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Genotoxicity evaluation of magnesium salts of isobutyrate and 2-methylbutyrate

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ABSTRACT

Results of genotoxicity studies for magnesium salts of isobutyrate and 2-methylbutyrate, two candidate ingredients for inclusion in animal feed, are described in this manuscript. Both substances were tested for mutagenicity in a bacterial reverse mutation assay and clastogenicity/aneugenicity in an *in vitro* micronucleus study in human lymphocytes, conducted according to Organisation for Economic Co-operation and Development (OECD) Guidelines. The substances were tested up to the limits of solubility in the tests. The results showed that that magnesium salts of isobutyrate and 2-methylbutyrate are not mutagenic, clastogenic or aneugenic. The tests were valid, as the negative and positive controls produced expected responses.

1. Introduction

Magnesium salts of isobutyrate and 2-methylbutyrate (collectively known as isoacids) are being developed as feed additives for livestock and poultry. Magnesium salts of fatty acids are expected to dissociate in the gastrointestinal tract into fatty acid carboxylates and their corresponding cations [1]. The volatile fatty acids (VFA) in the ingredient are normal components of the rumen of cattle and are normally metabolized and directly absorbed from the rumen and large intestine [2] or are metabolized by bacteria in the rumen to amino acids and fatty acids which are absorbed along with dietary nutrients. While cellulolytic bacteria require branched chain VFAs, both cellulolytic and non-cellulolytic bacteria in the rumen utilize branch chain VFA. By providing isoacids in diets, more amino acids and fatty acids will be synthesized and made available for the ruminant's intermediary metabolism. Addition of isoacids to rumen fluid also has been shown to improve digestion of ground barley straw, alfalfa hay, cottonseed meal and corn gluten meal *in vitro* [3,4].

In order to be used as feed ingredients for production animals, substances need to be safe for the animals ingesting the ingredient and humans that are ingesting tissues from the animals, which may contain residuals. If genetic toxicity testing has not been performed, it is recommended. No studies have been published regarding the genotoxicity of magnesium salts of isobutyrate and 2-methylbutyrate.

A stepwise approach is recommended for the generation and evaluation of data on genotoxic potential, beginning with a basic battery of *in vitro* tests, comprising a bacterial reverse mutation assay and an *in vitro* micronucleus assay [5]. In the event of negative results in a basic battery of *in vitro* tests comprised of a bacterial reverse mutation assay and an *in vitro* micronucleus assay, it can be concluded that the substance has no genotoxic potential [5]. If a substance produces a positive response in either of these tests, additional testing is warranted. The purpose of the studies described herein is to determine whether the magnesium salts of isobutyrate and 2-methylbutyrate formulations described in this manuscript are genotoxic, using the recommended basic battery.

2. Materials and methods

2.1. Test articles

The test materials were magnesium salts of isobutyrate (batch number VDA 19239, 30% isobutyrate) and 2-methylbutyrate (batch number VDA19301, 33.9% 2-methylbutyrate) from Zinpro Corporation, MN 55344, USA. The balance of both test materials is ground corn cobs carrier. The positive control chemicals colchicine, cyclophosphamide monohydrate and 9-aminoacridine were obtained from Sigma Chemical,

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and sodium azide, 2-nitrofluorene and 2-aminoanthracene were obtained from Moltox, Inc. Methylmethanesulfonate (MMS) and dimethylsulfoxide (DMSO) were sourced from Aldrich and Honeywell, respectively. The S9 tissue fraction used in the studies was isolated from livers of Sprague Dawley rats pretreated with phenobarbital-5,6-benzoflavone, produced by MOLTOX Molecular Toxicology, Inc. and provided by Trinova Biochem GmbH.

2.2. Guidelines

The studies were conducted in compliance with ENV/MC/CHEM(98) 17 OECD principles on Good Laboratory Practice (GLP). The bacterial reverse mutation and micronucleus studies were performed according to OECD Guidelines No. 471 (Adopted July 1997) and No. 487 (29 July 2016), respectively [6,7]. The micronucleus studies were performed at the European Research Biology Center S.r.l., Via Tito Speri, 12/14, 00071 Pomezia, Italy.

