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FINAL REPORT

Proprietary Information of MONSANTO Company

Report No.: MSL-12949

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Date: 10/5/93

Title: Assessment of the *in vitro* digestive fate of CP4 EPSP synthase

Authors: Joel E. Ream, Michele R. Bailey, John N. Leach and Stephen R. Padgette

Abstract:

Purpose

The protein being investigated in this study is 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). This enzyme has been expressed in crop plants to confer tolerance to glyphosate, the active ingredient of Roundup® herbicide, and has also been used for glyphosate selection in a variety of plant species. Glyphosate exerts its herbicidal activity due to inhibition of EPSPS, an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms. The EPSPS from *Agrobacterium* sp. strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate-tolerant EPSPSs (1,2). Upon glyphosate treatment, plants or plant cells expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds.

A study was conducted to assess the degradation of the CP4 EPSPS protein using *in vitro* mammalian digestion models consisting of simulated gastric and intestinal fluids. EPSPS enzymes, found in plants, bacteria, and fungi, are typically present in protein fractions of plant-derived food sources. The purpose of this study was to assess the degradation of CP4 EPSPS, as detected by Western blot analysis and enzymatic activity, using simulated gastric and intestinal fluids.

Rept.No.: MSL-12949 **Authors:** Joel E. Ream, Michele R. Bailey, John N. Leach and Stephen R. Padgette

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The information contained in this study is being supplied to the [redacted] Food and Drug Administration (FDA) as part of a food safety assessment program by Monsanto Company, in accord with the FDA Policy: Foods Derived from New Plant Varieties, May 29, 1992.

Justification of Test System

Simulated digestion models are commonly used as models of animal digestion. They have been used to investigate the digestibility of plant and animal proteins, food additives, to assess protein quality and to assess biodegradation of pharmaceuticals.

Materials and Methods

The test material, *E. coli*-expressed CP4 EPSPS (lot #5192245) was produced by fermentation in *E. coli* GB100 pMON21104 (3) and purified to greater than 90% purity (4). Studies to address the equivalence of plant-produced CP4 EPSPSs with the *E.coli*-produced enzyme are described separately. The characterization of the CP4 EPSPS test material is also described in a separate study (5).

CP4 EPSPS was incubated at approximately 37°C in simulated mammalian gastric and intestinal fluids (SGF and SIF, respectively). At defined incubation time periods the digestions were terminated and the levels of CP4 EPSPS protein were measured by Western blot analysis and enzymatic activity assay. The final concentration of CP4 EPSPS protein in gastric fluid was approximately 2 µg/ml for Western blot analysis and approximately 50 µg/ml for enzymatic activity measurements. For intestinal fluid, the final CP4 EPSPS protein concentration was approximately 50 µg/ml for both Western blot and enzymatic activity analyses.

Results

CP4 EPSPS protein degraded readily in gastric and intestinal fluids. The half-life in gastric fluid was < 15 seconds by Western blot analysis. CP4 EPSPS enzymatic activity decreased by > 84% by the first timepoint, which was a two-minute incubation in gastric fluid. In intestinal fluid, CP4 EPSPS protein half-life was < 10 minutes by Western blot analysis. CP4 EPSPS enzymatic activity decreased approximately 5% after 10 minutes of incubation in intestinal fluid and decreased > 91 to 94% after an approximately 4.5 hour incubation period.

Conclusion

The results of this study suggest that the CP4 EPSPS protein and its associated enzymatic activity will readily degrade in the mammalian digestive tract. CP4 EPSPS protein degrades very rapidly in gastric fluid as detected by both Western blot and enzymatic activity analyses. CP4 EPSPS protein degrades very rapidly in intestinal fluid when detected using Western blot analysis. CP4 EPSPS protein enzymatic activity does dissipate in intestinal fluid, but not as readily as when detected by Western blot analysis. This suggests the antigenic sites on the CP4 EPSPS protein for the particular antibody used in this study are more sensitive to proteolytic degradation under these conditions than is the functional active site of CP4 EPSPS. Despite this difference, it is clear that the

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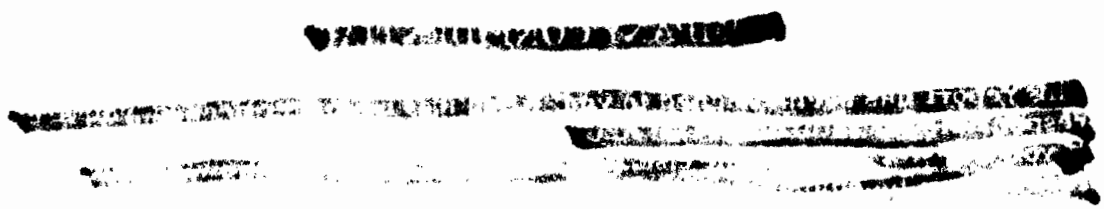
CP4 EPSPS enzymatic activity did dissipate substantially (>91 to 94%) after an approximately 4.5 hour incubation in SIF, a time period that would allow for substantial CP4 EPSPS enzyme activity dissipation in the typical human intestine. Thus, using both SGF and SIF model systems, CP4 EPSPS is predicted to be rapidly digested in the mammalian digestive tract.



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Study Title

Assessment of the *in vitro* digestive fate of CP4 EPSP synthase

Study Director
Stephen R. Padgette

Contributing Scientists
Joel E. Ream, Michele R.Bailey, John N. Leach, and Nancy Biest

Study Completed on
10/5/93

Performing Laboratories
The Agricultural Group of Monsanto Company
New Products Division
700 Chesterfield Parkway North
St. Louis, MO 63198

Laboratory Project ID
Study No. 92-01-30-15

Experiment No. 92-419-721

Statement of compliance

This study meets the requirements for 40 CFR Part 160 and 21 CFR 58.

Submitter _____ 10/6/93

Sponsor _____ 10/6/93

Study Director Stephen R Padgett 10/5/93

Quality Assurance Statement

Study Number: 92-01-30-15

This signed statement indicates that the ESH Q&CA Quality Assurance Unit has monitored this study and reviewed the study data and final report. These reviews indicate that the final report accurately presents the raw data as developed during the study.

Dates of reviews as well as dates that findings were reported to testing facility management and the study director are listed below.

Dates of Quality Assurance reviews:

December 11, 15, 28, 30, 1992
June 22, 1993
August 11, 12, 1993
September 1, 28, 1993

Dates findings were reported to management and study director:

January 8, 1993
April 21, 1993
June 22, 1993
July 29, 1993
August 11, 1993
September 1, 23, 28, 1993

Quality Assurance reviews conducted by:

M. D. Hoffman
D. E. McKinney
K. F. Yount

Quality Assurance Representative
Monsanto Company

October 4, 1993
Date

Study Number: 92-01-30-15

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Title: Assessment of the *in vitro* digestive fate of CP4 EPSP synthase

Facilities: The Agricultural Group of Monsanto Company
New Products Division
700 Chesterfield Parkway North
St. Louis, MO 63198

Principle Investigator: Joel E. Ream
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO 63198
Tel: 314-537-6678, Fax: 314-537-6880

Study Director: Stephen R. Padgette, Ph.D.
Associate Fellow
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO 63198
Tel: 314-537-6386, Fax: 314-537-6759

Contributors: Michele Bailey
John Leach
Nancy Biest

Study Start Date: December 14, 1992

Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Monsanto - St. Louis

Signatures of Approval

Stephen R. Padgett Date: 10/5/93
Study Director

Date: 10-5-93
Principal Investigator

Ray Fleck Date: 10/6/93
Sponsor

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I. Summary

Purpose

The protein being investigated in this study is 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). This enzyme has been expressed in crop plants to confer tolerance to glyphosate, the active ingredient of Roundup® herbicide, and has also been used for glyphosate selection in a variety of plant species. Glyphosate exerts its herbicidal activity due to inhibition of EPSPS, an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms. The EPSPS from *Agrobacterium* sp. strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate-tolerant EPSPSs (1,2). Upon glyphosate treatment, plants or plant cells expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds.

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CP4 EPSPS protein degraded readily in gastric and intestinal fluids. The half-life in gastric fluid was < 15 seconds by Western blot analysis. CP4 EPSPS enzymatic activity decreased by > 84% by the first timepoint, which was a two-minute incubation in gastric fluid. In intestinal fluid, CP4 EPSPS protein half-life was < 10 minutes by Western blot analysis. CP4 EPSPS enzymatic activity decreased approximetely 5% after 10 minutes of incubation in intestinal fluid and decreased > 91 to 94% after an approximately 4.5 hour incubation period.

