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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
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Dear Dr. Gaynor,

The following comments are submitted on behalf of the more than two million members and supporters of People for the Ethical Treatment of Animals (PETA) in regard to GRAS notices nos. 252 and 253 for rebaudioside A purified from Stevia rebaudiana. In particular, our comments are in response to those of Michael F. Jacobson, Ph.D., Executive Director of the Center for Science in the Public Interest (CSPI). PETA is committed to using the best available science to protect animals from suffering and to promote the acceptance of human-relevant methods for risk assessment.

CSPI asserts that rebaudioside A cannot be considered generally recognized as safe (GRAS) based on its purported genotoxic effects. Further, CSPI calls on FDA to request that the National Toxicology Program (NTP) conduct chronic toxicity tests on rats and mice, as well as a battery of genotoxicity tests. These tests would kill thousands of animals. In support of its claim, CSPI cites the opinions of “impartial” toxicologists from UCLA. In fact, the report it cites, Toxicology of Rebaudioside A: A Review by Sarah Kobylewski, a doctoral student, and Curtis D. Eckhert, Ph.D., was prepared specifically for CSPI and does not appear to have been published in a peer-reviewed journal. Moreover, not only does CSPI fail in its challenge of this GRAS claim; its report also contains numerous, worrisome misrepresentations of scientific opinion and relevant data regarding the safety of rebaudioside A.

Before addressing the specific claims made by CSPI, we note recent developments regarding the regulation of stevia that have transpired apparently since the preparation of its report (no publication date is provided). Kobylewski and Eckhert state that the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that there were insufficient data to give steviol glycosides a permanent acceptable daily intake (ADI) and requested additional human studies to evaluate potential effects on blood pressure and glucose homeostasis. This research has since been completed and at its sixty-ninth meeting in June 2008, JECFA established a permanent ADI of 0–4 mg/kg bw, based on a no observed effects level (NOEL) of 970 mg/kg bw per day (383 mg/kg bw per day expressed as steviol) from a long-term study with stevioside and a safety factor of 100 (JECFA 2008). Further, JECFA noted that the results of the new studies showed no adverse effects of steviol glycosides when taken at doses of about 4 mg/kg bw per day for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal or low-normal blood pressure for 4 weeks. It is also noteworthy that in 2004, JECFA concluded that stevioside and rebaudioside A are not genotoxic in vitro or in vivo and that the genotoxicity of stevioside and some of its oxidative derivatives in vitro is not expressed in vivo (JECFA 2004). Subsequently, in August 2008, Food Standards Australia
New Zealand (FSANZ) concluded that there are no public health and safety concerns for steviol glycosides when used as a food additive (FSANZ 2008), paving the way for products sweetened with them to enter the Australian market. Other countries which allow the use of steviol glycosides include Japan, China, Russia, Korea, Brazil, Paraguay, Argentina, Indonesia and Israel. In fact, the plant, *Stevia rebaudiana*, is native to South America and has been used for sweetening beverages and foods for more than 400 years, and more than 750 tons of stevia leaves per year are used as crude extract for consumption (Suttajit et al. 1993).

Kobylewski and Eckhert conclude that while several carcinogenicity studies have not found stevioside to be carcinogenic in rats, further studies on rebaudioside A, including a chronic feeding study on mice, are needed for the reasons below. We address each of these claims in turn.

**CSPI Claim: The rat is an imperfect model for evaluating steviol glycoside toxicity and carcinogenicity risks in humans because of the differences in metabolism in the two species.**

While this statement is certainly true, it would be expected to apply to the mouse as well for precisely the same reasons. As the authors note, steviol glycosides are hydrolyzed to their aglycone, steviol, by intestinal microflora in rats and humans. Steviol is subsequently conjugated with glucuronide in the liver. In rats, steviol glucuronide enters the bile and ultimately returns to the gastrointestinal tract where it may be de-conjugated releasing steviol to be reabsorbed or eliminated in the feces. In humans, however, steviol glucuronide does not enter the bile, because humans have a higher molecular weight threshold for biliary excretion than do rats; instead, it is excreted in the urine. Since biliary excretion is similar in rats and mice, mice would be equally imperfect “models” for evaluating steviol glycoside toxicity in humans. Further, as a result of this enterohepatic circulation, rats and mice will be exposed to higher concentrations of steviol per given dose than will humans.