2.3. Bacterial reverse mutation assay

The ability of magnesium salts of isobutyrate and 2-methylbutyrate to cause mutations was assessed in bacterial reverse mutation assays using a plate incorporation method (Experiment 1) and preincubation method (Experiment 2). Bacterial strains utilized in all experiments were *S. typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA, kept at the test site. Both experiments were conducted in the absence and presence of an S9 metabolizing system. Three replicate plates were used at each test point. In addition, plates were prepared to check the sterility of the test item suspensions and S9 preparations.

Suspensions of the test items were prepared immediately before use in DMSO and were maintained under magnetic stirring until use (approximately 2 h after preparation). A preliminary solubility trial was performed for both test items using water, DMSO, ethanol and acetone, and DMSO was chosen as the vehicle, although solubility was limited. Preliminary experiments were also performed in each strain to examine the potential of the test material to cause toxicity and determine optimal concentrations to use in the studies. Toxicity was assessed by observing a decline in the number of spontaneous revertants, a thinning of the background lawn, or microcolony formation. For Experiment 2, the test item is in direct contact with the bacteria before the subsequent plate incorporation, so the potential for cytotoxicity from DMSO would be greater in this Experiment than in Experiment 1. The volumes of test formulations added to the cultures in Experiment 2 were halved compared to Experiment 1 to limit cytotoxicity, resulting in 50% lower test concentrations in Experiment 2 *vs*. Experiment 1. The concentrations of the isobutyrate form used in Experiments 1 and 2 (188, 93.9, 46.8, 23.4 and 11.7 μg/plate, and 94.0, 47.0, 23.4, 11.7 and 5.85 μg/plate, respectively) and the 2-methylbutyrate form used in Experiments 1 and 2 (157, 78.6, 39.3, 19.6 and 9.80 μg/plate, and 78.5, 39.3, 19.7, 9.80 and 4.90 μg/plate) were chosen based on the results of solubility and toxicity experiments. The positive controls in the absence of S9 mix were 2-nitrofluorene for TA98, sodium azide for TA100 and TA1535, 9-aminoacridine for TA1537 and MMS for *E. coli* WP2uvrA. The positive control for all bacterial strains in the presence of S9 mix was 2-aminoanthracene (2-AA) and the negative control for all strains in the presence or absence of S9 mix was DMSO.

For Experiment 1, the following substances were mixed in a test tube and poured over the surface of a minimal medium agar plate (1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% glucose): (1) the positive or negative control solutions or test formulations (0.1 mL each); (2) 0.5 mL of 0.1 M sodium phosphate buffer (pH 7.4) or 0.5 mL of the S9 mix (for metabolic activation); (3) 0.1 mL of bacterial suspension; and (4) 2.0 mL of overlay agar (0.6% Difco Bacto-agar containing histidine (or tryptophan for *E. coli* cultures)). For Experiment 2, all substances except for the overlay agar were mixed together and incubated for 30 min at 37 C. The test formulations were added at 0.05 mL instead of 0.1 mL for this experiment. Two mL of overlay agar was then added to the cell mixture and the preparation was vortexed again and poured onto the surface of a minimal medium agar plate. All plates were inverted after solidification and incubated at 37 C for approximately 72 h prior to counting.

The assay was considered valid if mean plate counts for untreated and positive control plates were within two standard deviations of the current historical mean values, the estimated numbers of viable bacteria/plate ranged from 100 to 500 million for each strain, and no more than 5% of the plates were lost through contamination or other unforeseen event. The test substance was mutagenic if two-fold (or more) increases in mean revertant numbers were observed at two consecutive dose levels or at the highest practicable dose level only, and if the numbers of mutant colonies increased according to a dose-response relationship.

