April 5, 2005

MEMORANDUM

SUBJECT: Transmittal of Minutes of the FIFRA Scientific Advisory Panel Meeting Held March 1-2, 2005: Scientific Issues Associated With The Human Health Assessment Of The Cry34Ab1 Protein

TO: James Jones, Director
Office of Pesticide Programs

FROM: Paul I. Lewis, Ph.D. Designated Federal Official
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

THRU: Larry C. Dorsey, Executive Secretary
FIFRA Scientific Advisory Panel
Office of Science Coordination and Policy

Clifford Gabriel, Ph.D. Director
Office of Science Coordination and Policy

Please find attached the minutes of the FIFRA Scientific Advisory Panel open meeting held in Arlington,Virginia from March 1-2, 2005. These meeting minutes address a set of scientific issues being considered by the U.S. Environmental Protection Agency regarding scientific issues associated with the human health assessment of the Cry34Ab1 protein.

Attachment

cc:

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Vanessa Vu (SAB)
Rebecca Edelstein

FIFRA SAP Members
Stephen M. Roberts, Ph.D.
Stuart Handwerger, M.D.
Steven Heeringa, Ph.D.
Kenneth Portier, Ph.D.

FQPA Science Review Board Members
Tong-Jen Fu, Ph.D.
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Simon Hogan, Ph.D.
Dean Metcalfe, MD,
Shridhar K Sathe, Ph.D.
Santiago Schnell, Ph.D.
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SAP Report No. 2005-02

MEETING MINUTES

March 1-2, 2005 FIFRA Scientific Advisory Panel Meeting held at the Holiday Inn-National Airport, Arlington, Virginia

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

SCIENTIFIC ISSUES ASSOCIATED WITH THE HUMAN HEALTH ASSESSMENT OF THE CRY34AB1 PROTEIN

NOTICE

These meeting minutes have been written as part of the activities of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP). This report has not been reviewed for approval by the United States Environmental Protection Agency (Agency) and, hence, the contents of this report do not necessarily represent the views and policies of the Agency, nor of other agencies in the Executive Branch of the Federal government, nor does mention of trade names or commercial products constitute a recommendation for use.

The FIFRA SAP was established under the provisions of FIFRA, as amended by the Food Quality Protection Act (FQPA) of 1996, to provide advice, information, and recommendations to the Agency Administrator on pesticides and pesticide-related issues regarding the impact of regulatory actions on health and the environment. The Panel serves as the primary scientific peer review mechanism of the EPA, Office of Pesticide Programs (OPP) and is structured to provide balanced expert assessment of pesticide and pesticide-related matters facing the Agency. Food Quality Protection Act Science Review Board members serve the FIFRA SAP on an ad hoc basis to assist in reviews conducted by the FIFRA SAP. Further information about FIFRA SAP reports and activities can be obtained from its website at http://www.epa.gov/scipoly/sap/ or the OPP Docket at (703) 305-5805. Interested persons are invited to contact Paul Lewis, Designated Federal Official, via e-mail at lewis.paul@epa.gov.

In preparing these meeting minutes, the Panel carefully considered all information provided and presented by the Agency presenters, as well as information presented by public commenters. This document addresses the information provided and presented within the structure of the charge by the Agency.

TABLE OF CONTENTS

Page

SAP Report No. 2005-02

MEETING MINUTES:
March 1-2, 2005 FIFRA Scientific Advisory Panel Meeting, held at the Holiday Inn-National Airport, Arlington, Virginia

A Set of Scientific Issues Being Considered by the Environmental Protection Agency Regarding:

SCIENTIFIC ISSUES ASSOCIATED WITH THE HUMAN HEALTH ASSESSMENT OF THE CRY34AB1 PROTEIN

Paul I. Lewis, Ph.D.
Designated Federal Official
FIFRA Scientific Advisory Panel
Date: April 5, 2005

Stephen M. Roberts, Ph.D.
FIFRA SAP Session Chair
FIFRA Scientific Advisory Panel
Date: April 5, 2005

Federal Insecticide, Fungicide, and Rodenticide Act
Scientific Advisory Panel Meeting
March 1-2, 2005

SCIENTIFIC ISSUES ASSOCIATED WITH THE HUMAN HEALTH ASSESSMENT OF THE CRY34AB1 PROTEIN

PARTICIPANTS

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Abraham R.Tzafriri, **Ph.D.,** Postdoctoral Fellow, Harvard-MIT Biomedical Engineering Center, Division of Health Science and Technology, Massachusetts Institute of Technology, Cambridge, MA

