as it severity was no different than controls, did not impact function, was concurrent with hyaline droplet induced changes, and was only seen in males. Chemical exacerbation of CPN usually occurs in conjunction with the induction of α 2u-g nephropathy (Hard et al, 1993), so this is not a surprising observation in the males. Regardless of the apparent increases,

exacerbation of CPN in rats should be regarded as an adverse effect, but not an indicator *per se* of chemically induced toxicity. This is because the incidence and severity of this spontaneous disease can be influenced by physiological factors (Hard and Khan, 2004). CPN can be modified by varying the protein content of the diet or the source of protein (Iwasaki *et al.*, 1988; Masoro and Yu, 1989; Rao *et al.*, 1993), by varying caloric intake (Keenan *et al.*, 2000), and by male sex steroid manipulation (Baylis, 1994). Furthermore, CPN is characterized by a spectrum of histopathology and clinical features that set it apart from the main causes of chronic renal disease in humans (Hard and Khan, 2004). Thus, rat CPN has no strict counterpart in humans and therefore appears to have no relevance to human hazard assessment (Hard and Khan, 2004).

Reference: A Thirteen-Week Dietary Toxicity Study of TXIB Plasticizer in Rats. Craig L. Study No.:777-005, MPI Research August 2005.

Baylis C (1994). Age-dependent glomerular damage in the rat. Dissociation between glomerular injury and both glomerular hypertension and hypertrophy. Male gender as a primary risk factor. *J Clin Invest* 94: 1823-1829.

Hard GC and Khan KN (2004). A contemporary overview of chronic progressive nephropathy in the laboratory rat, and its significance for human risk assessment. *Toxicologic Pathol* 32: 171-180.

Hard GC, Rodgers IS, Baetcke KP, Richards WL, McGaughy RE, and Valcovic LR (1993). Hazard evaluation of chemicals that cause accumulation of α_{2u} -globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidneys of male rats. *Environ Hlth Perspect* 99: 313-349.

Iwasaki K, Gleiser CA, Masoro EJ, McMahan CA, Seo EJ, and Yu BP (1998). The influence of dietary protein source on longevity and age-related disease processes of Fischer rats. *J Gerontol*43: B5-12.

Keenan KP, Coleman JB, McCoy CL, Hoe CM, Soper KA, and Laroque P (2000). Chronic nephropathy in ad libitum overfed Sprague-Dawley rats and its early attenuation by increasing degrees of dietary (caloric) restriction to control growth. *Toxicologic Pathol* 28: 788-798.

Masoro EJ and Yu BP (1989). Diet and nephropathy. *Lab Invest* 60: 165-167.

Rao GN, Edmondson J, and Elwell MR (1993). Influence of dietary protein concentration on severity of nephropathy in Fischer-344 (F-344/N) rats. *Toxicologic Pathol* 21: 353-361.

90-Day feeding study in dogs

Result: All dogs survived with no evidence of any clinical signs, or effects on growth and food consumption at any dose. No treatment-related toxicities were noted in

hematology, clinical chemistries, or urinalysis. No changes in organ weights were deemed to be of toxicological relevance (relative increases in liver and pituitary weights, especially at the high dose, were deemed not relevant as they were within normal limits for beagles and there was no histological or clinical chemistry changes noted).

Method: Four beagle dogs/sex/dose were fed a diet containing 0.0%, 0.1%, 0.35%, or 1.0% TXIB for 90 days. Hematology (Hb, Hct, Prothrombin time, and white cell count and differentials), clinical chemistry (BUN, glu, SGOT, LDH, and SAP) and urinalysis were conducted at study initiation and after 6 and 12 weeks. Organs weighed and histologically examined included the liver, kidney, spleen, heart, pituitary, adrenal, gonads, and thyroid were weighed and histologically examined. Other tissues histologically examined included: stomach, lg & sm intestine, pancreas, bladder, thymus, salivary glands, mesenteric lymph nodes, aorta, lungs, bone marrow, skeletal muscle with attached nerves, spinal cord, brain, gall bladder.

Discussion and Interpretation: TXIB was well-tolerated by the animals with a NOAEL of 1.0%.