2.4. Mammalian cell micronucleus assay

Magnesium salts of isobutyrate and 2-methylbutyrate were assayed for the ability to induce micronuclei in human lymphocytes, following *in vitro* treatment in the presence and absence of S9 metabolic activation. The lymphocytes were obtained from anticoagulated (sodium heparin) whole blood samples collected from healthy, nonsmoking individuals (a male for isobutyrate and a female for 2-methylbutyrate forms) without any recent exposure to drugs or radiation. Three treatment conditions were utilized for each test substance. A short-term treatment, where the cells were treated for 3 h, was performed in the absence and presence of S9 metabolism (Experiment 1). A harvest time of approximately 32 h, corresponding to approximately two cell cycle lengths, was used for Experiment 1. A long term (continuous) treatment also was performed only in the absence of S9 metabolism, until harvest at 31 h (Experiment 2).

Test item suspensions were prepared in culture medium (vehicle). All test item suspensions were maintained under magnetic stirring until use and utilized within approximately 2 h of preparation. The culture medium had the following composition: 500 mL RPMI 1640 1x (Dutch modification), 100 mL heat-inactivated fetal calf serum, 6.25 mL L-glutamine (200 mM), and 1.25 mL antibiotic solution (type not specified). Phytohemagglutinin (PHA, 10 mL) was added to 500 mL of medium to stimulate proliferation of lymphocytes. Lymphocyte cultures were prepared by adding whole blood (0.5 mL) to 4.5 mL of the culture medium containing PHA. The cultures were incubated at 37 °C for approximately 48 h before treatment. At time of treatment, the cells were spun in a centrifuge at 1000 rpm for 10 min and the culture medium was decanted and replaced with treatment medium (4.0 mL test item (or control) suspension +1.0 mL S9 mix for Experiment 1 or with 4.0 mL test item suspension +1.0 mL culture medium (without PHA) for Experiments 1 and 2). The medium served as the negative control and positive controls were cyclophosphamide (15 or 20 μg/mL depending on substance tested) for Experiment 1 and colchicine (0.04 μg/mL) for Experiment 2. Two replicate cell cultures were prepared for each test concentration. Preliminary solubility tests were performed to determine the highest concentration used in each study. For the isobutyrate form, nine concentrations ranging from 9.84–252 *ug*/mL were tested in Experiment 1 and ten concentrations ranging from 6.55–252 *ug*/mL were tested in Experiment 2. For the 2-methylbutyrate form, eight concentrations ranging from 19.9–339 *ug*/mL were tested in Experiment 1 and nine concentrations ranging from 13.2–339 *ug*/mL were tested in Experiment 2. Since no cytotoxicity occurred at any concentration, 252 *ug*/mL and 339 *ug*/mL were used as the highest concentrations for scoring for the isobutyrate and 2-methylbutyrate forms, respectively, as well as two lower doses separated by appropriate intervals.

For Experiment 1, the treatment media were added to the tubes and the cultures were incubated for 3 h at 37 °C. At the end of treatment time, the cell cultures were spun in a centrifuge and washed twice with

Phosphate Buffered Saline Solution. Fresh medium was added, and the cultures were incubated for an additional 28 h (Recovery Period) before harvesting. At the same time, Cytochalasin B was added (final concentration of 6 μg/mL) to inhibit mitosis. The cytokinesis block proliferation index (CBPI) was calculated to evaluate cytotoxicity. For Experiment 2, CB was added 3 h after treatment and the cultures were incubated at 37 °C for an additional 28 h before harvesting. At the end of each experiment, the lymphocyte cultures were spun in a centrifuge for 10 min at 1000 rpm and the supernatant was removed. The cells were suspended in hypotonic solution and fresh methanol/acetic acid fixative was added and changed several times by centrifugation and resuspension. A few drops of each cell suspension were placed onto clean, labelled slides. The slides were air dried and then stained with acridine orange (0.1 mg/mL in PBS). One thousand binucleated cells per culture (500/slide) were scored to assess the frequency of micronucleated cells. The criteria for identifying micronuclei were as follows:

1. The micronucleus diameter was less than 1/3 of the nucleus diameter.
2. The micronucleus diameter was greater than 1/16 of the nucleus diameter.
3. No overlapping with the nucleus was observed.
4. Micronuclei were non-refractile and had the same staining intensity as the main nuclei.