PUBLIC COMMENTERS

Oral statements were provided by:
Ken Narva, Ph.D., on behalf of Dow AgroSciences
Nick Storer, Ph.D., on behalf of Dow AgroSciences
Mr. Rod Herman on behalf of Dow AgroSciences
Ray Layton, Ph.D., on behalf of Pioneer Hi-Bred
Mr. Bill Freese on behalf of Friends of the Earth
Doug Gurian-Sherman, Ph.D., on behalf of the Center For Food Safety
Mr. Martin Barbre on behalf of the National Corn Growers Association

Written statements were provided by:
Mr. Dave Ahlers, private citizen
Mr. Damon Bahnson, private citizen
Mr. Allyn Buhrow, private citizen
Mr. Robert Collister, private citizen
Dow AgroSciences LLC and Pioneer Hi-Bred International, Inc.
Mr. John Foster, private citizen
Mr. Bill Freese on behalf of Friends of the Earth
Mr. Michael Hass, private citizen
Mr. Tom Hooper on behalf of Beck's Superior Hybrids
Mr. Jeff Housman, private citizen
Mr. Kelly Hulstein, private citizen
David Humes, M.D. on behalf of the University of Michigan
Mr. Marvin Krohn, private citizen
Mr. Michael Lindberg, private citizen
Mr. Ron Ortman, private citizen
Doug Gurian-Sherman, Ph.D., on behalf of the Center For Food Safety
Mr. Robert Short, private citizen
Mr. Ron Thurston and Ms. Peggy Vesterby on behalf of Thurston Genetics
Gerald Wilde, Ph.D., on behalf of Kansas State University
Jeff Wolt, Ph.D., on behalf of Iowa State University

INTRODUCTION

The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), Scientific Advisory Panel (SAP) has completed its review of a set of scientific issues being considered by the Agency pertaining to the human health assessment of the Cry 34Ab1 protein. Advance notice of the meeting was published in the *Federal Register* on December 8, 2004. The review was conducted in an open Panel meeting held in Arlington, Virginia, from March 1-2, 2005. The meeting was chaired by Stephen Roberts, Ph.D. Paul Lewis, Ph.D., served as the Designated Federal Official. Clifford Gabriel, Ph.D. (Director, Office of Science Coordination and Policy, EPA) welcomed the Panel to the meeting. Janet Andersen, Ph.D. (Director, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, EPA) offered opening remarks at the meeting. Rebecca Edelstein, Ph.D. (Chemist, Biopesticides and Pollution Prevention Division, Office of Pesticide Programs, EPA) summarized the scientific issues associated with the human health assessment of the Cry34Ab1 protein.

SUMMARY OF PANEL DISCUSSION AND RECOMMENDATIONS

Protocols for Digestibility Assays

- The Panel believed that the explanations offered for omitting early time points for application of the first order kinetics model were not adequately justified.

- It is impossible to comment on the registrant statements on: (1) first-order decay or (2) how much pepsin or protein substrate concentrations can vary without affecting the kinetics of pepsin digestion and first-order rate constants, without knowing the value of the Michaelis-Menten constant, $K_{M,}$ (or the affinity of the substrate for the enzyme).

- Some Panel members recommended the comparison of test proteins on a weight basis, while others recommended a molar basis.

- The digestibility of proteins, as determined by the simulated gastric fluid assay, is greatly influenced by the ratio of pepsin and test protein used in the assay.

- The Panel reviewed the pros and cons of Western blotting, SDS-PAGE and HPLC to monitor digestion reactions and the pros and cons of having single versus separate digestion reactions (vials).

- The kinetic approach is useful for proteins that are not rapidly degraded since several time points are fit to a trend-line and it is inherently more accurate than the single point assay.

Allergenicity Assessment Factors

- Digestibility of proteins is of some value (weight), but of less significance, than the source of the protein, sequence homology, or a validated animal model in the assessment

of allergenic potential. The use of digestibility data is also only of value in the context of a weight of the evidence approach.

- The Panel noted that the setting of limits on results of digestibility is difficult if not impossible given the lack of consistency in digestibility or resistance to digestion of allergens and non allergens. Digestibility must be considered in the context of the entire weight of evidence approach.

- The Panel recommended that fragment digestion rates should be determined for all fragments of a molecular mass > 1500 Da (~ 10-15 amino acids). These determined rates should have significant weight in the assessment of pepsin digestibility.

Cry34Ab1 and Cry35Ab1 Allergenicity Assessment

Considering all of the available information on Cry34Ab1 and based on the weight of evidence approach as recommended by the 2003 Codex Alimentarius Commission guidelines and the methodology used at previous FIFRA SAP meetings, the Panel determined that Cry34Ab1 was unlikely to be a food allergen. As with previous FIFRA SAPs reviewing allergenicity with plant incorporated protectants, the Panel strongly supported development of a validated animal model. Such a model would be significantly more valuable in risk assessment than digestibility. Together, these studies would strengthen the ability to assess allergenic potential. Finally, the Panel again encouraged ring studies on digestibility using a constant acceptable protocol.