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The results of this study suggest that the CP4 EPSPS protein and its associated enzymatic activity will readily degrade in the mammalian digestive tract. CP4 EPSPS protein degrades very rapidly in gastric fluid as detected by both Western blot and enzymatic activity analyses. CP4 EPSPS protein degrades very rapidly in intestinal fluid when detected using Western blot analysis. CP4 EPSPS protein enzymatic activity does dissipate in intestinal fluid, but not as readily as when detected by Western blot analysis. This suggests the antigenic sites on the CP4 EPSPS protein for the particular antibody used in this study are more sensitive to proteolytic degradation under these conditions than is the functional active site of CP4 EPSPS. Despite this difference, it is clear that the CP4 EPSPS enzymatic activity did dissipate substantially (>91 to 94%) after an approximately 4.5 hour incubation in SIF, a time period that would allow for substantial CP4 EPSPS enzyme activity dissipation in the typical human intestine. Thus, using both SGF and SIF model systems, CP4 EPSPS is predicted to be rapidly digested in the mammalian digestive tract.

II. Purpose

A. General: The protein being investigated in this study is 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). This enzyme has been expressed in crop plants to confer tolerance to glyphosate, the active ingredient of Roundup® herbicide, and has also been used for glyphosate selection in a variety of plant species. Glyphosate exerts its herbicidal activity due to inhibition of EPSPS, an enzyme of the shikimate pathway for aromatic amino acid biosynthesis in plants and microorganisms. The EPSPS from *Agrobacterium sp.* strain CP4 is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most glyphosate-tolerant EPSPSs (1,2). Upon glyphosate treatment, plants or plant cells expressing the CP4 EPSPS are unaffected since the continued action of the glyphosate-tolerant EPSPS enzyme meets the plant's need for aromatic compounds.

Some of the potentially commercial plant varieties expressing CP4 EPSPS (soybean, potato, tomato, corn, canola) are commonly used as a source of animal feed, as well as food for human consumption. Thus, animals and humans would be exposed to trace levels of CP4 EPSPS. EPSPS enzymes, found in plants, bacteria, and fungi, are typically present in protein fractions of plant-derived food sources. The CP4 EPSPS gene, although of microbial origin, is functionally and structurally related to the plant-expressed EPSPS proteins. Upon consumption, most proteins are readily degraded upon exposure to the mammalian digestive tract (6). The purpose of this study is to assess the degradation of the CP4 EPSPS protein using *in vitro* mammalian digestion models consisting of simulated gastric and intestinal fluids. In this study, the degree of degradation of CP4 EPSPS protein upon incubation in simulated gastric and intestinal fluids was assessed using Western blot analysis and enzymatic activity assay. The experimental protocol for this study is provided as Attachment 1.

B. Protocol amendments:

Two protocol amendments to this study are discussed below. For complete details, the amendments are provided as Attachment 2 to this report.

1. The concentration of CP4 EPSPS in SGF and SIF was approximately 2 µg/ml and 50 µg/ml, respectively- CP4 EPSPS protein concentration for SIF assays was increased from 2 µg/ml to 50 µg/ml to correspond to the level that was needed to measure CP4 enzymatic activity in SIF incubation treatments. Also, the procedure used to terminate CP4 EPSPS incubation in SIF for Western blots was changed because the original conditions described did not effectively terminate the reaction, and the incubation time in SIF was increased to 24 h.

2. **The incubation time of CP4 EPSPS in SIF was increased for enzymatic assays** - This amendment reflects the increased incubation times for the enzymatic assays to allow a comparison to results from the Western blots

III. Justification of Test System

In vitro studies with simulated digestion solutions are widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (7,8), animal proteins (9) and food additives (10); to assess the protein quality (11); to study digestion in pigs and poultry (12); to measure tablet dissolutions rates to assess biodegradation for pharmaceutical (13); and to investigate the controlled-release properties of experimental pharmaceuticals (14). The method of preparation of the simulated digestion solutions used in this study is described in the United States Pharmacopeia (15), a frequently cited reference for *in vitro* digestion studies.

IV. Materials

A. Test Substance (CP4 EPSPS from *E. coli*, lot #5192245): The test substance was purified microbially-expressed CP4 EPSPS produced in *E. coli* GB100 from plasmid pMON21104 (3) and purified to greater than 90% purity (4), based on SDS-PAGE analysis (5). The test substance was characterized (5) and shown to be equivalent to the CP4 EPSPS protein expressed in genetically modified plants (21). The protein was obtained as a 20 mg/ml and 12 mg/ml solution in buffer (50 mM sodium bicarbonate [pH 8.5], 10 mM cysteine, 5% (w/v) sucrose). The test substance was stored at approximately - 80°C until used for the preparation of dosing solutions. Since the current study was specifically designed to assess the stability of the test material in the simulated digestive fluid test systems, no additional test substance stability data was deemed necessary.

B. Simulated Digestion Fluids: Simulated gastric and intestinal fluids were prepared as described in the United States Pharmacopeia (15). Solutions were used within 24 hours of preparation and were stored at approximately 4°C until used. The intestinal fluid was mixed before each use to resuspend insoluble material that settled after preparation. Each digestion solution was assayed for activity before use. Simulated gastric fluid (SGF) was assayed for pepsin activity, using hemoglobin as substrate, by measuring the increase in absorbance at 280 nm following TCA precipitation (16). Protease activity of simulated intestinal fluid (SIF) was assayed by measuring spectrophotometrically the increase in supernatant absorbance at 574 nm following trichloroacetic acid (TCA) precipitation of SIF incubations using resorufin-labeled casein as substrate (17).

C. Reagents: Pepsin (porcine, Product Number P-7000), pancreatin (porcine, Product Number P-1500), and hemoglobin (bovine, Product Number H-2625) were obtained from Sigma Chemical Company (St. Louis, MO). Resorufin-labeled casein (Product Number 1080733) and phosphoenolpyruvate (Product Number 108-294) were obtained from Boehringer Mannheim Corporation (Indianapolis, IN).

V. Methods

A. Assessment of Digestion by Western Blot Analysis:

1. Digestions: Dosing solutions were prepared by dilution of the CP4 EPSPS protein with buffer (50 mM sodium bicarbonate [pH 8.5], 10 mM cysteine, 5 % (w/v) sucrose). CP4 EPSPS protein was added to temperature-equilibrated (approximately 37°C) digestion solutions by pipetting from these concentrated CP4 EPSPS dosing solutions. This method of administration of the test substance was selected to provide reproducible dosing of small amounts of the test material. One ml volumes of digestion fluids were incubated in 15 ml plastic test tubes in a Gyrotory® water bath shaker (Model G76, New Brunswick Scientific, Edison, NJ) with the following exceptions: 1) a 0.1 ml incubation volume in a 1.5 ml plastic test tube was used for the t = 0 samples of CP4 EPSPS in SGF and SIF; and 2) a 0.1 ml incubation volume in a 1.5 ml plastic test tube was used for a whole incubation sample control. Both of these incubations were conducted using a separate water bath at approximately 37°C without continuous shaking. The reduced-volume treatments were used to keep final quenched sample volumes compatible with equipment used for aliquots sampled from 1 ml incubations. One ml digestion solutions were agitated as much as practical. For SGF incubations, digestion solutions were agitated by hand at each sampling interval instead of continuously due to the very short total incubation time (2 minutes). For SIF incubations, digestion solutions were agitated continuously except for brief intervals for sampling. CP4 EPSPS was added to 1 ml of buffer to allow an assessment of the recovery of CP4 EPSPS protein from the digestion fluids. For these samples, they were mixed thoroughly upon addition of CP4 EPSPS and then again just before quenching the digestion.