**CSPI Claim: Several genotoxicity studies that found that stevioside and steviol cause mutations, chromosomal damage, and DNA breakage indicate the need for greater reassurance of noncarcinogenicity.**

In his letter, Jacobson claims that “[c]onsidering the genotoxic effects of rebaudioside A… this ingredient cannot be considered generally recognized as safe.” In their report for CSPI, however, Kobylewski and Eckhert cite two studies showing potential genotoxic activity for stevioside but note that “[r]ebaudioside A was not found to cause mutations, chromosome damage, or DNA strand breakage in several *in vitro* and *in vivo* studies.” It is therefore unclear to what genotoxic effects of rebaudioside A Jacobson is referring. As shown below, Kobylewski and Eckhert cite differences between rebaudioside A and stevioside to support their contention that chronic toxicity studies for stevioside should be repeated for rebaudioside A and yet they question the completely negative genotoxicity results for rebaudioside A on the basis of its *similarity* to stevioside.
Kobylewski and Eckhart cite the *in vitro* mutagenicity study of Suttajit et al. (1993) as evidence of the potential genotoxicity of stevioside. However, their conclusions are opposite to those of the studies’ authors:

We have shown the lack of mutagenicity of stevioside and steviol at limited doses (up to 20 mg) toward *Salmonella typhimurium* strains TA98 and TA100 with or without metabolic activation. This confirmed the findings previously reported… that crude stevia extract and stevioside were negative toward TA98 and TA100 mutation.

Regarding their positive result, Suttajit et al. state:

Generally, stevioside *per se* is not a mutagen toward bacterial cells or a genotoxin to cultured mammalian cells, and it is not carcinogenic to experimental animals either.

Only at an unusually high dose, 50 mg/plate, was stevioside mutagenic to TA98 but not to TA100. The mutagenicity might be due to some impurities in the sample.

Rather than demonstrating stevioside’s genotoxicity, the results of Suttajit et al. instead appear to show the lengths to which CSPI needed to go in searching for any evidence to support its claim.

The other study cited by Kobylewski and Eckhart in support of stevioside’s potential genotoxicity is a comet assay conducted by Nunes et al. (2007). In this study, a stevioside aqueous solution (4 mg/ml) was made available as drinking water to a group of five male Wistar rats. The authors reported lesions in peripheral blood, liver, brain and spleen cells, the most pronounced effects being in the animals’ livers.

This study prompted letters by Gary M. Williams, M.D., New York Medical College and Jan M.C. Geuns, Catholic University of Leuven which raise numerous concerns. Both Williams (2007) and Geuns (2007) point out that Nunes et al. used stevioside with a purity of only 88.6 percent; administered only one concentration – consequently neglecting dose-dependence; and failed to include a positive control. Williams raises additional concerns regarding methodology, noting that cytotoxicity, which can contribute to increased DNA fragmentation, was not assessed and that the manual method of scoring was poorly characterized and employed a criteria for comets that did not conform to usual practice. Geuns is even more critical noting that the structure shown for steviol is incorrect, that the reported standard deviations are very large – sometimes much larger than the means themselves – and that a $p$ value referred to in the discussion is ten-fold lower than reported in a table. Nunes et al. report scores of control blood cells that vary from $0.6 \pm 1.34$ to up to $27 \pm 13.3$ at 6 weeks and increase and decrease at different time points, suggesting stress as the possible cause. Geuns regards this explanation as “unbelievable,” offering instead that a lack of standardization by use of an internal standard and a lack of quality control of the feed – which might contain mutagenic compounds – seem more likely. Finally, Nunes et al. fail to cite two independent comet assays by Sekihashi et al. (2002), in which mice were given up to 2000 mg/kg bw – substantially more than was used in their own study – with no evidence of DNA damage found in their livers, stomachs, colons, kidneys or testes.
Regarding the genotoxicity of steviol, Kobylewski and Eckhert cite *in vitro* studies by Matsui et al. (1989, 1996a) and Pezzuto et al. (1985). As noted above, JECFA has considered the genotoxicity of steviol and some of its oxidative derivatives *in vitro* and concluded that this genotoxicity is not expressed *in vivo* since no evidence of DNA damage or micronucleus formation is observed in rats, mice or hamsters at doses of up to 8000mg/kg bw.