PANEL DELIBERATIONS AND RESPONSE TO THE CHARGE

The specific issues to be addressed by the Panel are keyed to the Agency's background documents, references and Agency's charge questions.

Protocols for Digestibility Assays

1) Dow has stated that enzyme kinetic theory predicts first order kinetics for pepsin hydrolysis under conditions of high enzyme and low substrate concentrations and has demonstrated that the rate of substrate disappearance under these conditions follows first-order kinetics for a number of proteins. However, for several proteins, initial time points were omitted to achieve a good fit to the model. Dow states that the data were not included "based on theoretical considerations, which include: potential zero-order or mixed order kinetics due to high substrate concentration, possible presence of denatured and highly digestible protein contaminating the native protein preparation, or the possibility of an initial burst phase or transient phase preceding the first-order phase of digestion (Schnell and Maini, 2000; Milgrom et al., 1998)."

The Panel is requested to comment on whether the explanation justifies omitting early time points or whether the poor fit of early time points indicates a problem with the model.

The Panel concluded that the explanations offered for omitting early time points for application of the first order kinetics model were not adequately justified. There may in fact be problems with the kinetic model, particularly at the early time points. It was agreed that modeling of the late first order decay was an adequate/conservative approach for a given simulated gastric fluid (SGF) degradation assay. This approach would provide a measure of the observed slow phase of substrate hydrolysis, provided that a significant fraction of the digestion takes place during this slow phase. However, it was not clear to the Panel how to extrapolate this assay to *in vivo* digestion. Furthermore, the Panel could not rule out the possibility that the pool of fast degrading substrate is of primary importance *in vivo*.

The Panel questioned the existence of a mechanistic model *per se*. The pesticide registrant presented empirical data to the effect that late decay follows first order kinetics. As noted by the Panel, the observation of first order kinetics during the final stages of the reaction does not permit identification of a specific kinetic mechanism. This is a typical chemical kinetics indistinguishability problem. Thus, the approach taken by registrant can be categorized as a "black box" model. However, the Panel did agree that measurement of the late slow phase of the reaction was important in terms of defining the total time dependence for complete disappearance of Cry34Ab1. This approach was considered an improvement over current single point assays.

The registrant invoked the single substrate-enzyme Michaelis-Menten mechanism

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} C \xrightarrow{k_2} E + P$$

and quasi-steady-state kinetics equation

$$v = -\frac{d[S]}{dt} = \frac{v_{\max}[S]}{K_M + [S]}$$

to explain their data. In these equations, E, S, C, and P are, respectively, the enzyme, substrate, enzyme-substrate complex and product. k_i are the rate constants, v is the velocity rate of the reaction, the square brackets denote concentration, v_{max} is the maximum velocity of the reaction, and K_M is the Michaelis-Menten constant.

A poor fit of early time points to Michaelis-Menten kinetics indicates a problem with the model. The Panel questioned the application of single substrate Michaelis-Menten kinetics to the registrant data. The registrant did not adequately address the problems of applying classical Michaelis-Menten kinetics to a situation where $[E_o] >> [S_o]$ (Segel 1975). They incorrectly applied the term V_{max}/K_M to this scenario (Schnell & Mendoza, 2004).

The Panel noted that at high enzyme to substrate ratios, the simple quasi-steady-state Michaelis-Menten equation cannot be applied (Schnell & Maini, 2000). Based on the analysis performed, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the formation of an enzyme substrate complex will not be discernable and cannot account for the observed initial fast transient. It should be noted that at the first published time point of 1 minute, approximately one half of the substrate had been consumed. Thus one does not know

whether or not the reaction follows first order kinetics during the initial 50% of the reaction (Herman et al. 2003) For the purpose of this study, this may not be important since one is interested in the time course for total substrate hydrolysis. Thus, in spite of any problems with the model proposed by the registrant, the data does appear to predict digestibility based on a series of time points rather than a single time point as done in other studies.

2) Dow has asserted that first-order decay is predicted based on enzyme theory as long as the pepsin concentration is high and the substrate concentration is low (<< K_m) and that the first-order rate constant determined under these conditions is equal to V_{max}/K_m. Dow has also stated that as long as first-order conditions are met, first-order rate constants and half-lives are unaffected by changes in substrate protein concentration and that first-order rate constants can be used to predict relative digestion efficiencies for proteins even if the protein concentration is varied among experiments. In addition, Dow has stated that at the USP concentration for pepsin of 0.32%, the enzyme concentration is saturating and can also be varied between experiments without affecting the first-order rate constant.