2. Gastric fluid assay: CP4 EPSPS protein was added to SGF to a final concentration of approximately 2 µg/ml. Five time periods, including t = 0, up to a total incubation period of 2 min were assayed by removing 50 µl aliquots from the digestion solution and adding them to 1.5 ml plastic test tubes containing a quench solution consisting of 15 µl of 0.2 M sodium carbonate. 0.1 ml samples were quenched with 30 µl of 0.2 M sodium carbonate. For the t = 0 incubation treatment, the sodium carbonate was added to the SGF before the addition of CP4 EPSPS. Quenched SGF incubation samples were diluted 1:1 with 2X SDS-PAGE sample buffer (18), incubated at approximately 100°C for

approximately 5 min and stored at approximately -20°C. For the whole incubation sample control, the entire 0.1 ml incubation mixture was quenched with 30 µl 0.2 M sodium carbonate and then diluted with 130 µl of 2X SDS-PAGE sample buffer. CP4 EPSPS protein levels in digestion samples were assessed by SDS-PAGE analysis followed by Western blot analysis. CP4 EPSPS levels were estimated by visual comparison of sample band intensities to band intensities of CP4 EPSPS standards included with each blot (standards were also prepared and heated with SDS).

3. Intestinal fluid assay: CP4 EPSPS protein was added to SIF to a final concentration of approximately 50 µg/ml. Six time periods, including t = 0, up to a total incubation period of 4.75 hours were assayed by removing 50 µl aliquots from the digestion solution, added to 1.5 ml plastic test tubes and immediately heating at approximately 100°C for approximately 5 min to terminate the digestion. Heat-quenched digestion samples were diluted with SDS-PAGE sample buffer, re-heated and stored as described for gastric fluid assays. For whole incubation sample controls, the entire 0.1 ml incubation sample was quenched by heating to approximately 100°C for 5 min and then diluted with 100 µl of 2X SDS-PAGE sample buffer. Before Western blot analysis as described for gastric fluid treatments, samples were further diluted with 1X SDS-PAGE sample buffer. CP4 EPSPS protein levels in each sample were estimated by visual comparison of sample band intensities to band intensities of CP4 EPSPS standards included with each blot (standards were also prepared and heated with SDS). CP4 EPSPS protein levels in some SIF digestion solutions were also assessed using a densitometer (LKB Ultroscan XL, using GelScan software) to evaluate the accuracy of the visual estimates.

B. Assessment of Digestion by Enzymatic Activity Assay:

1. Digestions: CP4 EPSPS was added to SGF and SIF and incubated as described for Part V.A with the following exceptions: 1) total digestion solution volume were 0.5 ml instead of 1 ml; 2) digestion samples were not agitated during incubation; 3) digestion solutions were incubated in 1.5 ml plastic tubes instead of 15 ml tubes; and 4) no 0.1 ml incubations for t = 0 and whole-sample incubations were done. These changes were adaptations of the procedure used for Western blot analysis of digestion to the CP4 EPSPS enzyme assay. They are not expected to affect the comparison of digestion to Western blot analysis.

2. Gastric fluid assay: CP4 EPSPS was added to SGF to a final concentration of approximately 50 µg/ml, incubated and the digestion quenched as described for Part A using 0.2 M sodium carbonate at a level of 15 µl per 50 µl of SGF digestion solution.

3. Intestinal fluid assay: CP4 EPSPS was added to SIF to a final concentration of approximately 50 µg/ml. The digestion was quenched by immediate dilution of 10 µl aliquots of digestion solution to total volume of 50 µl with EPSPS enzymatic activity assay reagents. For t = 0 treatments, the CP4 EPSPS was added to chilled (approximately 4°C) SIF, mixed and immediately added to enzymatic assay reagents.

4. CP4 EPSPS enzymatic assay: The procedure utilized for determining the amount of functionally active EPSPS entailed the use of an HPLC with radioactivity detector, and has been previously described (19,20). Labelled substrate ^{14}C-phosphoenolpyruvate (^{14}C-PEP) is converted to ^{14}C-5-enolpyruvylshikimate-3-phosphate (^{14}C-EPSP) in the presence of shikimate-3-phosphate (S3P) by EPSPS, and the resultant ^{14}C-EPSP is detected using HPLC and radioactive flow detection. The final reagent concentrations in the assay were 50 mM Hepes, 0.1 mM ammonium molybdate, 5 mM potassium flouride, 1 mM ^{14}C-PEP, and 2 mM S3P, pH 7.0. Reactions were run at approximately 25°C. The reactions were quenched after 2 to 5 minutes with an equal volume of 9:1 ethanol: 0.1 M acetic acid, pH 4.5. The samples were then centrifuged and chromatographed by HPLC anion exchange using approximately 0.25 M potassium phosphate eluent, pH 6.5, at 1 ml/min flow rate. For EPSPS, 1 unit (U) is defined to be 1 µmol EPSP produced/ min at approximately 25°C, under the assay conditions described.

VI. Results

A. Assessment of Digestion by Western Blot:

1. Gastric fluid: CP4 EPSPS protein degradation in simulated gastric fluid was assessed by Western blot analysis (Fig. 1). No CP4 EPSPS protein was detected after a 15 s incubation at approximately 37°C (lane 6), the earliest time interval evaluated. SGF alone did not contain bands that may interfere with the assessment of CP4 EPSPS protein levels at either t = 0 s (lane 3) or after 120 s of incubation at approximately 37°C (lane 10). The recovery of added CP4 EPSPS from SGF was estimated by visual comparison of CP4 EPSPS levels detected when CP4 EPSPS was added to buffer (lane 4) to that amount of CP4 EPSPS recovered when added to SGF at t = 0 s (lane 5). The recovery was estimated by visual inspection to be approximately 100%. To assess whether the 50 µl aliquot of CP4 EPSPS in SGF removed from the approximately 1 ml digestion solution is representative of the whole digestion solution, the CP4 EPSPS protein level indicated with an aliquot after 120 s incubation (lane 9) was compared to the CP4 EPSPS protein level indicated in a sample in which the entire incubation sample was quenched and diluted with SDS-PAGE sample buffer (lane 11). No CP4 EPSPS was detected by Western blot analysis by either method. The dose of CP4 EPSPS to the SGF was confirmed by assessment of CP4 EPSPS detected by Western blot at t = 0

(approximately 10 ng CP4 EPSPS/lane) compared to the expected level if the final CP4 EPSPS protein added to SGF was 2 µg/ml (10 ng/lane).

2. Intestinal fluid: CP4 EPSPS degradation in simulated intestinal fluid was assessed by Western blot analysis (Fig. 2). More than 50% of the CP4 EPSPS was degraded after a 10 min incubation in SIF at 37°C (lane 6) as compared to the level detected in the t = 0 sample (lane 5) by visual assessment of Western blots [This assessment was corroborated by scanning densitometry (data not included in this report)]. No CP4 EPSPS protein was detected after 100 min incubation in SIF (lane 8). SIF alone did not contain bands that may interfere with the assessment of CP4 EPSPS protein levels at either t = 0 min (lane 3) or after 19.5 h (lane 11) incubation. The recovery of added CP4 EPSPS from SIF was estimated to be approximately 100% by visual comparison of CP4 EPSPS added to buffer (lane 4) to that in unincubated SIF (lane 5). The 50 µl aliquots taken from CP4 EPSPS protein incubations in SIF after 19.7 h incubation (lane 10) were found to be representative of the whole digestion solution by comparison to a sample incubated for 19.3 h in which the entire incubation sample was quenched and diluted with SDS-PAGE sample buffer (lane 12). No CP4 EPSPS protein was detected by either method. The dose of CP4 EPSPS to the SIF was confirmed by assessment of CP4 EPSPS detected by Western blot at t = 0 (approximately 10 ng/lane) compared to the expected level if the final CP4 EPSPS protein added to SIF was 50 µg/ml (10 ng/ml, after dilution). The visual assessment of CP4 EPSPS protein levels by Western blot analysis was compared to levels quantified by densitometry and were found to be comparable.

B. Assessment of Digestion by Enzymatic Activity:

1. Investigation of potential assay interference: The degree to which SGF and SIF interfere with the determination of CP4 EPSPS protein enzymatic activity was investigated. No substantial interference was observed; significant CP4 EPSPS activity was measured in the presence of both SGF and SIF at the level (10 µl/assay) used for subsequent digestion assays (Table 1). No turnover of PEP to EPSP was observed when SGF and SIF were incubated in assay reagents without CP4 EPSPS added and when SGF and SIF were added with CP4 EPSPS but without S3P (data not shown, but in raw data file). These results support the appropriateness of this assay to assess the degradation of CP4 EPSPS enzymatic activity in digestion solutions.