We must conclude that CSPI has greatly exaggerated its case for the potential genotoxicity of stevioside. Presenting only two studies in support of their conclusion, Kobylewski and Eckhert have overlooked the authors’ own conclusions in one of these studies and significant scientific criticisms of the other.

**CSPI Claim: The differences in pharmacokinetics between rebaudioside A and stevioside indicate the need to test rebaudioside A itself in two rodent species.**

Kobylewski and Eckhert note that in a study of the transformation of stevioside and rebaudioside A *in vitro* upon incubation with human fecal microflora, Gardana et al. (2003) found that stevioside was completely hydrolyzed to steviol after 10 hours of incubation while rebaudioside A was completely hydrolyzed to steviol after 24 hours of incubation. They also note that in a human metabolism study, Wheeler et al. (2008) found that “there was a longer T\text{max} and a lower C\text{max} of steviol glucuronide and steviol when volunteers were administered rebaudioside A compared to stevioside.” Kobylewski and Eckhert conclude that because stevioside and rebaudioside A are metabolized at different rates, toxicity assessments of stevioside cannot definitively be extrapolated to assess the risk of rebaudioside A.

On page 3 of their report, Kobylewski and Eckhert reproduce Table 1 of Wheeler et al. (2008) changing the title from “Summary of the mean (SD) pharmacokinetic data for steviol” to “Mean pharmacokinetic parameters for steviol in men.” It is important to note that steviol was detected in plasma at levels above the lower limit of quantitation in only one out of eight subjects following the administration of both compounds (Wheeler et al. 2008). Therefore, the pharmacokinetic data reported for steviol is that for single plasma samples (\(N = 1\)). Wheeler et al. do not discuss these results other than to remark that “very limited amounts of steviol” were observed. Kobylewski and Eckhert also reproduce Table 2 summarizing the mean pharmacokinetic data for steviol glucuronide. It is interesting that they claim there was a lower C\text{max} of steviol glucuronide and steviol with rebaudioside A compared to stevioside when the tables they reproduce clearly show a higher C\text{max} for steviol (227 vs. 121ng/mL). Perhaps this illustrates the danger of drawing conclusions from a single sample. Wheeler et al. draw their conclusions from the geometric mean C\text{max} values (1472 ng/mL for rebaudioside A and 1886 ng/mL for stevioside) and the geometric mean AUC\text{0-1} values (30,788 ng h/mL for rebaudioside A and 34,090 ng h/mL for 34,090 ng h/mL) presented in a third table which Kobylewski and Eckhert did not reproduce. These values correspond to differences of only 22 percent and 9.7 percent, respectively.

Once again, the conclusions of Kobylewski and Eckhert are at odds with those of the studies’ authors. Wheeler et al. observe:
Rebaudioside A has one additional glucose moiety that must be removed… and therefore, it is not unexpected that the formation of steviol from stevioside would be more rapid than that of rebaudioside A.

Wheeler et al. conclude:

There were no meaningful differences observed in urinary recovery after administration of either rebaudioside A or stevioside…

On the basis of the similarity in human metabolism to the primary metabolite steviol glucuronide following administration of rebaudioside A or stevioside…, it can be concluded that previous human studies and rodent toxicological studies conducted with stevioside are relevant for assessing the human safety of rebaudioside A.

Likewise, Gardana et al. (2003) point out that “after an initial lag phase of 6-7 h, rebaudioside A was hydrolyzed to steviolbioside (Cmax = 12-15 h), and this was rapidly converted to steviol.”

Again, CSPI has exaggerated its case. The differences in pharmacokinetics between rebaudioside A and stevioside, while significant, are small and easily explained on the basis of their chemical structures. A much more significant difference is that in elimination between rodents and humans mentioned above, the effect of which is to cast doubt on the relevance of the chronic carcinogenicity tests that CSPI proposes.