The Panel is asked to comment on these statements. How much can the pepsin or protein substrate concentrations vary without affecting the kinetics of pepsin digestion and first-order rate constants?

It is impossible to comment on the registrant statements on first-order decay or how much pepsin or protein substrate concentrations can vary without affecting the kinetics of pepsin digestion and first-order rate constants without knowing the value of the Michaelis-Menten constant, $K_{M,}$ (or affinity of the substrate for the enzyme). The registrant used a simplified assumption that the rate at high enzyme to substrate ratios approximates simple Michaelis-Menten kinetics such that when $[S_0] << K_M$, the velocity equation reduces to $v = V_{max} / [S]/K_M$. The pseudo-first order approximation is not necessarily valid at high enzyme to substrate ratios to characterize time-independent and time-dependent data (Schnell and Mendoza 2004). The Panel believed that the registrant did not validate the assumption that $[S_0] << K_M$, because they did not estimate K_M.

Since the K_M for Cry34Ab1 and its fragments are unknown, it cannot be established that the substrate concentration is less than the K_M. The Panel emphasized the importance of estimating the K_M for Cry34Ab1 and suggested the use of Cry34Ab1 as an alternate substrate inhibitor as a method to assess its K_M.

One of the major concerns of the Panel is that the registrant did not appropriately distinguish between first-order and pseudo-first order kinetics. For the enzyme-substrate association pathway, the rate will be $v_a = k_1[E][S]$. It is generally considered that if $[E_0] >> [S_0]$, then $[E_0]$ will remain essentially constant during the reaction and the rate can be written as a pseudo-first order one, that is $v_a = k'[S]$, where $k' = k_1[E_0]$. From these equations it can be seen that if [S] changes, but $[E_0]$ does not, the pseudo-first order rate constant ($k_1[E_0]$), which is proportional to the half-time will not change. However, a change in the pepsin concentration will change the pseudo-first order rate constant and the half-life.

For time-course data, Schnell & Mendoza (2004) derived the same pseudo-first order rate constant for the enzyme-substrate association. The time-evolution of the substrate depletion obeys a biphasic exponential curve if pseudo-first order kinetics is valid ($[S_0] << K_M$). The timescale of the exponential decays (i.e. the constant in the exponential term) is given by the quadratic expression $\lambda = \frac{k'}{2}\left((1+K_M^{'}) \pm \sqrt{(1+K_M^{'})^2 - 4K'}\right)$, where $k' = k_1[E_0], K' = k_2 / k'$ and $K_M^{'} = \frac{k_{-1}}{k'} + K'$. Clearly, if $[E_0]$ varies, the constant in the exponential will vary.

In enzyme kinetics studies of the total substrate (free substrate + enzyme-substrate) depletion, the Michaelis-Menten mechanism is indeed well approximated as first order decay at high enzyme/substrate ratios (Tzafriri, 2003). However, the first order rate constant is $k_2 E_0 / (E_0 + K_M) = V_{\max} / (E_0 + K_M)$ where $V_{\max} \equiv k_2 E_0$. When $E_0 > K_M$, the apparent first order rate constant is weakly dependent on enzyme concentration only if $E_0 >> K_M$. Therefore, determination of K_m and validation of the predicted enzyme dependence of the observed first order rate constant are important. Taking into account that the first order rate constant is equal to $k_2 E_0 / (E_0 + K_M)$, any deviation from this expression would suggest that the Michaelis-Menten scheme is inappropriate for the specific enzyme-substrate pair. However, it is highly questionable that the pepsin hydrolysis follows a simple single substrate Michaelis-Menten reaction mechanism.

3) Typically, for comparing the *in vitro* digestibility of different proteins, researchers have used fixed concentrations of pepsin and substrate protein on a weight basis (mg/mL) rather than adjusting for molecular weight of the substrate protein, presumably because larger proteins likely have more potential pepsin cleavage sites. However, Dow states that "while multiple pepsin-labile sites may occur within a protein, a single site is often responsible for limiting digestion rates, and thus the number of molecules, rather than total weight, is most often more influential in determining the kinetics that describe decay."

The Panel is asked to comment on Dow's statement. To compare the rate of pepsin digestion of different proteins, is it more appropriate for the concentration of test protein to be constant on a weight basis (mg/mL) or a mole basis (mol/L)?

The Panel was divided on these issues. Some Panel members recommended the comparison of test proteins on a weight/ml, while others recommended a mole/ml. The customary approach in enzyme catalyzed reactions is using the molar basis because the rate of the reactions is experimentally and theoretically found to be proportional to the concentration of the reactants. The Panel emphasized that both a weight basis and molar basis are equivalent, the conversion from weight to mole basis can be easily computed if the molecular weight of test protein is known. The Panel strongly recommended reporting the molecular weight of the test proteins if the tests are made on weight basis.