2. Digestions: None of the CP4 EPSPS enzymatic activity added to SGF was detected after a 2 min incubation in SGF (Table 2). The enzyme assay utilized would have detected approximately 15% of the added EPSPS. Two experiments were done to assess CP4 EPSPS activity degradation in SIF (Table 3). In both experiments, 93 to 95% of added CP4 EPSPS was still

present after 10 min incubation in SIF. CP4 EPSPS activity had decreased to <9% and <6% of the initial level after incubations of 285 min and 270 min, respectively.

VII. Discussion

Simulated mammalian digestion fluids were used to assess the degradation of CP4 EPSPS, a protein expressed in plants to confer tolerance to the herbicide Roundup® or to select transformed plant cells using glyphosate. EPSPS enzymes are typically present in protein fractions of plant-derived food sources. Degradation of CP4 EPSPS was assessed by two methods, Western blot analysis and enzymatic activity assay. These methods were selected to assess the degradation of the CP4 EPSPS protein *per se* as well as the dissipation of its associated functional activity. Western blot analysis was selected because of its high selectivity and sensitivity for the CP4 EPSPS protein. The decrease in CP4 EPSPS protein band intensity on Western blots from digestion samples indicates that there is CP4 EPSPS protein degradation due to the activity of the digestion fluids. The decrease in CP4 EPSPS functional activity also indicates that the digestion fluids are degrading the protein. By both the Western blot and functional activity assays, CP4 EPSPS protein degraded readily in this study.

CP4 EPSPS protein purified from *E. coli* is an appropriate test material for this study. The *E. coli* CP4 EPSPS protein has been shown to be equivalent to the protein expressed in glyphosate-tolerant plants evaluated to date, *e.g.* soybean (21) and canola (22). The use of a highly purified sample of CP4 EPSPS allows a direct assessment of the digestibility of this protein without the complication of additional components in the test material that could influence protein degradation.

No half-life calculations were done for CP4 EPSPS in digestion fluids as detected by Western blot analysis. The degree of degradation in both SGF and SIF was such that only a limit on half-life could be estimated. Since no CP4 EPSPS protein was detected by Western blot analysis after 15 s of incubation in SGF, the half-life is less than 15 s. In SIF, more than 50% of the CP4 EPSPS synthase protein degraded after 10 min incubation, so the half-life is less than 10 minutes. A more refined determination of half-life would require shorter incubation time intervals. The current estimates of half-life reported in this study appear appropriate for assessing the degradation of CP4 EPSPS in these model digestion fluids.

CP4 EPSPS protein was dosed to SGF to a level of approximately 2 µg/ml for assessment of degradation by Western blot analysis and 50 µg/ml for the functional activity assessment. The 2 µg/ml level was selected based on the

sensitivity of the Western blot analysis - an aliquot withdrawn from the digestion solution, when diluted 1:1 with 2X SDS-PAGE sample buffer, could be loaded directly on gels to provide a strong signal from which to use as a reference for degradation. Due to the lower sensitivity of the CP4 EPSPS enzymatic assay, the CP4 EPSPS protein concentration had to be raised to 50 µg/ml in digestion fluids to provide good enzymatic activity at the start of the digestion incubation period. The CP4 EPSPS protein degradation in SIF experiment, as measured using Western blot analysis, was done using a dose of 50 µg/ml. This dose was selected to provide a direct correlation with the degradation data collected by measuring CP4 EPSPS enzymatic activity. Due to the extremely rapid degradation of CP4 EPSPS protein in SGF, we did not feel it was necessary to repeat this experiment at the 50 µg/ml dose level used for assessing the degradation of enzymatic activity.

There is a good correlation between the Western blot analysis (VI.A) and enzymatic activity (VI.B) results for the degradation of CP4 EPSPS in SGF. No band corresponding to CP4 EPSPS protein was present after 2 min incubation in SGF (Fig. 1) and no CP4 EPSPS enzymatic activity was measured after a 2 min incubation in SGF (Table 2). In SIF, the level of CP4 EPSPS detected by Western blot analysis after 10 min incubation was less than 50% of the initial level (Fig. 2); however, when dissipation of enzymatic activity was monitored, 93 to 95% of the initial activity remains after 10 min incubation (Table 3). This result was unexpected. It suggests the antigenic sites on the CP4 EPSPS protein for the particular antibody used in this study are more sensitive to proteolytic degradation under these conditions than is the functional active site of CP4 EPSPS. Despite this difference, the CP4 EPSPS enzymatic activity dissipated substantially (>91 to 94%) after an approximately 4.5 hour incubation in SIF.

The very fast degradation of CP4 EPSPS protein in SGF as measured by both Western blot analysis and enzymatic activity strongly suggests that this protein will degrade readily in the stomach when ingested by mammals. Solid food has been measured, using isotopically-labelled meal, to empty from the human stomach about 50% in 2 hr, while liquid empties 50% in approximately 25 min (23). In the human intestine, isotopically-labelled chromate, which is not absorbed by the intestine, first appears in the feces in 4 to 10 hours but takes 68 to 165 hours to clear totally from the GI tract (24). Based on these results and the results in this report, it is likely most of CP4 EPSPS protein ingested by humans will degrade in the digestive tract. Furthermore, the dose levels in the SGF and SIF are likely to be high relative to the amount of CP4 EPSPS that will be ingested by animals and humans (25). For instance, the CP4 EPSPS protein is expressed at approximately 0.3 µg CP4 EPSPS/mg seed tissue fresh weight in soybean seeds (26). This concentration would likely be

diluted by mixing with other foods, and then diluted again by the addition of digestion fluids in the stomach and intestine. Also, in many food crops the plant material expressing CP4 EPSPS protein will be heat and/or solvent processed, which can substantially reduce the CP4 EPSPS protein content in processed food derived from plant material (26)

VIII. Conclusion

CP4 EPSPS protein degrades readily in simulated gastric and intestinal fluids when measured using both Western blot analysis and enzymatic activity assay. The doses used and degree of degradation observed in this study suggest a high level of degradation of the CP4 EPSPS protein and its associated enzymatic activity is likely in the mammalian digestive tract upon ingestion as a component of food or feed.

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X. Abbreviations

C	Centigrade
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
E. coli	*Escherichia coli*
Fig.	Figure
h	hours
HPLC	high performance liquid chromatography
M	molar
mg	milligram
min	minute
ml	milliliter
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PEP	phosphoenolpyruvate
s	second
SDS	sodium dodecyl sulfate
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
SOP	Standard Operating Procedure
S3P	shikimate-3-phosphate
TCA	trichloroacetic acid
µg	microgram
µl	microliter

Table 1. Lack of interference of gastric and intestinal fluids on the CP4 EPSPS activity assay. For gastric fluid, 10 µl of 2.5 mg/ml CP4 EPSPS protein in 50 mM Hepes (pH 7.0) was added to quenched SGF (0.5 ml SGF + 0.15 ml 0.2 M carbonate), and a 10 µl aliquot of the mixture was assayed for CP4 EPSPS enzyme activity in a 50 µl total reaction volume. For intestinal fluid, 10 µl of 2.5 mg/ml CP4 EPSPS protein in 50 mM Hepes (pH 7.0) was added to SIF, and a 10 µl aliquot of the mixture was immediately assayed for CP4 EPSPS enzyme activity in a 50 µl total reaction volume. For the control reactions without quenched SGF or SIF, 10 µl 50 mM Hepes (pH 7.0) was added instead of the digestion fluid mixture. Values listed represent the mean from two replicate assays. Standard errors of the mean are given in parentheses.

Treatment, CP4 EPSPS	CP4 EPSPS activity, % turnover of 1 mM ^{14}C-PEP to ^{14}C-EPSP in the assay	Std. Error
- SGF	8.83%	(0.55)
+ SGF	9.55%	(0.61)
- SIF	11.13%	(0.35)
+ SIF	15.91%	(1.07)

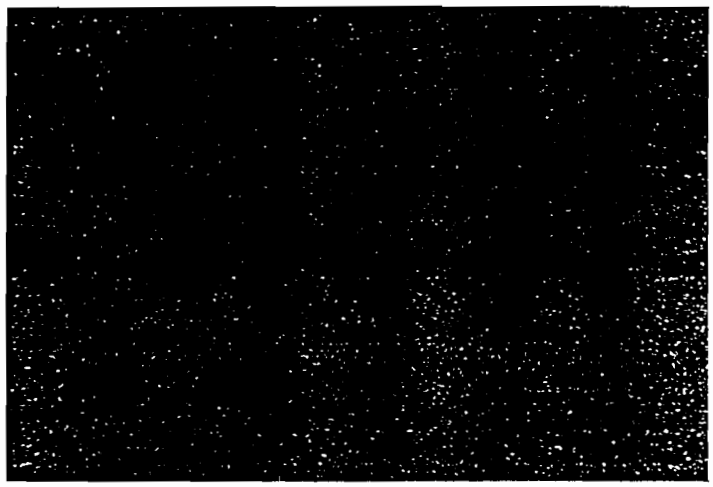
Table 2. Dissipation of CP4 EPSPS enzymatic activity in gastric fluid. CP4 EPSPS protein was added to SGF to a final concentration of 50 µg/ml, incubated at approximately 37°C and quenched with 0.2 M sodium bicarbonate. 10 µl of quenched digestion solution was assayed for CP4 EPSPS enzymatic activity. Values listed represent the mean from three replicate assays. Standard errors of the mean are given in parentheses.