A modified $\chi 2$ test was used to compare the number of cells with micronuclei in control and treated cultures and a Cochran-Armitage Trend Test (one-sided) was performed to analyze the concentration response relationship. The test was considered valid if the incidences of micronucleated cells for the negative and positive controls were within the distribution ranges of historical control values, concurrent positive controls produced statistically significant increases compared with the concurrent negative control, adequate cell proliferation was observed in solvent control cultures, and appropriate numbers of doses and cells were analyzed. The test item was considered clearly negative if none of the dose levels showed a statistically significant increase in the incidence of micronucleated cells, there was no concentration-related increase when evaluated with the Cochran-Armitage trend test, and all the results were inside the distribution of the historical control data (95% control limits). The test item was considered clearly positive if significant increases in the proportion of micronucleated cells occurred compared to concurrent negative controls at one or more concentrations, the proportion of micronucleated cells at such data points exceeded the normal range based on historical control values (95% control limits), and there was a significant dose effect relationship.

3. Results and discussion

The magnesium salts of isobutyrate or 2-methylbutyrate did not induce reverse mutations in *Salmonella typhimurium* or *Escherichia coli* in the absence or presence of S9 metabolism, under the reported experimental conditions (Tables 1 and 2). None of the tested concentrations were toxic in either experiment. For both test materials, a precipitate was observed in all strains at the highest concentration used in Experiment 1. In both the experiments performed with magnesium salts of isobutyrate, microbial contamination was seen in the sterility plates and with all tester strains at higher concentrations, in a dose-dependent manner. Since the microbial contamination did not interfere with the growth of tester strains and the scoring of revertant colonies, it was not considered to have affected the results of the study. The sterility of the S9 mix was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in each experiment following treatment with the positive control items, indicating that the assay systems

Table 1
Reverse mutation assay of magnesium salt of isobutyrate in *Salmonella typhimurium* and *Escherichia coli*: mean number of revertants/plate.

Concentration (μg/plate)	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Experiment 1										
Untreated	35	34	121	132	19	21	19	23	27	28
0[a]	27	32	123	168	21	21	17	23	28	32
11.7	34	39	119	137	19	19	20	22	29	32
23.4	34	34	133	139	20	20	18	18	25	30
46.8	29	39	134	142	18	20	22	21	31	27
93.9	25	41	158	137	18	19	20	19	25	30
188	29*	40*	159*	140*	19*	18*	17*	20*	29*	37*
Positive control	176[b]	477[c]	527[d]	1081[c]	421[d]	133[c]	147[e]	125[c]	154[f]	188[c]
Historical negative control (-S9, range)	23–43		109–182		13–26		11–27		21–38	
Historical negative control (+S9, range)	29–53		86–196		12–22		15–33		26–47	
Experiment 2										
Untreated	33	36	153	147	18	17	18	21	33	29
0[a]	29	40	139	134	20	18	20	20	29	32
5.85	28	40	126	128	19	20	17	20	24	31
11.7	30	37	121	137	22	21	19	19	28	28
23.4	34	34	132	145	22	20	20	17	31	33
47.0	32	39	124	154	20	22	19	21	29	32
94.0	27	39	131	157	21	18	18	20	27	30
Positive control	197[b]	578[c]	692[d]	1148[c]	426[d]	114[c]	112[e]	92[c]	214[f]	164[c]
Historical negative control (-S9, range)	24–42		112–192		12–25		12–27		20–38	
Historical negative control (+S9, range)	33–50		119–197		12–23		15–27		24–44	

Substance was tested using the standardized plate incorporation assay (Experiment 1) and the pre-incubation method (Experiment 2). Results are means of three replicates per test condition.

[a] DMSO vehicle.
[b] 2-nitrofluorene.
[c] 2-aminoanthracene.
[d] Sodium azide.
[e] 9-aminoacridine.
[f] MMS.
* Precipitate observed.

Table 2
Reverse mutation assay of magnesium salt of 2-methylbutyrate in *Salmonella typhimurium* and *Escherichia coli*: mean number of revertants/plate.