Reference: Subacute (90-Day) Feeding Study of Texanol Isobutyrate in Dogs. Food Drug Research Laboratories Inc., Maspeth NY, June 15, 1966. These data were published in the manuscript "The toxicology and fate of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate" Toxicol. and Appl. Pharmacol 22, 387-399 (1972).

100-Day feeding study in rats

Result: All but one high-dose rat survived with no toxicologically significant changes seen in growth, behavior, hematology, or in tissues examined grossly or microscopically. At 0.1% there were no effects in male organ weights, while females had significantly reduced kidney weights. At 1.0% males had significantly elevated liver weights (both absolute and relative) and females showed a significant decrease in absolute kidney weight. (Note: The manuscript in which these data were published indicated female liver/BW ratios were significantly increased. However, this is believed to be an error as the effect was not noted in the laboratory report as being significant). The high dose animal was sacrificed on Day 55 after showing weight loss and marked symptoms of a respiratory infection. The average amount of dose received over the 100 days for the 0.1% group was 75.5 mg/kg (M) and 83.5 mg/kg (F); the 1.0% group was exposed to 772 mg/kg (M) and 858.5 mg/kg (F).

Method: 10 albino (Holtzman) rats/sex/dose were fed a diet containing 0.0%, 0.1%, or 1.0% TXIB for 103 days. Hematology (Hb, cell volume, white cell count and differentials) was conducted prior to treatment and at study termination. Tissues taken for histopathology included: esophagus, stomach, lg & sm intestine, liver, trachea, lung, thyroid, parathyroid, spleen, brain, heart, kidney, bladder, adrenal, gonads, and bone. The only organs weighed were the liver and kidney.

Discussion and Interpretation: TXIB was well-tolerated by the animals with a NOAEL of 1.0%. This is equivalent to 772 mg/kg in males and 858.5 mg/kg in females. The study

authors stated that the low kidney weights seen in females were due to unusually high values seen in controls and that they were more comparable to historical control values. Since they lacked any evidence of histopathology (nor was any seen in males) the effect was not deemed toxicologically relevant

Reference: Texanol isobutyrate: 100 day feeding study. David Fassett, Eastman Kodak Company, March 14, 1963. These data were published in the manuscript "The toxicology and fate of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate" Toxicol. and Appl. Pharmacol 22, 387-399 (1972).

Repeat dose and combined reproductive/developmental toxicity study

Result: All animals survived with no treatment-related clinical signs noted. There were no significant effects on body weight at any dose at any time. Food consumption was normal between groups except high dose females who showed an increase in the level of feed consumed. There were no significant treatment-related effects in hematology in either sex. Changes in clinical chemistries were indicative of effects on the liver and kidney with increased albumin (and A/G ratio), creatinine, and total bilirubin at 150 and 750 mg/kg. Interestingly, significant decreases were noted in serum levels of the GOT, GPT, GGT enzymes at these doses. Calcium (mid and high dose), inorganic phosphate (all dose levels) were significantly increased. A slight, but significant, decrease was noted in serum chloride concentration. Organ weight changes seen at 750 mg/kg included absolute and relative liver weight increases in both sexes and kidney weight increase in males only. At 150 mg/kg, males showed an increase relative liver weight. At 30 mg/kg there was a decrease in absolute, but not relative, weight of the thymus. There were no effects noted on the thymus weight or histologically at the highest dose so this weight change was not considered a treatment-related effect. Various findings were noted on gross examination of organs in the two highest dose levels. Histologically the only effect noted due to TXIB exposure (sporadic non-treatment related changes were noted in some organs) was necrosis of the kidney tubules in high dose males along with an accumulation of alpha-2-u-globulin at the mid (5/11) and high dose (10/11). Also seen was centrilobular hypertrophy in the liver of high dose males. No histological changes were reported in the testes.

Method: OECD test guideline 422 using 12 rats/sex with daily gavage dosing at levels of 30, 150 and 750 mg/kg. Males and females received test material for 28 days (2 weeks before mating and 2 weeks during mating). In addition males received it for an additional 16 days for a total of 44 days; while females received test material throughout gestation and until Day 3 of lactation. In males, complete hematology and clinical chemistry profiles were conducted. A robust number of tissues were harvested for analysis commensurate with a modern OECD guideline repeat dose study. The study utilized GLP assurances.