Incubation time (min)	CP4 EPSPS activity, % turnover of 1 mM ^{14}C-PEP to ^{14}C-EPSP in the assay	Std. Error	% Initial Activity Remaining
0	12.8%	(0.6)	100%
2	<2.0%	(0.0)	<16%

Table 3. Dissipation of CP4 EPSPS enzymatic activity in intestinal fluid. CP4 EPSPS protein was added to SIF to a final concentration of 50 µg/ml, incubated at approximately 37°C and quenched by heating at approximately 100°C for 5 min. 10 µl of quenched digestion solution was assayed for CP4 EPSPS enzymatic activity. Values listed represent the mean from three replicate assays. Standard errors of the mean are given in parentheses.

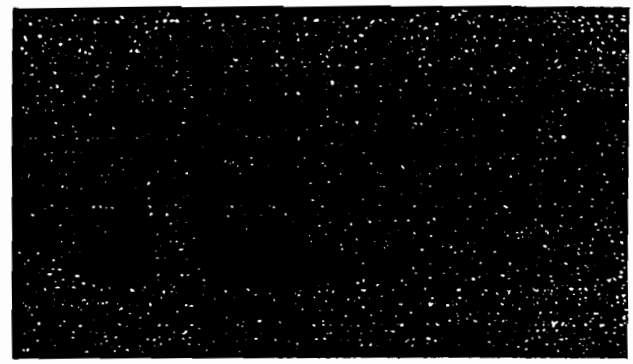
Incubation time (min)	Experiment 1			Experiment 2		
	CP4 EPSPS activity, % turnover of 1 mM ^{14}C-PEP to ^{14}C-EPSP in the assay		% Initial Activity Remaining	CP4 EPSPS activity, % turnover of 1 mM ^{14}C-PEP to ^{14}C-EPSP		% Initial Activity Remaining
0	21.3	(0.8)	100	32.2	(0.5)	100
10	20.3	(0.6)	95	30.0	(0.7)	93
270	-	-	-	<2.0	(1.1)	<6
285	<2.0	(0.1)	<9	-	-	-

Figure 1. Western blot analysis of CP4 EPSPS degradation in gastric fluid. Legend: Western blot shown is one of three replicate digestions. CP4 EPSPS was added to SGF to a final concentration of 2 µg/ml, incubated at 37°C, quenched by neutralization with 0.2 M sodium carbonate and evaluated by Western blot analysis. Lane 1 and 2 are CP4 EPSPS standards at 5 and 10 ng/lane, respectively. Lane 3 and 10 are SGF after 0 and 120 s incubation, respectively. Lane 4 is buffer plus CP4 EPSPS at t = 0 s. Lane 5 through 9 are CP4 EPSPS after 0, 15, 30, 60 and 120 s incubation in SGF, respectively. Lane 11 is SGF plus CP4 EPSPS after 120 s incubation where the entire incubation sample was quenched and prepared for Western blot analysis. Lane 12 was used for molecular weight markers. The gel shown is gel #2 from 12/17/92.



1 2 3 4 5 6 7 8 9 10 11 12

Figure 2. Western blot analysis of CP4 EPSPS degradation in intestinal fluid. Legend: Western blot shown is one of three replicate digestions. CP4 EPSPS was added to SIF to a final concentration of 50 µg/ml, incubated at approximately 37°C, quenched by heating to approximately 100°C for 5 min and evaluated by Western blot analysis. Lane 1 and 2 are CP4 EPSPS standards at 5 and 10 ng/lane, respectively. Lane 3 and 11 are SIF after 0 and 1171 min incubation, respectively. Lane 4 is buffer plus CP4 EPSPS at t = 0 s. Lanes 5 through 10 are CP4 EPSPS after 0, 10, 32, 100, 270 and 1181 min incubation in SIF, respectively. Lane 12 is CP4 EPSPS after 1160 min incubation is SIF where the entire incubation was quenched and prepared for Western blot analysis. The gel shown is gel #3 from 3/18/93.



1 2 3 4 5 6 7 8 9 10 11 12

Attachment 1. Protocol for Study 92-01-30-15, Experiment 92-419-721: Assessment of the *in vitro* digestive fate of CP4 EPSP synthase

Study #:	92-01-30-15
Experiment #:	92-419-721
Study Title:	Assessment of the *in vitro* digestive fate of CP4 EPSP synthase
Sponsor:	Monsanto Company 800 N. Lindbergh Blvd. St. Louis, Missouri 63167
Study Director:	Stephen R. Padgette, Ph.D. Senior Research Specialist Monsanto AG AA3I 700 Chesterfield Village Parkway North St. Louis, Missouri 63198 Tel: 314-537-6386 Fax: 314-537-6759
Principal Investigator:	Joel E. Ream Research Specialist The Agricultural Group of Monsanto Company 700 Chesterfield Village Parkway North St. Louis, Missouri 63198 (314) 537-6678
Test Facility:	The Agricultural Group of Monsanto Company New Products Division 700 Chesterfield Village Parkway St. Louis, Missouri 63198

SPONSOR APPROVAL

Study Director: Stephen R. Padgette Date: 12/14/92

Stephen R. Padgette, Ph.D.
(AA3I, 314-537-6386)

Sponsor: Roy Fuchs Date: 12/14/92

Roy L. Fuchs, Ph. D.
(GG4G, 314-537-6438)

[redacted]

Principle Investigator:

Joel E. Ream (GG4K, 314-537-6678) Date: 12-14-92

REVIEWED BY:

Kevin F. Yount (GG4F, 314-537-6945) Date: 12/28/92

1. Purpose:

The purpose of this study is to assess the rate of degradation of CP4 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase (hereafter called "CP4") using *in vitro* mammalian digestion models. CP4 is a protein introduced into plants to confer tolerance to Roundup® herbicide (glyphosate).

2. Records and retention:

All data and information generated in this study will be recorded directly and promptly onto appropriate forms and/or notebooks. The exceptions are electronically captured data, for which a printout will be generated and included with other study data. All data and information will be written legibly in indelible ink, preferably black. No records will be recorded in pencil. All entries will be dated on the day of entry and signed or initialed by the person entering the information. Computer printouts will be dated and signed or initialed by person responsible for their generation. Any changes in entries will be made so as not to obscure the original entry, will indicate the reason for the change and will be dated and signed (or initialed) at the time of change. All raw data such as microtiter plate reader printouts, chromatographs from western blots, photos of SDS-PAGE gels, etc. will be saved. All raw and final data will be archived at the conclusion of the study.

3. Proposed experimental start date: December, 1992

[redacted]

4. Proposed experimental termination date: January, 1993

5. Test materials:

The CP4 protein (Lot Number 5192245) was purified from *E. coli* cells engineered to express it. It will be demonstrated to be similar to the protein expressed in glyphosate tolerant plants; *e.g.* soybean (Study Number 92-01-30-11, Experiment Number 92-419-718) and canola (Study Number 92-02-30-03, Experiment Number 92-447-704).

6. Test system:

CP4 protein will be added to simulated digestion fluids and incubated at approximately 37°C. *In vitro* digestion models are used widely to assess the digestive fate of ingested material. These systems will be used in this study due to their relative ease of analysis and reproducibility relative to *in vivo* systems. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) will be prepared at the testing facility as described in The United States Pharmacopeia, 1990, pp 1788-1789 (attached). These fluids will be used within 24 hours of preparation, stored at approximately 4°C and assayed for protease activity before use.