Regarding the registrant's statement "while multiple pepsin-labile sites may occur within a protein, a single site is often responsible for limiting digestion rates, and thus the number of molecules, rather than total weight, is most often more influential in determining the kinetics that describe decay", some members of the Panel pointed out that it is incorrect to assume a direct correlation between a protein molecular weight and the number of cleavage sites. In addition, some Panel members disagreed with the registrant's statement that "…a single site is often responsible for limiting digestion rate." in their reactions. Although this certainty holds for some pepsin substrates, in the present case the reaction mechanism is unknown.

4) Typically, researchers have looked at the effect of pepsin to substrate ratio rather than concentrations on digestion (Karamac, *et al.*, 2002). How do varying the ratios and/or concentrations affect the rate of hydrolysis?

The digestibility of proteins, as determined by the SGF assay, is greatly influenced by the ratio of pepsin and test protein used in the assay. A protein can appear to be resistant or labile to digestion depending on the relative amount of pepsin and test protein used (Fu et al., 2002 and Astwood et al., 1996). The pepsin to test protein ratio is also important to place the digestion reaction in the correct kinetic models. The concentrations of pepsin and protein substrate will also have a pronounced effect on digestion rates as described above.

5) Different assays exist for determining pepsin activity. A pepsin activity assay based on measuring the trichloracetic acid-soluble products of pepsin hydrolysis of hemoglobin is provided in USP, 2004 under the entry for pepsin. However, the entry in USP, 2004 for "gastric fluid, simulated" references the Food Chemicals Codex for pepsin activity, which provides an assay that measures pepsin digestion of egg albumen.

The Panel is asked to comment on the appropriateness of using a fixed concentration of pepsin versus using a fixed specific activity of pepsin in digestibility protocols. How would the use of different pepsin activity assays affect the measured pepsin activity units?

Determination of an activity of any enzyme depends on the substrate used for such quantification. Assay conditions also will influence this number. The Panel recommended a standard assay be established to define pepsin activity and preferred using a fixed amount of enzyme units in digestion studies. The reasons for using a fixed activity are to: a) allow replication of the study; b) account for "inactive enzyme molecules"; c) provide better control of "enzyme activity" in relation to substrate concentration; and d) eliminate effects of batch-to-batch variations in enzyme preparations (with respect to enzyme activity) on experimental results.

Enzyme activity is affected by the method used to assess activity. Depending on the assay conditions and substrate used, the number that represents enzyme activity may vary (thus the activity units may appear to be different). It is important to have a standardized protocol to determine enzyme activity and the Panel encouraged the Agency to work with interested stakeholders in developing such a protocol.

Since specific activity refers to enzyme purity, using pepsin preparations within a defined limited range of specific activity would greatly assist in comparative analysis among studies. It is important to remember that the specific activity should be determined using a defined substrate under specified reaction conditions as specific activity is dependent on the substrate, purity of the enzyme and assay conditions (i.e. pH, temperature and buffer).

The Panel raised the need for verifying that pepsin activity does not diminish with time under assay conditions. The registrant reported that the pepsin was stable during the time course they employed.

6) Typically, scientists have used SDS-PAGE with staining or western blot analysis for monitoring digestion reactions. HPLC is also sometimes used.

The Panel is asked to comment on the pros and cons of the different methods that could be used for monitoring digestion reactions.

The Panel reviewed the pros and cons of Western blotting, SDS-PAGE and (high pressure liquid chromatography (HPLC) to monitor digestion reactions. Their analysis is provided below.

Western blotting

Pros: 1) allows visualization based on the ability to bind antigen-specific antibodies, 2) breakdown products that bind antibodies may be identified, 3) allows estimation of molecular weight(s), and 4) allows multiple samples on the same gel.

Cons: 1) will not track a breakdown product when it no longer has sufficient epitopes to be visualized. A corollary of this is that the original molecule will contain all epitopes, most of which will be available to bind antibody; and some fragments will not be seen if they do not contain epitopes, 2) if blotting is from a reducing and denaturing gel, native epitopes may not be identified, and 3) the detection sensitivity depends on the visualization method used and the protein load applied. [Note: the above assumes that Western blots utilize satisfactory polyclonal antisera.]

SDS-PAGE

Pros: 1) long history of use; allows comparison with historical data, 2) allows visualization of proteins regardless of presence of epitopes, 3) can be semi-quantitated by employing densitometry, and 4) allows multiple samples on the same gel.

Cons: 1) denatures proteins, 2) dissociates sub-units, 3) not as sensitive as HPLC, 4) does not always follow antigenicity, and 5) sensitivity is dependent on the staining method used and protein load applied.

HPLC

Pros: 1) does not require denaturation of proteins, 2) may be performed preserving interactions, and 3) most sensitive among the listed techniques for monitoring the intact protein.

Cons: 1) molecular weights may not agree with SDS-PAGE, 2) more time-consuming, 3) does not allow head-to-head comparisons, 4) resolution may be a problem, and 5) does not follow antigenic determinants. [Note: the above assumes HPLC uses sizing columns and that extraction procedures are adequate.]

7) Some researchers have used one digestion reaction and removed aliquots at various times for monitoring, while others have set up separate reactions for each of the time points.

What are the pros and cons of these approaches?

Most Panel members concluded that there would be no difference between using single homogenous vials or separate vials as long as there are no pipetting errors and the reactions are homogeneous. The advantages of using single vial are: 1) ease of operation and 2) reduction in number of pipettings needed, thus a reduction in corresponding pipetting errors. Several Panel members indicated that single vial experiments seem to have slightly better ability to control extraneous factors that could affect experimental variability. A major disadvantage is that it can only be used for homogenous single phase reactions.

The advantages of using multiple vials are: 1) more degrees of freedom, 2) does not negatively influence non-homogenous multiple phase reactions; and 3) provides independent indication of each data point. The disadvantage of this approach is an increased chance for pipetting errors as the number of pipettings increase.

From a statistical point of view, the single vial and multiple vial experiments require different statistical models for analysis of the density readings. The specific issue is that the single vial experimental responses are repeated measures data and as such the expected correlations among the measured values must be taken into account in the process of estimating the uncertainty in the decay rate. In the multiple vial experiment, vials are randomly assigned to assessment times. This randomization theoretically removes correlations among responses and hence traditional independent sample residual variability estimates can be used in the uncertainty estimate. The different models can also result in different rate estimates although the differences in the rate estimate would be small. In addition, the statistical tests of the hypothesis of zero decay rate will be different for the two approaches and involve more than just differences in the degrees of freedom associated with the residual variability estimates. While different statistical analysis approaches are used for the different protocols, these statistical analysis models are not particularly useful in informing choice of protocols.

8) Under the current protocol, Dow's kinetic approach is only applicable to moderately digestible proteins (i.e., using Dow's protocol, many proteins digest too quickly and some too slowly to obtain an adequate number of data points for quantitative kinetic analysis).

Please comment on the usefulness of the kinetic approach for proteins that are not rapidly degraded.

The kinetic approach is useful for proteins that are not rapidly degraded since several time points are fit to a trend-line and it is inherently more accurate than the single point assay.

The fit to a kinetic plot becomes a problem if a protein is digested too quickly. However, one could easily establish a criterion for placing a given protein in a "rapidly digested" category. Since SDS-PAGE is used to obtain time points until the protein is fully digested, the kinetics approach would give the same results as obtained by the "standard assay", even if the data do not fit a pseudo-first order reaction. The same holds true for slowly digested proteins; here a time limit for a "slowly digested category" could be easily established. With the slowly digested proteins, one might extend the time of digestion to generate useful data.

The Panel emphasized the importance of standardized conditions that would help ensure reproducibility. It was noted that the specific kinetic approach advocated by the registrant that focuses on the late phase of protein degradation is only relevant if a significant fraction of the digestion process occurs during this late phase. Otherwise the late phase is not representative of the overall degradation process. Moreover the late phase of protein degradation may be hard to define in a practical setting and consequently impossible to standardize.

Allergenicity Assessment Factors

9) The 2001 FAO/WHO report and 2003 Codex guidelines both recommend using in vitro digestibility in assessing the allergenicity potential of a protein. The FAO/WHO report provides a "decision tree" approach, while the Codex guidelines suggest a weight of evidence approach. Codex guidelines state "resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic," and "it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen." The Codex guidelines, however, don't specify how a protein should be further evaluated if it is "resistant" to degradation, and "resistant" is not defined.

a) What weight should *in vitro* digestibility studies be given in the overall assessment compared with other criteria such as sequence homology?

Digestibility of proteins is of some value (weight), but of less significance, than the source of the protein, sequence homology, or a validated animal model in the assessment of allergenic potential. The use of digestibility data also is only of value in the context of a total weight of the evidence approach.

Sequence homology and *in vitro* digestibility are the criteria available for risk assessment when the source of the protein is one with no history of allergenicity. In the case of Cry34Ab1, the Panel was not aware of any report of *Bacillus thuringiensis* being an allergen. For example,

the Bernstein et al. study (2003) reported positive skin tests in some individuals exposed to an intact source organism (*B. thuringiensis*), not a crystal protein *per se*, and there was no demonstration of clinical allergy to *B. thuringiensis* through challenge studies. In such a case, a search for sequence homology becomes the first step in risk assessment. For Cry34Ab1, there was no sequence identity over 8 amino acids or more than 35% identity in a segment of over 80 amino acids with known inhalant and ingested allergens with known allergens. Although not recommended as per 2003 Codex Alimentarius Commission guidelines, sequence similarity data (rather than identity data) would be more reassuring to the Panel for its review.

Given no sequence identity by the above criteria, the remaining risk assessment consists in large part of assessment of digestibility, given that there is no validated animal model for testing of allergic potential. While digestibility results are difficult to use in risk assessment due to the number of exceptions of digestibility correlating with allergenic potential, they remain useful. In addition, susceptibility to degradation is reassuring. While resistance to degradation would be a concern, moderate digestibility of Cry34Ab1 suggests this protein is less likely to have allergenic potential.

These conclusions take into account the registrant's statements that the Cry proteins in question do not auto aggregate one with another or with each other in the fluid phase. If aggregation was to occur, protease binding sites could be masked and the proteins could then be resistant to digestion.

One Panel member noted that for the purpose of comparison of digestibility between laboratories, one specific pH is preferred. There are no data conclusively demonstrating the use of more than one pH is needed to predict allergenicity or compare allergenicity of specific proteins. The time specified should also be standardized with one hour being an acceptable approach.

However, one Panel member provided a different perspective on the importance of *in vitro* digestibility studies. The Agency presented data for the half-lives of the most persistent fragments of seven known allergens and eight known non-allergens following pepsin hydrolysis. Three of the seven fragments resulting from the hydrolysis of the allergens had half-lives that were less than 10 minutes and overlapped the values obtained for many of the non-allergens. Two of the eight most persistent fragments resulting from hydrolysis of the non-allergens had half-lives much greater than 10 minutes. Since there was a significant overlap in the ability of pepsin hydrolysis to distinguish between allergens and non-allergens, members of the Panel agreed that protein hydrolysis data should not be considered alone in assessing the potential of a protein to be an allergen, but must be considered along with other criteria in the weight of the evidence approach for an allergenicity assessment. Because three of the seven known allergens were rapidly hydrolyzed, this Panel member concluded that protein hydrolysis data should not be considered at all in the assessment of potential allergenicity.

Both the weight of evidence and the decision tree approach can be formulated statistically as a discriminate analysis. One Panel member commented that quantification of the different characteristics used in these models across multiple proteins, both known allergens and

non-allergens, are only now becoming available and in the future as formal statistical discriminate analysis should be possible. This discriminate analysis, using either a linear model or a non-linear (classification tree) model will allow assessment of the relative importance of the specific components. In particular, the true strength or utility of digestibility assay results in the decision process can be gauged quantitatively. This analysis needs clear, reproducible protocols for quantifying all criteria or as a binary variable for other characteristics.

b) The Panel is asked to comment on the appropriateness of setting acceptable/unacceptable limits for digestibility in assessing the safety of a protein.

The Panel noted that the setting of limits on results of digestibility is difficult if not impossible given the lack of consistency between digestibility and allergenicity. Digestibility must be considered in the context of the entire weight of evidence approach.

The relative digestibility of proteins depends on the assay conditions used. It is important to use standardized assay conditions to determine pepsin resistance. There was no consensus of how stable a protein needs to be in order to be considered as having high allergenic potential. The usual approach would be to compare the digestibility of test proteins with those of known allergens. Therefore a database consisting of the digestibility of a group of known allergens under standard conditions needs to be established.

Although they would not constitute thresholds, descriptors (e.g. “degradable”, “moderately resistant to degradation”, “resistance to degradation”) based on defined ranges of digestibility rates could be used in characterizing digestibility of a protein. Data developed under defined protocols would be necessary to assign such descriptors.

10) Stable digestion fragments are often formed during pepsin digestion of proteins, and Dow has used the kinetic approach to estimate the half-lives of several digestion fragments.

Please comment on the significance of the rate of digestion of protein fragments for allergenicity assessments.

The Panel recommended that fragment digestion rates should be determined for all fragments of a molecular mass > 1500 Da (~ 10-15 amino acids). These determined rates should have significant weight in the assessment of pepsin digestibility.

Understanding the rate of digestion of small protein fragments is important in the assessment of pepsin digestibility as a number of allergens have been shown to be peptides (linear epitopes-B and T cell epitopes) that have allergenic activity. Thus the Panel concluded that fragment digestion rates should be determined for all protein fragments and that these determined rates should have significant weight in the assessment of pepsin digestibility. However, the Panel recommended that the rate of digestion should be determined for all protein fragments of 10-15 amino acids as peptides of this size range are of the minimal molecular mass that can be accurately analyzed using conventional detection methods (denaturing SDS-PAGE) and that may possess allergen potential. The Panel did recognize that smaller fragments may have allergenic

potential. However, the technology required for detection of these smaller fragments (MALDI-TOF mass spectrometry) may not always be feasible.

Cry34Ab1 and Cry35Ab1 Allergenicity Assessment

11) Cry34Ab1 appears to be moderately digested in SGF, rather than rapidly digested. Considering all of the available information– Cry34Ab1 originates from a non-allergenic source, has no sequence similarity with known allergens, is not glycoslyated, is inactivated by heat, is moderately digested in SGF, and will only be present at low levels in food– EPA has concluded that Cry34Ab1 is unlikely to be a food allergen.

Please comment on the Agency's conclusions regarding the allergenicity of Cry34Ab1.

Considering all of the available information on Cry34Ab1 and based on the weight of evidence approach as recommended by the 2003 Codex Alimentarius Commission guidelines in addition to the methodology used at previous FIFRA SAP meetings, the Panel determined that Cry34Ab1 was unlikely to be a food allergen. As with previous FIFRA SAPs reviewing allergenicity with plant incorporated protectants, the Panel strongly supported development of a validated animal model. Such a model would be significantly more valuable in risk assessment than digestibility. Together these studies would strengthen the ability to assess allergenic potential. Finally, the Panel again encouraged ring studies on digestibility using a constant acceptable protocol.

The Panel reached its decision based on the following points.

1) Cry34Ab1 appears to be moderately digested in SGF, rather than rapidly digested, or totally resistant to digestion. This conclusion is accepted in part because the Panel has been reassured that Cry34Ab1 does not self aggregate or aggregate with Cry35Ab1 in physiologic solutions. Were either self aggregation or aggregation with Cry35Ab1 to happen, such an event could mask protease sensitive sites and alter digestion.

2) Cry34Ab1 originates from a non-allergenic source. There is no convincing evidence that *B. thuringiensis* is an allergen in that it has never conclusively been documented to provoke an allergic reaction. There are no data indicating that crystal proteins are allergens including demonstration of individuals who form IgE to crystal proteins and experience documented allergic reactions upon challenge. In addition, approximately 27 % of corn raised in the US for human consumption express crystal proteins (ERS 2004) and there are no documented reports known to the Panel of individuals who react specifically to corn expressing a crystal protein. The conclusion that Cry34Ab1 originated from a non-allergenic source means that there is no evidence that *B. thuringiensis* or Cry proteins induce an IgE response. Thus, there is no serum from sensitized individuals allergic to Cry34Ab1 that can be employed in risk assessment.

3) Cry34Ab1 has no sequence similarity with known allergens based on the data presented on the lack of identity of 8 contiguous amino acids or more than 35% identity over 80 amino acids with known inhalant and ingested allergens. The Panel did have some concern that the use of 8

contiguous amino acids would have been better performed searching for similarity, rather than identity.

4) Cry34Ab1 is not glycosylated when expressed in maize. Western blots presented support this conclusion in that the expressed protein is of the same molecular weight as the *E. coli* product, and MALTI-TOF analysis did not show glycosylation.

5) Cry34Ab1 is heat labile as defined by inactivation of biological activity after exposing the protein to 90^0 C for 30 minutes. This feature of heat lability is not part of the FAO/WHO risk assessment strategy and not a major feature of the Codex recommendations. The Panel concluded, with the exception of one Panel member, that the determination of heat sensitivity is of minimal to no value in considering the possibility of Cry34Ab1 being a potential allergen. The exceptions noted by the particular Panel member were:

The inactivation of Cry34Ab1 by heat (i.e. the loss of bioactivity and the loss of tertiary structure of Cry34Ab1 after heating) may not be relevant with respect to an allergenicity assessment. There are data showing that heat treatment increased the allergenicity of peanut proteins due to expose to additional allergenic binding sites or due to covalent modification of the protein [Nordlee et al. 1981; Maleki et al. 2000]). Therefore the data provided by the registrant in this aspect would not be useful for assessing the allergenic potential of Cry34Ab1. Thus, this Panel member also concluded that the assessment of the heat stability of the protein should focus on the immunological aspects.

6) Cry34Ab1 is present at low levels in food. The Panel noted that the 2001 joint FAO/WHO Expert Consultancy indicated that it was not possible to define a level of expression below which a protein can be considered safe from the allergenicity point of view.

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