Discussion and Interpretation: TXIB was well-tolerated by the animals with a NOEL of 30 mg/kg in males and 150 mg/kg in females. Effects noted in males at 150 mg/kg should be deemed as being adaptive in nature and as a result of the alpha-2u-globulin accumulation, and thus 150 mg/kg should be considered a NOAEL.

Reference: Combined repeat dose and reproductive/developmental toxicity screening test of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate by oral administration in rats. Hagita, K., Tanaka, R., Watari, N., Shojiu, A., Hirouchi, Y., and Kobayashi, K. Biosafety Research Center, Foods, Drugs, Pesticides. Shizuoka, Japan. 1993. <http://wwwdb.mhlw.go.jp/ginc/html/db1.html>

Developmental and Reproductive Toxicity

Study 1

Repeat dose and combined reproductive/developmental toxicity study

Result: All animals in the control and low dose successfully copulated. One mating pair at the mid and high dose failed to copulate. All successfully copulated females became pregnant except for one low dose female. The mean estrous cycle of the high dose (4.1 days) was significantly shorter than the control value (4.6 days). This change was still within historical controls (4.0 days). There were no abnormalities during delivery. The gestation period, numbers of CL, implantation sites, pups born, live pups born, sex ratio, number live on Day 4, and number of stillborns were similar across all dose groups. Similarly there was no effect on gestation index, implantation index, delivery index, live birth index, or variability on Day 4 among any of the groups. None of the pups showed any external abnormalities. The Day 4 lactation body weights of the pups of both sexes tended to be higher in the 750 mg/kg dose group.

Method: OECD test guideline 422 using 12 rats/sex with daily gavage dosing at levels of 30, 150 and 750 mg/kg. Males and females received test material for 28 days (2 weeks before mating and 2 weeks during mating). In addition males received it for an additional 16 days for a total of 44 days; while females received test material throughout gestation and until Day 3 of lactation. The study utilized GLP assurances.

Discussion and Interpretation: TXIB was well-tolerated by the animals with a NOEL of 750 mg/kg for developmental and reproductive toxicity.

Reference: Combined repeat dose and reproductive/developmental toxicity screening test of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate by oral administration in rats. Hagita, K., Tanaka, R., Watari, N., Shojiu, A., Hirouchi, Y., and Kobayashi, K. Biosafety Research Center, Foods, Drugs, Pesticides. Shizuoka, Japan. 1993. <http://wwwdb.mhlw.go.jp/ginc/html/db1.html>

Study 2

Combined reproductive/developmental toxicity study

Result: There were no mortalities and the only consistent clinical observation noted was reduced and soft feces most notably in the high-dose animals. High-dose males had reduced feed intake on Day 7 while females showed reductions on Day 7 (pre-mating), Day 7 (gestation), and Day 4 (lactation). Reductions in BW were also occasionally seen in high-dose exposed animals occurring on Day 7 (pre-mating) in

both sexes and Day 20 gestation. There were no effects noted on organ weights (testes and epididymides), nor were any treatment-related observations noted grossly or histologically in any of the reproductive organs examined (testes, epididymides, and ovaries). Sperm analysis indicated no effect on motility. The mean total count of epididymal spermatozoa was significantly lower in all dose groups. However, the weight adjusted counts were not significantly different. Other changes of significance noted in the high dose group consisted of a reduction in the number of sperm/tissue and weight adjusted testicular spermatids heads. A reduction in the weight adjusted count was also noted in the low dose but the lack of effect in the mid-dose group suggested that the effects noted for testicular sperm counts in the low-dose group were not related to TXIB exposure. There were no neonatal observations of significance and none of the pups showed any external abnormalities. Statistically significant reproductive effects observed in the high dose group included a reduced number of implantation sites, mean litter weights on Days 0 and 4, and the number of live pups on Day 4. The mean number of pups dying between Days 0 and 4 was higher in the mid-dose group. There were no abnormalities in any other parameters: reproductive performance fertility index, fecundity index, precoital interval, gestation duration, pup survival, post-implantation loss, number of implants, live and dead pups, sex ratio, and pup body weight and body weight change.