7. Conduct of the Study:

Part 1. Measurement of protein degradation by western blot analysis

Primary treatments:

A. SGF
B. SGF plus CP4
C. SIF
D. SIF plus CP4

[redacted]

General procedure. Three replicate one-ml samples will be prepared for each treatment. All samples will incubated at approximately 37°C in individually labelled 15-ml test tubes. Purified protein samples of CP4 will be added to temperature-equilibrated (approximately 37°C) digestion fluids to a final concentration of approximately 2 µg ml^{-1}. Incubation solutions will be agitated briefly at each sampling time instead of agitated continuously due to the very short total incubation time. At specified time points during the incubation, 50-µl aliquots of treatment solutions will be removed from each tube, added to individually labeled tubes and the reaction process immediately terminated as described below. Samples will be diluted 1:1 with 2X SDS-PAGE sample buffer, heated to approximately 100°C for approximately five minutes, cooled in an ice bath and stored in capped 1.5-ml microcentrifuge tubes at approximately -20°C. CP4 protein levels in samples will be estimated by western blot analysis.

Gastric fluid assays. CP4 will be incubated in SGF for a total time period of approximately two minutes. At least five samples will collected between zero and approximately two minutes for each treatment tube. To terminate the reaction, 50-µl aliquots of incubation solutions will be neutralized by addition to individually-labeled 1.5-ml tubes containing 15 µl of 0.2M sodium carbonate. Time-course assays will be carried out sequentially due to the short incubation intervals.

Intestinal fluid assays. CP4 will be incubated in SIF for a total time period of approximately two minutes. At least five samples will collected between zero and approximately two minutes for each treatment tube. To terminate the reaction, 50-µl incubation solution samples will be added to individually-labeled 1.5 ml tubes containing 50 µl 2X SDS-PAGE sample buffer and immediately heated to approximately 100 °C for approximately five minutes, as described in the "General procedure" section. Time-course assays will be carried out sequentially due to the short incubation intervals.

Controls. Incubated SGF and SIF without added CP4 will be incubated, terminated and processed as for the "+ CP4" samples to serve as negative controls. For these treatments, an equal volume of buffer will be added in place of the CP4 protein in the same buffer. CP4 recovery from both of the digestive fluids will be determined by a comparison of the CP4 protein levels detected in the "t=0" digestive fluid samples to those detected in buffer. The "t=0" incubation sample will be generated by spiking CP4 into already-terminated assay samples. A treatment in which the entire incubation solution is mixed into SDS-PAGE sample buffer will be prepared to allow a comparison with treatments in which an aliquot from the incubation solution was analyzed by western blotting.

[redacted]

Data analysis. Specific protein levels in each sample will be estimated by comparison of intensities of bands on western blots to corresponding standards for each protein. The mean value for three replicates will be determined. An approximate half-life will be estimated by kinetic evaluation of the rate data, if appropriate. In some cases, limits on half-life (e.g. "less than 10 seconds") may be reported instead of an actual half-life.

Part II. Measurement of degradation of protein activity.

CP4 protein incubations will be carried out generally as described for Part I except no SDS-PAGE sample buffer will be added. CP4 incubation solutions will be assayed for 5-enolpyruvylshikimate-3-phosphate synthase activity if the digestion solutions do not interfere with the assay. Intestinal fluid assays will be terminated by immediately freezing incubation samples. Gastric fluid assays will be terminated generally as described for Part I. The mean values of three replicates will be calculated. The degree of degradation of the CP4 protein will be calculated as a percentage of activity remaining after approximately two minutes incubation compared to the activity measured in the t = 0 incubation samples.

8. Protocol amendments:

Any changes in or deviations from this approved protocol and the reasons for the changes/deviations will be documented, dated, and signed by the Study Director.

9. GLP Compliance:

This study will be performed in compliance with FDA 21CFR58, EPA 40CFR160 and OECD Good Laboratory Practice Standards and principles.

[redacted]

For use in staining endocrine tissue, dilute this test solution with an equal volume of water.

Denigès' Reagent—See *Mercuric Sulfate TS*.

Diazobenzenesulfonic Acid TS—Place in a beaker 1.57 g of sulfanilic acid, previously dried at 105° for 3 hours, add 80 mL of water and 10 mL of diluted hydrochloric acid, and warm on a steam bath until dissolved. Cool to 15° (some of the sulfanilic acid may separate but will dissolve later), and add slowly, with constant stirring, 6.5 mL of sodium nitrite solution (1 in 10). Then dilute with water to 100 mL.

Dichlorofluorescein TS—Dissolve 100 mg of dichlorofluorescein in 60 mL of alcohol, add 2.5 mL of 0.1 *N* sodium hydroxide, mix, and dilute with water to 100 mL.

Dicyclohexylamine Acetate TS—Dissolve 50 g of dicyclohexylamine in 150 mL of acetone, cool in an ice bath, and add, with stirring, a solution consisting of 18 mL of glacial acetic acid in 150 mL of acetone. Recrystallize the precipitate that forms, by heating the mixture to boiling and allowing it to cool in an ice bath, then collect the crystals on a filtering funnel, wash with a small volume of acetone, and air-dry. Dissolve 300 mg of the dicyclohexylamine acetate so obtained in 200 mL of a mixture of 6 volumes of chloroform and 4 volumes of water-saturated ether. Use immediately.

2,7-Dihydroxynaphthalene TS—Dissolve 100 mg of 2,7-dihydroxynaphthalene in 1000 mL of sulfuric acid, and allow the solution to stand until the yellow color disappears. If the solution is very dark, discard it and prepare a new solution from a different supply of sulfuric acid. This solution is stable for approximately one month if stored in a dark bottle.

Diiodofluorescein TS—Dissolve 500 mg of diiodofluorescein in a mixture of 75 mL of alcohol and 30 mL of water.

Diluted Lead Subacetate TS—See *Lead Subacetate TS, Diluted*.

***p*-Dimethylaminobenzaldehyde TS**—Dissolve 125 mg of *p*-dimethylaminobenzaldehyde in a cooled mixture of 65 mL of sulfuric acid and 35 mL of water, and add 0.05 mL of ferric chloride TS. Use within 7 days.

Dinitrophenylhydrazine TS—Carefully mix 10 mL of water and 10 mL of sulfuric acid, and cool. To the mixture, contained in a glass-stoppered flask, add 2 g of 2,4-dinitrophenylhydrazine, and shake until dissolved. To the solution add 35 mL of water, mix, cool, and filter.

Diphenylamine TS—Dissolve 1.0 g of diphenylamine in 100 mL of sulfuric acid. The solution should be colorless.

Diphenylcarbazone TS—Dissolve 1 g of crystalline diphenylcarbazone in 75 mL of alcohol, then add alcohol to make 100 mL. Store in a brown bottle.

Disodium Ethylenediaminetetraacetate TS—Dissolve 1 g of disodium ethylenediaminetetraacetate in 950 mL of water, add 50 mL of alcohol, and mix.

Dithizone TS—Dissolve 25.6 mg of dithizone in 100 mL of alcohol. Store in a cold place, and use within 2 months.

Eosin Y TS (adsorption indicator)—Dissolve 50 mg of eosin Y in 10 mL of water.

Eriochrome Black TS—Dissolve 200 mg of eriochrome black T and 2 g of hydroxylamine hydrochloride in methanol to make 50 mL.

Eriochrome Cyanine TS—Dissolve 750 mg of eriochrome cyanine R in 200 mL of water, add 25 g of sodium chloride, 25 g of ammonium nitrate, and 2 mL of nitric acid, and dilute with water to 1000 mL.

Fehling's Solution—See *Cupric Tartrate TS, Alkaline*.

Ferric Ammonium Sulfate TS—Dissolve 8 g of ferric ammonium sulfate in water to make 100 mL.

Ferric Chloride TS—Dissolve 9 g of ferric chloride in water to make 100 mL.

Ferrous Sulfate TS—Dissolve 8 g of clear crystals of ferrous sulfate in about 100 mL of recently boiled and thoroughly cooled water. Prepare this solution fresh.

Ferrous Sulfate, Acid, TS—Dissolve 7 g of ferrous sulfate crystals in 90 mL of recently boiled and thoroughly cooled water, and add sulfuric acid to make 100 mL. Prepare this solution immediately prior to use.