**CSPI Claim: Based on a maximum estimated intake level of steviol glycosides of 1.7 mg/kg bw/day (steviol equivalent), steviol glycosides should be considered a concern level III chemical, for which the FDA recommends carcinogenicity studies in two rodent species (usually mice and rats) (FDA Redbook, 2000).**

The concern levels and recommended toxicity tests referred to apply to direct food additives not to substances for which GRAS notices have been submitted. CSPI is calling for FDA to advise the sponsors that they should submit a full food additive petition. To claim that these concerns and recommendations should apply to the GRAS status of rebaudioside A begs the question. As noted on the Center for Food Safety and Applied Nutrition’s web site (http://www.cfsan.fda.gov/~dms/grasov2.html) “substances that are generally recognized, among experts qualified by scientific training and experience to evaluate their safety as having been adequately shown . . . to be safe under the conditions of their intended use, are excluded from the definition [of food additive].” It is precisely this claim for rebaudioside A which CSPI is attempting to dispute, but the concerns expressed appear to be largely those of Jacobson, Kobylewski and Eckhert. It is perfectly appropriate for a consensus of expert opinion based on weight-of-evidence – including human experience of safe use over centuries – to form the basis for these GRAS notices.

Even so, we note that Suttajit et al. (1993) cite chronic toxicity studies in both rats and hamsters showing no evidence of carcinogenicity. In addition, we take this opportunity to note that in preparing the current International Conference on Harmonization (ICH) Guidance on
Testing for Carcinogenic Potential for Pharmaceuticals, the ICH Safety Working Group found that data from the second species (usually mouse) gave very little information for a definitive regulatory decision on a compound following its careful retrospective evaluation of several databases (Jena et al. 2005).

**CSPI Claim:** The value of testing chemicals in two species is indicated by the fact that bioassays of chemicals with a variety of structures that did not find carcinogenicity in rats did find carcinogenicity in mice (see appendix C).

While we dispute this conclusion below, we must first point out the obvious fact that carcinogenicity cannot be found in rats if it has not been tested for in rats. Kobylewski and Eckhert state that NTP found no evidence of carcinogenicity in rats for 1,3-butadiene – interestingly, the only one of these 15 chemicals that NTP lists in its 11th Report on Carcinogens (RoC) as a known human carcinogen. While this is indeed true, it is because NTP did not test 1,3-butadiene in rats! NTP (1984) explained that this chemical was tested only in mice because a 2-year inhalation study in rats sponsored by the International Institute of Synthetic Rubber Producers, Inc. (IISRP) was already in progress at the time of chemical selection. Further, the results of the IISRP study showed that 1,3-butadiene is carcinogenic at multiple organ sites in Sprague-Dawley rats (NTP 1993). Several additional discrepancies were found in Kobylewski and Eckhert’s appendix C, but these were relatively minor and will not be discussed.

As noted, of the 15 chemicals considered – all showing evidence of carcinogenicity in either male or female mice or both, NTP lists only 1,3-butadiene as a known human carcinogen. In its 11th RoC, NTP lists four others (diphenylhydantoin, 4-chloro-\(o\)-toluidine hydrochloride, DDE and toxaphene) as reasonably anticipated to be human carcinogens. Ten chemicals – two-thirds of those considered – are not listed at all. Regarding 1,3-butadiene, NTP bases its judgment “on sufficient evidence of carcinogenicity from studies in humans, including epidemiological and mechanistic information, which indicate a causal relationship between occupational exposure to 1,3-butadiene and excess mortality from lymphatic and/or hematopoietic cancers (emphasis added).”

The conclusion to be drawn from this table is not that there is value in testing chemicals in two species, as Kobylewski and Eckhert suggest. Rather, it is that chronic carcinogenicity testing in animals simply is not a valid method for predicting human carcinogenicity. Since the five chemicals mentioned above are the only ones known or reasonably anticipated to be human carcinogens, 18 of the 30 tests reported in mice – 60 percent – produced false positive results. (Interestingly, the test in male mice for diphenylhydantoin, listed by NTP as reasonably anticipated to be human carcinogen, produced a false negative result.) This absurdly high false positive rate is not unusual. NTP itself has acknowledged that about half the chemicals it has tested have produced evidence of cancer in rodents (Fung et al. 1995). A review by cancer researchers at UC Berkeley found that closer to two-thirds of 800 chemicals tested positive in rodent cancer studies (Gold and Slone 1993). Other scientists have suggested that the true false positive rate could be upwards of 90 percent (Gaylor 2005), indicating that nearly all chemicals cause cancer in rodents at high enough doses.
Nor is it unusual for results to vary between mice and rats or females and males. In our report, *Wasted Money, Wasted Lives* (http://www.stopanimaltests.com/pdfs/Wasted$$.pdf), PETA analyzed all 502 cancer studies published on NTP’s web site as of January 2006. We found that results in one species-gender group failed to predict cancer incidence in the other species-gender groups 27.5 percent of the time on average; in one case, the rate was 40 percent. It is also not unusual for NTP to discount its own results in animal tests when choosing chemicals for listing in the RoC. In our analysis, of the 114 chemicals that caused cancer in *both rats and mice* in NTP tests, only 62 (53 percent) were listed in the RoC. Conversely, only three of the nine chemicals that NTP had classified as known human carcinogens caused cancer in both rats and mice. Of the chemicals classified by NTP as reasonably anticipated to be human carcinogens, 20 percent caused cancer in only one species tested, and four of these produced no evidence of carcinogenicity in either rats or mice.

Finally with regard to chronic carcinogenicity testing, we note that CSPI is currently promoting an extended chronic carcinogenicity test that would expose animals to test substances *in utero* and for 30 months or until their natural deaths, claiming that this would increase the test’s sensitivity, etc (Huff et al 2008). In our correspondence to *Environmental Health Perspectives* (in press; attached), we argue that longer exposures are instead likely to result in increased numbers of spontaneously arising tumors – as well as increased cost and animal suffering – while failing to address the bioassay’s fundamental flaws. Indeed, this was the opinion of the U.K. Food Standards Agency regarding three year tests conducted by the Ramazzini Foundation on aspartame cited by Huff et al (2008). It is surprising that Jacobson mentions aspartame and especially saccharin in the current context, since the latter is perhaps the best known example of the problems that may arise when carcinogenicity tests in animals falsely predict carcinogenic risk for humans as a result of species-specific modes of action.

According to the NTP’s own estimates, each chronic carcinogenicity test already requires five years to plan, conduct, and evaluate, 860 animals are killed, and $2 – $4 million is spent. Further, extending this test runs counter to current trends in regulatory testing with concern for the suffering of animals resulting in the elimination of the one year dog toxicity test (EPA 2007) and an international effort to replace the two-generation reproductive toxicity test (Cooper et al. 2006). The time has come for such antiquated animal tests to be abandoned – not extended – in favor of modern, human-relevant methods such as epidemiological studies, high-throughput *in vitro* methods, and computational toxicology, including those currently being implemented by FDA’s own Center for Drug Evaluation and Research.

In summary, CSPI has failed to convincingly challenge the claim in GRAS notices nos. 252 and 253 that rebaudioside A is generally recognized to be safe among qualified experts. Moreover, Jacobson, Kobylewski and Eckhert have ignored relevant information, presented flawed arguments, overlooked the conclusions of authors they themselves have cited as well as relevant scientific criticism, and misreported data. We urge FDA to dismiss CSPI’s comments and to accept rebaudioside A as GRAS without further animal testing based on a consensus of expert opinion and taking into account its extremely long and extensive history of safe use.
Thank you for your attention to these comments. I can be reached at (757) 622-7382, ext. 8001, or by e-mail at josephm@peta.org.

Sincerely,

Joseph Manuppello, MS
Research Associate

Nancy Douglas, Ph.D.
Science and Regulatory Policy Advisor

Attachment: Longer Bioassay Fails to Address 2-Year Bioassay’s Flaws

References