Method: OECD test guideline 421 with additional sperm motility assessment experiments. Twelve Sprague-Dawley rats of each sex were fed test diets containing 1.5, 4.5, or 15.0 ppm TXIB. This resulted in daily dose of 91, 276, 905 mg/kg in males and 120, 359, 1135 mg/kg in females. The study consisted of pre-mating (14 days), mating (1 to 8), gestation (21-23 days), and early lactation (4 to 5 days). The study utilized GLP assurances.

Discussion and Interpretation: The NOAEL for reproductive and developmental toxicity was deemed by the study director to be 276 mg/kg in males and 359 mg/kg in females. These NOAELs were also confirmed in a review by Dr. Willem Faber. Please see Appendix I ("Expert Review of TXIB Reproductive/Developmental Toxicity Screening Study (Report TX-2001-031) in Rats") where a thorough discussion is provided about the significance of the observed findings.

Reference: Gearhart, S and David, RM. 2,2,4-trimethyl-1,3-pentanediol diisobutyrate
Synonym: TXIB; Reproductive/developmental toxicity screening test in the rat. Eastman Kodak Company. Report Date: August 10, 2001

Adsorption, Distribution, Metabolism, and Excretion Studies

Study 1

Result: The major route of elimination was urine (47 – 72% total dose) within 10 days and the majority of this occurring in the first 72 hours. Radioactivity in feces accounted for 14 – 31% of the dose with elimination being essentially complete by 7 days. Radiolabeled CO₂ was not detected. At 8 days the carcass and organs combined

accounted for 2.9% of the dose with this decreasing to less than 1% on Days 15 and 22.

Method: Three Sprague-Dawley rats received radiolabeled TXIB by gavage. A single animal was sacrificed on Day 8, 15, and 22 after being administered 250, 236, or 283 mg/kg TXIB-¹⁴C. Urine, feces, and cage washes were collected in 24 hour intervals. Air was analyzed for ¹⁴CO₂. At termination liver, brain, kidneys, spleen, lungs, GI tract, fat, and carcass were examined for radioactivity.

Discussion and Interpretation: TXIB is readily absorbed, metabolized, and rapidly eliminated from the body with urine being the primary excretion route. The vast majority was eliminated within 72 hours.

Reference: The physiological disposition of the diisobutyrate ester of 2,2,4-trimethyl-1,3-pentanediol-3-¹⁴C (TXIB-3-¹⁴C). Ethel Cantor, Eastman Kodak Company, Report Date: April 11, 1966.

Study 2

Result: The major route of elimination was urine (47 – 72% total dose) within 5 - 10 days and the majority of this occurring in the first 72 hours. Radioactivity in feces accounted for 14 – 31% of the dose with elimination being essentially complete by 7 days with the majority isolated after 48 hours. Radiolabeled CO₂ was not detected. In total, excretions accounted for 95-99% of the dose. Residual radioactivity of treated animals approached control by two weeks. Identification of metabolites showed the feces to contain both 2,2,4-trimethyl pentanediol (TMPD) and TXIB-3-¹⁴C indicating esterase cleavage of the two isobutyrate esters. A small portion of the absorbed material in the urine was unchanged TXIB-3-¹⁴C while the majority consisted of metabolites consistent with complete cleavage to the glycol (TMPD) parent molecule. Although much of the urinary metabolite was unidentified it does, nonetheless, represent rapidly cleared material.

Method: Five Sprague-Dawley rats received radiolabeled TXIB by gavage. A single animal was sacrificed on Day 8, two on Day 14, one on Day 15, and one on Day 22 after being administered 236, 250, 283, 350, or 895 mg/kg TXIB-3-¹⁴C. Urine, feces, and cage washes were collected in 24 hour intervals. Air was analyzed for ¹⁴CO₂. At termination liver, brain, kidneys, lungs, fat, and carcass were examined for radioactivity.

Discussion and Interpretation: TXIB is readily absorbed, metabolized, and rapidly eliminated from the body with urine being the primary excretion route. The vast majority was eliminated within 72 hours with no accumulation in any specific tissue.

Reference: The metabolic fate and physiological disposition of the diisobutyrate ester of 2,2,4-trimethyl-1,3-pentanediol-3-¹⁴C (TXIB-3-¹⁴C). Cantor, E. and Astill, BD. Eastman Kodak Company, Report Date: July 20, 1966.

Remark

Results from the two ADME studies described above were summarized in an internal

Eastman Kodak Company report entitled: The physiological disposition of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate (Kodaflex TXIB) in rats: A Summary. Astill, BD and Cantor, EE. Report Date: June 20, 1967. These data were also part of a publication "The toxicology and fate of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate" *Toxicol. and Appl. Pharmacol* 22, 387-399 (1972).

Specialized and Experimental Studies to demonstrate reversibility of liver effects

Study 1

Reversibility of weight change

Result: There were no mortalities or statistically significant changes noted in body weights, growth rates, or in food consumption and efficiency in any of the three experiments. There were no differences in absolute organ weights in any of the animals in any of the three experiments. All organs microscopically examined in all experiments appeared normal. However, all animals (M&F) fed diets containing 1.0% TXIB for 51 days, 99 days, or the last 47 days of experiment 3 showed significant increases in relative liver weight. Other relative organ weight effects were noted in the kidneys of males and females fed 1.0% TXIB for 51 days (but not 99 days or the last 47 days in experiment 3). Females also showed increases in relative thyroid and brain weights after 99 days of exposure. There were no statistically significant effects noted in the hematology or clinical chemistry parameters analyzed (Note: The manuscript in which these data were published indicated that the SGOT values were elevated in males fed 0.1 and 1.0% TXIB for either 52 or 99 straight days and for females exposed for 99 days. Enzyme levels were still elevated at both doses in males and females in experiment 3 under both the exposure scenarios i.e., test diet for 52 days than control diet for 47 days or control for 52 days than test diet. The manuscript noted their elevation although significant was not manifested in a dose or time related manner and were within historical control values for all groups.) Dose levels of material consumed in experiment one for 51 days at 1.0% were 708 mg/kg (M) and 747 mg/kg (F); while 0.1% animals received either 70 mg/kg (M) or 68 mg/kg (F). In experiment two animals on the 1.0% diet received 824 mg/kg (M) and 853 mg/kg (F); the 0.1% test diet animals received 79 mg/kg (M) and 87 mg/kg. In experiment three animals on the 1.0% diet for the first 52 days received 959 mg/kg (M) and 947 mg/kg (F) while those on the 0.1% test diet received 94 mg/kg (M) and 79 mg/kg. Animals who received 1.0% test diets for the second half of the experiment (Days 52-99) received 558 mg/kg (M) and 614 mg/kg (F); those on the 0.1% test diet averaged 55 mg/kg (M) and 59 mg/kg.

Method: Three dietary studies were carried out to assess the reproducibility and reversibility of previously seen changes in liver weights. 10 albino (Holtzman) rats/sex/dose were fed diets containing 0.0%, 0.1%, or 1.0% TXIB. In study number one groups were fed test diets for 51 days while in study two they received TXIB for 99 days. In study three two groups of animals received 0.1% or 1.0 % TXIB diets for 52 days than were placed back on control diet till termination at Day 99, while another two

groups were treated in an opposite manner, they received control diet for 52 days followed by TXIB test diets till Day 99. Body weights and feed consumption were recorded weekly while clinical signs were daily during the first month of the studies and weekly thereafter. Hematology (Hb, cell volume, white cell count and differentials) was conducted on the 43 and 91st days of feeding. Blood for assessing AP and SGOT was collected just prior to sacrifice. Tissues taken for histopathology included: trachea, lung, heart, tongue, esophagus, stomach, lg & sm intestine, liver*, kidney*, bladder, adrenal*, pancreas, thyroid*, parathyroid, pituitary*, gonads*, spleen, brain* (cerebrum and cerebellum), eye, and femoral bone marrow ("*" denotes organs that were also weighed).

Discussion and Interpretation: Similar to the 103 day feeding study in rats previously performed TXIB was well-tolerated by the animals with a NOAEL of 1.0%. The results of this study, with three different exposure scenarios, demonstrated that the increase in relative liver weights seen in the first 103 day study could be reproduced (experiment two 99 day exposure), could be manifested after only receiving diet for 52 days (experiment 1), and was an adaptive effect that was readily reversed (experiment three). Therefore, it should be of no toxicological relevance. Similarly, the significant changes noted in the other relative organ weights (brain and thyroid) seen in this study at 1.0% were not considered to be toxicologically relevant as they were only noted in one sex and was only noted as a relative effect with body weights slightly lower in these animals. Importantly, there was no change in absolute weight and no change in histological appearance. Effects on these organs were also not noted in the first repeated dose study either. The change in male and female kidney weights also was not considered relevant as it was only noted in experiment one and was also only a relative effect that occurred in the absence of any histopathological changes.

Reference: Texanol isobutyrate a three part dietary feeding study
David Fassett, Eastman Kodak Company, Report Date: June 11, 1969.

Study 2

Reversibility of liver enzyme changes

Result: Males and females fed 1.0% TXIB for either 52 or 99 days showed significant increases in p-nitroanisole demethylase. Males and females fed TXIB for 52 days also had elevated UDP-bilirubin-glucuronyl transferase and UDP-p-aminophenol glucuronyl transferase levels increased. Interestingly only the UDP-bilirubin-glucuronyl transferase level was increased after 99 days of feeding and only in females. Importantly, none of the four enzymes were elevated in experiment three in which animals were fed control diets for 47 days after being fed 1.0% TXIB for the first 52 days. Seven daily IP injections of 100 mg/kg TXIB resulted in elevated levels of UDP-p-aminophenol glucuronyl transferase only.

Method: Liver microsomal enzyme activities for the following enzymes: glucose-6-phosphatase, p-nitroanisole demethylase, UDP-p-aminophenol glucuronyl transferase, UDP-bilirubin-glucuronyl transferase were measured from the livers harvested in study one described above. In addition to the three feeding studies, levels of the two

glucuronyl transferase enzymes were evaluated in rats (males only) after receiving seven daily IP injections of TXIB in corn oil at a rate of either 25 or 100 mg/kg.

Discussion and Interpretation: The results of this study demonstrated that TXIB exposure in the diet at 1.0% or following 7 IP doses of 100 mg/kg could induce the activity of certain metabolic enzymes in the liver. These increased levels of enzymes activities were readily reversible after animals were removed from TXIB diets for 47 days.

References:

- 1.) Rat liver enzyme activities after feeding and intraperitoneal exposure to TXIB. Tischer, KS. Eastman Kodak Company. Report Date: October 24, 1969.
- 2.) TXIB: An example of the application of inducible oxidative and conjugative enzymes, and glucose-6-phosphatase to safety evaluation. Tischer, KS. Eastman Kodak Company. Report Date: December 1, 1969.

Remark

These data were published in the manuscript: "The reversibility of increased rat liver weights and microsomal processing enzymes after feeding high levels of 2,2,4-trimethyl-1,3-pentanediol diisobutyrate" Toxicol. Appl. Pharmacol 22, 400-408 (1972).

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Appendix I.

EXPERT REVIEW OF TXIB REPRODUCTIVE/DEVELOPMENTAL TOXICITY SCREENING STUDY (REPORT TX-2001-031) IN RATS

April 2004

Provided by:

Willem Faber, Ph.D., D.A.B.T.

Willem Faber Toxicology Consulting, L.L.C

Reproductive & Developmental Toxicology Specialist

1338 Strong Road, Victor, NY 14564

wfaber@msn.com

585-742-1568

The potential for TXIB to cause reproductive and developmental toxicity was evaluated in an OECD 421 screening assay. There was no evidence of developmental toxicity in this study. However, several endpoints of reproductive toxicity in the high-dose group treated with 905-1135 mg/kg/day of TXIB for 50 days were statistically significantly different from the control group values. In addition, isolated endpoints of reproductive function were also statistically significantly different in the low and mid-dose groups (91-120 and 276-359 mg/kg/day, respectively).

Statistically significant reproductive effects observed in the high dose group included a reduced number of implantation sites, reduced mean litter weights on postnatal (PND) 0, reduced mean number of live pups on PND 4, decreased mean absolute epididymal sperm counts, and reduced absolute and relative (to body weight) testicular sperm counts. The mean number of live pups per litter on PND 0 was also reduced (12.2 vs. 14.5 for the control group) but this difference was not statistically significant.

Effects observed in the mid-dose group were restricted to decreased mean absolute epididymal sperm counts while the low-dose group had decreased mean absolute epididymal sperm counts and reduced absolute and relative (to body weight) testicular sperm counts. Absolute and relative (to body weight) testicular sperm counts for the mid-dose group were slightly higher than the mean control group value. The lack of effect in the mid-dose group suggested that the effects noted for testicular sperm counts in the low-dose group were not related to TXIB exposure. There was no effect on absolute or relative (to body weight) testes or epididymides weights in any of the treated groups. There were also no changes in the histopathology of the testes or epididymides in the high-dose group animals.

In order to interpret these findings, it is important to understand what each endpoint represents. This is relatively straightforward for the effects on number of implantation sites, mean litter weights, and mean number of live pups. However, a more detailed understanding of sperm parameters is needed to evaluate the meaning and significance of the effects on absolute epididymal sperm counts, and absolute and relative (to body weight) testicular sperm counts seen in this study.

The testes produce spermatids that are then stored in the epididymides while undergoing maturation. The method used to measure the gamete count within the testes in this study was based upon the fact that spermatid (immature sperm) within the testes are resistant to homogenization during (approximately) the last 6 days of residence in the testes (Chapin, R.E. and Conner, M.W. (1999) "Testicular Histology and Sperm Parameters" in "An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment", ILSI Press,

International Life Sciences Institute, 1126 Sixteenth St., N.W., Washington, D.C. 20036-4810, USA.) The spermatozoa within the epididymides are also resistant to homogenization and differences in count within this tissue reflect changes in either testicular production or the storage function of the epididymides.

Spermatogenesis requires about 4.5 cycles of the seminiferous epithelium of the testes, with a cycle length of 12.9 days in the rat (Thomas, J.A. (1996) "Toxic Responses of the Reproductive System" in Casarett & Doull's Toxicology: The Basic Science of Poisons, Fifth Edition, McGraw Hill Publishers). Therefore, one entire cycle of spermatogenesis in the rat takes approximately 58 days. Maturation of spermatid to spermatozoa within the epididymides takes another 5 days for a total cycle length of 63 days. Protocols for definitive reproductive toxicity studies typically require treatment for approximately 70 days prior to breeding to allow for test article and/or metabolites to achieve significant blood levels thereby allowing for the possibility of affecting the entire cycle of spermatogenesis.

The male rats in the TXIB study were exposed for 50 days prior to collection of the sperm parameters. Therefore, the sperm count collected from the epididymides was determined by testicular cell division that occurred from 7-13 days prior to exposure to TXIB. Testicular sperm counts reflect cell division that occurred from 2-7 days prior to TXIB exposure. Since the stages of spermatogenesis responsible for these counts occurred prior to TXIB exposure, it is highly unlikely that the reductions in these counts were related to altered cell division within the testes.

Another function of the testes and epididymides is to provide an environment for sperm maturation. Theoretically, a chemical could affect sperm maturation within the testes leading to increased phagocytosis of damaged immature sperm by the Sertoli cells within the seminiferous tubules. The problem with this explanation for the results from the TXIB study is that there was no evidence of damaged immature sperm observed histologically nor was there an effect on sperm motility, a function certain to be affected in damaged sperm. In addition, the epididymal counts were similar to control values when the mass of the organ was considered in the analysis (no effects observed on counts/gram of tissue). Consideration of historical control values from the same laboratory do not add anything to the analysis, since the concurrent control counts were unusually low compared to the historical values for these same parameters.

In conclusion, the finding of decreased number of implantation sites and decreased live pups on PND 0 in the high-dose group suggest a treatment-related effect of TXIB exposure. However, it is not clear if the male or female (or both) of the species were affected. The sperm count parameters (both testicular and epididymidal) do not contribute to this analysis since the primary biological determinant of these values occurred prior to TXIB exposure and the sperm motility values suggest there was no functional problem with the sperm. For these reasons, the No-Observed-Adverse-Effect Level (NOAEL) for this study should be considered to be the 0.45% TXIB in the diet (approximately 276-359 mg/kg/day).