Folin-Ciocalteu Phenol TS—Into a 1500-mL flask introduce 100 g of sodium tungstate, 25 g of sodium molybdate, 700 mL of water, 50 mL of phosphoric acid, and 100 mL of hydrochloric acid. Reflux the mixture gently for about 10 hours, and add 150 g of lithium sulfate, 50 mL of water, and a few drops of bromine. Boil the mixture, without the condenser, for about 15 minutes, or until the excess bromine is expelled. Cool, dilute with water to 1 liter, and filter: the filtrate has no greenish tint. Before use, dilute 1 part of filtrate with 1 part of water.

Formaldehyde TS—Use *Formaldehyde Solution* (see in the section, *Reagents*).

Fuchsin-Pyrogallol TS—Dissolve 100 mg of basic fuchsin in 50 mL of water that previously has been boiled for 15 minutes and allowed to cool slightly. Cool, add 2 mL of a saturated solution of sodium bisulfite, mix, and allow to stand for not less than 3 hours. Add 0.9 mL of hydrochloric acid, mix, and allow to stand overnight. Add 100 mg of pyrogallol, shake until solution is effected, and dilute with water to 100 mL. Store in an amber-glass bottle in a refrigerator.

Fuchsin-Sulfurous Acid TS—Dissolve 200 mg of basic fuchsin in 120 mL of hot water, and allow the solution to cool. Add a solution of 2 g of anhydrous sodium sulfite in 20 mL of water, then add 2 mL of hydrochloric acid. Dilute the solution with water to 200 mL, and allow to stand for at least 1 hour. Prepare this solution fresh.

Gastric Fluid, Simulated, TS—Dissolve 2.0 g of sodium chloride and 3.2 g of pepsin in 7.0 mL of hydrochloric acid and sufficient water to make 1000 mL. This test solution has a pH of about 1.2.

Gelatin TS (for the assay of *Corticotropin Injection*)—Dissolve 340 g of acid-treated precursor gelatin (Type A) in water to make 1000 mL. Heat the solution in an autoclave at 115° for 30 minutes after the exhaust line temperature has reached 115°. Cool the solution, and add 10 g of phenol and 1000 mL of water. Store in tight containers in a refrigerator.

Glacial Acetic Acid TS—See *Acetic Acid, Glacial, TS*.

Glucose oxidase–chromogen TS—A solution containing, in each mL, 0.5 µmol of 4-aminoantipyrine, 22.0 µmol of sodium *p*-hydroxybenzoate, not less than 7.0 units of glucose oxidase, and not less than 0.5 units of peroxidase, and buffered to a pH of 7.0 ± 0.1.[%]

Suitability—When used for determining glucose in Inulin, ascertain that no significant color results by reaction with fructose, and that a suitable absorbance-versus-concentration slope is obtained with glucose.

Gold Chloride TS—Dissolve 1 g of gold chloride in 35 mL of water.

Hydrogen Peroxide TS—Use *Hydrogen Peroxide Topical Solution* (USP monograph).

Hydrogen Sulfide TS—A saturated solution of hydrogen sulfide, made by passing H_2S into cold water. Store it in small, dark amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H_2S, and unless it produces at once a copious precipitate of sulfur when added to an equal volume of ferric chloride TS. Store in a cold, dark place.

Hydroxylamine Hydrochloride TS—Dissolve 3.5 g of hydroxylamine hydrochloride in 95 mL of 60 percent alcohol, and add 0.5 mL of bromophenol blue solution (1 in 1000) and 0.5 *N* alcoholic potassium hydroxide until a greenish tint develops in the solution. Then add 60 percent alcohol to make 100 mL.

8-Hydroxyquinoline TS—Dissolve 5 g of 8-hydroxyquinoline in alcohol to make 100 mL.

Indigo Carmine TS (*Sodium Indigotindisulfonate TS*)—Dissolve a quantity of sodium indigotindisulfonate, equivalent to 180 mg of $C_{16}H_8N_2O_2(SO_3Na)_2$, in water to make 100 mL. Use within 60 days.

Indophenol-Acetate TS (for the assay of *Corticotropin Injection*)—To 60 mL of standard dichlorophenol-indophenol solution

...section, *Volumetric Solutions*) add water to make 250 ...dd to the resulting solution an equal volume of sodium ...olution freshly prepared by dissolving 13.66 g of anhy...odium acetate in water to make 500 mL and adjusting ...h 0.5 *N* acetic acid to a pH of 7. Store in a refrigerator, and within 2 weeks.

...estinal Fluid, Simulated, TS—Dissolve 6.8 g of monobasic ...ssium phosphate in 250 mL of water, mix, and add 190 mL 0.2 *N* sodium hydroxide and 400 mL of water. Add 10.0 g ...ancreatin, mix, and adjust the resulting solution with 0.2 *N* ...ium hydroxide to a pH of 7.5 ± 0.1. Dilute with water to ...0 mL.

...dine TS—Use *0.1 N Iodine* (see in the section, *Volumetric ...utions*).

...odine Monochloride TS—Dissolve 10 g of potassium iodide ...d 6.44 g of potassium iodate in 75 mL of water in a glass-...ppered container. Add 75 mL of hydrochloric acid and 5 mL ...chloroform, and adjust to a faint iodine color (in the chloro...m) by adding dilute potassium iodide or potassium iodate so...ion. If much iodine is liberated, use a stronger solution of ...assium iodate than 0.01 *M* at first, making the final adjust...nt with the 0.01 *M* potassium iodate. Store in a dark place, ...d readjust to a faint iodine color as necessary.

...Iodine and Potassium Iodide TS—Dissolve 500 mg of iodine ...d 1.5 g of potassium iodide in 25 mL of water.

...Iodobromide TS—Dissolve 13.615 g of iodine, with the aid of ...at, in 825 mL of glacial acetic acid that shows no reduction ...th dichromate and sulfuric acid. Cool, and titrate 25.0 mL of ...e solution with 0.1 *N* sodium thiosulfate VS, recording the ...lume consumed as *B*. Prepare another solution containing 3 ...L of bromine in 200 mL of glacial acetic acid. To 5.0 mL of ...is solution add 10 mL of potassium iodide TS, and titrate with ...e 0.1 *N* sodium thiosulfate VS, recording the volume consumed *C*. Calculate the quantity, *A*, of the bromine solution needed ...ble the halogen content of the remaining 800 mL of iodine ...n by the formula:

$$800B/5C$$

...dd the calculated volume of bromine solution to the iodine ...lution, mix, and store in glass containers, protected from light.

...Iodochloride TS—Dissolve 16.5 g of iodine monochloride in ...00 mL of glacial acetic acid.

...Iodoplatinate TS—Dissolve 300 mg of platinic chloride in 97 ...L of water. Immediately prior to use, add 3.5 mL of potassium ...dide TS, and mix.

Iron-Phenol TS (*Iron-Kober Reagent*)—Dissolve 1.054 g of ...errous ammonium sulfate in 20 mL of water, and add 1 mL of ...ulfuric acid and 1 mL of 30 percent hydrogen peroxide. Mix, ...eat until effervescence ceases, and dilute with water to 50 mL. ...o 3 volumes of this solution contained in a volumetric flask add ...ulfuric acid, with cooling, to make 100 volumes. Purify phenol ...y distillation, discarding the first 10% and the last 5%, collecting ...he distillate, with exclusion of moisture, in a dry, tared glass-...oppered flask of about twice the volume of the phenol. Solidify ...he phenol in an ice bath, breaking the top crust with a glass rod ...o ensure complete crystallization. Weigh the flask and its con...ents, add to the phenol 1.13 times its weight of the iron–sulfuric ...cid solution prepared as directed, insert the stopper in the flask, ...nd allow to stand, without cooling but with occasional mixing, ...ntil the phenol is liquefied. Shake the mixture vigorously until ...mixed, allow to stand in the dark for 16 to 24 hours, and again ...eigh the flask and its contents. To the mixture add 23.5% of ...s weight of a solution of 100 volumes of sulfuric acid in 110 ...olumes of water, mix, transfer to dry glass-stoppered bottles, ...nd store in the dark, protected from atmospheric moisture. Use ...within 6 months. Dispense the reagent from a small-bore buret, ...nged to exclude moisture, capable of delivering 1 mL in 30 ...nds or less, and having no lubricant, other than reagent, on ...s stopcock. Wipe the buret tip with tissue before each addition.