Concentration (μg/plate)	TA98		TA100		TA1535		TA1537		WP2*uvrA*	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Experiment 1										
Untreated	32	42	145	143	16	16	19	17	29	32
0[a]	30	34	136	134	17	20	19	24	26	32
9.8	28	34	134	143	18	19	17	21	29	32
19.6	30	36	138	150	14	15	22	20	30	31
39.3	31	42	133	130	16	17	20	20	30	29
78.6	28	36	154	142	17	18	17	18	29	33
157	31*	34*	145*	136*	19*	20*	16*	19*	27*	31*
Positive control	184[b]	625[c]	597[d]	1061[c]	432[d]	123[c]	189[e]	114[c]	146[f]	169[c]
Historical negative control (-S9, range)	23–43		109–182		13–26		11–27		21–38	
Historical negative control (+S9, range)	29–53		86–196		12–22		15–33		26–47	
Experiment 2										
Untreated	27	40	156	178	18	16	15	19	23	36
0[a]	29	42	159	172	17	15	18	19	26	35
4.9	27	44	181	182	18	15	14	23	30	34
9.8	31	40	183	193	15	16	16	20	31	36
19.7	32	40	180	185	19	17	14	22	31	33
39.3	30	41	184	166	18	18	16	23	27	33
78.5	35	42	180	171	14	17	16	19	24	34
Positive control	188[b]	577[c]	700[d]	1137[c]	450[d]	107[c]	188[e]	102[c]	186[f]	220[c]
Historical negative control (-S9, range)	24–42		112–192		12–25		12–27		20–38	
Historical negative control (+S9, range)	33–50		119–197		12–23		15–27		24–44	

Substance was tested using the standardized plate incorporation assay (Experiment 1) and the pre-incubation method (Experiment 2). Results are means of three replicates per test condition.

[a] DMSO vehicle.
[b] 2-nitrofluorene.
[c] 2-aminoanthracene.
[d] sodium azide.
[e] 9-aminoacridine.
[f] MMS.
* Precipitate observed.

Table 3
In vitro micronucleus assay of magnesium salt of isobutyrate in human lymphocytes.

Concentration (μg/mL)	Cytotoxicity (%)		Mean CBPI		Incidence of Micronucleated Cells (%)	
	-S9	+S9	-S9	+S9	-S9	+S9
Experiment 1						
Negative control[a]	CP	CP	1.814	1.849	0.35	0.35
112	0	8	1.812	1.778	0.30	0.40
168	−3	11	1.837	1.757	0.25	0.25
252	−2	5	1.834	1.803	0.60	0.30
Positive control[b]	NP	57	NP	1.363	NP	2.95*
Historical negative control	ND	ND	ND	ND	0.00–0.77	0.00–0.95
Experiment 2						
0	CP	NP	1.813	NP	0.45	NP
112	1	NP	1.802	NP	0.40	NP
168	5	NP	1.769	NP	0.20	NP
252	4	NP	1.781	NP	0.40	NP
Positive control[c]	97	NP	1.022	NP	2.25*	NP
Historical negative control	ND	ND	ND	ND	0.00–1.00	ND

Experiment 1: Cells were treated for 3 h and harvested at 32 h.
Experiment 2: Continuous 31-h treatment followed by harvest. Results are means of two plates per treatment. Historical control values for both experiments are ranges of upper and lower confidence limits (mean ± 2 standard deviations). Ranges of values were not provided in the study report.
CBPI = cytokinesis block proliferation index; CP = comparator; ND = no data; NP = not performed.

[a] Culture medium.
[b] Cyclophosphamide (15 μg/mL).
[c] Colchicine (0.04 μg/mL).
* $p < 0.001$.

were functioning correctly. The studies were considered valid because the mean plate counts for untreated control plates fell within the range of historical control data, positive controls induced expected responses, the estimated numbers of viable bacteria/plate fell in the range of 100–500 million for each strain, and no plates were lost in the studies.

Following treatment with the magnesium salts of isobutyrate or 2-methylbutyrate, no statistically significant increases in the incidences of micronucleated cells were observed at any dose level compared to concurrent negative control values, no dose-response relationship occurred, and all incidences of micronucleated cells were within the distribution of historical negative controls (95% confidence limits) (Tables 3 and 4). For 2-methylbutyrate, precipitation visible by eye was noticed by the end of treatment of Experiment 1 at all dose levels, in the absence and presence of S9 metabolic activation. No precipitation or opacity of the medium occurred in Experiment 2 at any dose level. No remarkable variations of pH or osmolality and no cytotoxicity were observed in any of the experiments with either test material (data not shown). The tests were valid, as the incidences of micronucleated cells of the negative controls were within the distribution ranges of historical control values, positive controls induced expected responses, adequate cell proliferation was observed in negative control cultures, and appropriate numbers of doses and cells were analyzed. The test items were not considered to induce micronuclei in human lymphocytes under the conditions of the tests.

There is a relative lack of published genetic toxicity information about structurally similar ingredients; however, the genotoxicity of isobutyric acid has been examined in an Ames test with/without activation. The test results were negative but study details were not supplied [8]. Magnesium stearate also is not genotoxic *in vivo* or *in vitro* [9]. Isoacids and magnesium are normal constituents of the body and as such, are not considered to pose a risk of genetic toxicity. The genotoxic potential of magnesium salts of isobutyrate or 2-methylbutyrate is

Table 4
In vitro micronucleus assay of magnesium salt of 2-methylbutyrate in human lymphocytes.

Concentration (µg/mL)	Cytotoxicity (%)		Mean CBPI		Incidence of Micronucleated Cells (%)	
	-S9	+S9	-S9	+S9	-S9	+S9
Experiment 1						
Negative control[a]	CP	CP	2.068	2.160	0.40	0.40
67[b]	1	11	2.057	2.035	0.40	0.20
151[b]	−5	8	2.119	2.069	0.55	0.40
339[b]	−2	3	2.089	2.120	0.40	0.35
Positive control[c]	NP	50	NP	1.577	NP	2.5*
Historical negative control	ND	ND	ND	ND	0.00−0.77	0.00−0.95
Experiment 2						
Negative control	CP	NP	1.948	NP	0.50	NP
67	0	NP	1.950	NP	0.55	NP
151	−3	NP	1.973	NP	0.65	NP
339	−5	NP	1.991	NP	0.45	NP
Positive control[d]	95	NP	1.047	NP	2.85*	NP
Historical negative control	ND	ND	ND	ND	0.00−1.00	ND

Experiment 1: Cells were treated for 3 h and harvested at 32 h.
Experiment 2: Continuous 31-h treatment followed by harvest. Results are means of two plates per treatment. Historical control values for both experiments are ranges of upper and lower confidence limits (mean ± 2 standard deviations). Ranges of values were not provided in the study report.
CBPI = cytokinesis block proliferation index; CP = comparator; NP = not performed.
[a] Culture medium.
[b] Precipitate observed.
[c] Cyclophosphamide (20 µg/mL).
[d] Colchicine (0.04 µg/mL).
* $p < 0.001$.

therefore expected to be driven by the concentrations of genotoxic contaminants in the ingredients. Short chain aldehydes which could potentially be in the ingredients (such as acetaldehyde, formaldehyde, propionaldehyde, butyraldehyde and isobutyraldehyde) are mutagenic and/or clastogenic *in vitro* [10]. The results of the studies described in the current manuscript indicate that neither test material was positive for genotoxicity *in vitro*. Therefore, the manufacturing process adequately controls for the production of genotoxic substances. In the event of negative *in vitro* results, it can be concluded that the substance has no genotoxic potential [5].

4. Conclusion

The results of the studies described in this manuscript demonstrate that magnesium salts of isobutyrate or 2-methylbutyrate are not mutagenic, clastogenic or aneugenic *in vitro,* in validated tests conducted according to GLP and established guidelines and are therefore highly unlikely to be genotoxic *in vivo*. Additional studies are planned to add to the body of evidence that these ingredients would be safe for addition to livestock or poultry feed.

Author statement

All authors contributed materially to the manuscript.
Laurie C. Dolan: Writing – original draft preparation, reviewing and editing
Paola Ciliutti, Laura Bisini and Cristina Marabottini: Investigation
Brian Curtin: Funding acquisition, project administration

Declaration of Competing Interest

All authors have a financial relationship with the sponsor of the study, Zinpro Animal Nutrition.

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