Iron Salicylate TS—Dissolve 500 mg of ferric ammonium sul...ate in 250 mL of water containing 10 mL of diluted sulfuric ...cid, and add water to make 500 mL. To 100 mL of the resulting ...olution add 50 mL of a 1.15% solution of sodium salicylate, 20 ...mL of diluted acetic acid, and 80 mL of a 13.6% solution of sodium acetate, then add water to make 500 mL. Store in a well-closed container. Protect from light. Use within two weeks.

Lead Acetate TS—Dissolve 9.5 g of clear, transparent crystals of lead acetate in recently boiled water to make 100 mL. Store in well-stoppered bottles.

Lead Acetate TS, Alcoholic—Dissolve 2 g of clear, transparent crystals of lead acetate in alcohol to make 100 mL. Store in tight containers.

Lead Subacetate TS—Triturate 14 g of lead monoxide to a smooth paste with 10 mL of water, and transfer the mixture to a bottle, using an additional 10 mL of water for rinsing. Dissolve 22 g of lead acetate in 70 mL of water, and add the solution to the lead oxide mixture. Shake it vigorously for 5 minutes, then set it aside, shaking it frequently, during 7 days. Finally filter, and add enough recently boiled water through the filter to make 100 mL.

Lead Subacetate TS, Diluted—Dilute 3.25 mL of lead subacetate TS with water, recently boiled and cooled, to make 100 mL. Store in small, well-filled, tight containers.

Litmus TS—Digest 25 g of powdered litmus with three successive, 100-mL portions of boiling alcohol, continuing each extraction for about 1 hour. Filter, wash with alcohol, and discard the alcohol filtrate. Macerate the residue with about 25 mL of cold water for 4 hours, filter, and discard the filtrate. Finally digest the residue with 125 mL of boiling water for 1 hour, cool, and filter.

Locke-Ringer's Solution—See *Locke-Ringer's TS*.

Locke-Ringer's TS (*Locke-Ringer's Solution*)—

Sodium Chloride	9.0 g
Potassium Chloride	0.42 g
Calcium Chloride	0.24 g
Magnesium Chloride	0.2 g
Sodium Bicarbonate	0.5 g
Dextrose	0.5 g
Water, recently distilled from a hard-glass flask, a sufficient quantity, to make	1000 mL

Prepare fresh each day. The constituents (except the dextrose and the sodium bicarbonate) may be made up in stock solutions and diluted as needed.

Magnesia Mixture TS—Dissolve 5.5 g of magnesium chloride and 7 g of ammonium chloride in 65 mL of water, add 35 mL of ammonia TS, set the mixture aside for a few days in a well-stoppered bottle, and filter. If the solution is not perfectly clear, filter it before using.

Magnesium Sulfate TS—Dissolve 12 g of crystals of magnesium sulfate, selected for freedom from efflorescence, in water to make 100 mL.

Malachite Green TS—Dissolve 1 g of malachite green oxalate in 100 mL of glacial acetic acid.

Mallory's Stain—Dissolve 500 mg of water-soluble aniline blue, 2 g of orange G, and 2 g of oxalic acid in 100 mL of water.

Mayer's Reagent—See *Mercuric-Potassium Iodide TS*.

Mercuric Acetate TS—Dissolve 6.0 g of mercuric acetate in glacial acetic acid to make 100 mL. Store in tight containers, protected from direct sunlight.

Mercuric-Ammonium Thiocyanate TS—Dissolve 30 g of ammonium thiocyanate and 27 g of mercuric chloride in water to make 1000 mL.

Mercuric Bromide TS, Alcoholic—Dissolve 5 g of mercuric bromide in 100 mL of alcohol, employing gentle heat to facilitate solution. Store in glass containers, protected from light.

Mercuric Chloride TS—Dissolve 6.5 g of mercuric chloride in water to make 100 mL.

Mercuric Iodide TS (*Valser's Reagent*)—Slowly add potassium iodide solution (1 in 10) to red mercuric iodide until almost all of the latter is dissolved, and filter off the excess. A solution containing 10 g of potassium iodide in 100 mL dissolves approximately 14 g of HgI_2 at 20°.

Attachment 2. Protocol amendments for Study 92-01-30-15, Experiment 92-419-721

Study #: 92-01-30-15 **Amendment #:** 1 **Date change implemented:** 03/15/93

Experiment's affected by this amendment:

92-419-721

Section 7. Conduct of the Study originally stated:

General procedure.
line 5: "...approximately 2 ug/ml. Incubation solutions will be agitated briefly at each sampling time instead of agitated continuously due to the very short total incubation time."

line 13: "...estimated by western blot analysis."

Intestinal fluid assays.

all of section: "CP4 will be incubated in SGF for a total time period of approximately two minutes. At least five samples will be collected between zero and approximately two minutes for each treatment tube. To terminate the reaction, 50-ul aliquots of incubation samples will be added to individually--labeled 1.5 ml tubes containing 50 ul 2X SDS-PAGE sample buffer and immediately heated to approximately 100 C for approximately five minutes, as described in the "General procedure" section. Time-course assays will be carried out sequentially due to the short incubation intervals."

This section is amended as follows:

General procedure.
line 5: "...approximately 2 ug/ml for SGF and approximately 50 ug/ml for SIF. For SGF, incubation solutions will be agitated briefly at each sampling time instead of agitated continuously due to the very short total incubation time. For SIF, incubation solutions will be agitated continuously except for brief intervals during sampling."

line 13: "...estimated by western blot analysis after dilution with 1X SDS-PAGE sample buffer, if dilution is appropriate."

Intestinal fluid assays.

all of section: "CP4 will be incubated in SIF for a total time period not exceeding 24 hours. Samples will be collected at six time intervals (including t = 0) for each treatment tube. To terminate the reaction, 50-ul incubation solution samples will be added to individually-labeled 1.5 ml tubes and immediately heated to approximately 100 C for five minutes. SDS-PAGE sample buffer addition, heating and storage will be done as described in the "General procedure" section.

Reason for amendment:

CP4 protein concentration for SIF assays was increased from 2ug/ml to 50 ug/ml to correspond to the level that was needed to measure CP4 enzymatic activity in SIF incubation treatments.

The procedure used to terminate CP4 incubation in SIF was changed because the original conditions described did not effectively terminate the reaction. A new procedure was developed, checked and is reflected in this amendment.

This change will impact the Study in the following ways:

The increase in CP4 protein concentration in the SIF incubations for analysis by western blot will allow a direct comparison between degradation of CP4 observed by western blot and enzymatic activity. The modification in the assay termination procedure will allow an assessment of the stability of CP4 in SIF.

Signature of Approval:

Study Director: _____ Date: 3/12/93

Signature's of Acknowledgement:

Principal Investigator: _____ Date: 3/12/93

Sponsor: _____ Date: 3/18/93

Quality Assurance: _____ Date: 4/6/93

Quality Control: _____ Date: 3/18/93

Statistical Support: _____ Date: 3/24/93

Monsanto
The Agricultural Group

Protocol Amendment
SOP Reference: GEN-POL-002

New Products Division - Regulatory Sciences

Study Number: 92-01-30-15 **Amendment #:** 2

Date change implemented: 05/05/93

Experiment's affected by this amendment:

92-419-721

Page No/s. &/or Section/s: **p.5, sect "Part II....."** **originally stated:**

The degree of of degradation of the CP4 protein will be calculated as a percentage of activity remaining after approximately two minutes incubation compared to the activity measured in the t = 0 incubation samples.

This section is amended as follows:

The degree of degradation of the CP4 protein activity will be calculated as a percentage of activity remaining after specified incubation period(s) relative to the activity measured in the t = 0 incubation samples. At least one incubation period will be selected to approximately correspond to an incubation period used in Part I.

Reason for amendment:

Protocol amendment #1 provided for increased incubation times beyond two minutes for assessment of CP4 protein stability in SIF. It did not provide for correspondingly increased incubation times for Part II. This amendment reflects the increased incubation times for Part II. to allow a comparison to results from Part I.

This change will impact the Study in the following ways:

This change will allow a comparison of CP4 protein stability in intestinal fluid assessed by western blot and enzymatic activity.

Signature of Approval:

Study Director: _____ Date: 5/10/93

Signatures of Acknowledgement:

Sponsor: _____ Date: 5/6/93

Principal Investigator: _____ Date: 5/11/93

Quality Assurance: _____ Date: 5/11/93

Not Applicable for this Protocol N/A Date: N/A

Not Applicable for this Protocol N/A Date: N/A

CC: