Study Title

Assessment of Degradation of Neomycin Phosphotransferase II in *in vitro* Mammalian Digestion Models

Author

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Performing Laboratory

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Laboratory Project ID

Study Number IRC-91-ANA-06

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Volume 4 of 5

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1) (A), (B), or (C).

Company Monsanto Company

Company Agent TERRY STONE Date 11/23/93

Title SR. REGULATORY SPEC. Signature

STATEMENT OF COMPLIANCE

This study meets the requirements for 40 CFR Part 160.

Appendix 2 is not considered part of the study and is not included in the Submitter's, Sponsor's, or Study Director's GLP compliance statement. The information included in this appendix is solely to support and facilitate the review process.

Explanatory Notes:

This study was originally conducted using two proteins: Neomycin phosphotransferase II (NPTII) and truncated *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) HD-1. The product for which that study was originally conducted was dropped from commercialization plans. Consequently, this study has been separated into two separate studies, each specific for one protein (Amendment #1 to the Study Protocol). Only the NPTII protein is addressed in this report. The truncated *B.t.k.* HD-1 protein study data will be reported under another study number. The separation of the two phases of the original study is intended to enhance the relevance of each study report to the regulatory data package in which it is included.

Submitter: _____ Date: 11/29/93

Sponsor: _____ Date: 11/29/93

Study Director: _____ Date: 11-29-93

QUALITY ASSURANCE STATEMENT

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:

August 21, 1991
September 4, 11, 1991
February 25, 1992
April 30, 1992
May 1, 8, 1992
July 8, 1992
August 8, 1992
October 15, 1993
November 1, 18, 19, 1993

Date findings were reported to management and/or study director:

August 22, 1991
September 4, 1991
February 25, 1992
April 30, 1992
May 1, 8, 14, 1992
July 8, 1992
April 14, 1993
October 15, 1993
November 1, 18, 19, 1993

Quality Assurance Review Conducted by:

D.E. McKinney
K.F. Yount

November 23, 1993

Quality Assurance Representative
Monsanto Company

Date

Study Number: IRC-91-ANA-6

Title: Assessment of Degradation of Neomycin Phosphotransferase II in *in vitro* Mammalian Digestion Models

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Study Initiation: May 1, 1992

Experimental Completion Date: July, 1992

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

Signatures of Approval:

Joel E. Ream — 11/23/93
Joel Ream, Study Director — Date

Roy Fuchs — 11/23/93
Roy Fuchs, Sponsor — Date

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SUMMARY

The gene encoding neomycin phosphotransferase II (NPTII) has been used routinely as a selectable marker in the production of new plant varieties. Prior to commercialization, studies assessing the safety and expression levels of the NPTII protein in plants have been conducted. The purpose of this study was to assess the degradation of NPTII using *in vitro* mammalian digestion models. Simulated digestion fluids 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 the biodegradation of pharmaceuticals.

Purified NPTII protein was added to simulated gastric and intestinal fluids and incubated at approximately 37°C. The degradation of this protein in digestion fluids was assessed over time by western blot analysis. NPTII enzymatic activity dissipation in digestion fluids was also measured after a single incubation period.

NPTII was found to readily degrade in simulated gastric (half-life < 10 seconds) and simulated intestinal fluid (half-life between 2 and 5 minutes) by western blot analysis. Greater than 99% of the NPTII enzymatic activity dissipated after 2 minutes incubation in gastric fluid. Greater than 99% of the NPTII enzymatic activity dissipated after 15 minutes incubation in intestinal fluid. NPTII recovery from the digestion fluids was at least 50% as measured using western blot analysis.

The results of this study suggest that the NPTII protein and its associated enzymatic activity will readily degrade in the mammalian digestive tract.

INTRODUCTION

The gene encoding neomycin phosphotransferase II (NPTII) has been used routinely as a selectable marker in the production of new plant varieties. NPTII is an antibiotic resistance protein necessary to allow selection of transformants from tissue culture. NPTII is present in the bacteria in the human gut (1). It is widely recognized that most proteins are readily degraded upon exposure to the mammalian digestive tract (2). A demonstration that the NPTII protein also degrades readily would further support the safety of this protein for human and animal consumption.

In vitro digestion solutions have been widely used as models of animal digestion. They have been used to investigate the digestibility of plant proteins (3,4), animal proteins (5) and food additives (6); to assess the protein quality (7); to study digestion in pigs and poultry (8); to measure tablet dissolution rates to assess pharmaceutical biodegradation (9); and to investigate the controlled-release properties of experimental pharmaceuticals (10).

The method of preparation of the simulated digestion solutions -- simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) -- used in this study is described in the United States Pharmacopiea (11), a frequently cited reference for *in vitro* digestion studies.

The purpose of this study was to assess the rate of degradation of NPTII using *in vitro* mammalian digestion models.

MATERIALS AND METHODS

Reagents. Pepsin (porcine, Product Number P-7000), pancreatin (porcine, Product Number P-1500), hemoglobin (bovine, Product Number H-2625), lactate dehydrogenase (rabbit muscle, Product Number L-2518), pyruvate kinase (rabbit muscle, Product Number P-1506) and neomycin sulfate (Product Number N-1876) were obtained from Sigma Chemical Company (St. Louis, MO). Resorufin-labeled casein (Product Number 1080733) was obtained from Boehringer Mannheim Corporation (Indianapolis, IN). All other reagents were reagent grade obtained from commercial sources.

Protein. Neomycin phosphotransferase II (NPTII) -- purified from *E. coli* (MSL 11560 included in Attachment 3, Volume 3 of this application), characterized (MSL 12692 included in Attachment 2, Volume 3 of this application), and demonstrated to be equivalent to NPTII protein expressed in plants (Study Number 92-01-37-08 included in Volume 2 of this application) -- was provided as a lyophilized powder (Lot Number NBP4821020). Protein solutions with appropriate final concentrations of NPTII were prepared using a buffer in

which NPTII is stable [50 mM Tris/HCl (pH 7.8), 20% (w/v) glycerol and 1 mM ß-mercaptoethanol] and used on the same day as preparation.

Digestion Fluids. Simulated gastric and intestinal fluids were prepared as described in the United States Pharmacopeia (11). Solutions were used the day they were prepared and stored at approximately 4°C. SIF was mixed before use to resuspend any insoluble material that may have settled after preparation. Digestive fluids were assayed for activity prior to use in digestion assays. Pepsin activity was measured by monitoring the increase in absorbance at 280 nanometers (nm) following trichloroacetic acid (TCA) precipitation of SGF incubations with hemoglobin (12). Protease activity of SIF was assayed by measuring spectrophotometrically the increase in supernatant absorbance at 574 nm following TCA precipitation of SIF incubations with resorufin-labelled casein(13).

Digestion Assay. NPTII was typically added to 1-ml solutions of temperature-equilibrated SGF and SIF to final concentration of 2 µg/ml and incubated in 15-ml test tubes at approximately 37°C with agitation when appropriate. The volume of the incubation solution was reduced to 0.1 ml for the t = 0 samples and for the whole incubation sample control to keep final quenched sample volumes compatible with equipment used for aliquots from 1-ml incubations. Very short incubation experiments (≤ 2 minutes) were not agitated. Agitation was stopped periodically to remove aliquots. Fifty microliter aliquots were removed from the digestion solutions at specific time intervals and the reaction was immediately quenched. For incubations in SGF the reaction was quenched by neutralization with 15 µl of 0.2 M sodium carbonate per 50 µl of SGF. For samples to be analyzed by western blot, quenched aliquots were kept on an ice bath until dilution (1:1, v/v) with 2X SDS-PAGE sample buffer [100 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) B-ME, and 0.2% (w/v) bromophenol blue]. Samples were heated for five minutes at approximately 100°C and stored at approximately -20°C until analysis. For incubations in SIF in which the analysis was going to be by western blot, the reaction was terminated by immediate 1:1 (v/v) dilution with 2X SDS-PAGE sample buffer, followed by immediate incubation at approximately 100°C for approximately five minutes. These samples were stored on an ice-bath until all incubations were complete and then transferred to approximately -20°C until analysis. For samples measuring protein levels with no incubation in digestion fluids (*i.e.* t=0), protein was added to SGF and SIF after addition of quenching reagent. To allow a calculation of the recovery of NPTII protein from digestion fluid, this protein was added to 1-ml solutions of buffer and then sampled and quenched as described above. An additional control was prepared to allow the determination if the 50-µl aliquot was representative of the entire digestion incubation solution. For this sample, the digestion incubation volume was reduced to approximately 0.1 ml and the entire sample was quenched.

Western Blot Analysis. Samples in 1X SDS-PAGE sample buffer were thawed and reheated at approximately 100°C, allowed to cool, and evaluated by SDS-PAGE and western blotting as described in SOP Numbers PST-91-EQP-005-00 and PST-91-PRO-002-01. Rabit antibodies raised against native NPTII (R373) were used for western blot analyses. Protein recoveries and half-lives were estimated based on visual comparison of relative intensities of bands corresponding to standards for three replicates for each treatment. When intermediate levels of protein were detected with increasing incubation time, the estimated level relative to initial levels were averaged and the half-life estimated. For some treatments, protein degradation was too fast or too slow to observe intermediate levels; for these, limits on half-life were estimated. A visual assessment of western blots was used because the results were sufficiently clear to be interpreted in this manner.

NPTII Enzymatic Activity. NPTII activity was measured before and after incubation in digestive fluids. NPTII was added to SGF and SIF to a final concentration of approximately 10 µg/ml and incubated as described previously. SGF incubations were quenched as described for the western blot analysis. SIF incubations were quenched by immediate placement of the incubation sample on an ice bath. Incubation solutions were assayed for NPTII enzymatic activity immediately after incubation by monitoring the neomycin-dependent change in absorbance at 340 nm using the continuous coupled spectrophotometric assay described by Goldman and Northrup (14).

RESULTS AND DISCUSSION

NPTII degrades extremely rapidly in SGF (Figure 1); no protein was detected after only ten seconds of incubation. In SIF, NPTII degrades readily with a half-life of between 2 to 5 minutes (Figure 2).

SGF and SIF themselves did not produce bands by western blot analysis that might interfere with the assessment of NPTII levels (Figures 1 and 2). The recovery of added protein from the digestive fluids could be estimated by comparison of protein added to digestive fluid with no incubation to protein added to buffer instead of digestive fluid (*e.g.* Figure 2, lane 4 versus lane 5). NPTII recovery from SGF was approximately 100% and approximately 50% from intestinal fluid. The decrease in recovery of NPTII from intestinal fluid is likely due to some degradation of NPTII occurring in the 1X SDS-PAGE sample buffer during heating to approximately 100°C used to terminate the SIF incubations.

A further control added to this study to assess whether the fifty microliter aliquot removed from the 1-ml incubation solution was representative of the

entire incubation solution. It is conceivable that rapid precipitation of an added protein in the digestive fluid could account for lack of protein in fifty microliter aliquots analyzed by western blot. This was addressed by adding sample buffer to the entire incubation solution and comparing the results from an aliquot of the incubation solution. For NPTII in both SGF and SIF, the western blot results were similar for aliquot and whole-sample treatments (data not shown). These results suggest that, for all incubations, the results for aliquot samples are representative of the entire incubated digestive fluids.

A good correlation was established between the disappearance of NPTII bands as determined by western blot analysis upon incubation in digestive fluids and the loss of the enzymatic activity of NPTII in digestion fluids. The enzymatic activity of NPTII was completely destroyed by a 2-minute incubation in SGF and 15-minute incubation in SIF (Table I), the only incubation time points measured. This correlates well with the loss of western blot band intensity under similar conditions (Figures 1 and 2).

CONCLUSION

The results of this study established that the NPTII protein and its associated activity is rapidly degraded in both gastric and intestinal models and suggests that the NPTII protein and its associated enzymatic activity will degrade readily in the mammalian digestive tract after ingested as a component of food or feed. The extremely rapid degradation of NPTII protein was expected and supports the safety of the NPTII protein for human and animal consumption.

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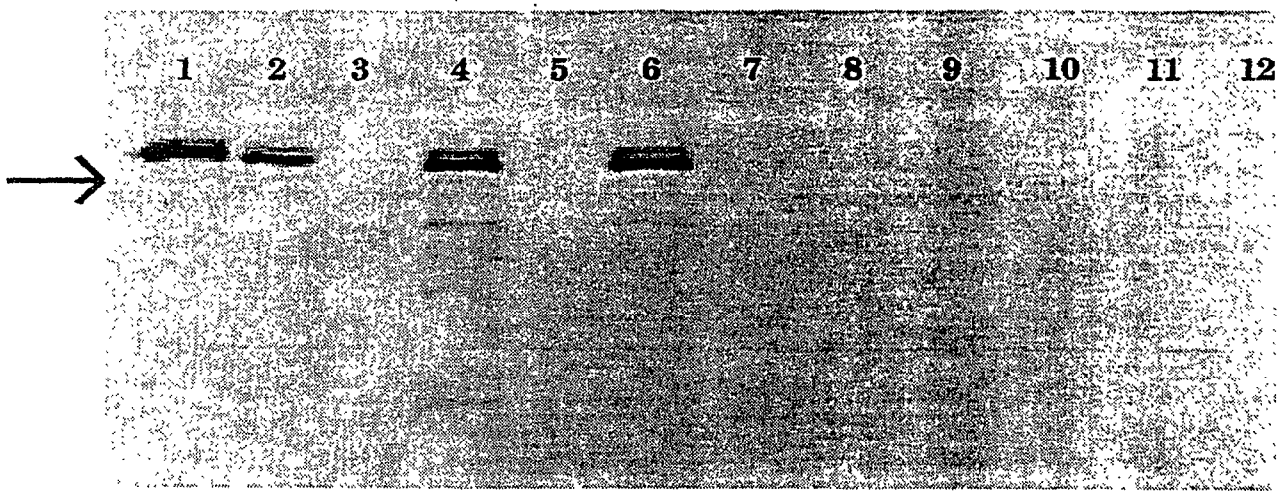

Figure 1. Degradation of NPTII in simulated gastric fluid as analyzed by western blot.

Legend:

Western blot shown is representative of results from three replicates per treatment. Lanes were loaded with 10 µl sample in 1X sample buffer. Lanes 1 and 2 are NPTII standards at 10 (lane 1) and 5 (lane 2) ng/lane. Lanes 3 and 4 are unincubated buffer with (lane 4) and without (lane 3) addition of NPTII. Lane 5 and 12 are SGF incubated for 0 and 120 seconds, respectively, at approximately 37°C. Lanes 6 through 11 are samples from NPTII incubation with SGF for 0 (lane 6), 10 (lane 7), 20 (lane 8), 30 (lane 9), 60 (lane 10) and 120 (lane 11) seconds at approximately 37°C.

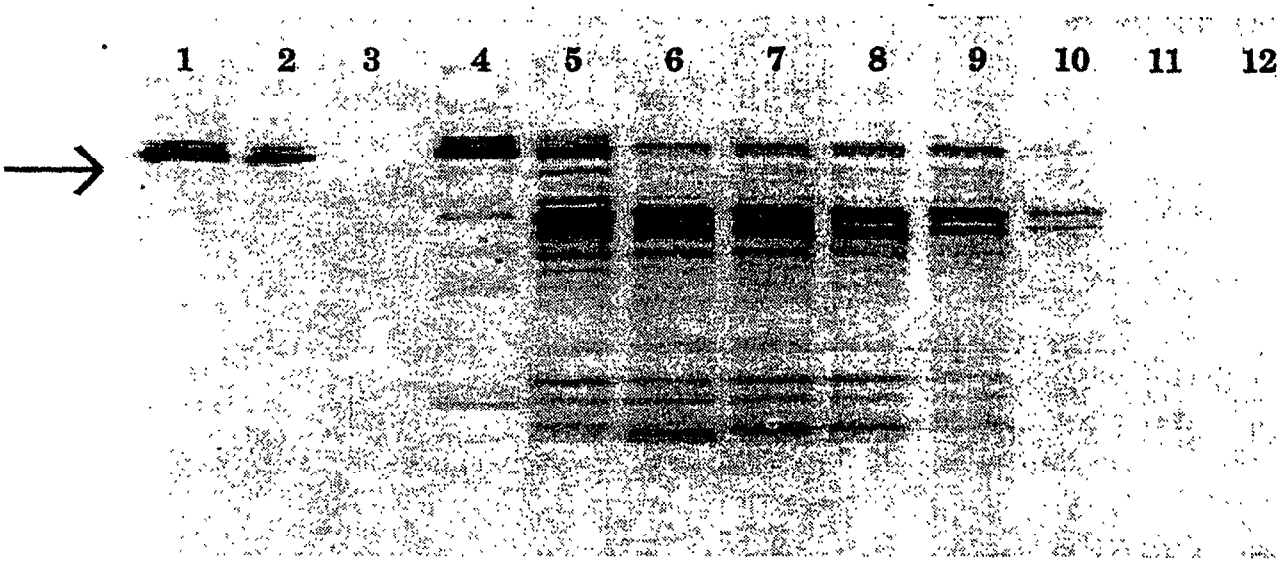

Figure 2. Degradation of NPTII in simulated intestinal fluid as analyzed by western blot.

Legend:

Western blot shown is representative of results from three replicates per treatment. Lanes were loaded with 10 µl sample in 1X sample buffer. Lanes 1 and 2 are NPTII standards at 10 (lane 1) and 5 (lane 2) ng/lane. Lanes 3 and 12 are SIF after 0 (lane 3) and 15 (lane 12) minutes incubation at approximately 37°C. Lane 4 is NPTII added to unincubated buffer. Lanes 5 through 11 are samples from NPTII incubation with SIF for 0 (lane 5), 10 seconds (lane 6), 30 seconds (lane 7), 1 min (lane 8), 2 min (lane 9), 5 min (lane 10) and 15 min (lane 11) at approximately 37°C.

Table 1. Degradation of NPTII enzymatic activity in simulated digestive fluids. NPTII activity values reported are the mean value from three replicate treatments. The standard deviations are in parentheses.

	Incubation time (minutes)	NPTII Activity (U[1]/mg)
Gastric fluid	0	6.55 (0.11)
	2	0.02 (0.03)
Intestinal fluid	0	7.67 (0.18)
	15	0.01 (0.05)

[1] One Unit (U) of activity is that amount providing one μmole of NPTII-dependent NADH consumption per minute at 37 °C.

APPENDIX 1

Protocol and Amendments - Study No. IRC-91-ANA-06

Protocol Number: IRC-91-ANA-6

Study Title: Assessment of Degradation of Truncated *B.t.k.* Delta Endotoxin HD-1 and Neomycin Phosphotransferase II in *In Vitro* Mammalian Digestion Models

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Study Director: Joel E. Ream Date: 5/1/92

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Purpose:

The purpose of this study is to measure the rate of degradation of truncated *B.t.k.* HD-1 delta endotoxin (hereafter called "HD-1") and neomycin phosphotransferase II (NPTII) using *in vitro* mammalian digestion models. HD-1 is the activated (tryptic) fragment of the *B.t.k.* HD-1 protein. This is equivalent to the activated fragment of the protein encoded by the *B.t.k.* gene introduced into cotton by genetic engineering to confer insect resistance. NPTII is the selection marker protein introduced into cotton to allow tissue culture selection of transformed cells.

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.

Proposed study start date: May, 1992

Proposed study termination date: June, 1992

Test materials:

Purified truncated *B.t.k.* HD-1 delta endotoxin from *E. coli* (pMAP40). The *B.t.k.* protein is expressed in *E. coli* as the full-length protein and then trypsinized to the activated fragment. This activated fragment is similar to the protein expressed in

insect resistant cotton plants.

Purified neomycin phosphotransferase II (NPTII) from *E. coli* (pMON1636). This protein is equivalent to NPTII expressed in insect resistant cotton plants.

Test system:

HD-1 and NPTII 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 relative to *in vivo* systems. Simulated gastric fluid and simulated intestinal fluid will be prepared at the testing facility as described in The United States Pharmacopeia, 1990, pp 1788-1789 (attached). These fluids will be evaluated for protease activity, used within 24 hours of preparation and stored at approximately 4 °C until used.

Conduct of the Study:

Part 1. Measurement of protein degradation by western blot analysis

Treatments:

A. Gastric fluid
B. Gastric fluid, plus HD-1
C. Gastric fluid, plus NPTII
D. Intestinal fluid
E. Intestinal fluid, plus HD-1
F. Intestinal fluid, plus NPTII

General procedure. Three replicate 1-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 HD-1 and NPTII will be added to temperature-equilibrated (approximately 37 °C) digestion fluids to a final concentration of approximately 2 μg ml^{-1} for each protein. Incubation solutions will agitated continuously throughout the incubation period with the following exceptions: Agitation may be interrupted briefly to facilitate sampling; and, for treatments where the total incubation time is two minutes or less, samples will be agitated briefly at each sampling time instead of agitated continuously. At specified time points during the incubation, aliquots (50 μl/aliquot) of treatment solutions will be removed, added to individually labeled tubes and the reaction

process immediately terminated. 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. HD-1 and NPTII protein levels in samples will be estimated by western blot analysis.

Gastric fluid assays. HD-1 and NPTII will be incubated in gastric fluid for a total time period of approximately two minutes. Six samples will collected between zero and approximately two minutes incubation for each treatment tube. To terminate the reaction, 50-µl aliquots of incubation solutions will be added 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. HD-1 will be incubated in intestinal fluid for up to 24 hours. Four samples will be collected between zero and up to 24 hours incubation time for each treatment tube. NPTII will be incubated in intestinal fluid for a total time period of approximately 15 minutes. Seven samples will be collected from each treatment tube. Time-course assays with NTPII will be carried out sequentially due to the short incubation time intervals. 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 specified in the "General procedure" section.

Controls. Incubated gastric and intestinal fluids without HD-1 or NPTII added will be prepared for negative controls. HD-1 and NPTII recoveries from the digestive fluids will be determined by comparison of the specific proteins detected in the "t=0" digestive fluid samples to those detected in buffer. The "t=0" incubation sample will be generated by spiking the specific protein into already terminated assay samples.

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.

HD-1 and NPTII incubations will be carried generally as described for Part I, except no SDS-PAGE sample buffer will be added. HD-1 incubation solutions will be evaluated for bioactivity using the tobacco budworm (TBW) diet incorporation bioassay. Intestinal fluid assays will be terminated by immediately freezing incubation samples. Gastric fluid assays will be terminated generally as described for Part I. NPTII enzymatic activity will be measured using the coupled spectrophotometric assay (Goldman and Northrop (1975) Biochem Biophys Res Comm 69: 230 - 236). Gastric fluid assays will be terminated generally as described for Part I. NPTII activity of intestinal fluid incubation solutions will measured if the intestinal fluid does not significantly interfere with the assay. The mean values of three replicates will be calculated. Degree of activity degradation between two incubation time intervals will be reported.

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.

GLP Compliance:

This study will be conducted according to the applicable provisions of EPA FIFRA Good Laboratory Practice Standards (40 CFR 160.).

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

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

✱ **Intestinal Fluid, Simulated, TS**—Dissolve 6.8 g of monobasic potassium phosphate in 250 mL of water, mix, and add 190 mL of 0.2 *N* sodium hydroxide and 400 mL of water. Add 10.0 g of pancreatin, mix, and adjust the resulting solution with 0.2 *N* sodium hydroxide to a pH of 7.5 ± 0.1. Dilute with water to 1000 mL.

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

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

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

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

$$800B/5C.$$

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

Iodochloride TS—Dissolve 16.5 g of iodine monochloride in 1000 mL of glacial acetic acid.

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

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

Iron Salicylate TS—Dissolve 500 mg of ferric ammonium sulfate in 250 mL of water containing 10 mL of diluted sulfuric acid, and add water to make 500 mL. To 100 mL of the resulting solution 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°.

Monsanto
The Agricultural Group

Protocol Amendment
SOP Reference: GEN-POL-005

New Products Division - Regulatory Sciences

Study Number: IRC-91-ANA-6 **Amendment #: 1**

Date change implemented: 10/06/93

Experiment's affected by this amendment:

Page No/s. &/or Section/s: entire protocol originally stated:

Original protocol addressed assessing degradation of two proteins, truncated *B.t.k.* HD-1 delta endotoxin (HD-1) and neomycin phosphotransferase II (NPTII).

This section is amended as follows:

The protocol is amended to separate the two test materials, HD-1 and NPTII, into separate studies. NPTII degradation will retain the originally assigned study number; HD-1 will be assigned a separate study number.

Reason for amendment:

These two proteins, HD-1and NPTII, were originally placed in the same study because they were components of a single product that was dropped from commercialization by Monsanto. They are being separated to allow the results for each protein to be reported separately. This separation will provide for reporting only the protein of relevance for separate data packages.

This change will impact the Study in the following ways:

No change to data or conclusions from data will occur because of this amendment. It will allow separate study reports for the two different proteins which will increase the relevance of each study report to the regulatory data package in which it is included.

Signature of Approval:

Study Director: _____ Date: 10-8-93

Signatures of Acknowledgement:

Sponsor: _____ Date: 10/11/93

Quality Assurance: _____ Date: 10/15/93

Not Applicable _____ Date: _____

Not Applicable _____ Date: _____

Not Applicable _____ Date: _____

CC:

APPENDIX 2

Copies of References Cited

SELECTABLE MARKER GENES: SAFE FOR PLANTS?

by Richard B. Flavell, Ed Dart, Roy L. Fuchs, & Robert T. Fraley

The introduction of new genes into crop plants promises to overcome some of the substantial agronomic and environmental problems that have not been solved using genes currently in plant breeders' germplasms. For example, genetically modified cotton and potato cultivars resistant to insects, and tomato and potato cultivars resistant to viruses under field conditions have already been created. A class of molecular aids essential in enabling the genetic construction of these plants—selectable and scoreable marker genes and their encoded proteins—stays with the crop and with the foods made from them. Selectable marker genes have a clear and vital function in the laboratory.

Regulatory committees and individuals worldwide are currently debating the safety of these markers. For instance, the Food and Drug Administration (FDA, Washington, DC) is looking at a document[1] submitted by Calgene Inc. (Davis, CA) entitled "Request for Advisory Opinion *kan*r gene: safety and use in the production of genetically engineered plants." This comprehensive document provides extensive background data intended to establish the food and environmental safety of one of the markers, the NPTII gene, and its gene product. NPTII (neomycin phosphotransferase II) is an enzyme which inactivates, and provides resistance to, the antibiotics neomycin and kanamycin. We want to communicate the major issues and arguments on the safety of NPTII to a wider scientific and regulatory audience. We will provide neither all the data nor all the answers; the Calgene document does a much fuller job. We will, however, outline the arguments which lead to our position—that there are no reasons to deny or restrict the use of the NPTII gene in genetically modified plants on safety grounds.

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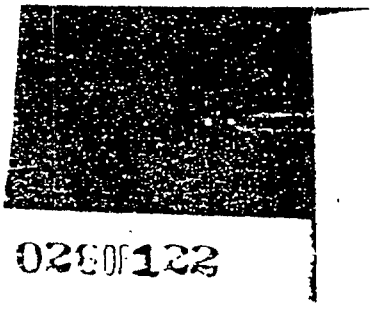
Safety of the NPTII gene and protein

There are four major questions that should be addressed in the safety appraisal: is the NPTII gene product toxic; does eating the NPTII protein compromise oral kanamycin and neomycin therapy; does the transfer of the NPTII gene from plant to pathogenic bacteria compromise kanamycin and neomycin therapy, and will the spread of the NPTII gene from genetically modified plants into the environment cause unacceptable damage?

All the human health analyses need to be viewed against the knowledge that humans continually ingest kanamycin-resistant microorganisms. The diet, especially raw salad, is the major source. A conservative estimate, each human ingests 1.2×10^8 kanamycin-resistant microorganisms daily.

Is NPTII toxic to humans or other organisms?

None of the plant, bacterial, and other species into which the active NPTII gene has been inserted has shown any deleterious effects that could be attributed to the NPTII protein. This conclusion is supported, for plants, *inter alia* by data from



yield trials in glasshouse and field for many of the crops in Table 1. In mammalian cells, NPTII is not toxic *in vitro*: the identical NPTII gene and gene product used in plants is used routinely in the introduction of genes into mammalian cell lines with no effects on viability or growth. The protein also seem innocuous *in vivo* in animals and humans. Calgene[11] established that NPTII is rapidly inactivated and degraded upon ingestion based on *in vitro* digestion analysis. Calgene[11] fed rodents with tomatoes that produced NPTII and observed no deleterious effects. Furthermore, in preliminary gene therapy experiments, cells containing the NPTII gene have been infused into human cancer patients: NPTII was produced intracellularly *in vivo*—the most extreme test of its safety in humans. No adverse effects attributable to NPTII were observed.

There have also been what might be regarded as long-term, continuous safety trials of NPTII in humans. At least since the introduction of the antibiotics into medicine, humans have been constantly exposed to both the NPTII gene and gene product. Natural populations of microorganisms highly resistant to kanamycin/neomycin occur ubiquitously on food (especially on raw vegetables) and in our digestive systems. Again, no adverse effects have been noted.

Does consumption of NPTII compromise the efficacy of oral kanamycin and neomycin in humans?

The oral use of the antibiotics would not be compromised by NPTII in the diet. In the first place, NPTII as a protein which does not contain any unusual amino acids, is rapidly inactivated and degraded during the digestive process[11]. Furthermore, proteins are rarely absorbed by the digestive system. These are precisely the reasons clinicians cannot administer therapeutic proteins like insulin and growth hormones orally. Secondly, NPTII requires ATP in order to catalyze the inactivation of kanamycin or neomycin. ATP is present in the digestive system at extremely low concentrations because it is unstable at low pH. Thirdly, only 0.36 percent of the kanamycin or neomycin administered was for oral or gastrointestinal tract use (U.S. figures for 1989).

Does the transfer of the NPTII gene from plant to bacteria pathogenic to humans (or other species) compromise the efficacy of kanamycin and neomycin in human therapy?

By far the highest concentration of potentially pathogenic bacteria occurs in the gut. Most, if not all, of ingested NPTII DNA would, however, be degraded in the stomach and the small intestine before it reached the regions where transformation of either microorganisms or post-duodenal intestinal cells could potentially occur. In any case, it is far more likely that any NPTII DNA taken up by and surviving in either microorganisms or gut epithelial cells would come from bacteria in the gut and not from plant material. Even if plant-derived NPTII DNA survived intact and reached those regions, the probability of transformation occurring would be remote. With 10^{12} kanamycin- or neomycin-resistant bacteria already in the gut of each person, any new transformation event would be of no significance. Calgene[11] calculated that, even with the most liberal assumptions, eating genetically modified tomatoes containing the NPTII gene, would increase the number of kanamycin-resistant microbes in the gut less than 0.000001 percent. Furthermore, to be of medical significance, the NPTII gene would have to be transferred to and expressed in a pathogenic microorganism being treated with kanamycin or neomycin. So the transfer of the NPTII gene to microorganisms from plant material in the gut is not of significant medical concern. The same arguments apply to animals that might consume plants containing the NPTII gene.

Will spread of the NPTII gene from genetically modified plants cause unacceptable environmental damage?

The selectable marker genes used in the genetic modification of plants are of bacterial origin, and kanamycin-resistant bacteria are ubiquitous in soils. Many of the genes conferring kanamycin-resistance are transferred between bacteria because they are on transposons and often on transmissible plasmids. The probability of a bacterium obtaining the NPTII gene from plant DNA compared to another bacterium is extremely low. Thus the effect of NPTII-containing plant material even when grown on large acreage, on bacterial NPTII gene frequencies is insignificant. Calgene[11] calculated that the increase in kanamycin-resistant bacteria in the soil caused by the extensive planting of genetically modified tomatoes, canola, and cotton (assuming that the gene could be transferred from plants to microbes) would be less than 0.0001 percent.

If the NPTII gene passed from crops to related plants by pollination, its effects on recipient plants would not be expected to be any different from those on the original host plant. Overall, the ubiquity of the gene in nature and its benign properties make it ideal as a selectable marker in plant transformation.

TABLE1. Plant species that have been genetically modified and express the NPTII gene

Tomato	Walnut
Potato	Arabidopsis
Lettuce	Muskmelon
Peas	Flax
Celery	Morning glory
Oil seed rape	Apple
Apple	Pear
Petunia	Poplar
Cauliflower	Cotton
Carnations	Carrot
Kiwi	Horse radish
Eggplant	Asparagus
Alfalfa	Tobacco
Soybean	Chrysanthemum
Sunflower	Strawberry
Cabbage	Papaya
Cucumber	Canola
Sugar beet	Corn

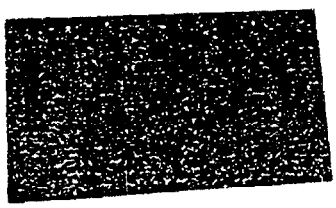
SELECTABLE MARKER GENES ARE USEFUL IN AGRICULTURE

These selectable marker genes not only are essential for those constructing the genetically modified plants but also are useful to plant breeders, legislative bodies, and monitoring agencies. Plant breeders can use selectable markers to identify progeny of crosses which contain the gene of agronomic (commercial) interest because the two are linked. This saves the breeder having to assay the gene of commercial interest by more complex and expensive methods. Very importantly, selectable markers can be used by breeders, and by regulatory and monitoring agencies to distinguish transgenic from non-transgenic plants by a simple test which does not involve advanced molecular biology.

CUTTING OUT MARKERS

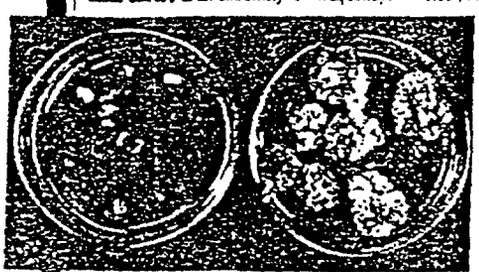
A recent article suggests that selectable markers could be readily eliminated by a *Cre/Lox* site-specific recombination[12]. This is a sophisticated, scientifically interesting and scientifically useful method of DNA manipulation. But to suggest that it should be used to remove marker genes is to fail to appreciate the implications of applying the method to agronomically important crops.

While the *Cre/Lox* system works in a model plant, tobacco, it has not been demonstrated in more agronomically important species. For vegetatively propagated crops, the system would be particu-



MARKER GENES

Selectable marker genes are essential for the introduction of agronomically important genes into important crop plants. The incorporation of genes into plant chromosomes during genetic modification processes occurs at an extremely low frequency: perhaps only one cell in thousands or millions stably carries the desired gene. Selectable marker genes are, therefore, joined to the agronomic gene(s) of interest. Only the plant cells that contain and express the selectable marker genes will survive the selective pressure imposed in the laboratory. Plants regenerated from the surviving cells will contain the selectable marker joined to the agronomic gene of interest.



The value of using NPTII as a selectable marker for cotton transformation. Cotton hypocotyl cells transformed with a vector lacking the gene encoding *NPTII* (left) cannot grow on media containing kanamycin whereas the rare hypocotyl cells that are transformed with a vector containing the gene encoding *NPTII* (right) can.

Two main classes of selectable traits have been used in genetic modification of plants. On the one hand, there are selectable markers that encode proteins that confer resistance to antibiotics—for instance, kanamycin[1], bygromycin B[2], methotrexate[3], gentamicin[4], and bleomycin[5]. On the other hand, there are genes whose products confer tolerance to herbicides such as phosphinothricin[6], the sulfonylureas[7], and 2,4 D[8]. By far the most commonly used selectable marker is the gene from Transposon 5 (Tn5) from *Escherichia coli* K12 encoding aminoglycoside 3-phosphotransferase II (APH (3') II, Chemical Abstracts Registry number 58943-39-8). This enzyme, also commonly known as neomycin phosphotransferase II ("NPTII"), inactivates kanamycin, G418, and neomycin by phosphorylation. Over 100 laboratories around the world have used NPTII in introducing genes into over 30 plant species (Table 1).

Other "scoreable" markers are also frequently introduced into genetically modified plants and provide valuable tools to identify and track genetic modifications in plants. Scoreable markers do not facilitate survival of transformed cells under particular laboratory conditions, rather they identify or tag transformed cells. They are particularly important where the genetically modified plants cannot be regenerated from single cells and direct selection is not feasible or effective. They can also be important in quantifying both transformation efficiency and gene expression in transformants. The two most frequently used scoreable markers are beta glucuronidase (GUS) and luciferase (LUC). Genetically modified plant tissue expressing the GUS gene turns blue when incubated with the substrate, 5-bromo-4-chloro-3-indoxyl-1-glucuronide[9]; tissue expressing the LUC gene produces a characteristic light emission upon incubation with the substrate luciferin[10].

The antibiotics and herbicides selective agents are used only in the laboratory in the initial stages of the genetic modification process to select individual cells containing genes coding for agronomic traits of interest. The selective agents are not applied after the regeneration of whole plants from those cells nor during the subsequent growth of the crop in the field. Therefore, these plants and all subsequent plant and plant products will neither have been exposed to, nor contain the selective antibiotic or herbicide. Similarly, the reagents used for the scoreable markers are applied only to plant cells or parts dissected from the plant.

lately cumbersome since the necessary sexual crosses and seed production scramble the elite genome. Therefore, if regulatory agencies decided *a priori* that selectable markers should be removed, potato, apples, strawberries, sweet potato, cassava, plantains, and many other crops would be much more difficult to improve using plant biotechnology.

For crops grown from seed, any products currently under development would have to be discarded and reconstructed using redesigned vectors. If regulatory agencies mandated the removal of the NPTII marker, commercialization of the most advanced crops would be delayed 5-6 years. For all products, 2-3 generations (2-10 years) would be added to the development time to allow for the sexual cross.

Selectable marker genes and their protein products will be integral components of crops and of our diets. The marker genes and their products pose, in our view, risks to neither humans nor the environment; however, they do provide the agricultural community with accurate, sensitive, and non-molecular methods for monitoring value-added genes in genetically modified plants. We believe, therefore, that the benefits of agronomically useful genes released in cultivars will vastly outweigh any concerns about associated NPTII marker genes. We conclude that there is no scientific reason to prohibit or limit the use of selectable markers, nor to encourage or require their removal from genetically modified plants.

We also believe that many other of the marker genes pose no additional risk to mankind or the environment. However, a case by-case examination of each will be necessary before bread use in agriculture is admissible.

ACKNOWLEDGEMENTS

We are grateful to Frank Serdy, Rob Horsch, and Dennis Hoerner for their help in the preparation of this manuscript.

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Digestion and Absorption in the Gastrointestinal Tract

The foods on which the body lives, with the exception of small quantities of substances such as vitamins and minerals, can be classified as carbohydrates, fats, and proteins. However, these generally cannot be absorbed in their natural forms through the gastrointestinal mucosa and, for this reason, are useless as nutrients without the preliminary process of digestion. Therefore, the present chapter discusses, first, the processes by which carbohydrates, fats, and proteins are digested into small enough compounds for absorption and, second, the mechanisms by which the digestive end-products, as well as water, electrolytes, and other substances, are absorbed.

DIGESTION OF THE VARIOUS FOODS

Hydrolysis as the Basic Process of Digestion. Almost all the carbohydrates of the diet are large *polysaccharides* or *disaccharides*, which are combinations of *monosaccharides* bound to each other by the process of *condensation*. This means that a hydrogen ion has been removed from one of the monosaccharides while a hydroxyl ion has been removed from the next one; the two monosaccharides then are combined with each other at these sites of removal, and the hydrogen and hydroxyl ions combine to form water. When the carbohydrates are digested back into monosaccharides, specific enzymes return the hydrogen and hydroxyl ions to the polysaccharides and thereby separate the monosaccharides from each other. This process, called *hydrolysis*, is the following:

$$R' - R'' + H_2O \xrightarrow{digestive\ enzyme} R'OH + R''H$$

Almost the entire fat portion of the diet consists of triglycerides (neutral fats), which are combinations of three *fatty acid* molecules condensed with a single *glycerol* molecule. In the process of condensation, three molecules of water had been removed. Digestion of the triglycerides consists of the reverse process, the digesting enzymes returning the three molecules of water to each molecule of neutral fat and thereby splitting the fatty acid molecules away from the glycerol. Here again, the process is one of hydrolysis.

Finally, proteins are formed from *amino acids* that are bound together by means of *peptide linkages*. In each linkage a hydroxyl ion is removed from one amino acid while a hydrogen ion is removed from the succeeding one; thus, the amino acids also combine together by the process of condensation while losing a molecule of water. Digestion of proteins, therefore, also involves the process of hydrolysis, the proteolytic enzymes returning the water to the protein molecules to split them into their constituent amino acids.

Therefore, the chemistry of digestion is really simple; for in the case of all three major types of food, the same basic process of *hydrolysis* is involved. The only difference lies in the enzymes required to promote the reactions for each type of food.

All the digestive enzymes are proteins. Their secretion by the different gastrointestinal glands is discussed in the preceding chapter.

DIGESTION OF CARBOHYDRATES

The Carbohydrate Foods of the Diet. Only three major sources of carbohydrates exist in the normal human diet. These are sucrose, which is the disaccharide known popularly as cane sugar; lactose, which is a disaccharide in milk; and starches, which are large polysaccharides present in almost all foods and particularly in the grains. Other types of carbohydrates ingested to a slight extent are glycogen, alcohol, lactic acid, pyruvic acid, pectins, dextrins, and minor quantities of other carbohydrate derivatives in meats. The diet also contains a large amount of cellulose, which is a carbohydrate. However, no enzymes capable of hydrolyzing cellulose are secreted by the human digestive tract. Consequently, cellulose cannot be considered to be a food for the human being, though it can be utilized by some lower animals.

Digestion of Carbohydrates in the Mouth. When food is chewed, it is mixed with the saliva, which contains the enzyme *ptyalin* (α-amylase) secreted mainly by the

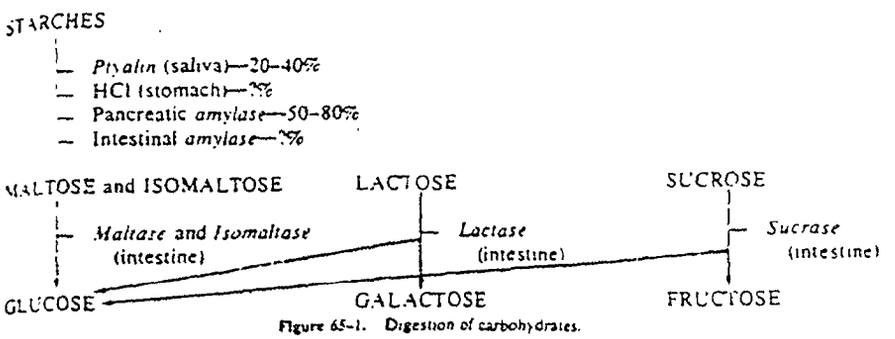


Figure 65–1. Digestion of carbohydrates.

salivary glands. This enzyme hydrolyzes starch into the disaccharides *maltose* and *isomaltose*, as shown in Figure 65–1; but the food remains in the mouth only a short time, and probably not more than 3 to 5 per cent of all the starches that are eaten will have become hydrolyzed into maltose and isomaltose by the time the food is swallowed. One can demonstrate the digestive action of ptyalin in the mouth by chewing a piece of bread for several minutes; after this time, the bread tastes sweet because of the maltose and isomaltose that have been liberated from the starches of the bread.

Most starches in their natural state, unfortunately, are present in the food in small globules, each of which has a thin protective cellulose covering. Therefore, most naturally occurring starches are digested only poorly by ptyalin unless the food is cooked to destroy the protective membrane.

Digestion of Carbohydrates in the Stomach. Even though food does not remain in the mouth long enough for ptyalin to complete the breakdown of starches into maltose, the action of ptyalin continues for as long as several hours after the food has entered the stomach, that is, until the contents of the fundus are mixed with the stomach secretions. Then the activity of the salivary amylase is blocked by the acid of the gastric secretions, for it is essentially nonactive as an enzyme once the pH of the medium falls below approximately 4.0. Nevertheless, on the average, before the food becomes completely mixed with the gastric secretions, as much as 30 to 40 per cent of the starches will have been changed into maltose and isomaltose.

Digestion of Carbohydrates in the Small Intestine. ***Digestion by Pancreatic Amylase.*** Pancreatic secretion, like saliva, contains a large quantity of α-amylase which is almost identical in its function with the α-amylase of saliva and is capable of splitting starches into *maltose* and *isomaltose*. Therefore, immediately after the chyme empties from the stomach into the duodenum and mixes with pancreatic juice, the starches that have not already been split are digested by amylase. In general, the starches are almost totally converted into maltose and isomaltose before they have passed beyond the jejunum.

Hydrolysis of Disaccharides into Monosaccharides by the Intestinal Epithelial Enzymes. The epithelial cells lining the small intestine contain the four enzymes *lactase*, *sucrase*, *maltase*, and *isomaltase*, which are capable of splitting the disaccharides lactose, sucrose, maltose, and isomaltose, respectively, into their constituent monosaccharides. These enzymes are located in the brush border of the cells lining the lumen of the intestine, and the disaccharides are digested as they come in contact with this border. The digested products, the monosaccharides, are then immediately absorbed into the portal blood. Lactose splits into a molecule of *galactose* and a molecule of *glucose*. Sucrose splits into a molecule of *fructose* and a molecule of *glucose*. Maltose and isomaltose each split into *two molecules of glucose*. Thus, the final products of carbohydrate digestion that are absorbed into the blood are all monosaccharides.

In the ordinary diet, which contains far more starches than either sucrose or lactose, glucose represents about 80 per cent of the final products of carbohydrate digestion, and galactose and fructose each represent, on the average, about 10 per cent of the products of carbohydrate digestion.

DIGESTION OF FATS

The Fats of the Diet. By far the most common fats of the diet are the neutral fats, also known as *triglycerides*, each molecule of which is composed of a glycerol nucleus and three fatty acids, as illustrated in Figure 65–2. Neutral fat is found in food of both animal origin and plant origin.

In the usual diet are also small quantities of phospholipids, cholesterol, and cholesterol esters. The phospholipids and cholesterol esters contain fatty acid, and, therefore, can be considered to be fats themselves. Cholesterol, on the other hand, is a sterol compound containing no fatty acid, but it does exhibit some of the physical and chemical characteristics of fats, it is derived from fats, and it is metabolized similarly to fats. Therefore, cholesterol is considered from a dietary point of view to be a fat.

Digestion of Fats in the Intestine. A small amount of short chain triglycerides of butter fat origin is digested in the stomach by gastric lipase (*tributyrase*). However, the amount of digestion is so slight that it is unimportant. Instead, essentially all fat digestion occurs in the small intestine as follows:

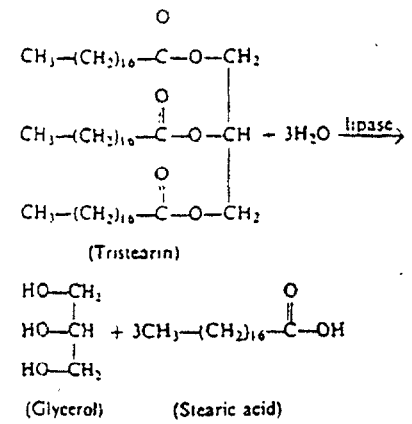


Figure 65–2. Hydrolysis of neutral fat catalyzed by lipase.

Emulsification of Fat by Bile Acids. The first step in fat digestion is to break the fat globules into small sizes so that the water-soluble digestive enzymes can act on the globule surfaces. This process is called emulsification of the fat, and it is achieved under the influence of *bile*, the secretion of the liver that does not contain any digestive enzymes. However, bile does contain a large quantity of *bile salts*, mainly in the form of ionized sodium salts, which are extremely important for the emulsification of fat. The carboxyl (or polar) part of the bile salt is highly soluble in water, whereas the sterol portion of the bile salt is highly soluble in fat. Therefore, the fat-soluble portion of the bile salt dissolves in the surface layer of the fat globule but with the carboxyl portion of the salt projecting outward and soluble in the surrounding fluids; this effect greatly decreases the interfacial tension of the fat

When the interfacial tension of a globule of nonmiscible fluid is low, this nonmiscible fluid, on agitation, can be broken up into many minute particles far more easily than it can when the interfacial tension is great. Consequently, a major function of the bile salts is to make the fat globules readily fragmentable by agitation in the small bowel. This action is the same as that of many detergents that are used widely in most household cleansers for removing grease.

Each time the diameters of the fat globules are decreased by a factor of 2 as a result of agitation in the small intestine, the total surface area of the fat increases 2 times. In other words, the total surface area of the fat particles in the intestinal contents is inversely proportional to the diameters of the particles

The lipases are water-soluble compounds and ca attack the fat globules only on their surfaces. Cor sequently, it can be readily understood how importar this detergent function of bile salts is for the digestion c fats.

Digestion of Fats by Pancreatic Lipase. By far th most important enzyme for the digestion of fats i *pancreatic lipase* in the pancreatic juice. However, th epithelial cells of the small intestine also contain a sma quantity of lipase known as *enteric lipase*. Both of thes act alike to cause hydrolysis of fat.

End-Products of Fat Digestion. Most of the triglycer ides of the diet are finally split into *monoglycerides*, *fre fatty acids*, and *glycerol*, as illustrated in Figure 65–: However, small portions are not digested at all e remain in a diglyceride state

Role of Bile Salts in Accelerating Fat Digestion — For mation of Micelles. The hydrolysis of triglycerides is highly reversible process; therefore, accumulation c monoglycerides and free fatty acids in the vicinity c digesting fats very quickly blocks further digestion Fortunately, the bile salts play an important role i removing the monoglycerides and free fatty acids fro the vicinity of the digesting fat globules almost a rapidly as these end-products of digestion are formed This occurs in the following way:

Bile salts have the propensity to form *micelles*, whic are small spherical globules about 25 Angstroms i diameter and composed of 20 to 50 molecules of bil salt. These develop because each bile salt molecule i composed of a sterol nucleus that is highly fat-solubl and a polar group that is highly water-soluble. The sterc nuclei of the 20 to 50 bile salt molecules of the micell aggregate together to form a small fat globule in th middle of the micelle. This aggregation causes the polc groups to project outward to cover the surface of th micelle. Since these polar groups are negatively charged, they allow the entire micelle globule to becom dissolved in the water of the digestive fluids and t remain in stable solution despite the very large size c the micelle.

During triglyceride digestion, as rapidly as the mor oglycerides and free fatty acids are formed they becom dissolved in the fatty portion of the micelles, whic immediately removes these end-products of digestio from the vicinity of the digesting fat globules. Cor sequently, the digestive process can proceed unaba ed.

The bile salt micelles also act as a transport mediu to carry the monoglycerides and the free fatty acid: both of which would otherwise be almost completel insoluble, to the brush borders of the epithelial cell: There the monoglycerides and free fatty acids ar absorbed, as will be discussed later. On delivery c these substances to the brush border, the bile salts ar again released back into the chyme to be used again an again for this "ferrying" process.

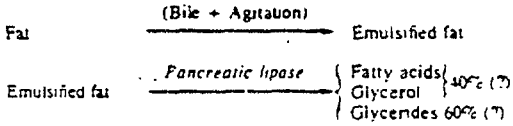
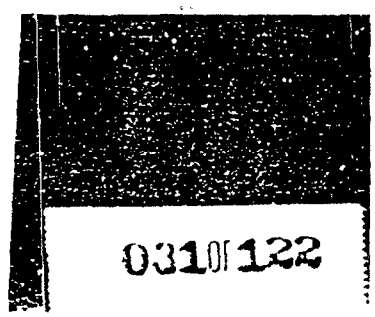


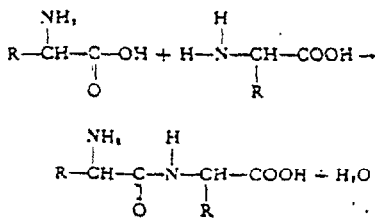
Figure 65–3. Digestion of fats.



Digestion of Cholesterol Esters. Most of the cholesterol in the diet is in the form of cholesterol esters, which cannot be absorbed in this form, though free cholesterol is readily absorbed. A *cholesterol esterase* in the pancreatic juice hydrolyzes the esters and thus frees the cholesterol. The bile salt micelles play identically the same role in "ferrying" cholesterol as they do in "ferrying" monoglycerides and free fatty acids. Indeed, this role of the bile salt micelles is absolutely essential to the absorpuon of cholesterol because essentially no cholesterol is absorbed without the presence of bile salts. On the other hand, as much as 60 per cent of the triglycerides can be digested and absorbed even in the absence of bile salts.

DIGESTION OF PROTEINS

The Proteins of the Diet. The dietary proteins are derived almost entirely from meats and vegetables. These proteins in turn are formed of long chains of amino acids bound together by *peptide linkages*. A typical linkage is the following:



The characteristics of each type of protein are determined by the types of amino acids in the protein molecule and by the arrangement of these amino acids. The physical and chemical characteristics of the different proteins will be discussed in Chapter 69.

Digestion of Proteins in the Stomach. *Pepsin*, the important peptic enzyme of the stomach, is most active at a pH of about 2 and is completely inactive at a pH above approximately 5. Consequently, for this enzyme to cause any digestive action on protein, the stomach juices must be acidic. It will be recalled from Chapter 64 that the gastric glands secrete a large quantity of hydrochloric acid. This hydrochloric acid is secreted by the oxyntic (parietal) cells at a pH of about 0.8, but, by the time it is mixed with the stomach contents and with the secretions from the nonoxyntic glandular cells of the stomach, the pH ranges around 2 to 3, a highly favorable range of acidity for pepsin activity.

Pepsin is capable of digesting essentially all the different types of proteins in the diet. One of the important features of pepsin digestion is its ability to digest collagen, an albuminoid that is affected little by other digestive enzymes. Collagen is a major constituent of the intercellular connective tissue of meats, and for the digestive enzymes of the digestive tract to penetrate meats and digest the cellular proteins it is first necessary that the collagen fibers be digested. Consequently, in persons lacking peptic activity in the stomach, the ingested meats are poorly penetrated by the digestive enzymes and, therefore, are poorly digested.

As illustrated in Figure 65–4, pepsin usually only

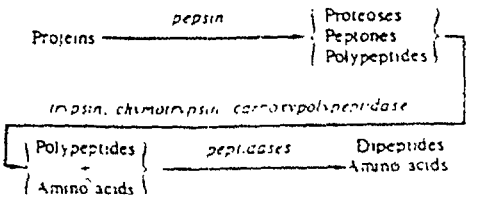


Figure 65–4. Digestion of proteins

begins the process of protein digestion, simply splitting the proteins into proteoses, peptones, and large polypeptides. Thus splitting of proteins is a process of hydrolysis occurring at the peptide linkages between the amino acids.

Digestion of Proteins by Pancreatic Secretions. When the proteins leave the stomach, they ordinarily are mainly in the form of proteoses, peptones, and large polypeptides. Immediately upon entering the small intestine, the partial breakdown products are attacked by the pancreatic enzymes *trypsin*, *chymotrypsin*, and *carboxypolypeptidase*. As illustrated in Figure 65–4, these enzymes are capable of hydrolyzing all the partial breakdown products of protein to peptides and many also to the final stage of amino acids.

Digestion of Peptides by the Epithelial Peptidases of the Small Intestine. The epithelial cells of the small intestine contain several different enzymes for hydrolyzing the final peptide linkages of the remaining dipeptides and other small polypeptides as they come in contact with the epithelium of the villi. The enzymes responsible for final hydrolysis of the peptides into amino acids are *amino-polypeptidase* and the *dipeptidases*.

All the proteolytic enzymes — including those of the gastric juice, the pancreatic juice, and the brush border of the intestinal epithelial cells — are very specific for hydrolyzing individual types of peptide linkages. The linkages between certain pairs of amino acids differ in their bond energy and other physical characteristics from the linkages between other pairs. Therefore, a specific enzyme is required for each specific type of linkage. This accounts for the multiplicity of proteolytic enzymes as well as for the fact that no one single enzyme can usually digest protein all the way to all its constituent amino acids.

When food has been properly masticated and is not eaten in too large a quantity at any one time, about 98 per cent of all the proteins finally become either amino acids or very small peptides, mainly dipeptides. A few molecules of protein are never digested at all, and some remain in the stages of proteoses, peptones, and varying sizes of polypeptides.

BASIC PRINCIPLES OF GASTROINTESTINAL ABSORPTION

ANATOMICAL BASIS OF ABSORPTION

The total quantity of fluid that must be absorbed each day is equal to the ingested fluid (about 1.5

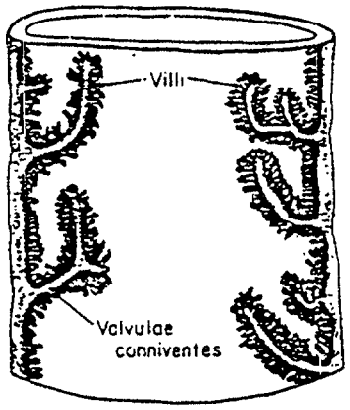


Figure 65–5. A longitudinal section of the small intestine, showing the valvulae conniventes covered by villi.

liters) plus that secreted in the various gastrointestinal secretions (about 7.5 liters). This comes to a total of approximately 9 liters. About 8 to 8.5 liters of this is absorbed in the small intestine, leaving only 0.5 to 1 liter to pass through the ileocecal valve into the colon each day.

The stomach is a poor absorptive area of the gastrointestinal tract because it lacks the typical villus type of absorptive membrane and also because the junctions between the epithelial cells are tight junctions. Only a few highly lipid-soluble substances, such as alcohol and some drugs such as aspirin, can be absorbed in small quantities.

The Absorptive Surface of the Intestinal Mucosa — The Villi. Figure 65–5 illustrates the absorptive surface of the intestinal mucosa, showing many folds called *valvulae conniventes* (or *folds of Kerckring*), which increase the surface area of the absorptive mucosa about three-fold. These folds extend circularly most of the way around the intestine and are especially well developed in the duodenum and jejunum, where they often protrude as much as 8 mm. into the lumen.

Located over the entire surface of the small intestine, from approximately the point at which the common bile duct empties into the duodenum down to the ileocecal valve, are literally millions of small *villi*, which project about 1 mm. from the surface of the mucosa, as shown on the surfaces of the valvulae conniventes in Figure 65–5 and in detail in Figure 65–6. These villi lie so close to each other in the upper small intestine that they actually touch in most areas, but their distribution is less profuse in the distal small intestine. The presence of villi on the mucosal surface enhances the absorptive area another 10-fold.

The intestinal epithelial cells are characterized by a brush border, consisting of about 600 *microvilli* 1 μm in length and 0.1 μm in diameter protruding from each cell; these are illustrated in the electron micrograph in Figure 65–7. This increases the surface area exposed to the intestinal materials another 20-fold. Thus, the combination of the folds of Kerckring, the villi, and the microvilli increases the absorptive area of the mucosa about 600-fold, making a tremendous total area of about 250 square meters for the entire small intestine — about the surface area of a tennis court.

Figure 65–6A illustrates the general organization of a villus, emphasizing especially the advantageous arrangement of the vascular system for

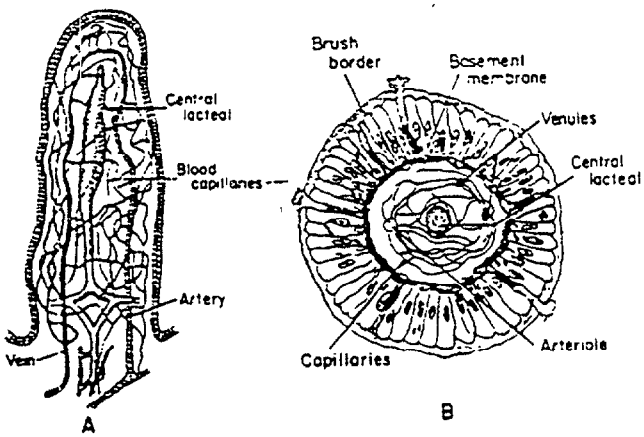


Figure 65–6. Functional organization of the villus: (A) Longitudinal section. (B) Cross-section showing the epithelial cells and basement membrane.

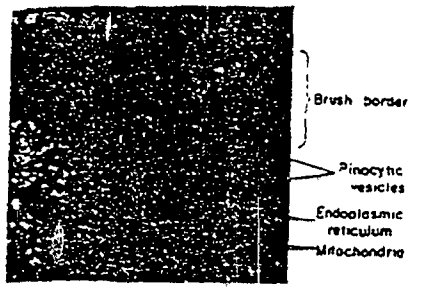


Figure 65–7. Brush border of the gastrointestinal epithelial cell showing, also, pinocytic vesicles, mitochondria, and endoplasmic reticulum lying immediately beneath the brush border. (Courtesy of Dr William Lockwood.)

absorption of fluid and dissolved material into the portal blood, and the arrangement of the *central lacteal* for absorption into the lymph. Figure 65–6B shows the cross-section of a villus, and Figure 65–7 shows many small *pinocytic* vesicles, which are pinched-off portions of infolded epithelium surrounding extracellular materials that have been entrapped inside the cells. Small amounts of substances are absorbed by this physical process of *pinocytosis*, though, as noted later in the chapter most absorption occurs by means of single molecular transfer. Located near the brush border of the epithelial cell are many *mitochondria*, which supply the cell with oxidative energy needed for *active transport* of materials through the intestinal epithelium; this also is discussed later in the chapter.

PHYSICAL MECHANISMS OF ABSORPTION

Absorption through the gastrointestinal mucosa occurs by *active transport* and by *diffusion*, as is also true for other membranes. The physical principles of these processes were explained in Chapter 4.

Briefly, active transport imparts energy to the substance as it is being transported for the purpose of concentrating it on the other side of the membrane or for moving it against an electrical potential. On the other hand, the term diffusion means simply transport of substances through the membrane as a result of molecular movement *along*, rather than against, an electrochemical gradient.

ABSORPTION IN THE SMALL INTESTINE

Normally, absorption from the small intestine each day consists of several hundred grams of carbohydrates, 100 or more grams of fat, 50 to 100 grams of amino acids, 50 to 100 grams of ions, and 8 or 9 liters of water. However, the absorptive *capacity* of the small intestine is far greater than this: as much as several kilograms of carbohydrates per day, 500 to 1000 grams of fat per day, 500 to 700 grams of amino acids per day, and 20 or more liters of water per day. In addition, the large intestine can absorb still more water and ions, though almost no nutrients.

ABSORPTION OF WATER

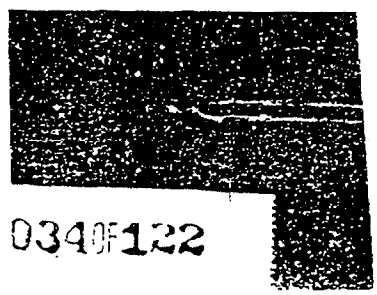
Isosmotic Absorption. Water is transported through the intestinal membrane entirely by the process of *diffusion*. Furthermore, this diffusion obeys the usual laws of osmosis. Therefore, when the chyme is dilute, water is absorbed through the intestinal mucosa into the blood of the villi by osmosis.

Water can also be transported by osmosis from the plasma into the chyme. This occurs whenever hyperosmotic solutions are discharged from the stomach into the duodenum. Usually within minutes, sufficient water is transferred by osmosis to make the chyme isosmotic with the plasma. On the other hand, if there is excess water in the chyme, the osmosis into the plasma also causes an isosmotic state within a few minutes. Thereafter, the chyme remains almost exactly isosmotic throughout its total passage through the small and large intestine.

As dissolved substances are absorbed from the lumen of the gut into the blood the absorption tends to decrease the osmotic pressure of the chyme, but water diffuses so readily through the intestinal membrane (because of large 7 to 15 Å intercellular pores) that it almost instantaneously "follows" the absorbed substances into the blood. Therefore, as ions and nutrients are absorbed, so also is an isosmotic equivalent of water absorbed. In this way not only are the ions and nutrients almost entirely absorbed before the chyme passes through the small intestine, but so also is almost 95 per cent of the water absorbed.

ABSORPTION OF IONS

Active Transport of Sodium. Twenty to 30 grams of sodium are secreted into the intestinal secretions each day. In addition, the normal person eats 5 to 8 grams of sodium each day. Combining these two, the small intestine absorbs 25 to 35 grams of sodium each day, which amounts to about one-seventh of all the sodium that is present in the body. One can well understand that whenever the intestinal secretions are lost to the exterior, as in extreme diarrhea, the sodium reserves of



the body can be depleted to a lethal level within hours. Normally, this sodium is secreted and reabsorbed continually with only about 1 milliequivalent lost in the feces each day. The sodium plays an important role in the absorption of sugars and amino acids, as we shall see in subsequent discussions.

The basic mechanism of sodium absorption from the intestine is illustrated in Figure 65–8. The *principles of this mechanism*, which were discussed in Chapter 4, are also essentially the same as those for absorption of sodium from the renal tubules, as discussed in Chapter 34. The motive power for the sodium absorption is provided by active transport of sodium from inside the epithelial cells through the side walls of these cells into the intercellular spaces. This is illustrated by the heavy black arrows in Figure 65–8. This active transport obeys the usual laws of active transport: it requires a carrier, it requires energy, and it is catalyzed by appropriate ATPase carrier enzymes in the cell membrane. Part of the sodium is transported along with chloride ions, part is transported in exchange for potassium ions, and part is transported without either of these.

The active transport of sodium reduces its concentration in the cell to a low value (about 50 mEq./liter), as also illustrated in Figure 65–8. Since the sodium concentration in the chyme is normally about 142 mEq./liter (that is, approximately equal to that in the plasma), sodium diffuses from the chyme through the brush border of the epithelial cell into the epithelial cell cytoplasm. This replaces the sodium that is actively transported out of the epithelial cells into the intercellular spaces.

The next step in the transport process is osmosis of water out of the epithelial cell into the intercellular spaces. This movement is caused by the osmotic gradient created by the reduced concentration of sodium inside the cell and the elevated concentration in the intercellular space. The osmotic movement of water creates a flow of fluid into the intercellular space, then through the ment membrane of the epithelium, and final the circulating blood of the villi. New wat fuses along with sodium through the brush of the epithelial cell to replenish the wat osmoses into the intercellular spaces.

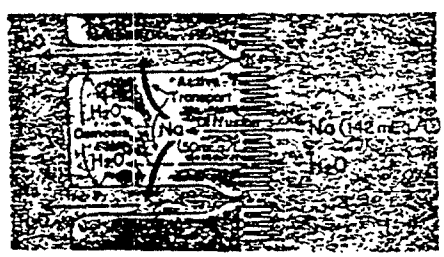


Figure 65–8. Absorption of sodium through the intestinal epithelium. Note also the osmotic absorption of water – that is, the water "follows" the sodium through the epithelial membrane.

Transport of Chloride. In the upper part small intestine chloride transport is mair passive diffusion. The transport of sodiun through the epithelium creates electronegati the chyme and electropositivity on the bas of the epithelial cells. Then chloride ions along this electrical gradient to "follow" sodium ions.

Active Absorption of Chloride Ions and Secretion of Bicarbonate Ions in the Lower Ileu in the Large Intestine. The epithelial cells c distal ileum and of the large intestine hav special capability of actively absorbing chl ions by means of a tightly coupled active tran mechanism in which an equivalent numbe bicarbonate ions are secreted. The functional of this mechanism is to provide bicarbonate for neutralization of acidic products forme bacteria — *especially in the large intestine.*

Various bacterial toxins, particularly thos cholera, colon bacilli, and staphylococci, strongly stimulate this chloride-bicarbonate change mechanism. The secreted bicarbonate carries with it sodium ions, and the two of th together carry an isosmotic equivalent of wate well. This results in rapid flow of fluid from distal part of the gut, thus causing diarrhea cholera, especially, the diarrhea can be so sev that it can cause death within 24 hours.

Absorption of Other Ions. Calcium ions actively absorbed, especially from the duodenu and calcium ion absorption is exactly controlle relation to the need of the body for calcium. (*important factor controlling calcium absorptio* parathyroid hormone secreted by the parathyr glands, and another is vitamin D. The parathyr hormone activates vitamin D in the kidneys, a the activated vitamin D in turn greatly enhanc calcium absorption. These effects are discussed Chapter 79.

Iron ions are also actively absorbed from t small intestine. The principles of iron absorptio and the regulation of its absorption in proporti to the body's need for iron were discussed Chapter 5.

Potassium, magnesium, phosphate, and prob bly still other ions can also be actively absorbe through the mucosa. In general, the monovale ions are absorbed with ease and in great quan ties. On the other hand, the bivalent ions a normally absorbed in only small amounts; fort nately, only small quantities of these are normal needed by the body.

ABSORPTION OF NUTRIENTS

Absorption of Carbohydrates

Essentially all the carbohydrates are absorbed in the form of monosaccharides, only a small fraction of a per cent being absorbed as disaccharides and almost none as larger carbohydrate compounds. Furthermore, little carbohydrate absorption results from simple diffusion, for the pores of the mucosa through which diffusion occurs are essentially impermeable to water-soluble solutes with molecular weights greater than 100.

That the transport of most monosaccharides through the intestinal membrane is an active process is demonstrated by several important experimental observations:

1. Transport of most of them, especially glucose and galactose, can be blocked by metabolic inhibitors, such as iodoacetic acid, cyanides, and phlorhizin.

2. The transport is selective, specifically transporting certain monosaccharides without transporting others. The order of preference for transporting different monosaccharides and their relative rates of transport in comparison with glucose are:

Galactose	1.1
Glucose	1.0
Fructose	0.4
Mannose	0.2
Xylose	0.15
Arabinose	0.1

3. There is a maximum rate of transport for each type of monosaccharide. The most rapidly transported monosaccharide is galactose, with glucose running a close second. Fructose, which is also one of the three important monosaccharides for nutrition, is absorbed less than half as rapidly as either galactose or glucose; also, its mechanism of absorption is different, as will be explained below.

4. There is competition between certain sugars for the respective carrier system. For instance, if large amounts of galactose are being transported, the amount of glucose that can be transported simultaneously is considerably reduced.

Mechanism of Glucose and Galactose Absorption. Glucose and galactose transport ceases whenever active sodium transport is blocked. Therefore, it is assumed that the energy required for transport of these two monosaccharides is actually provided by the sodium transport system. A theory that attempts to explain this is the following: It is known that the carrier for transport of glucose (which is the carrier for galactose as well) is present in the brush border of the epithelial cell. However, this carrier will not transport the glucose in the absence of sodium transport. Therefore, it is believed that the carrier has receptor sites for both a glucose molecule and a sodium ion, and that it will not transport either of these to the interior of the epithelial cell until both receptor sites are simultaneously filled. The energy to cause movement of the carrier from the exterior of the membrane to the interior is derived from the difference in sodium concentration between the outside and inside. That is, as sodium diffuses to the inside of the cell it "drags" the carrier, and therefore the glucose as well, along with it, thus providing the energy for transport of the glucose. For obvious reasons, this explanation is called the *sodium co-transport theory* for glucose transport.

Subsequently, we will see that sodium transport is also required for transport of amino acids, suggesting a similar "carrier-drag" mechanism for amino acid transport.

Absorption of Fructose. Transport of fructose is slightly different from that of most other monosaccharides. It is not blocked by some of the same metabolic poisons — specifically, phlorhizin — and it does not require metabolic energy for transport, even though it does require a specific carrier. Therefore, it is transported by *facilitated diffusion* rather than active transport. Also, it is mainly converted into glucose inside the epithelial cell before entering the portal blood, the fructose first becoming phosphorylated, then converted to glucose, and finally released from the epithelial cell into the blood.

Absorption of Proteins

Most proteins are absorbed in the form of amino acids. However, small quantities of dipeptides and even tripeptides are also absorbed, and extremely minute quantities of whole proteins can at times be absorbed by the process of pinocytosis, though not by the usual absorptive mechanisms.

The absorption of amino acids also obeys the principles listed above for active absorption of glucose; that is, the different types of amino acids are absorbed selectively and certain ones interfere with the absorption of others, illustrating that common carrier systems exist. Finally, metabolic poisons block the absorption of amino acids in the same way that they block the absorption of glucose.

Absorption of amino acids through the intestinal mucosa can occur far more rapidly than can protein digestion in the lumen of the intestine. As a result, the normal rate of absorption is determined not by the rate at which they can be absorbed but by the rate at which they can be

released from the proteins during digestion. For these reasons, essentially no free amino acids can be found in the intestine during digestion — that is, they are absorbed as rapidly as they are formed. Since most protein digestion occurs in the upper small intestine, most protein absorption occurs in the duodenum and jejunum.

Basic Mechanisms of Amino Acid Transport. As is true for monosaccharide absorption, very little is known about the basic mechanisms of amino acid transport. However, at least four different carrier systems transport different amino acids — one transports *neutral amino acids*, a second transports *basic amino acids*, a third transports *acidic amino acids*, and a fourth has specificity for the two imino acids *proline* and *hydroxyproline*. Also, the transport mechanisms have far greater affinity for transporting L-stereoisomers of amino acids than D-stereoisomers. And experiments have demonstrated that *pyridoxal phosphate*, a derivative of the vitamin pyridoxine, is required for transport of many amino acids.

Amino acid transport, like glucose transport, occurs only in the presence of simultaneous sodium transport. Furthermore, the carrier systems for amino acid transport, like those for glucose transport, are in the brush border of the epithelial cell. It is believed that amino acids are transported by the same *sodium co-transport* mechanism as that explained above for glucose transport. That is, the theory postulates that the carrier has receptor sites for both an amino acid molecule and a sodium ion. Only when both of the sites are filled will the carrier move to the interior of the cell. Because of the sodium gradient across the brush border, the sodium diffusion to the cell interior pulls the carrier and its attached amino acid to the interior where the amino acid becomes trapped. Therefore, amino acid concentrations increase within the cell, and they then diffuse through the sides or base of the cell into the portal blood.

Absorption of Fats

Earlier in this chapter it was pointed out that as fats are digested to form monoglycerides and free fatty acids, both of these digestive end-products become dissolved in the lipid portion of the bile acid micelles. Because of the molecular dimensions of these micelles and also because of their highly charged exterior, they are soluble in the chyme. In this form the monoglycerides and the fatty acids are transported to the surfaces of the epithelial cells. On coming in contact with these surfaces, both the monoglycerides and the fatty acids immediately diffuse through the epithelial membrane, leaving the bile acid micelles still in the chyme. The micelles then diffuse back into the chyme and absorb still more monoglycerides and fatty acids, and similarly transport these ab the epithelial cells. Thus, the bile acids perfo "ferrying" function, which is highly importar fat absorption. In the presence of an abundan bile acids, approximately 97 per cent of the absorbed. In the absence of bile acids, only 60 per cent is normally absorbed.

The mechanism for absorption of the monog erides and fatty acids through the brush bord based on the fact that both of these substances highly lipid-soluble. Therefore, they become solved in the membrane and simply diffuse to interior of the cell.

The undigested triglycerides and the dig erides are both also highly soluble in the membrane of the epithelial cell. However, small quantities of these are normally absor because the bile acid micelles will not disso either triglycerides or diglycerides and there will not ferry them to the epithelial membrane

During entry into the epithelial cell, many of monoglycerides are further digested into glyce and fatty acids by an epithelial cell lipase. Th the free fatty acids are reconstituted by smooth endoplasmic reticulum into triglyceri Almost all of the glycerol that is utilized for t purpose is synthesized *de novo* from alp glycerophosphate, this synthesis requiring b energy from ATP and a complex of enzymes catalyze the reactions.

Once formed, the triglycerides aggregate wit the endoplasmic reticulum into globules al with absorbed cholesterol, absorbed phosp pids, and newly synthesized cholesterol and ph pholipids. Each of these is then encased in protein coat, utilizing protein also synthesiz by the endoplasmic reticulum. This globu mass, along with its protein coat, is extruded fr the sides of the epithelial cells into the intercellu spaces, and from here it passes into the cent lacteal of the villi. Such globules are called *chy microns*

The protein coat of the chylomicrons mak them hydrophilic, allowing a reasonable gree of suspension stability in the extracellu fluids. Poisons or genetic disorders that preve formation of the protein for coating the chy microns cause the fat to accumulate in the e thelial cell and not to be extruded into the ext cellular fluid.

Transport of the Chylomicrons in Lymph. From the sides of the epithelial cells t chylomicrons wend their way through the ba ment membrane and into the central lacteal of t villi and from here are propelled, along with t lymph, by the lymphatic pump upward throu the thoracic duct to be emptied into the great vei of the neck. Between 80 and 90 per cent of all absorbed from the gut is absorbed in this manr

and is transported to the blood by way of the thoracic lymph in the form of chylomicrons.

Direct Absorption of Fatty Acids into the Portal Blood. Small quantities of short chain fatty acids such as those from butterfat, are absorbed directly into the portal blood rather than being converted into triglycerides and absorbed into the lymphatics. The cause of this difference between short and long chain fatty acid absorption is presumably that the shorter chain fatty acids are more water-soluble, which allows direct diffusion of fatty acids from the epithelial cells into the capillary blood of the villus.

Absorption of Bile Salts. In the upper portion of the small intestine the bile salts are not absorbed, this failure to be absorbed requires them to remain in the chyme and to continue their function of "ferrying" free fatty acids and monoglycerides to the intestinal mucosa through the entire extent of the small intestine. Once the processes of fat digestion and fat absorption have been accomplished in the upper and mid-intestinal levels, however, the bile salts themselves are then absorbed from the distal ileum before the chyme empties into the large intestine. This absorption is an active process and is carrier-mediated.

After being absorbed from the distal ileum, the bile salts are again secreted in the bile by the liver and returned once more to the upper intestine. Thus, the same bile salts are re-secreted several times each day and are used again and again in the process of fat absorption. Only a small portion of the bile salts (approximately 5 per cent) is lost during each cycle of this "bile salt circulation."

On occasion, the bile salts fail to be absorbed in the ileum and instead empty with the chyme into the large intestine; this occurs especially in patients whose distal ileum has been removed because of ileitus. The presence of bile salts in the large intestine frequently causes severe diarrhea, presumably because of the detergent effect of these salts acting on and irritating the large intestinal mucosa.

ABSORPTION IN THE LARGE INTESTINE: FORMATION OF THE FECES

Approximately 500 to 1000 ml. of chyme passes through the ileocecal valve into the large intestine each day. Most of the water and electrolytes in this are absorbed in the colon, usually leaving less than 100 ml. of fluid to be excreted in the feces. Also, essentially all the ions are also absorbed, leaving less than 1 milliequivalent each of sodium and chloride ions to be lost in the feces.

Most of the absorption in the large intestine occurs in the proximal half of the colon, giving this portion the name *absorbing colon*, while the distal colon functions principally for storage and is therefore called the *storage colon*.

Absorption and Secretion of Electrolytes and Water. The mucosa of the large intestine, like that of the small intestine, has a very high capacity for active absorption of sodium, and the electrical potential created by the absorption of the sodium causes chloride absorption as well. In addition, as in the distal portion of the small intestine, the mucosa of the large intestine actively secretes bicarbonate ions while it simultaneously actively absorbs an equal amount of chloride ions in an exchange transport process. The bicarbonate helps to neutralize the acidic end-products of bacterial action in the colon.

The absorption of sodium and chloride ions creates an osmotic gradient across the large intestinal mucosa, which in turn causes absorption of water.

Bacterial Action in the Colon. Numerous bacteria, especially colon bacilli, are present in the absorbing colon. These are capable of digesting small amounts of cellulose, in this way providing a few calories of nutrition to the body each day. In herbivorous animals this source of energy is very significant, though it is of negligible importance in the human being. Other substances formed as a result of bacterial activity are vitamin K, vitamin B_{12}, thiamin, riboflavin, and various gases that contribute to *flatus* in the colon. Vitamin K is especially important, for the amount of this vitamin in the ingested foods is normally insufficient to maintain adequate blood coagulation.

Composition of the Feces. The feces normally are about three-fourths water and one-fourth solid matter composed of about 30 per cent dead bacteria, 10 to 20 per cent fat, 10 to 20 per cent inorganic matter, 2 to 3 per cent protein, and 30 per cent undigested roughage of the food and dried constituents of digestive juices, such as bile pigment and sloughed epithelial cells. The large amount of fat derives from unabsorbed fatty acids from the diet, fat formed by bacteria, and fat in the sloughed epithelial cells.

The brown color of feces is caused by *stercobilin* and *urobilin*, which are derivatives of bilirubin. The odor is caused principally by the products of bacterial action, these vary from one person to another, depending on each person's colonic bacterial flora and on the type of food eaten. The actual odoriferous products include *indole*, *skatole*, *mercaptans*, and *hydrogen sulfide*.

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Degradation of Bean Proteins by Endogenous and Exogenous Proteases—A Review[1]

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ABSTRACT

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Bean proteins are degraded by proteases when humans and insects utilize the proteins for food and during germination of the seed. Whereas human digestive enzymes generally act on heat-denatured bean proteins, enzymes in the germinated seed and in the bean weevil must degrade native bean proteins. Unlike the germinated seed and the bean weevil, which utilize a cysteine proteinase along with other types of enzymes to degrade bean proteins, humans depend largely on acid and serine proteases. Comparing the breakdown of bean proteins by various proteases in these three systems leads to a better understanding of bean protein structure and nutritive value.

The proteins of *Phaseolus vulgaris* are subjected to degradation by proteases from a variety of sources. These proteins are a source of amino acids for the growing plant, for insects that infest the seeds, and for humans who consume the legumes as an economical source of protein. During germination of the seed, storage proteins are degraded and mobilized. They are broken down to amino acids, which are used for the synthesis of various enzymes, structural proteins, non-nitrogenous compounds, or for energy (Ashton 1976, Mayer and Marbach 1981, Wilson 1986, Shotov and Vaintraub 1987). The bean weevil *Acanthoscelides obtectus* is also able to degrade the proteins of *P. vulgaris* using proteolytic enzymes in its gut. This bruchid beetle can cause severe loss of stored legume seeds intended for human consumption or planting (Southgate 1979, Harin 1981). Whereas enzymes in the germinating seed and in the bean weevil gut must degrade native bean proteins, the digestive enzymes of humans presumably have an easier task since the proteins have generally been heat denatured. The discussion to follow will consider these endogenous and exogenous proteases and their degradation of *P. vulgaris* proteins. The structure and nutritive value of bean proteins can be better understood when one compares the breakdown of bean proteins by proteases in various systems.

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GERMINATION

Degradation of Phaseolin and Other Bean Proteins

Much of the literature on the degradation of *P. vulgaris* proteins concerns the major storage protein that constitutes 50–75% of the total seed protein (Romero et al 1975). Confusion exists concerning the identity of this protein, since it has been referred to by various names, such as phaseolin, euphaseolin, glycoprotein II, G1 globulin, and vicilin (Liener and Thompson 1960). Since all of these preparations seem to share the same properties, it will be referred to as phaseolin in this paper.

Numerous studies have examined the degradation of phaseolin during germination (Table I). The degradation of this protein proceeds slowly and is nearly complete by eight to 10 days of germination (Racusen and Foote 1971, 1973, Bollini and Chrispeels 1978; Sathe et al 1983; Nielsen and Liener 1984). Reserve protein degradation is also slow during germination of other legumes including chickpea (Ganesh Kumar and Venkataraman 1978), pea (Konopska 1979, Basha and Beevers 1975), soybean (Catsimpoolas et al 1968), and mung bean (Chrispeels and Boulter 1975). Gel electrophoresis shows that germination of *P. vulgaris* for several days has little effect on phaseolin, but this is followed by a gradual degradation (Racusen and Foote 1971, 1973; Nielsen and Liener 1984; Pusztai et al 1977). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicates that the three subunits of phaseolin (50–52, 47–49, and 44–46 kDa) are degraded during germination into components with molecular weights (M_r) in the range of 20,000–30,000 (Bollini and Chrispeels 1978, Murray 1982, Sathe et al 1983, Nielsen and Liener 1984). The smallest phaseolin subunit is degraded more rapidly than the two larger subunits (Sathe et al

...ence and Lerner 1984). This pattern differs from that reported for the pea (Basha and Beevers 1975) and mung bean (Boulter et al 1973), where the largest subunit of the major storage protein is the first to be broken down during germination.

Little work has been reported on the breakdown during germination of *P. vulgaris* proteins other than phaseolin. The results of studies with these bean proteins listed in Table I are difficult to compare because of differences in methods of protein fractionation and other factors discussed below.

Proteolytic Activity

Proteolytic enzymes play a key role in the biochemical mechanism of germination. Protein synthesis in the developing seedling depends on the hydrolysis of seed storage proteins. This review covers the literature concerning the proteolytic enzyme activities in the cotyledons of germinating *P. vulgaris* and does not discuss proteolytic activities in other parts of the seedling.

Reports of changes in enzyme activities in the cotyledons of beans during germination are not all in agreement, and some inconsistencies can be attributed to differences in the way data are expressed. As noted by Crump and Murray (1979), to be accurate, activities must be expressed on a per organ basis and not on a parameter that changes with time, such as weight or protein. Discrepancies in the literature are also attributable to different conditions of germination such as soaking before germination, temperature, light, and seed size. Disappearance of total nitrogen or dry weight from the cotyledons more accurately determines the stage of germination than does time expressed as days of germination. Therefore, when adequate data are provided, enzyme activities reported are best compared between studies at points where equal total nitrogen (or dry weight) has been mobilized.

Table II summarizes reports of protease activities for germinating *P. vulgaris* cotyledons and the conditions used to measure the activities. The cotyledons of germinating *P. vulgaris* seeds contain aminopeptidase (as measured by L-leucine-*p*-nitroanilide or leucine-tyrosine), carboxypeptidase (as measured by *N*-carbobenzoxy-L-phenylalanine-alanine), and dipeptidase (as measured by alanine-glycine) activities. They also contain enzymes capable of hydrolyzing low molecular weight substrates typically used to measure trypsinlike enzyme activity (α-*N*-benzoyl-L-

TABLE I
Studies Reporting Degradation of Reserve Proteins During Germination of *Phaseolus vulgaris*

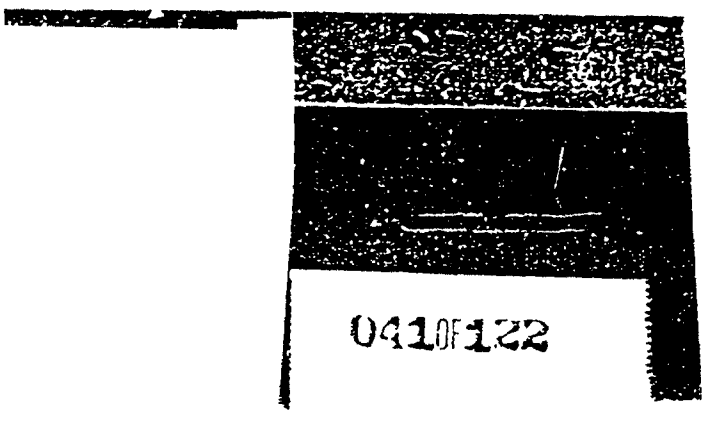
Reference	Cultivar	Protein Studied*
Bollini and Chrispeels 1978	Guatemalan	*6.9 Vicilin
El-Hag et al 1978	Red Kidney	Globulin fraction E (crude globulin isolate)
Jaw and Stanley 1970	Red Kidney	Globulin proteins
		Albumin proteins
		Basic proteins
Murray 1982	Hawkesbury Wonder	*Euphaseolin
Nielsen and Lerner 1984	Improved Tendergreen	*Q1
Pusztai and Duncan 1971	Dwarf Bean Harvester	Globulin proteins
		Albumin proteins
Pusztai et al 1977	Processor	*Major storage protein
Racusen and Foote 1971	Pencil Pod Wax	*Glycoprotein II
Racusen and Foote 1973	Pencil Pod Wax	*Glycoprotein II
Sathe et al 1983	Great Northern	*Major storage protein and other bean proteins

*Those marked with an asterisk presumably refer to the major storage protein, phaseolin.

TABLE II
Studies Reporting Proteinase and Peptidase Activities in Germinating *Phaseolus vulgaris* Cotyledons

Reference	Cultivar	Substrate Utilized*	pH Utilized	Enzyme Activity Described*
Boyce and Simon 1967	Taylor's Horticultural	^{3}H-phaseolin	5.9	Cysteine endopeptidase
Crump and Murray 1979	Hawkesbury Wonder	LPA	7.25	Aminopeptidase, AP1 and AP2
		BTEE	7.8	BTEE hydrolase
		N-CBZ-L-Tyr-*p*NP	8.0	*N*-CBZ-L-Tyr-*p*NP hydrolase
		BAPA	8.2	BAPA hydrolase
Cuoma and Poljak 1984	Jelinka, Cherokee	Azocasein	6.5	Cysteine endopeptidase
Feller 1979, 1981	Saxa	LPA	7.0	Aminopeptidase
		N-CBZ-L-Phe-L-Ala	5.0	Carboxypeptidase
		Azocasein	5.4, 7.5	Endopeptidase
Geyssens and Ilse 1980	Brittle Wax	Casein	5.5	Protease
		Endogenous proteins	5.5	Protease
Meunier and Prabhu 1980	Canoca	Casein	5.5	Protease
Mikkonen 1976	Processor	Leu-Tyr	9.2	Alkaline aminopeptidase
		Ala-Gly	8.5	Dipeptidase
		Leu-β-naphthylamide	6.4	Neutral aminopeptidase (leucinebetanaphthylamidase)
	—	*N*-CBZ-L-Phe-L-Ala	5.9	Carboxypeptidase
		Hemoglobin	3.7	Protease
		Casein	5.4, 7.8	Protease
Nielsen and Lerner 1984	Improved Tendergreen	BAPNA	5.0–9.5	Trypsinlike enzyme
		Azocasein	5.0–9.5	Azocaseinase
		Phaseolin	5.0–6.8	Protease
Pusztai and Duncan 1971	Dwarf Bean	LPA	7.0	Aminopeptidase
	Harvester (kidney)	*N*-CBZ-L-Tyr-*p*NP	8.05	Chymotrypsinlike enzyme
		BAPA	7.8	Trypsinlike enzyme
		Hemoglobin	4.5–6.85	Protease
		Casein	5.5	Protease
		Endogenous proteins	4.5–6.85	Autodigesting activity
Yoshe and Srinivasan 1973	Kentucky Wonder	Casein	5.5	Protease
Yoshe and Taylor 1973	Kentucky Wonder	Gelatin	5.5	Protease

*LPA, L-leucine-*p*-nitroanilide; *N*-CBZ-L-phe-L-Ala, *N*-carbobenzoxy-L-phenylalanine-L-alanine; BAPNA or BAPA, α-*N*-benzoyl-L-arginine-*p*-nitroanilide; BTEE, *N*-benzoyl-L-tyrosine ethyl ester; *N*-CBZ-L-Tyr-*p*NP, α-*N*-carboxy-L-tyrosine-*p*-nitrophenyl ester.



arginine-*p*-nitroanilide, BAPNA) and chymotrypsinlike enzyme activity (*N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester, *N*-CBZ-L-Tyr-*p*NP; *N*-benzoyl-L-tyrosine ethyl ester, BTEE). Protease activities have been reported using protein substrates such as casein, azocasein, or hemoglobin.

Chrispeels and co-workers (1979) hypothesized a mechanism to control the catabolism of reserve proteins in legumes, based on their work with mung bean seedlings. They suggested that the protein bodies of the resting bean contain reserve proteins and enzymes such as carboxypeptidases, but not endopeptidases required for reserve protein degradation. However, such endopeptidases are synthesized during germination in the cytoplasm and transported into the protein bodies, where they catalyze the degradation of reserve proteins in cooperation with the carboxypeptidases. Mikkonen (1986) reported a low carboxypeptidase activity in resting seeds of *P. vulgaris*, but both Mikkonen (1986) and Chrispeels and Boulter (1975) found that carboxypeptidase activity increases during germination. This implies that, like the endopeptidases, carboxypeptidases are synthesized during germination and transported into the protein bodies. The carboxypeptidase activity peaked at day 6 of germination in a study by Feller (1979) and at day 10 in a study by Mikkonen (1986). However, the peak carboxypeptidase activity in both experiments occurred when approximately half of the total nitrogen had been mobilized.

Crump and Murray (1979) found two different aminopeptidase activities in dry beans that peaked between days 2 and 4 during germination. However, aminopeptidase activity has most often been reported to be highest in the nongerminated seed and to decrease during germination (Pusztai and Duncan 1971, Feller 1979, Mikkonen 1986). The activities of an alkaline aminopeptidase, an alkaline dipeptidase, and a neutral aminopeptidase (naphthylamidase) have been shown to be highest in the nongerminated cotyledons, then decrease during germination (Mikkonen 1986, Mikkonen and Mikola 1986, Mikkonen et al 1986).

Pusztai and Duncan (1971) found the activity on *N*-CBZ-L-Tyr-*p*NP to be stable to day 7, then increase to a maximum at day 14 (approximately 50% nitrogen mobilization), when expressed on a dry weight basis. Crump and Murray (1979), who expressed data on a cotyledon basis, reported a fourfold increase in activity on *N*-CBZ-L-Tyr-*p*NP from day 4 to its maximum at day 10 (approximately 55% nitrogen mobilization) and maximum activity on BTEE at day 6. However, Crump and Murray (1979) suggested the activity against these two substrates is due to a peptide hydrolase or esterase and not to a chymotrypsinlike enzyme.

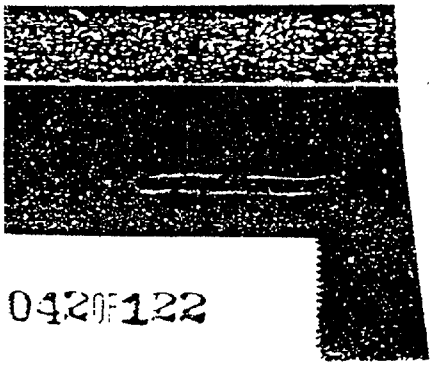
Activity on BAPNA during germination was reported to remain constant for 16 days when calculated on a dry weight basis, by which time approximately 70% of the nitrogen had been mobilized (Pusztai and Duncan 1971). When calculated on a cotyledon basis, it was reported to remain at a maximum level from day 2 to 10 (approximately 55% nitrogen mobilization) before decreasing (Crump and Murray 1979). Nielsen and Liener (1984) showed a small increase in activity on BAPNA during 10 days of germination when calculated on a dry weight or protein basis and a decrease in activity on a cotyledon basis. The optimum pH for hydrolysis of BAPNA by bean extract is 8–9, and cysteine proteinase enhancers or inhibitors have no effect on this activity (Nielsen and Liener 1984).

Numerous research groups have found that the activity (on a seed or cotyledon basis) on casein or azocasein is optimized at pH 5–6 and peaks at days 5–7 of germination (Pusztai and Duncan 1971, Yomo and Srinivasan 1973, Gepstein and Ilan 1980, Meuwer and Paulillo 1980, Feller 1979, Nielsen 1982, Nielsen and Liener 1984). Nitrogen mobilization for this time period of germination ranged from approximately 15% to 90% in these studies. Mikkonen (1986) reported the activity on casein at pH 5.4 on a seed basis peaked at day 10 of germination (approximately 50% nitrogen mobilization). However, when calculated on a dry weight or protein basis, Nielsen and Liener (1984) reported that this activity on azocasein at pH 5.5 increased through day 10 of germination, at which time 50% of the nitrogen had been mobilized (Nielsen 1982). Proteolytic activity on casein or azocasein at pH 5.5 has been shown by several research groups to be enhanced at least twofold by mercaptoethanol (Yomo and Srinivasan 1973, Feller 1979, Nielsen and Liener 1984) and inhibited by *p*-chloromercuribenzoate (*p*-CMB) (Yomo and Srinivasan 1973) or *N*-ethylmaleimide (Nielsen and Liener 1984). Extracts of the germinated beans break down phaseolin at pH 5–6 (Nielsen and Liener 1984). This activity is enhanced by mercaptoethanol and completely eliminated by the cysteine proteinase inhibitors, *N*-ethylmaleimide, leupeptin, and E-64 (*trans*-epoxysuccinyl-L-leucylamide [4-guanidino] butane). These observations led to the conclusion that a cysteine proteinase with an acid pH optimum is largely responsible for the breakdown of the major storage protein during germination.

Results concerning the involvement of an apparent cysteine proteinase in germination have been confirmed by two research groups. Csoma and Polgar (1984) purified a cysteine proteinase synthesized during germination of *P. vulgaris* seeds. The purified enzyme had a M_r of 23,400 and resembled papain in that its thiol group is part of an interactive system where its nucleophilic reactivity is enhanced. However, it differs from papain in the pK_a values for acylations and in the magnitudes of the pH-independent rate constants for alkylations with iodoacetate. Boylan and Sussex (1987) purified and characterized an endopeptidase containing cysteine from germinated seeds of *P. vulgaris* cv. Taylor's Horticultural. The enzyme had a M_r of 30,000, an optimum activity on ^{3}H-phaseolin at pH 5, and showed maximum activity at five days of germination, when approximately 60% of the total nitrogen had been mobilized. No endopeptidase activity was detected in the cotyledons until after the first day of seedling growth, when nitrogen mobilization was yet unapparent. The endopeptidase cleaved the three subunits of phaseolin into three clusters of fragments having M_r of 27,000, 25,000, and 23,000. The cleavage site was not determined, but the size distribution of the fragments suggested that each of the subunits is cleaved at sites near the middle of the polypeptide chain. Boylan and Sussex (1987) suggested that the endopeptidase catalyzes the initial endoproteolytic cleavages of phaseolin during germination, then other proteases can further degrade the protein. It should be noted that Vavrinova and Turkova (1975) reported the isolation of a sulfhydryl endopeptidase from *P. vulgaris*, but it is unclear if this enzyme was obtained from the seed or the leaves.

The enzyme purified from germinating *P. vulgaris* cotyledons is closely related to other plant endopeptidases that are generally monomeric, cysteine enzymes with M_r of 23,000–37,000 (Ryan and Walker-Simmons 1981). The best known cysteine enzymes from plants are papain (*Carica papaya*), ficin (*Ficus glabrata*), bromelain (*Ananas comosus*), and actinidin (*Actinidia chinensis*). Of leguminous species, the cotyledons of peas (*Pisum sativum*) (Basha and Beevers 1975), castor beans (*Ricinus communis* L.) (Tully and Beevers 1978, Alpi and Beevers 1981 a,b), black gram (*Vigna mungo*) (Mitsuhashi et al 1986), and mung beans (*Vigna radiata*) (Chrispeels and Boulter 1975, Baumgartner and Chrispeels 1977) contain cysteine enzymes, as suggested by enhancement and inhibition patterns. The best characterized of these proteinases is vicilin peptidohydrolase, which was purified from germinating mung bean cotyledons (Baumgartner and Chrispeels 1977). The enzyme consists of a single polypeptide chain with a M_r of 23,000. This cysteine proteinase, with optimum activity at pH 5.1, accounts for 95% of the endopeptidase activity in the cotyledons and digests vicilin, the major storage protein of *V. radiata* seeds.

In summary, the literature reports indicate that phaseolin and other bean proteins are degraded slowly during germination. Several types of enzyme activity have been detected in the germinating cotyledon. The literature on changes in enzyme activities contains numerous apparent discrepancies, some of which can be accounted for by differences in the conditions of germination and the method of reporting data. The enzyme in germinating bean cotyledons most thoroughly studied is a cysteine endopeptidase. This proteinase appears to cleave at a site near the middle of phaseolin subunits, but the exact cleavage site has not



been determined. Other enzymes must be present in the germinating cotyledon to further degrade phaseolin to peptides and amino acids that can be utilized by the growing plant. Wilson (1986) has suggested that degradation of the storage protein in dicots is initiated by an endopeptidase that inflicts a limited number of specific proteolytic cleavages to make the protein susceptible to further attack by the same enzyme or other proteases. It is likely that the cysteine endopeptidase is capable of making this initial cleavage in phaseolin. It remains to be determined how this cysteine proteinase acts on other bean proteins, which other proteases are involved in the further attack of bean proteins, and at what point during germination these proteases act.

INSECT GUT PROTEASES

Species of the insect family Bruchidae are most closely associated with the damage to legume seeds caused by insects. Within this family, some 20 species belonging to six genera attack grain legumes (Southgate 1979). Infestation by bruchids starts in the field and continues in storage, leading to severe losses. The bruchids of most concern in the storage of *P. vulgaris* are the bean weevil, *A. obtectus* (Say), and the Mexican bean weevil, *Zabrotes subfasciatus* (Boheman) (Schoonhoven and Cardona 1982). While insects have been used to evaluate the nutritional quality of *P. vulgaris* proteins (Shariff et al 1981), very little information is available in the literature explaining how insects digest the protein of legumes.

An early study suggested that the larvae of *A. obtectus* and the related bruchid *Callosobruchus maculatus* (cowpea weevil), possess only very low levels of protease activity (Applebaum 1964). These assays were performed at neutral-alkaline pH and were primarily designed to detect serine endopeptidase activity. Recently there have been reports of a cysteine proteinase with an acidic pH optimum in the gut of the bruchid, *C. maculatus* (Gatehouse et al 1985, Kitch and Murdock 1986, Murdock et al 1987).

The literature on *A. obtectus* midgut enzymes reports α- and β-glucosidases, α- and β-galactosidases, and high activity against starch and pectin (Leroi et al 1984). Applebaum and Gotz (1972) found that *A. obtectus* was able to digest a toxic bioactive heteropolysaccharide containing arabinose, xylose, rhamnose, glucose, and galactose. Only recently has the proteolytic activity of the *A. obtectus* gut been examined. The proteolytic enzymes in the guts of *A. obtectus* are of interest, not only to compare to proteases in the germinating bean and human digestive system but also as a vulnerable site in this major storage pest. Characterizing the gut protease of *A. obtectus* and evaluating its importance in the life of the insect could reveal a new target for directed plant breeding work to confer bruchid resistance upon *P. vulgaris*.

Wiemann and Nielsen (1988) isolated and partially characterized a major digestive protease from the gut of *A. obtectus*. The enzyme appears to have a cysteine group and an acidic group at or near the active site. The thiol enhancers, cysteine and dithiothreitol, enhanced activity, whereas the thiol inhibitors *p*-CMB and E-64 and the acidic protease inhibitor Pepstatin A eliminated all activity. The molecular weight of the protease was estimated to be 3,500 by gel filtration and 25,300 by SDS-PAGE. The pH range or optimum activity of this protease was pH 4.5–7.0 using the substrate benzoyl-DL-arginine-2-naphthylamide (BANA). Native phaseolin was somewhat resistant to degradation by a crude gut extract of *A. obtectus* and the purified protease. When native phaseolin was subjected to the purified insect protease, the molecular weights of breakdown products were in the M_r range of 21,000–24,000. Heated phaseolin was readily degraded by the purified protease to small molecular weight polypeptides. Whole bean extract and the globulin fraction of *P. vulgaris* appeared to be somewhat degraded by a crude gut extract and by the purified protease, while the albumin fraction was more resistant. Some evidence was obtained suggesting the presence of other proteolytic enzymes in the crude gut extract (Wiemann 1986), but they have not been identified or characterized. Such proteinases and peptidases would be required to complete the degradation of phaseolin to amino acids or peptides small enough to be utilized by the insect, but these have not been identified.

MAMMALIAN DIGESTIVE PROTEASES

Digestion of Phaseolin

Most studies designed to answer questions related to the nutritional quality of *P. vulgaris* proteins have focused on proteins in the globulin fraction (Table III). In particular, considerable attention has been directed toward phaseolin. Heated phaseolin is highly susceptible to digestion by mammalian digestive enzymes (Liener and Thompson 1980, Bradbear and Boulter 1984, Deshpande and Nielsen 1987a). However, the resistance of native phaseolin to proteolytic attack by mammalian digestive enzymes contributes to the poor nutritive value of the unheated bean (Waterman and Johns 1921, Vaintraub et al 1976, Romero and Ryan 1978, Vaintraub et al 1979, Liener and Thompson 1980, Chang and Satterlee 1981, Bradbear and Boulter 1984, Deshpande and Nielsen 1987a). The inaccessibility of phaseolin to enzymes has been attributed to certain of its structural properties (Romero and Ryan 1978; Valetraub et al 1976, 1979; Bradbear and Boulter 1984), in particular its compact structure (Chang and Satterlee 1981) and the stability and steric hinderances imparted by its carbohydrate moieties (Chang and Satterlee 1981, Sernino et al 1985).

The degradation of native and heated phaseolin has been studied in vitro by examining the protein banding patterns on SDS-PAGE, and by treating the protein digest with trichloroacetic acid then analyzing the supernatant for free amino groups using trinitrobenzene sulfonic acid. Native phaseolin has been shown to be largely resistant to in vitro hydrolysis by pepsin, trypsin, and chymotrypsin (Vaintraub et al 1976, 1979; Romero and Ryan 1978; Liener and Thompson 1980; Bradbear and Boulter 1984; Deshpande and Nielsen 1987a). Digestion with a combination of these enzymes does little or nothing to overcome the resistance of native phaseolin to hydrolysis (Romero and Ryan 1978, Vaintraub et al 1979, Liener and Thompson 1980). Trypsin more readily degrades native phaseolin than does pepsin or chymotrypsin (Romero and Ryan 1978, Vaintraub et al 1979, Liener and Thompson 1980, Deshpande and Nielsen 1987a). Pepsin hydrolysis of native phaseolin reportedly stops after only 2.4% of the peptide bonds have been cleaved (Vaintraub et al 1979) and causes no change in electrophoretic mobility of the protein (Liener and Thompson 1980, Deshpande and Nielsen 1987a). The low in vitro digestibility of phaseolin by trypsin as compared to trypsin digestion of bovine serum albumin appears to be partially related to the lower concentration of trypsin-susceptible bonds (Romero and Ryan 1978). Vaintraub et al (1976) found that trypsin cleaved only 15% of all the trypsin-hydrolyzable bonds in native phaseolin, whereas this value would be 21% using the data of Romero and Ryan (1978) and the known lysine plus arginine content of phaseolin.

Trypsin and chymotrypsin cleave native phaseolin in such a way that the main part of the molecule remains intact. The major degradation products from trypsin and chymotrypsin digestion of native phaseolin subunits are in the range of M_r 22,000–30,000 (Liener and Thompson 1980, Bradbear and Boulter 1984, Deshpande and Nielsen 1987a). The major degradation products are also of similar size when digested by subtilisin, pronase E, or papain (Deshpande and Nielsen 1987a). The patterns of native phaseolin disappearance and the appearance of degradation products suggest that each subunit is cleaved in a similar position near the center of the subunit (Romero and Ryan 1978, Deshpande and Nielsen 1987a). The hydrolysis of phaseolin by enzymes is similar between cultivars of *P. vulgaris* (Deshpande and Nielsen 1987a). *N*-terminal sequence analysis of the major breakdown products from phaseolin digestion indicates that trypsin, chymotrypsin, and papain all cleave native phaseolin in the same region of the molecule (Fig. 1) (Nielsen et al, *in press*). Such cleavage would produce breakdown products of approximately 24,700 and 21,300 Da, which is consistent with those observed by

SDS-PAGE. The major cleavage sites in native phaseolin for these proteases occur in an extended region of hydrophobic amino acid residues that are predicted to occur on the protein surface and would be accessible to protease action.

While native phaseolin has been shown to be largely resistant to breakdown by pure endopeptidases from the stomach or small intestine (e.g. trypsin, chymotrypsin, pepsin), one report from the literature (Sgarbieri et al 1982) suggests that these pure endopeptidases, either alone or in combination, are poor models for studying the in vivo digestibility of this protein. Nearly 90% of native phaseolin was digested in vitro to small, trichloroacetic acid soluble segments by sequential treatments with stomach and small intestine extracts from rats and with pepsin and pancreatin. Heating this protein for 10 min at 100° C had no effect on the rate or extent of its in vitro proteolysis. Additional experiments with rat or human stomach and small intestine extracts are needed to confirm

TABLE III

Studies Reporting Digestion of Phaseolin and Other *Phaseolus vulgaris* Protein Fractions by Various Proteases and Peptidases

Reference	Cultivar	Protein Studied*	Enzymes Tested In Vitro	Parameters Measured In Vivo
Antunes and Sgarbieri 1980	Rosinha G2	Whole bean flour, Six water-soluble or salt-soluble fractions		Protein efficiency ratio, Digestibility
Brachvar and Souther 1984	Dwarf White	Phaseolin	Aminopeptidase Pepsin Prolidase Pronase Proteinase-K Trypsin	
Chang and Satterlee 1981	Great Northern	*Major storage protein	Combination of bacterial protease, chymotrypsin, peptidase, and trypsin	
Deshpande and Nielsen 1987a	17 Cultivars	Phaseolin	Aminopeptidase Chymotrypsin Pepsin Pepsin Pronase Subtilisin Trypsin	
Deshpande and Nielsen 1987b	11 Cultivars	Whole bean flour, Water-soluble fraction, Salt-soluble fraction	Trypsin	
Lorenz and Thompson 1980	Sanilac	*G1	Chymotrypsin Pepsin Trypsin Chymotrypsin-pepsin-trypsin combination	True digestibility, Weight of pancreas
Marquez and Lajolo 1981	Canoca	Albumin *Globulin G1 Globulin G2 Glutelin	Trypsin Pancreatin Pepsin-pancreatin	
Nielsen 1982	Improved Tendergreen	Phaseolin	Combination of bacterial protease, chymotrypsin, peptidase, and trypsin	
Romero and Ryan 1978	Improved Tendergreen	*G1	Chymotrypsin Pepsin Trypsin Pepsin-chymotrypsin Pepsin-trypsin	
Satter et al 1972	Great Northern	Whole bean flour Albumin Globulin Protein conc. Protein isolate	Combination of chymotrypsin, peptidase, and trypsin	
Small et al 1969	Cranberry	One globulin fraction	Chymotrypsin Ficin Papain Pepsin Subtilisin Trypsin	
Sgarbieri et al 1982	Processor	*Glycoprotein II	Pepsin-pancreatin, or rat stomach extract then rat intestinal contents	Digestibility for whole digestive tract or for stomach plus small intestine
Yamamoto et al 1976		*7S protein	Chymotrypsin Trypsin	
Yamamoto et al 1979		*7S protein	Pepsin Pepsin-trypsin	
Westerman and Jukes 1921	Navy	Phaseolin	Pepsin Trypsin	

* Proteins marked with an asterisk presumably refer to the major storage protein, phaseolin.

these results, because they are not in agreement with other in vitro studies using native phaseolin.

Although native phaseolin is very resistant to hydrolysis by individual treatment with trypsin, chymotrypsin, or pepsin, the hydrolysis is greatly increased by heat treatment (Romero and Ryan 1973, Liener and Thompson 1980, Deshpande and Nielsen 1987a). Vaintraub et al (1979) found that phaseolin is completely digested by pepsin only after denaturation by heat treatment or the action of guanidine hydrochloride. Heated phaseolin is more or less completely digested by trypsin (Bradbear and Boulter 1984, Deshpande and Nielsen 1987a). After heating, the degree of digestion by trypsin and chymotrypsin has been shown to approach that of casein, and actually exceed it for pepsin (Liener and Thompson 1980). The increases in hydrolysis by each enzyme caused by heat treatment of phaseolin have been shown to be substantially larger in than the increases seen after heat treatment of bovine serum albumin (Romero and Ryan 1973).

The digestibilities of native and heated phaseolin can be compared between various in vitro and in vivo studies. Bradbear and Boulter (1984) compared the yield of amino acids from phaseolin preparations after complete acid hydrolysis to that after enzymatic hydrolysis with a combination of pepsin, prolidase, and aminopeptidase M. The mean recovery of amino acids was 91 and 13% after enzymatic hydrolysis of heated phaseolin and native phaseolin, respectively. The in vitro digestibility of native and heated phaseolin also has been determined by two groups according to the four-enzyme procedure of Satterlee et al (1979), which utilizes trypsin, chymotrypsin, peptidase, and bacterial protease. Chang and Satterlee (1981) reported values of 74 and 91% for native and heated protein, respectively, which are very similar to the values of 79 and 92% reported by Nielsen (1982). Liener and Thompson (1980) found the true digestibility in rats in a four-week study to be 57% for native phaseolin and 92% after heat treatment (diet not supplemented with methionine). When Sgarbieri et al (1982) fed native phaseolin to previously fasted rats in a single dose by intubation, the digestibility was between 80 and 90%. However there was also increased production of insoluble intestinal secretions containing relatively large amounts of nitrogen. Because this nitrogen passed into the caecum, it reduced the apparent digestibility of native phaseolin to about 35%.

The *in vivo* and *in vitro* digestibility values reported above are similar for heated phaseolin, but results are not in agreement for native phaseolin. The large differences in in vitro digestibility values reported for native phaseolin are likely attributed to the method of measuring protein breakdown and the assay conditions. While Bradbear and Boulter (1984) apparently used the ninhydrin reagent to measure free amino groups after enzymatic hydrolysis, the four-enzyme procedure of Satterlee et al (1979) uses the change in pH due to enzymatic hydrolysis to calculate the digestibility. Both in vitro methods utilize not only endopeptidases, but also exopeptidases, which should more closely simulate the conditions required for complete digestion to the point at which they can be utilized by humans. However, the proteases used and time and temperature conditions differ between the two assays. The inconsistencies between in vivo digestibility results for native phaseolin can be explained at least partially by the differences between a four-week study and a single-dose treatment. While many in vitro and in vivo tests work well to estimate the nutritive value of heat-treated and high-quality proteins, all tests have their disadvantages when testing unheated and low-quality proteins.

Residue #	234	235	236	237	238	239	240	241	242
Amin. Acid	Ser	Arg	Lys	Ser	Leu	Ser	Lys	Gln	Asp
		↑				↑			
		Trypsin				Chymotrypsin			
						Peptic			

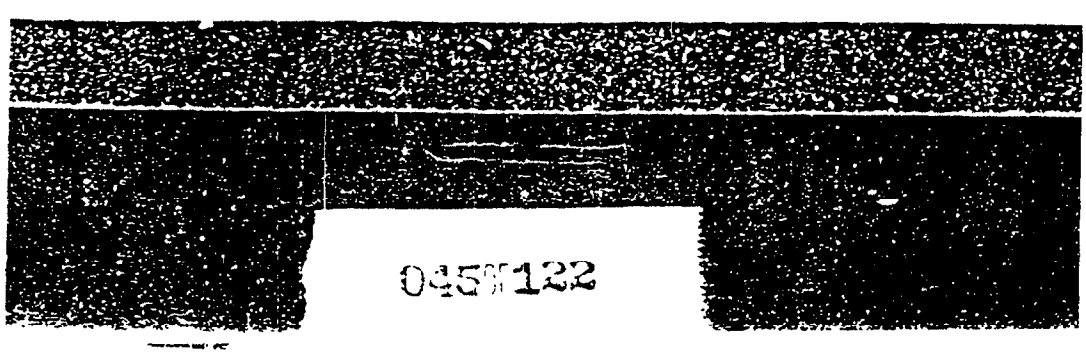
Fig. 1. Region of the phaseolin amino acid sequence cleaved when trypsin, chymotrypsin, or pepsin are reacted with native phaseolin. Phaseolin sequence used is that derived from the nucleotide sequence reported by Slightom et al (1983).

Digestibility of Protein Fractions

In addition to the studies with pure phaseolin, several studies have examined the in vitro and in vivo digestibilities of various *P. vulgaris* fractions (Table III). Antunes and Sgarbieri (1980) found that unheated bean flour and six isolated fractions were toxic when fed to weanling rats, but heat treatment improved the nutritive value of all samples, including albumin and globulin fractions. In a study by Sathe et al (1982), the in vitro digestibility of unheated bean flour, albumins, globulins, protein concentrates, and protein isolates was 9, 3, 10, 14, and 29%, respectively. Dry heat increased the digestibility to 69, 24, 30, 24, and 34%, respectively, but the values were raised even higher by moist heat. Marquez and Lajolo (1981) found that heating improved the in vitro digestibilities of globulin and glutelin fractions. However, unlike the albumin results of the two previously cited studies, Marquez and Lajolo (1981) found that heat treatment reduced the digestibility of the albumin fraction. They found that peptides with *M*, of 14,000 and 20,000 remained in the residue after digestion of autoclaved albumin and heating caused the appearance of high molecular weight aggregates. These researchers reported evidence for a relatively heat-stable trypsin inhibitor in the albumin fraction, which may have affected its digestibility.

Deshpande and Nielsen (1987b) also obtained evidence that protein-protein interactions and heat-stable inhibitors influence the digestibility of proteins in the albumin fraction, especially in certain bean varieties. The salt-soluble protein fractions of 11 *P. vulgaris* cultivars tested were more readily and completely hydrolyzed by trypsin than were the water-soluble proteins. Phaseolin was highly susceptible to trypsin when present in the salt-soluble fractions but was less readily hydrolyzed in the presence of certain water-soluble proteins. Phaseolin, present as a contaminant in the water-soluble fraction of Light Red Kidney, Pinto, Black Beauty, Small White, Small Red, and Great Northern beans, was much less hydrolyzed by trypsin compared to the remaining varieties investigated. Further work with the Great Northern bean albumin fraction showed that it contained heat-stable trypsin and chymotrypsin inhibitory activity. Like Marquez and Lajolo (1981), Deshpande and Nielsen (1987b) observed that some proteins in the heat-treated, water-soluble fraction failed to enter the gel, suggesting that protein aggregates of very high molecular weight were formed during heating and were not degraded by trypsin.

To summarize the literature reports examining the susceptibility of phaseolin and other bean protein fractions to mammalian digestive enzymes, numerous studies show that native phaseolin is quite resistant to proteolysis. The size of the degradation products from native phaseolin suggests that, with the likely exception of some potential cleavage sites for endopeptidases near the amino or carboxy terminal regions, only a small segment near the center of each subunit is accessible to proteases such as trypsin and chymotrypsin. Discrepancies in digestibility values exist between and among various in vitro and in vivo studies, but assay conditions vary considerably between many of these studies. However, upon heat treatment of phaseolin most in vitro and in vivo studies have shown drastic improvements in digestibility. The in vitro digestibility of the bean globulin fraction is increased by heat treatment, but data are conflicting for the effect of heat treatment on the digestibility of the albumin fraction. The high molecular weight aggregates formed when the albumin fraction is heated may not be readily degraded by mammalian digestive proteases. Further in vitro and in vivo studies are necessary to better understand the digestion of albumin proteins and their influence on the breakdown of other bean proteins. Heat-stable trypsin-chymotrypsin inhibitory activity has been detected in the albumin fraction of some *P. vulgaris* cultivars. It seems unlikely that such inhibitors are present in large enough quantities to cause any negative nutritional effects to humans under normal dietary conditions, but their effects have not yet been tested in any in vivo studies.



SUMMARY

The proteins of *P. vulgaris* are subjected to degradation by proteinases formed in the germinating cotyledons, the insect gut, and the human digestive system. The germinating bean cotyledon and the bean weevil gut both contain a cysteine proteinase with an acidic pH optimum. These proteinases seem to cleave native phaseolin at a site near the middle of the polypeptide chain. In both systems, other enzymes must be present to further degrade this major storage protein to peptides and amino acids that can be utilized for growth and development of the plant or insect. These other enzymes have not been clearly identified or characterized with regard to their role in protein degradation. Unlike germinating cotyledons and insects, humans do not utilize a cysteine proteinase in their digestive system. Humans also have the easier task of degrading heat-denatured protein rather than native protein. The serine proteinases, trypsin and chymotrypsin, cleave native phaseolin subunits in the same region of the amino acid sequence as does the thiol proteinase papain. Breakdown patterns of native phaseolin reacted with the cysteine proteinases of the germinated bean cotyledon or the gut of larval bean weevil suggest that they cleave phaseolin in the same regions near the center of each protein subunit. Heated phaseolin is readily hydrolyzed by mammalian digestive proteases to small molecular weight peptides. While the salt-soluble fraction of bean proteins appears to be readily digested upon heat treatment, there are still some questions about the effect of heat denaturation on the digestibility of the water-soluble fraction. The albumin proteins require further study to determine how they affect the digestibility of other bean proteins. Changes in the structure of proteins in the albumin fraction upon heat treatment and their susceptibility to mammalian digestive proteases also need further investigation.

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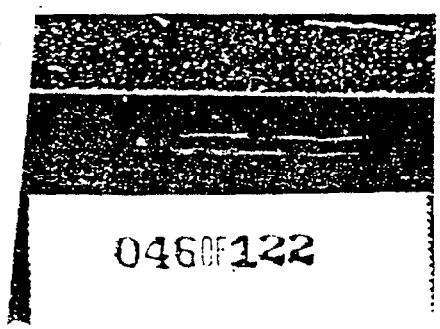

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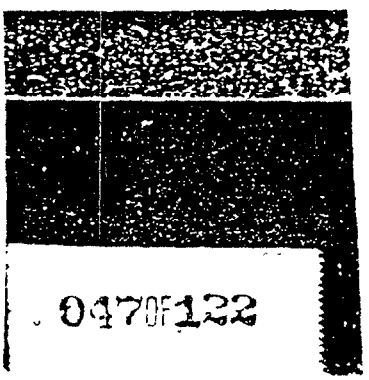

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Composition and Digestibility of Albumin, Globulins, and Glutelins from *Phaseolus vulgaris*

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Fifteen Brazilian varieties of *Phaseolus vulgaris* were tested for digestibility in vitro, trypsin inhibitor, and protein content. Four varieties with extreme digestibility values were assayed in rats and showed similar digestibilities in vivo. The protein from the Carioca variety fractionated for detailed studies yielded the following: albumin, 31.5% (richest in sulfur amino acids and trypsin inhibitors); globulin G_1, 38.5%; globulin G_2, 13.8%; glutelin, 22.4%. The in vitro digestibilities of the unheated globulins and glutelins were low but improved by heating. The albumins were well digested in the raw state but after heating digestibility dropped; the effect was pH dependent. The residue left after digestion of autoclaved albumin contained peptides with molecular weights of 14 000 and 20 000. Evidences on a relatively heat stable trypsin inhibitor in the albumin fraction are presented. The extent of digestion of the four fractions was tested by using either trypsin, pancreatin, or pepsin–pancreatin.

Beans (*Phaseolus vulgaris*) are an important source of protein in Brazil where they are usually consumed together with rice. However, several biochemical problems limit the optimal biological utilization of the amino acids (Kakade, 1974; Bressani and Elias, 1979).

The low digestibility of bean protein has been documented, but the reasons for it are not well understood and are probably due to a combination of factors. Improper storage at high relative humidities is known to increase cooking time and to reduce the protein digestibility and the biological utilization of bean amino acids (Molina et al., 1975; Antunes and Sgarbieri, 1979). Excessive heating to inactivate antinutritional factors is also prejudicial to the digestibility and amino acid availability; processes such as dry roasting and extrusion cooking are better for maintaining the biological value of beans (Molina et al., 1975; Yadav and Liener, 1978). Antiphysiological factors such as hemagglutinins and trypsin inhibitors are inactivated by proper heat treatment and can probably be excluded as a cause. The exception may be the heat-stable protease (enzyme) inhibitors which seem to be phenolic in nature and present in the seed coat of some colored beans (Elias et al., 1979).

Protein-complexing substances such as tannins appear to be partly implicated. They can either be extracted in the cooking water or migrate to the center of the cotyledon, thus reducing digestibility directly by reacting with the proteins or indirectly by inactivating digestive enzymes. The low biological value of the cooking broth has been attributed to the influence of these phenolic pigments (Elias et al., 1979; Mondragon and Gonzales, 1978).

Little is known about the influence of the protein itself. Seidl et al. (1969) isolated a globulin from kidney beans which was resistant even after heating to digestion by 10 different proteolytic enzymes, and Romero and Ryan (1978) also observed low digestibility of an isolated G_1 globulin when compared to that of denaturated bovine albumin. The beneficial effect of denaturation was also recently observed by Liener and Thompson (1980), who studied the digestibility of the G_1 fraction both in vitro and with rats. Evans et al. (1974) and Sgarbieri et al. (1979) observed reduced availability of the sulfur amino acids in rats fed autoclaved beans. Evans and Bauer (1978) also indicated the existence of a dialyzable toxic compound in the soluble fraction of the cooking broth.

This paper reports research on the composition and digestibility behavior of different bean protein fractions and the effect of heat on them. Results indicating the existence of a heat-stable trypsin inhibitor are also reported.

EXPERIMENTAL SECTION

Materials. Beans (*P. vulgaris*) of different varieties were obtained from the Agronomy School of Lavras. Bovine trypsin twice crystallized (Type III; 10 000 BAEE units/mg), pepsin of hog stomach (twice crystallized, 2500 units/mg), pancreatin from hog pancreas (Grade VI), and Pronase (Type VI) were purchased from Sigma Chemical Co. All other compounds were reagent grade. Deionized

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and glass-distilled water was used.

Methods. Extraction and Fractionations of the Proteins. The whole beans ground to a flour in a laboratory mill to pass a 0.297-mm screen were fractionated as described below. So that the albumins could be obtained, a 20% suspension of the flour in water was extracted for 1 h under agitation at room temperature, filtered through a cheesecloth, and centrifuged at 30000*g* for 30 min. The supernatant was dialyzed for 48 h against distilled water, followed by a 24-h dialysis against deionized water at 4 °C. This last step eliminated most of the contaminating globulins.

The globulins were extracted and separated essentially as described by McLeester et al. (1973). Ten grams of the flour was extracted with 100 mL of a 0.5 N NaCl–0.25 M ascorbic acid solution (pH 2.2) for 1 h at room temperature. The supernatant obtained after centrifugation of the extract (23000*g*, 30 min) was diluted 5 times with deionized water to reduce the ionic strength to 0.08 M and to precipitate the G_1 globulin which was then collected by centrifugation at 23000*g* for 30 min at 0–4 °C. The supernatant left was again dialyzed at 0–4 °C for 24 h to precipitate the G_2 fraction which was collected in the same way.

The glutelin and prolamin were obtained from the residue left after the NaCl–ascorbic acid solution extraction. Ten grams of the residue (dry weight) was extracted with 50 mL of 70% ethanol to separate the prolamin. The residue was reextracted twice with 0.1 N NaOH for 1 h at room temperature. The extracts were centrifuged at 23000*g*, and the combined supernatants, dialyzed and freeze-dried, represented the glutelin fraction. For the preparation of the glutelin, beans were used which had the seed coat previously removed: the seeds were soaked for 8 h in water (1:1.5 beans to water ratio) to facilitate the operation and then were dried at 45 °C in a circulating air oven and ground to a flour as previously described.

Digestibility Tests. Digestibility in vitro was studied by using trypsin, pancreatin, or pepsin–pancreatin according to the experiment.

For trypsin or pancreatin hydrolysis, a dispersion of the proteins under study containing 5 mg of protein/mL in 0.06 M phosphate buffer (pH 7.0) was incubated at 37 °C with a solution of the enzyme in 10^{-3} N HCl. The enzyme to substrate ratio was maintained at 1:40. At different incubation intervals, aliquots where taken, Cl_3AcOH (5% final concentration) was added, and after standing 1 h, the undigested material that precipitated out was separated at 23000*g* for 30 min.

Split peptide links were evaluated in the supernatant by measuring the α-amino nitrogen produced by the ninhydrin reaction according to Spies (1957) and using leucine as the standard.

For the pepsin–pancreatin sequential hydrolysis, the Akeson and Stahmann (1964) technique was used with a 2 mg/mL protein concentration and an enzyme to substrate ratio of 1:40. The digestion time for the pepsin (first enzyme) was 3 h, followed by the pancreatin digestion for 3 h more. Liberation of the α-amino nitrogen was evaluated as described above. Preliminary testing indicated that more prolonged digestion time did not significantly enhance the amount hydrolyzed.

Digestibility was expressed as milligrams of leucine produced per gram of protein and was compared to Hammerstein soluble casein which was used as a control.

The screening test for digestibility of the different bean varieties (Table I) was done on beans previously heated in water (2:10 bean to water ratio) for 30 min (time needed for softening). The resulting material, homogenized with the cooking broth and properly diluted, was digested as described above.

When digestibility of unheated proteins was tested, the trypsin inhibitor was previously complexed by titration with trypsin, and after 10 min to allow complete coupling, the hydrolysis was started by adding the enzyme solution.

Digestibility in vivo was evaluated in young rats fed twice daily a diet containing 10% of the protein under study following a restriction technique used by Kakade and Evans (1966). The feces were collected each day over a period of 5 days, pooled, and analyzed for N by the micro-Kjeldahl technique.

Analytical Techniques. Protein concentration was determined by either the micro-Kjeldahl (AOAC, 1970) or Lowry (Lowry et al., 1951) procedures.

Trypsin inhibitor activity was measured according to Kakade et al. (1969b); the protein-bound sugars were measured by the phenol-sulfuric acid method of Dubois et al. (1956) after precipitation of the protein with 10% Cl_3AcOH. Electrophoreses in polyacrylamide gel were run according to Davies (1964); both acrylamide and bis(acrylamide) were recrystallized (Schuster, 1971). Monomer concentration varied from 5 to 10% according to the experiment. The runs were conducted at 3 mA/tube with Tris–glycine buffer (pH 8.3), using bromphenol blue as the front marker, and the protein bands were visualized with Coomassie brilliant blue. Molecular weight distribution of proteins before and after digestion was estimated by electrophoresis in 1% $NaDodSO_4$ (Weber and Osborn, 1969). The runs was at 7 mA/tube, and cytochrome *c* (M_r = 12500), trypsin (M_r = 23300), aldolase (M_r = 40000), ovalbumin (M_r = 45000), and bovine serum albumin (M_r = 67000) were used as standards.

Amino acid analysis was done in a Beckman 120 °C analyzer with the standard resin (Moore and Stein, 1963). Sulfur amino acid was determined as cysteic acid and methionine sulfone by oxidation of the samples with performic acids before acid hydrolysis. For tryptophan determination, the Spies and Chambers (1949) technique was used.

RESULTS AND DISCUSSION

Protein Contents and Digestibility of Different Bean Varieties. Table I provides the results of the initial screening tests of 15 Brazilian varieties of *P. vulgaris* for total and soluble protein contents, in vitro digestibility by pepsin–pancreatin, and trypsin inhibitor (TI) activity. These results are in agreement with previous published data (Moraes and Angelucci, 1971; Sgarbieri et al., 1979). The Kjeldahl measurement of total protein ($N \times 6.25$) ranged from 17.4 to 27.4% while the salt-soluble extractable protein was lower, reaching in one case (Pintado variety) only 62% of the total measured protein. It is interesting to note that heating decreased the solubility of the salt-soluble fraction by different amounts, depending on the variety of bean. The use of polyvinylpyrrolidone, a phenol-complexing agent, or the previous removal of the pigmented seed coat did not change the yield of extraction or lability to heat.

The digestibility recorded varied from 17 to 40% of the maximum theoretically possible obtained after acid hydrolysis, (with the variety exhibiting the most digestion (Rosinha G-2) reaching only 50% of the value of casein (casein digestion was ~82% of the theoretical). Also worth noting is the large variation in TI content, ranging from 4.4 to 13.9 g/kg of bean.

No correlation could be established between digestibility and soluble proteins or trypsin inhibitor content. However,

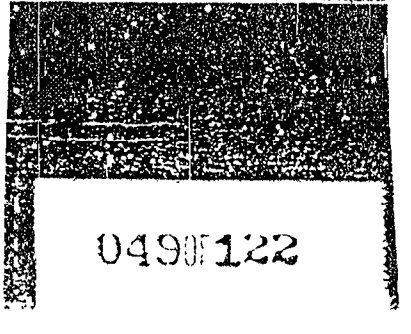


Table I. Total and Salt-Soluble Protein Content, Percent Digestion by Pepsin-Pancreatin and Trypsin Inhibitor Activity in Brazilian Varieties of *P. vulgaris*

variety	protein, % total	protein, % soluble[a] unheated	protein, % soluble[a] heated	digestibility,[b] %	TI, g/kg
Venez wila 350	21.1	15.3	13.6	28	8.6
Rico 23	27.4	19.9	12.6	31	7.4
Mantegão fosco	21.5	20.0	11.7	26	4.4
Rico pardo	20.7	16.8	10.0	25	13.9
Jalo	19.8	17.3	12.8	27	12.0
Lerma	17.8	15.9	11.0	29	12.2
Carioca	17.6	16.3	10.0	21	6.8
Pintado	20.8	12.9	8.9	17	10.0
Purutu	20.4	15.2	11.3	23	9.0
Rosinha G-2	20.3	16.8	11.0	40	7.0
Costa Rica	20.3	15.6	10.7	33	10.5
Bico de ouro	17.4	14.8	12.8		8.4
Pirata 1	21.1	18.3	9.9	24	11.3
Rosinha	21.0	17.4	12.6	23	6.3
Mulatinho precoce	20.8	15.4	11.0	28	9.0
casein	nd[c]	nd[c]	nd[c]	82	nd[c]

[a] Soluble in 0.85% NaCl solution. Heated at 121 °C for 30 min. [b] Percent of the total peptide bonds split. The total peptide bonds were measured with ninhidrin after acid hydrolysis by boiling under reflux with 6 N HCl; the hydrolysate was rotary evaporated at 40 °C to dry ness, washed twice, and dissolved in 0.2 M citrate buffer, pH 2.2. [c] nd, not determined.

Table II. Digestibility of the Protein of Four Bean Varieties for Rats[a]

variety	rat weight,[b] g initial	rat weight,[b] g final	feed consumption, g/day	digestibility, %
casein	61.9	78.3	7.8	88
Rosinha G-2	64.6	80.7	5.1	69
Carioca	63.3	83.8	5.7	71
Rosinha	60.1	60.5	5.4	70
Rico 23	61.0	61.1	5.6	72

[a] Beans heated for 30 min at 121 °C in a water to bean ratio of 10:2. [b] Period of 5 days.

the lower values were found among the red-brown beans, a fact already observed by Elias et al. (1979), who suggested that this might be caused by the higher tannin content of those varieties.

Four of the fifteen varieties tested in vitro were selected to be studied in vivo with rats. Unlike the observed differences in digestibility among the varieties in vitro, Table II demonstrates the similarities in digestibility in rats (~70%). This was probably due to the restricted feeding technique used—the rats were fed twice a day for 1-h periods. In these extreme conditions, digestibility was still lower than that for casein which was 88% absorbed.

Isolation and Partial Characterization of the Major Protein Fraction of the Carioca Variety. For identification of a specific protein component which might be responsible for the low digestibility observed in the varieties studied, five major protein fractions, albumin, globulins G_1 and G_2, prolamins, and glutelins were isolated from the Carioca bean. The NaCl-ascorbate system of McLeester et al. (1973) was used for the fractionation because it reduces the cross contamination of globulins with the albumins (only the acid-soluble albumins are coextracted) and also because it allows for easy separation of G_1 and G_2 by a simple reduction of the ionic strength, thus avoiding the need for prolonged dialysis.

Table III shows the yield obtained for each fraction, the bound carbohydrate, and the trypsin inhibitor contents, and Table IV provides data on the amino acid compositions.

The carbohydrate contents varied according to the fraction. Results presented in Table III on the G_2 globulin are comparable to those obtained by Pusztai (1966) in the vicilin of *P. vulgaris*, while the values for the albumin and total globulins are similar to values reported by Satterlee et al. (1975) in the great Northern beans. The presence of glycoproteins was confirmed in all fractions and observed in most of the bands obtained by electrophoresis by specific staining with fuchsine–sulfite after periodic acid oxidation. The presence of bound carbohydrates is important as a possible barrier to enzyme action and also because reactions such as β-elimination can occur during processing. This is especially true in alkaline media (Whitaker and Feeney, 1977) which is sometimes used in the preparation of beans to soften the seed coats and shorten the cooking time.

The trypsin inhibitors were primarily extracted with the albumins (73% of the total) although the glutelins still had a significant portion (14% of the total). The seed coats of the variety used did not show a significant inhibitory action.

The first nutritional limiting factor of bean proteins is the low content of sulfur amino acids. The albumins and glutelins are the best sources of those amino acids while both globulins, G_1 and G_2, have negligible amounts (Table IV). The negligible content of cysteine in the G_1 globulin was also observed by Millerd et al. (1971) in *Vicia faba* and Padhye and Salunkhe (1979) in the black gram. The

Table III. Yield, Carbohydrate Content, and Trypsin Inhibitor Activity of Protein Fractions Isolated from Carioca Bean

	albumins	globulins G_1	globulins G_2	glutelins	prolamins
yield, %[a]	31.5	38.1	13.8	22.4	1.7
carbohydrate, %[b]	8.0	11.7	13.8	nd[c]	nd[c]
TI, %[b]	6.1	0.4	0.7	1.2	nd[c]

[a] Expressed as percent of the total protein of the bean (the bean contained 17.6% protein). [b] Expressed as percent of the fraction. [c] nd, not determined.

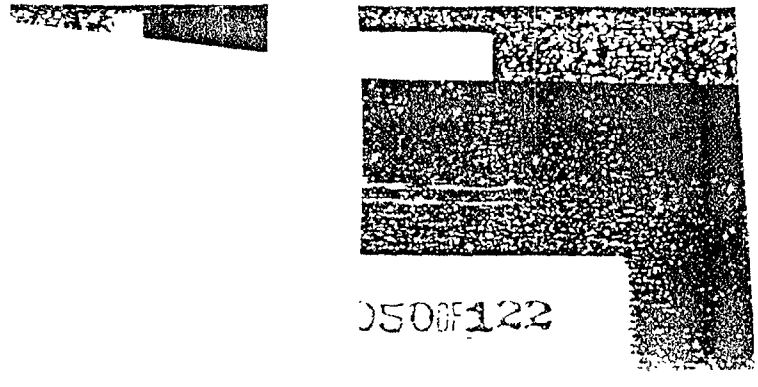


Table IV. Amino Acid Composition of the Albumins, Globulins (G_1 and G_2) and Glutelins[a] Isolated from Carioca Bean

amino	albumins	globulins G_1	globulins G_2	glutelins
Lys	8.6	6.8	6.4	9.5
Hys	2.8	3.0	1.8	3.8
Arg	5.9	5.7	3.5	6.3
Asp	14.1	12.8	13.4	10.7
Thr	7.3	3.2	7.2	5.3
Ser	7.1	6.4	8.2	8.0
Glu	13.1	19.0	12.2	10.0
Pro	4.8	3.8	4.2	0.3
Gly	4.3	3.6	4.3	8.5
Ala	4.7	3.3	4.4	7.5
Cys	3.9	0.0	t[b]	0.0
Val	7.7	6.6	7.7	7.1
Met	0.6	0.3	0.0	0.7
Iso	4.6	4.7	4.9	5.7
Leu	7.3	11.3	11.4	9.3
Tyr	2.8	3.2	3.3	3.9
Phe	6.7	6.7	6.7	2.9
Try	2.6	0.3	0.6	nd[c]

[a] Grams of amino acid/100 g of protein. [b] t, traces. [c] nd, not determined.

Table V. Inactivation of Trypsin Inhibitors as a Function of pH[a]

pH	residual act., %: crude extract,[a] boiled	albumins: boiled[a]	albumins: autoclaved[b]
control (unheated)	100	100	100
1.8	75 (74)	77	78
3.0	56	78	42
5.0	52	91	36
7.0	25 (21)	83	18
8.5	15	81	10

[a] 1% protein suspensions were heated for 30 min in a boiling water bath at the indicated pHs. Numbers in parentheses were obtained after 1 h of boiling. (Unheated controls were held at the same pH as heated samples.) [b] Autoclaved 30 min at 121 °C.

chemical scores (CS) for sulfur amino acids, as calculated in relation to egg protein, were 0.7 for albumin, 0.05 for G_1, 0.0 for G_2, and 0.12 for the glutelins.

The second limiting factor varied according to the fraction, it was leucine/isoleucine for albumin (CS = 0.8), threonine for G_1 (CS = 0.68), histidine for G_2 (CS = 0.8), and the aromatic amino acids for the glutelins (CS = 0.7). It should also be noted that histidine is in high excess in the glutelins (CS = 1.73). From the nutritional point of view, it appears that the best fraction is albumin, followed by glutelins, although more information is needed on the availability of the amino acids.

Inactivations of the Trypsin Inhibitors. It has already been shown (Table III) that the greater amount of trypsin inhibitor activity is associated with the albumin fraction. We also observed that the TI demonstrated great dependence of pH for inactivation and appears to be relatively heat resistant (Table V). Even after boiling crude extracts containing albumins and globulins for 1 h at pH 1.8, 75% of the original TI activity was still present. However, when the pH was near neutrality, boiling for 30 min resulted in the lowering of activity to 25%, and at the end of 1 h, the activity was measured at 21%. In the case of albumin fractions, boiling eliminated only 20–25% of the activity, depending on the pH. A heat-resistant TI that resisted boiling for 2 h was described by Puztai (1968) in *P. vulgaris* and by Warsy and Stein (1973) in *V. faba*.

It was observed when preparing the controls that the simple raise of pH before any heating enhances the activity of the TI: at pH 3.0, the TI content was 200 μg/g of albumin, at pH 5.0 it was 250 μg/g, and at pH 9.0, it was 300 μg/g. This observation aids in explaining why there was a higher retention of TI activity for boiled albumin when compared to that of the crude extract; i.e., it was in part actually due to an initial activation by some unknown factor related to pH. On the other hand, heating the crude extract also helped to accelerate inactivation, probably because of the interaction between other components. Only autoclaving at 121 °C for 30 min was able to reduce the TI content of the albumin fraction to the extent of resembling that accomplished by boiling of the crude extract.

In relation to the above, it is interesting to refer the paper of Ellenrieder et al. (1980), who observed higher thermal stability of the soybean TI when aqueous suspensions were diluted, they explained the fact by the presence of a high molecular weight compound which could accelerate the thermal inactivation.

These results are significant when considering industrial or home processing techniques and the production of isolated proteins in relation to the variable content of TI in different bean varieties. Curiously, the dependence of TI inactivation on pH in beans is reversed to that observed in soybean in which the lability is higher at acidic pHs (Boonvisut and Whitaker, 1976).

Digestibility of the Separated Fractions. *Digestion with Trypsin.* The results are depicted in Figures 1 and 2 and are compared to those for casein, considered an easily digested protein.

When digested with trypsin, the raw globulins showed a reduced digestibility in relation to that of casein (12% for G_1 and 35% for G_2), while the unheated albumins were well digested, reaching 81% of the value observed for casein; only the initial velocity of hydrolysis in albumin was slower, as the shape of the curve indicates (Figure 1). Since the trypsin inhibitor had been previously titrated, the low hydrolysis in the globulins may be attributed to their structural conformations, a barrier which does not exist in albumins.

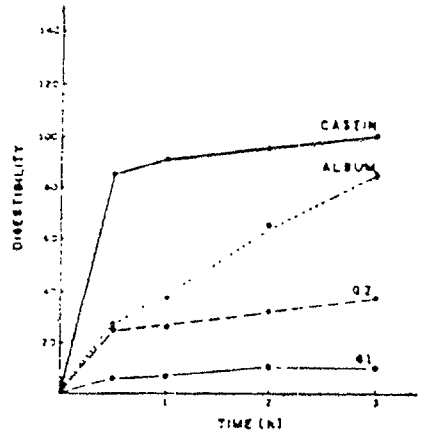


Figure 1. Digestibility (milligrams of leucine per gram of protein) by trypsin of unheated albumins, globulins, and casein. Trypsin inhibitor was previously titrated.

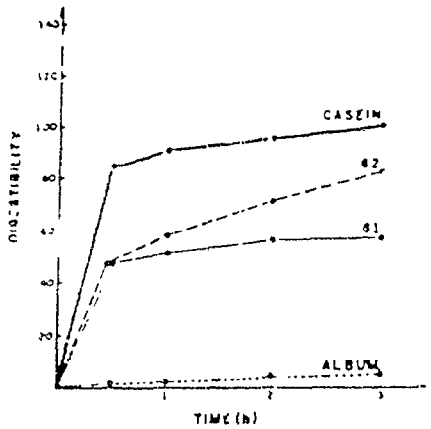


Figure 2. Digestibility (milligrams of leucine per gram of protein) by trypsin of albumin and globulins boiled for 30 min. Residual trypsin inhibitor was previously titrated.

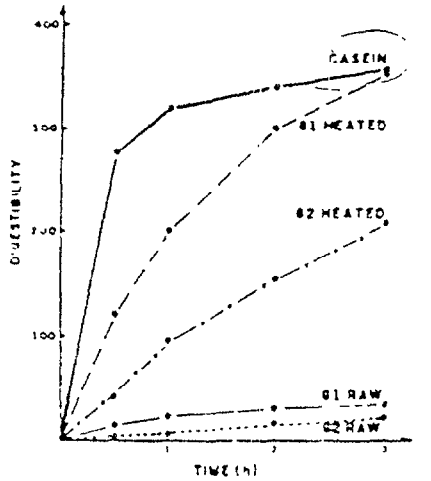


Figure 3. Digestibility (milligrams of leucine per gram of protein) of the globulins by pancreatin either boiled for 30 min or not boiled.

Heating the fractions (Figure 3) substantially increased the hydrolysis of G_1 and G_2 which reached respectively 56 and 82% of the casein value. Surprisingly, heating reduced the digestibility of albumins by trypsin from 81 to 8%. This may be explained by a steric impediment or a blockage of amino acid residues needed for enzyme action, though the possibility of participation by some phenol or quinone, free or complexed with the protein, cannot be excluded. Similar effect of heating on the G_1 globulin was observed by Liener and Thompson (1980), either in vitro or in vivo.

Digestion by Pancreatin and Pepsin-Pancreatin. The results are presented in Figures 3 and 4. The unheated globulins showed reduced hydrolysis (10% for G_1 and 7% for G_2) in relation to casein hydrolysis which was considered as 100%. This apparent reduction in the percentage of digestibility of G_1 and G_2 when compared to the previous results obtained with trypsin (Figure 1) is actually due to an absolute increase from 100 to 360 mg of leucine/g of protein of the number of split peptide bonds in casein (Figure 3). The thermal treatment enhanced the digestibility of G_1 to a level approximating that of casein (Figure 3) and G_2 to a lesser extent (59%).

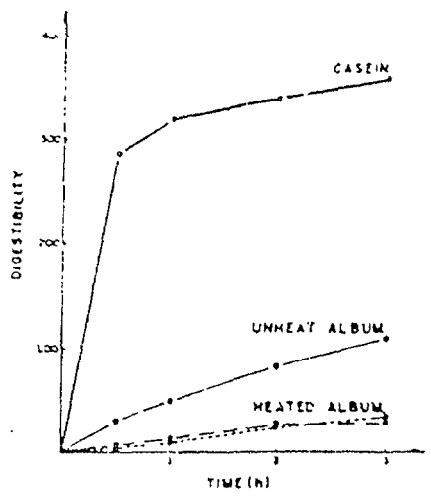


Figure 4. Digestibility of the albumins either raw or heated (boiling for 30 min) by pancreatin.

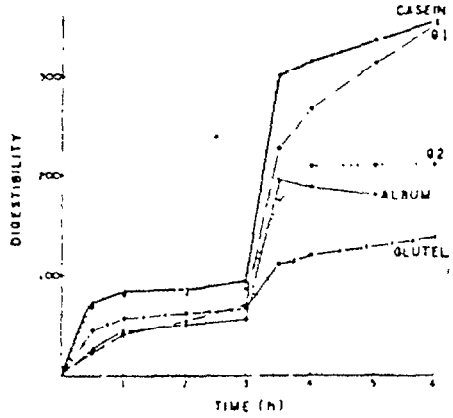


Figure 5. Digestibility of the autoclaved fractions submitted to pepsin-pancreatin.

These higher values achieved with pancreatin can be attributed to the presence of chymotrypsin and carboxypeptidase activity in the pancreatin, in addition to the presence of trypsin which was previously tested alone. The globulin G_1 fraction is a very well digested protein after denaturation. In the case of albumins, heating causes a reduction in hydrolysis which the attempted digestion by pancreatin did not alter. Pronase was also tested, but it

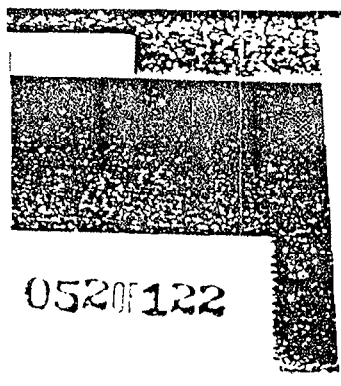


Table VI. Digestion of Heat-Treated Albumins as a Function of pH by Different Protease Systems

	digestibility, %[a]			
	boiled (30 min)	autoclaved (121 °C/30 min)		
pH	trypsin	trypsin	pancreatin	pepsin-pancreatin
1.8	33	27	nd[c]	58
4.0	nd[c]	nd[c]	9	
6.0	5	18	nd[c]	nd[c]
7.0	nd[c]	28	10	52
9.0	52	82	nd[c]	nd[c]
10.0	nd[c]	46	51	88
raw albumin[b]	82	82	27	100
casein[b]	100	100	350	350

[a] Expressed as percent of the hydrolysis observed for casein as shown in the bottom of the table. [b] Hydrolysis observed for casein expressed as milligrams of leucine per gram of casein and considered as 100% for the calculation of percent digestion of the albumins. [c] nd, not determined.

did not significantly alter the digestibility of the heated albumins.

Figure 5 shows data for the four fractions, including the glutelins, autoclaved at 121 °C for 30 min and subjected to pepsin–pancreatin sequential action. The globulin G_1 was again the best digested protein, equal to casein after 6 h of incubation. Measurements at 4 and 5 h recorded decreased rates of hydrolysis relative to those of casein, but it is difficult to assess what this represents in terms of velocity of liberation and absorption in vivo. The digestibility figures for the unheated albumins (not shown in Figure 5) expressed in milligrams of leucine per gram of protein were with pepsin: 40, 60, 75, and 80, respectively, after 0.5, 1, 2, and 3 h, following the addition of pancreatin the figures were 310, 320, 340, and 340, respectively after 0.5, 1, 2, and 3 h. The albumin had the percent of digestion increased by the pepsin treatment to ~52%, but it was still much lower than that for the raw state. The total digestions, at 6 h for the other proteins relative to that for casein were 60 and 40% for G_1 and glutelin, respectively.

The pH of the protein suspension during the thermal treatment had a marked effect on the influence of heating on digestion of albumins (Table VI). At pHs between 4 and 7, the digestibility fell, indicating that the effect of heating is dependent on the ionization of amino acid residues. It was suspected that this was related to cross-links involving amino acid groups of lysine or asparagine and glutamine residues. In fact, as was observed by electrophoresis (Figure 6), heating causes the appearance of high molecular weight aggregates which did not penetrate the gel even at low (5%) acrylamide concentration, and only at more alkaline pHs did smaller fragments appear. At higher pHs, fragmentation occurred, and the smaller aggregates produced help to explain the observed increased digestibility (see Table VI).

It should also be noted in Table VI that the pancreatin action increased the digestion of casein from 100 mg of leucine/g recorded with trypsin to 350 mg to leucine/g to protein but that albumin remained at the same level (~90 mg of leucine/g of protein) until pepsin was used in sequence with pancreatin.

Since the pepsin–pancreatin technique demonstrates a good correlation with in vivo digestibility (Saunders et al., 1973), it would be expected that rats would show similar results. In fact, calculating the percentage of each fraction digested by pepsin–pancreatin as a part of the total bean protein and in relation to casein resulted in a total digestion of 70% of the protein, which is in agreement with the rat assay (Table II) that gave a similar relative figure.

Figure 6. Electrophoretic behavior of albumins (5% polyacrylamide). From left to right: unheated albumins followed by albumin heated respectively at pH 7.0, 8.0, and 9.0 in boiling water.

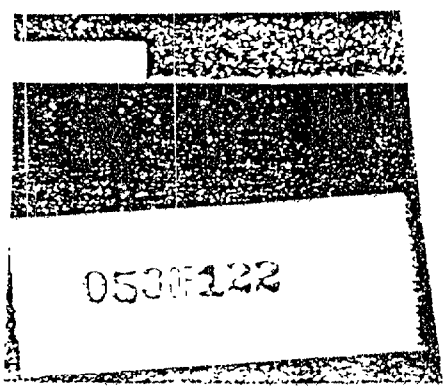
Table VII. Digestibility of Each Amino Acid of the Autoclaved Albumin Fraction by Treatment with Pepsin–Pancreatin

amino acid	albumin[a] autoclaved	soluble fraction after digestion	digestibility, %
Lys	8.8	10.5	56.0
His	2.8	2.1	37.0
Arg	5.9	7.1	56.0
Asp	12.8	13.6	44.0
Thr	7.4	7.9	50.0
Ser	6.9	7.2	46.1
Glu	8.5	11.4	39.7
Pro	4.0	4.9	45.8
Gly	3.7	5.1	53.5
Ala	4.3	6.7	55.3
Cys	2.9	1.3	48.0
Val	6.8	6.9	38.2
Met	0.5	0.4	60.0
Iso	4.4	5.7	36.9
Leu	6.5	7.3	46.5
Tyr	2.3	2.4	50.0
Phe	5.1	5.6	60.1
Try	2.5	1.3	24.0
protein	100	46.0	46.0

[a] Expressed as grams/100 g of protein.

Amino Acids Liberated from Albumin by Digestion with Pepsin–Pancreatin. The amino acids liberated by the action of pepsin followed by pancreatin were obtained by precipitating the undigested peptides from the incubation media with Cl_3AcOH (10% final concentration). The resulting supernatant was extracted with ethyl ether until neutrality to eliminate the Cl_3AcOH. The solution, containing the digested peptides and amino acids, was dried under vacuum and resuspended in 6 N HCl for hydrolysis and amino acid analysis. After ninhydrin treatment, the ratio of the absorbance obtained for the acid hydrolysate of the undigested albumin to the absorbance for the acid hydrolysate of the supernatant (after digestion) indicated a digestibility of 46%. The ninhydrin value of the supernatant before and after acid hydrolysis indicated that its average protein peptide composition was in the form of tripeptides.

Table VII shows that some amino acids, lysine, arginine, threonine, glycine, alanine, and tyrosine, have higher digestibilities than the protein average (46% of hydrolysis),



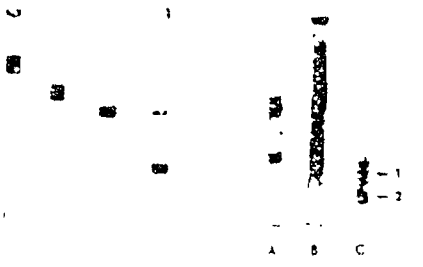


Figure 7. Electrophoretic behavior of albumins after digestion (10% polyacrylamide–1% NaDodSO$_4$). From left to right: bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 45,000), aldolase (M_r = 40,000); trypsin (M_r = 23,200); unheated albumins (A), autoclaved albumins before (B) and after digestion with pepsin–pancreatin (C). The arrows show the peptides bands (1) M_r = 20,000 and (2) M_r = 14,000.

while tryptophan, glutamic acid, valine, and isoleucine demonstrated lower digestibility. However, the differences among them were small and are probably not nutritionally significant with the exception of tryptophan which is slowly liberated (50% slower than the average) and lysine with a digestibility 25% higher than the average. This was expected in view of the specificity of trypsin and the high amount of lysine in the albumin. In view of the lowering of digestibility of albumin when heat treated (Figures 1 and 4), the presence of aggregates in the heated albumin (Figure 6), and the low availability of methionine for rats (Evans and Bauer, 1978; Sgarbieri et al., 1979), it was initially supposed that some undigestible peptide containing a large proton of methionine and cysteine might have been formed. But contrary to the expected, after digestion of the albumin, the distribution of sulfurated amino acids between the supernatant and the residues was similar to the average digestibility of the whole protein. However, this does not exclude the possibility of the existence of small peptides left in the supernatant after Cl$_3$AcOH precipitation which are not available to enzymes because the supernatant was hydrolyzed with acid before analysis.

Methionine content was not affected by auto-lasing (Table VII), which is in accordance with data provided by Hernandez Infante et al. (1979) showing that cooking has no effect on the availability of methionine added as a nutritional enrichment to beans.

The greater portion (54%) of the amino acids were precipitated as larger peptides from the incubation media after digestion of the heated albumin. This is especially important for sulfur amino acids that are already scarce and causes a reduction in the chemical score from 0.70 to ~0.30. Electrophoresis in PAA (Figure 7) showed the undigested material resulted in two major bands composed of large peptides with molecular weights of 14,000 and 20,000 that were present even after treatment with Pronase (not shown).

These bands appear to be related to the observed heat-resistant TI. In fact, the molecular weight of TI in beans has been reported to be between 10,000 and 15,000 and with an abundance of disulfide bridges which help to stabilize the structure (Pusztai, 1968). This may explain the low availability of the sulfur amino acids in beans (Kakade et al., 1969a). On the other hand, aggregates and undigestible compounds formed during heating of the albumin are present and are most likely responsible for part of the decreased digestibility.

More research directed at this last area and at explaining the low digestibility of glutelin, an important fraction of beans, is currently under way in this laboratory.

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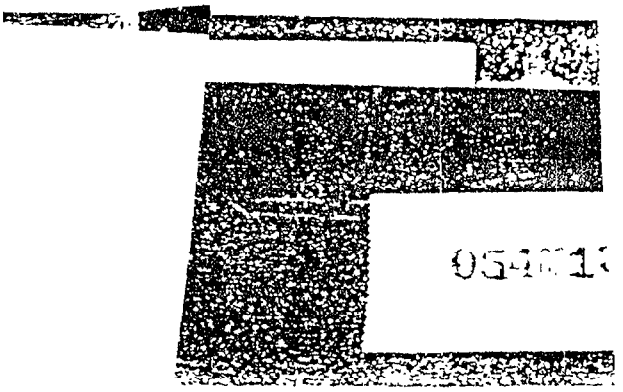

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Persistence of Bovine Milk Xanthine Oxidase Activity After Gastric Digestion In Vivo and In Vitro[1]

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ABSTRACT

Seventy rats were fasted for 20 h and used in two experiments. In the first experiment fasted rats were intubated with 5 ml marketed milk of known xanthine oxidase activity. After dosing, food was withheld and rats were sacrificed at intervals. The stomach of each rat was excised and its contents assayed polarographically for xanthine oxidase activity. Activity decreased with time. At 5 and 1.5 h the recovered activities were 62 and 45%, and in some rats activity persisted up to 8 h. In the second experiment, fasted rats received 2 ml marketed milk. Enzyme activity dropped faster than in the first experiment. Typical gastric transit times for ingested milk were 30 to 40 min. Enzyme activity in milk began to decrease at pH 6.70, below pH 3.90 the enzyme was inactivated completely. Inactivation was partly reversible at pH 2.50 and above by adjusting the pH to 7.3. When simulated gastric juice and milk were combined in ratios of 3:1, 2:1, 1:1, 1:2, and 1:3, pH's were 1.85, 2.08, 3.57, 5.16, and 5.56, and respective activities were 0, 0, 0, 14.2, and 23.8%. In one in vitro experiment, when juice and milk (1:2) were incubated at 37 C for 0, 5, 8, and 24 h, their pH's were 5.20, 5.26, 5.13, and 5.15 while recovered activities were 19.2, 18.7, 14.9, and 9.9%. When juice and milk (1:2) were incubated for 5 h and followed by pancreatin solution for 0, 5, 3.5, and 7.5 h, the recovered activities were 13.5, 11.9, 7.2, and 6.1%. Considerable xanthine oxidase in milk is not inactivated in the gastrointestinal tract but is available for absorption.

INTRODUCTION

Milk xanthine oxidase (XO, E.C. 1.2.3.2) is an enzyme abundant in cow's milk (2, 7, 28). The enzyme also is in lower concentrations in the milk of other ruminants such as the goat and sheep (15, 31). In monogastric mammals, Modi et al. (15) found XO in milk of the rabbit and absent from human, mare's, and sow's milk. However, in recent studies Zikakis et al. (30) demonstrated XO in human milk and colostrum. Furthermore, milk from the mouse, rat, guinea pig, and donkey contained high XO activity while milk from the mare, cat, dog and patas monkey (*Erythrocebus patas*) contained moderate to low activity (31).

Attention has been given to XO of milk because of a theory which involves XO of bovine milk in development of atherosclerosis (17, 18, 19, 20). This concept holds that the enzyme can survive passage through the gastrointestinal tract and be absorbed in an enzymatically active form. Once in circulation XO is presumed deposited in the myocardium and arterial wall where it destroys aldehydes liberated from the cell membrane-based plasmalogens as they are turned over metabolically. This initial damage to the cell membranes of the arterial intima and the myocardium then would stimulate cell proliferation, scar formation in the site, deposition of cholesterol esters, and ultimate development of atherosclerotic lesions. Because homogenization of milk may increase the bioavailability of XO, consumption of homogenized milk may be a predisposing factor in the development of this disease (18, 19).

Prevalent criticisms (4, 12) of this concept hold that all XO in ingested milk will be destroyed by the acidic environment and enzymatic action in the stomach. Should some of the enzyme escape gastric destruction, it would be hydrolyzed by proteolytic enzymes in the

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small intestine. Furthermore, critics assume that the normal adult gastrointestinal tract is an impermeable and impenetrable barrier to the uptake and transport of XO because of its high molecular weight of 300,000 to 340,000 daltons [although in recent studies (3, 12, 16) active XO with a molecular weight in the range of 50,000 to 85,000 daltons was isolated from whole milk and fat globule membrane]. Contrary to this opinion, normal adult intestines is permeable to undigested egg protein (21), milk protein (10), food proteins (27), enzymes (9, 23, 25), and emulsified olive oil droplets (6). Recently Warnaw et al. (26) and Walker and Isselbacher (24) provided evidence for absorption of intact macromolecules. Finally, in a series of studies with animals and healthy adult human subjects, Volkheimer (22) demonstrated the uptake of ingested raw starch granules which were found in the circulation within minutes after ingestion and later in the heart muscle, lungs, heart, and brain. The starch granules (approximately 40 μm in diameter) were taken up by a paracellular process referred to as persorption.

Our intentions were to determine if ingested XO via commercially available pasteurized-homogenized milk could survive exposure in the stomach of the rat, to determine gastric transit time in the rat, to ascertain the rate of inactivation of XO of milk in an in vitro system of simulated gastric juice, to test the effect of pH on XO activity in processed whole milk, and to test the effect of successive incubation of milk with pepsin-activated gastric juice and pancreatin on activity of XO.

EXPERIMENTAL PROCEDURES

In Vivo Experiments

A total of 70 Wistar albino rats[2] (36 males and 34 females) ranging in weight from 200 to 250 g and in age from 8 to 12 wk were in this study. The animals were in an air-conditioned building with a temperature of 22 C and a relative humidity of about 50%. Five rats of the same sex were in each galvanized cage (25 x 20 x 18 cm) with raised wire mesh floors. Purina pellet rat chow[3] and water were provided ad libitum, and all rats were fasted for 20 h prior to testing.

In the first of two experiments, 13 male and 13 female rats were assigned at random to a treatment group and an equal number of males and females were assigned to a control group. Each treatment animal received 5 ml of commercially produced Grade A pasteurized-homogenized bovine whole milk (Vitamin D added 400 IU/947 ml) purchased from a local Acme supermarket. The milk was administered by gavage with 14 gauge, 6.4 cm long blunt stainless steel needles and 5-ml disposable syringes. Activity of XO in the 5-ml milk received by each rat was 499 μl O_2/h or 326 fmU (International milliunits) (33). By the same route of administration, each control rat received 5 ml of .05 M sodium phosphate buffer, pH 7.2. All rats were dosed within 10 min and remained without food and water until sacrificed.

One male and one female rat from the treated and control groups were killed by an intracardial injection of 2 to 3 ml sodium pentobarbital[4] at .5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, and 9 h intervals. The stomachs of the rats were excised, and the stomach contents were placed in a 4 ml graduated homogenizer tube[5] measured, and described. Contents of each stomach were brought up to a volume of 4 ml with .05 M phosphate buffer and were homogenized. Three milliliters of homogenate were tested polarographically for activity of XO (28). Minor modifications of the procedure included omission of the .2 ml of .1 M phosphate buffer, assay volume was 3 ml, and the assay was at 37 C. The experiment was repeated with 10 male and 8 female rats, and the protocol was modified as follows. Six males and six females received 2 ml of pasteurized-homogenized milk whose total XO activity was 420 μl O_2/h or 275 fmU. Four males and two females served as controls and received 2 ml of the same milk after it was heated at 90 to 95 C for 5 min to inactivate the enzyme. One male and one female from the treated group and either a male or a female from the control group were killed with sodium pentobarbital at

[2] Hilltop Lab Animals, Inc., P. O. Box 195, Scottdale, PA 15683.
[3] Correll's Feed Service, Kirkwood, DE 19708.
[4] A. J. Buck and Sons, 10534 York Road, Cockeysville, MD 21030.
[5] Size AA obtained from Arthur H. Thomas, Inc., Philadelphia, PA.

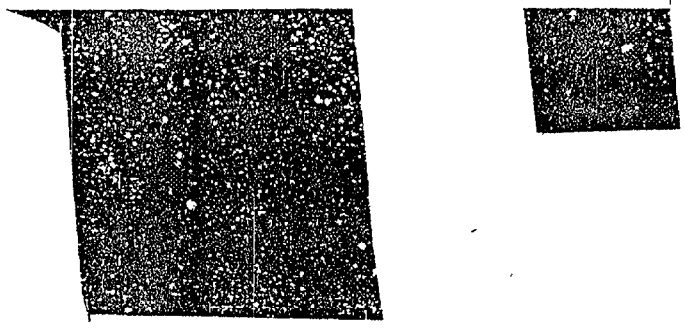


TABLE 1. Effect of pH on xanthine oxidase (XO) activity in commercially produced pasteurized homogenized and bovine whole milk

pH of milk sample	XO activity[a]		% XO activity[b] recovered	XO activity[a] at pH 7.3		% XO activity recovered at pH 7.3[c]
	$\bar{X}$	SE		$\bar{X}$	SE	
6.78[d]	1460.9	0		202.1	8	100.0
6.70	1435.3	1	98.3	183.3	6	91.7
6.60	1288.8	4	88.1	168.2	6	83.2
6.45	1152.7	1	69.4	151.8	6	75.1
5.10	35.1	1	19.5	39.7	1	19.4
4.65	8.5	2	5.1	126.2	5	62.4
3.90	0	0	0	71.2	2	35.2
2.95	0	0	0	13.1	1	6.5
2.50	0	0	0	3.1	1	1.5
2.10	0	0	0	0	0	0
1.50	0	0	0	0	0	0

[a] Average XO activity of duplicate samples was determined polarographically and is expressed as μl O_2/ml per hr.

[b] Calculated by dividing XO activity in the treatment sample by activity in starting milk (1460.9 μl O_2/ml per 81 minutes [OA] times 100.

[c] Calculated by dividing XO activity in the pH adjusted sample by activity in starting milk after its pH was adjusted to 7.3 times 100.

[d] Starting milk.

5, 1, 2, 3, 4, 5 and 7 h after dosing. Contents of excised stomachs were measured, described, brought up to 4 ml with milk heated to destroy activity (in lieu of buffer), homogenized and measured for activity. The activities of XO in stomach contents were not adjusted for dilution by the buffer in the first experiment or by heated milk in the second experiment.

Effect of pH on XO Activity in Milk

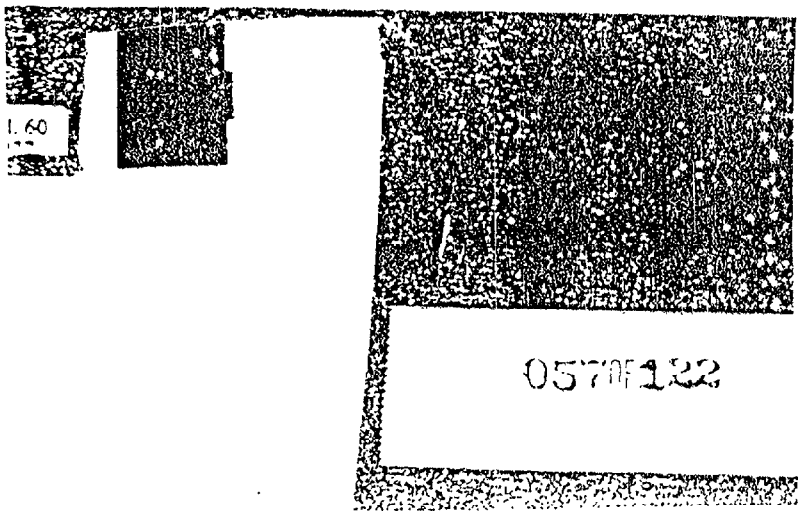
To determine the effect of pH on activity of XO in pasteurized-homogenized whole milk, milk samples were adjusted to pH ranging from 6.70 to 1.50 with 1N HCl, held at 37 C, and activity was determined in duplicate samples within 20 to 30 min. The percent activity recovered was calculated by dividing XO activity times 100 at a given pH by activity in the starting milk. To determine the degree of restoration of XO in milk after the pH of a milk sample was adjusted according to the schedule in Table 1, the pH of the sample was readjusted to 7.3 with 1N NaOH and the sample assayed for XO activity within 20 to 30 min. In this case, percent XO activity recovered at pH 7.3 was calculated by dividing activity times 100 in the pH adjusted milk sample by activity in the starting milk when its pH also was adjusted to 7.3.

In other tests, simulated gastric juice (SGJ) was combined with pasteurized-homogenized whole milk in ratios of 3:1, 2:1, 1:1, 1:2, and 1:3 (vol/vol), and the mixtures were analyzed for changes of XO activity and pH. The SGJ was made according to a formula (1) approved by the U.S. Food and Drug Administration. While stirring 1000 ml distilled water, 2 g NaCl, 3.2 g purified pepsin[1] and 7 ml concentrated HCl (12.1N) were added. The resulting SGJ had a pH of 1.45.

In Vitro Experiments

This portion of the study involved combining SGJ with milk. The mixture was incubated, and at various intervals samples were drawn and analyzed for activity of XO and pH. A 1:2 ratio of SGJ to milk was chosen the desirable

[1] Fisher Scientific Co., 191 South Gulph Road, King of Prussia, PA 19406.



combination even though this ratio is drastic in comparison to in vivo (e.g., in man, ingestion of 250 ml milk does not produce immediately 125 ml gastric juice). In a 1:2 ratio, 50 ml SCJ were combined with 100 ml whole milk whose activity of XO was 165.8 µl O_2/ml per h and its pH 6.81. One control consisted of an 1:2 ratio of 50 ml sodium phosphate buffer (.05 M, pH 7.2) to 100 ml milk to test changes in XO activity due to dilution of milk. The second control consisted of a 1:2 ratio SCJ to phosphate buffer to test for nonspecific reactions in the SCJ or buffer. Mixtures in separate beakers with 100 mg of thymol added to each to prevent bacterial growth were placed in a 37 C water bath and mixed continuously. At 0, .5, 1, 2, 3, 4, 6, 8, and 24 h, 3-ml aliquots were drawn from each mixture and analyzed for pH and activity of XO. Prior to analysis, the aliquots from SCJ to milk combinations were homogenized in a homogenizer tube because the milk curdled under these acidic conditions.

The experiment was repeated with milk whose initial XO activity and pH were 126.7 µl O_2/ml per h and 6.85. In addition, to obtain an indication whether XO would survive the action of intestinal enzymes, the 1:2 SCJ:milk combination was incubated for 5 h at 37° C. It then was combined with a 2% pancreatin[1] (a preparation of mainly proteolytic enzymes isolated from the pancreatin juice of the cow) solution (pH = 9.2) in a 2:1 ratio and incubated at 37 C with continuous mixing. At 0, .5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, and 24 h intervals, 3-ml aliquots were drawn and analyzed for activity of XO and for pH. The percent activity of XO recovered in the SCJ:milk and (SCJ:milk):pancreatin treatments was calculated by dividing activity times 100 at a given time interval by the corresponding activity in the buffer:milk control.

RESULTS

In Vitro Experiments

Results are in Fig. 1 and 2. The first experiment shows that at the 5- and 1-h intervals, approximately 62% of the original activity of XO in the milk administered was in the stomach contents. After 1.5 h, 46% of the

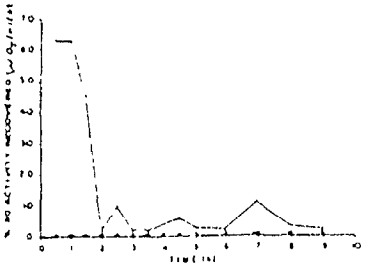


FIG. 1. Xanthine oxidase (XO) activity recovered from the stomachs of rats intubated with 5 ml whole milk (•——•) and with 5 ml of phosphate buffer (○ - - ○).

activity was recovered. In subsequent intervals, activity dropped sharply and between 2 to 9 h, the recoverable activity ranged between 0 to 11%. Stomach contents of the control rats showed essentially no enzyme activity (Fig. 1).

In the second experiment the trend was toward a gradual reduction of activity of XO (Fig. 2). However, this time activity dropped faster than in the first experiment. At .5, 1, and 2 h of digestion, recovered activities of XO were 23, 13, and 5%. These lower values compared to those in the first experiment are attributed primarily to the smaller volume of milk administered (2 ml). Judging from the contents in the excised stomachs, we found that typical gastric transit time of incubated

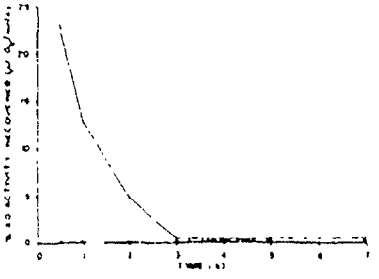


FIG. 2. Xanthine oxidase (XO) activity recovered from the stomachs of rats intubated with 2 ml whole milk (•——•) and with 2 ml heated whole milk (○ - - ○).

[1] Merck & Co., Inc., Rahway, NJ 07065.

cold (4 C) milk was between 30 to 40 min for the rat. However, large activity was evident at .5 h (62% and 23%, Fig. 1 and 2) and activity of XO decreased to little at 2 h (Fig. 2), although in some rats activity still was slight for as long as 8 h (Fig. 1). At the .5 interval, stomachs contained little or no fluid milk. Any activity of XO between .5 and 8 h was localized in milk curds which were most abundant at .5 h and least abundant at 8 h. These results suggest that a considerable amount of XO in ingested milk (about 40%) escapes gastric digestion and passes unaltered into the intestinal tract. We observed no difference due to sex in activity of XO in the stomach contents of rats.

Effect of pH on XO Activity

The pH of commercially processed bovine whole milk varies from 6.62 to 6.89 depending on the brand, batch, and freshness. As in Table 1, activity of XO in pasteurized-homogenized bovine whole milk with a pH of 6.78 started to decrease at a pH of about 6.70. As the pH was lowered further, the enzyme was inactivated rapidly and was inactivated completely at about pH 3.90. The reactivation of XO in milk was determined at pH 7.3 as described under Experimental Procedures. At pH 7.3 activity in the starting milk (pH 6.78) was increased by about 20% (Table 1). Recovery was highest at pH 8.3 (pH optimum for bovine milk XO (5)), but we decided to measure XO reactivation at pH 7.3, a pH near physiological pH. Between pH 6.70 and 4.65, 90.3 and 5.3% of activity of XO in milk was recoverable. Within pH 6.70 to 6.45, the difference in the recovery at these pH's and at pH 7.3 was small. But recovery at pH 7.3 was one-fold and elevenfold higher than at pH's 5.30 and 4.65. At pH's 3.90, 2.95, and 2.50, all activity in milk was lost. When the pH of these mixtures was adjusted to 7.3 (Table 1), 35.2, 6.5, and 1.5% of XO was reactivated. At pH below 2.50, there was no reactivation by increasing the pH of milk up to 8.3. Thus, we assumed that at pH below 2.50, XO was denatured irreversibly.

Immediately after combining SGJ and milk in ratios of 3:1, 2:1, 1:1, 1:2, and 1:3, the pH's of the mixtures were 1.85, 2.08, 3.57, 5.16, and 5.56, and the recovered XO activities were 0, 0, 0, 15.2, and 23.8%. When the pH of the 3:1, 2:1, 1:1, and 1:2 ratios was adjusted to 7.3 with .1 N NaOH, approximately 36 and 56% in the 1:1 and 1:2 ratios was reactivated. But, no activity was restored in the 3:1 and 2:1 ratios. In similar ratios of buffer and milk combinations, the pH stayed near neutrality and activity of XO showed little change between dilutions. The pH and activity of XO of starting milk for these tests were 6.85 and 126.7 μl O_2/ml per h.

In Vitro Experiments

In the first experiment immediately after SGJ and milk were combined, activity of XO dropped from 165.8 to 29.1 μl O_2/ml per h giving a recovery of 19.2% (Table 2). Thereafter activity followed a gradual reduction; at 8- and 24-h incubations, the recovered enzyme activities were 14.9 and 9.9%. The initial pH of this treatment was 5.20 and remained relatively unchanged throughout the 24 h incubation. Activity in the buffer:milk control also showed a gradual reduction with time, and a substantial change in pH occurred between 8 to 24 h intervals (Table 2). No activity was detected in the SGJ buffer control.

The results of the second experiment are in Tables 3 and 4. Although the percent recovered activities were lower, the characteristic sharp reduction in activity of XO at zero time in the first experiment (Table 2) was also in the second experiment (Table 3). At 8 h incubation, 9.8% of activity of XO was recovered. At 24 h interval, a substantial activity (7.6 μl O_2/ml per h) was in the SGJ milk treatment. However, we were unable to calculate percent activity of XO recovered for the 24 h. This was due to bacterial respiration in the buffer:milk control at the 24 h interval which interfered with the assay. The 7.2% recovered activity (not shown in Table 3) for this interval was estimated by the 8-h activity value for the buffer:milk control, a conservative estimate.

Table 4 shows the change in activity of XO and pH following successive incubation of milk with SGJ and pancreatin. A mixture of SGJ milk (1:2) which had been incubated for .5 h was combined with a 2% pancreatin solution (pH 5.93) in a 2:1 ratio. At zero time there was an increase in pH (due to the higher pH of pancreatin solution) and a slight decrease in activity of XO (due to the dilution effect of the SGJ milk with pancreatin). As in Table 4, activity of XO and pH of the treatment

followed a gradual reduction. After 24 h incubation, 3.4 μl O_2/ml per h of activity was still evident in the (SGJ milk) pancreatin digest.

DISCUSSION

The in vivo data indicate that in the rat a large portion of XO in ingested milk escapes gastric digestion. In the first experiment, over 60% of the apparent activity of XO of milk was recovered at 30 min of digestion in the stomach. Since most of the stomach emptied within 30 to 40 min, we assumed that a good deal (about 40%) of active enzyme passed into the small intestine shortly after it was ingested. The lower values of recovered activity of XO in the second in vivo experiment are probably due to the smaller volume of milk intubated (2 ml). In this experiment after 2 h of digestion, the excised rat stomachs contained less curds than stomachs of rats in the first experiment, which received 5 ml of milk. This may explain why activity of XO was longer in the stomach of some rats in the first experiment (Fig. 1). Apparently the enzyme is protected well by its close association with the milk fat globule membrane (34). Also, due to the buffering capacity of milk, pepsin with a pH optimum between 1.5 and 2.0 (11) would have little effect on XO. In humans, the pH of the stomach contents may range from 1.0 (fasted) to 5.0 (immediately after a meal).

Data in Table 1 agree with those from various ratios of SGJ milk tests. At pH 3.90, no XO activity was evident and upon adjusting the pH to 7.3 about 35% of XO activity was recovered. This observation is important because it suggests that even if the enzyme were inactivated by the low pH of the stomach, it could be reactivated again upon entering the more alkaline environment of the small intestine. Furthermore, the pH of 3:1 and 2:1 ratios of SGJ milk were 1.85 and 2.08, and both mixtures were devoid of XO activity. Adjusting the pH to 7.3 did not reactivate XO in these mixtures. As in Table 1, inactivation is irreversible at pH below 2.50, and the 3:1 and 2:1 SGJ milk ratios were below that pH. These findings disagree with Mangino and Brunner (13), who found that XO activity in commercially purchased homogenized bovine milk was not reactivated below pH 3.2. That they deter-

TABLE 2. Persistence of xanthine oxidase (XO) activity following in vitro incubation a... related gastric juice (SGJ) with marketed bovine whole milka

Sampling time, h	XO activityb in SGJ milk (1:2) x	XO activityb in buffer:milk control (1:2) x	XO activityb in buffer:milk control (1:2) pH	pH	% XO activityb,c recovered
0	29.1	1510	6.99	6.90	19.2
5	28.3	1506	7.00	6.52	18.7
1	24.5	1480	6.99	6.52	16.5
2	24.5	1542	6.98	6.51	15.5
3	22.9	1420	6.95	6.52	15.5
4	19.6	1418	6.93	6.50	13.7
6	20.1	1341	6.95	6.50	14.9
8	19.7	1315	6.94	6.51	14.9
24	12.8	1282	6.03	6.55	9.9

aXanthine utilizer activity and pH of starting milk were 165.8 μl O_2/ml per h and 6.81.

bAverage activity of duplicate samples was determined polarographically and expressed in μl O_2/ml per h.

cCalculated by dividing activity in SGJ milk treatment at a given time interval by the corresponding activity in the buffer:milk control times 100.

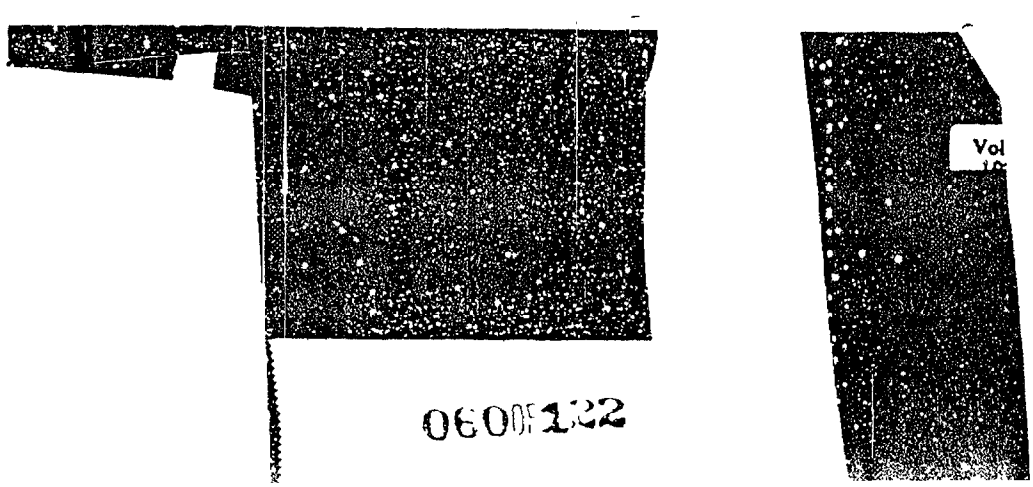


TABLE 3. Persistence of xanthine oxidase (XO) activity following in vitro incubation of simulated gastric juice (SGJ) with marketed low-fat whole milk[a]

Sampling time, h	XO activity[b] in SGJ milk (1:2)			XO activity[b] in buffer milk control (1:2)			XO activity[b] in SGJ buffer control (1:2)		% XO activity[b,c] recovered
	x	SD	pH	x	SD	pH		pH	
0	13.9	4	5.16	112.1	1	7.08	0	6.51	14.2
.5	14.5	4	5.23	110.1	3	7.10	0	6.52	12.9
1	11.4	3	5.16	108.9	3	7.09	0	6.53	10.4
2	11.0	3	5.10	113.4	4	7.08	0	6.53	9.8
3	10.5	3	5.10	112.0	3	7.05	0	6.51	9.3
4	9.4	2	5.09	110.6	2	7.05	0	6.52	8.4
6	10.6	3	5.08	107.1	2	7.05	0	6.51	9.9
8	10.5	3	5.10	106.3	2	7.05	0	6.50	9.8
24	7.6	2	5.13			6.90	0	6.54	

[a]Xanthine oxidase activity and pH of starting milk were 126.7 μl O_2/ml per h and 6.81.

[b]Average activity of duplicate samples was determined polarographically and is expressed in μl O_2/ml per h.

[c]Calculated by dividing activity in SGJ milk treatment at a given time interval by the corresponding activity in the buffer milk control times 100.

*High autoxidation due to bacteria respiration did not permit an activity reading.

TABLE 4. Persistence of xanthine oxidase (XO) activity following successive incubation of marketed bovine whole milk with simulated gastric juice (SGJ) and pancreatin.

Sampling time, h	XO activity[a] in (SGJ milk) PD (1:2)			XO activity[a] in buffer milk control (1:2)			% XO activity[a,c] recovered
	$\bar{x}$	SE	pH	$\bar{x}$	SE	pH	
0[d]	14.9	4	5.40	110.2	3	7.10	13.5
.5	13.0	3	5.12	108.9	3	7.09	11.9
1.5	7.3	1	5.03	114.6	4	7.08	6.2
2.5	7.0	1	5.00	112.0	2	7.05	6.3
3.5	7.7	2	5.00	106.6	2	7.05	7.2
5.3	7.0	1	4.92	107.1	2	7.05	6.5
7.5	6.6	1	4.91	106.5	2	7.05	6.1
24	3.4	1	4.91	*		6.00	

[a]Average activity of duplicate samples determined polarographically and is expressed in $\mu l\ O_2$/ml per h.

[b]2% solution of pancreatin, pH 5.93.

[c]Calculated by dividing activity in (SGJ milk) pancreatin treatment at a given time interval by the corresponding activity in the buffer-milk control times 100.

[d]At this point, the SGJ milk (1:2) combination had been incubated for 5 h and at this point its activity and pH were 15.9 $\mu l\ O_2$/ml per h and 5.14. Then the mixture was adjusted to a ratio of 2:1 with 2% pancreatin solution and activity and pH determined as shown.

*High autooxidation, due to bacteria respiration did not permit an activity reading.

mined XO activity in milk spectrophotometrically may account for the discrepancy.

Presently, there is no direct in vivo information on the fate of active XO after passage into the small intestine. The data in Table 4 show that activity of XO in milk persisted for up to 24 h after successive incubation with SGJ and pancreatin. After presentation of portions of these data (29), Ho et al. (8) reported on in vitro studies which support our findings. They incubated freshly aspirated human gastric juice with homogenized-pasteurized bovine milk in various ratios. Then they followed with pancreatin digestion for times comparable to transit times in the human gastrointestinal tract (time periods were not stated). Under these conditions, they recovered 27% of the total activity in the starting milk (8), a value which is comparable but higher than we found in the in vitro experiments. This higher value of XO activity recovered may be attributed to differences in the concentration of pancreatin, the pH of incubated mixture, or other unstated parameters.

A crucial question in assessing Oster's theory is whether XO can be absorbed from the small intestine in an enzymatically active form. No one has demonstrated the uptake of the active enzyme either by the absorptive epithelial cells or by a paracellular process (22). McCarthy and Long (14) recently concluded from feeding studies involving three pigs and 25 humans that no positive relationship exists between XO activity in blood serum and milk consumption. However, three factors may have prevented detection of such a relationship. First, (.39 lmU/ml) was used. Activity of XO in commercially pasteurized-homogenized bovine milk is variable and normally ranges from 32 to 122 lmU/ml with an average of 55 lmU/ml (13). Secondly, the radiochemical assay used was approximately 20 times less sensitive than a recently developed radiochemical assay (5). Finally, pig blood serum contained low molecular weight inhibitors which when removed increased XO activity elevenfold (5). Additional research is needed to evaluate whether XO absorption occurs through the gastrointestinal tract.

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Investigation of the mutagenicity of ethylphenylglycidate

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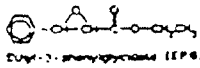
Summary

EPG and an in vitro digest of EPG by pepsin and pancreatin simulating mammalian digestion have been examined for genotoxicity in 4 mutagenicity tests employing different genetic endpoints.

In the Salmonella reverse mutation assay, EPG showed only slight mutagenic activity against TA100, a strain responsive to base-pair exchange activity, in the presence of S9 mix. In vitro EPG was mutagenic for CHO-K_1 BH4 cells with or without metabolic activation, the activity being greater in the presence of metabolic activation. In the in vitro SCE test, EPG was clastogenic for CHO-K_1-BH4 cells independent of metabolic activation. EPG also induced transformation of C3H T10½ mouse fibroblasts in vitro, producing both type II and type III foci. Subjecting an EPG solution to a simulated mammalian digestion process lowers the genotoxic activity of the solution.

Ethyl-3-phenylglycidate (EPG), is an artificial flavouring substance which does not occur in nature. It imparts a floral or fruity flavour note



Ethyl-3-phenylglycidate (EPG)

(Fenaroli, 1968) and is used at levels of 4.6–70 ppm in sweet drinks and food products (Hall and Carr, 1965). It is listed under No. 11544 in the 1981 publication of the Council of Europe dealing with flavouring substances not fully evaluated because of the lack of relevant data to support their safety in use.

Structurally, many of the flavouring substances appearing in natural material and many of those not found in natural products are potentially reactive with cell constituents following ingestion and absorption. EPG belongs to the group of substituted ethylene oxides which would be expected to display biological activity (Wade et al., 1978). Although the vast majority of these flavouring substances are present in food in minute amounts only, there is nevertheless a need for reassurance regarding their safety. The most important concern relates to the possible carcinogenic or mutagenic potential of flavouring substances. Hence studies of individual flavouring substances have been undertaken to determine their toxicological profiles.

It is now accepted that the determination of th mutagenic/carcinogenic potential of a chemic substance requires the performance of a battery short-term tests, some of which should involve vivo exposure. A recent study of 76 artifici flavouring substances used a battery consisting the Salmonella/microsome test, the sex-linked cessive lethal test in Drosophila and a mic

Abbreviations: BUdR, 5-bromodeoxyuridine; CPA, cyclophosphamide; DMSO, dimethyl sulphoxide; EPG, ethylphenylglycidate; MCA, methylcholanthrene; MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; NAD⁺, nicotinic acid diphosphate; SCE, sister-chromatid exchange; 6-TG, 6-thioguanine.

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nucleus test in mouse (Wild et al., 1983). In our study we have used 4 in vitro tests covering gene mutations, chromosome mutations and malignant transformation in somatic cells.

Materials and methods

Calf sera, antibiotics, trypsin and culture media were purchased from Gibco (Glasgow, Scotland); NADP and glucose 6-phosphate from Boehringer Mannheim (Germany); methylcholanthrene, cyclophosphamide, 6-thioguanine and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Serva (Heidelberg, Germany); pepsin, pancreatin, colchicine and BUdR from Carl Roth KG (Karlsruhe, Germany).

Rat-liver homogenate fraction S9 prepared from the liver of Sprague–Dawley rats treated with Aroclor 1254 was purchased as a KCl homogenate from Litton Bionetics (Kensington, MD). This preparation was used in all tests except the Salmonella reverse mutation assay, in which freshly prepared material from the liver of Sprague–Dawley rats treated i.p. with Aroclor were employed.

Sample preparation

Ethylphenylglycidate (for specification see Table 6) was kindly supplied by I.O.F.I. (Geneva, Switzerland). The EPG digest was prepared by adding 6 g of the substance to 30 ml distilled water. This solution was treated according to the in vitro digestion procedure described by Phillips et al. (1980).

The pH of the solution was adjusted to 1.8 by 37–39% HCl, 100 mg porcine pepsin was added, and the mixture was shaken for 3 h at 37 °C. The pH was then adjusted to 7.5 with N NaOH, 300 mg porcine pancreatin and 17 mg sodium taurocholate were added, and the mixture was shaken for 5 h at 37 °C. The mixture was then centrifuged for 20 min at 5000 rev/min and ultrafiltered through a Diaflo ultrafiltration membrane at 10000 dalton molecular weight. The osmolarity of the final EPG digest was determined after ultracentrifugation and filtration by a Knauer osmometer. It was then adjusted to 350 osmoles/kg and the EPG digest was sterilised by filtration. The final concentration of EPG in the digest was 41 mg/ml. The standard EPG solution contained 50 mg EPG/ml DMSO, appropriate dilutions being prepared for the individual tests as indicated.

The Salmonella reverse mutation assay was carried out by Mr. R.B. Varley at Toxicol. Laboratories, Ledbury, Herefordshire (England). EPG and EPG digest were tested for mutagenic potential according to the method of Ames et al. (1975), with and without the addition of 10% metabolic activation mixture as S9 mix. The following bacterial strains were used: *Salmonella typhimurium* strains TA98, TA1537, TA1538, TA100 and TA1535, all kept at Toxicol. Laboratories. Preliminary tests for cytotoxicity revealed that EPG doses exceeding 1000 μg/plate were toxic to the tester strains used. EPG digest, however, was tolerated in concentrations of up to 10 mg/plate. EPG was therefore used at doses of 80, 400 and 2000 μg/plate, while the EPG digest was used at 400, 2000 and 10000 μg/plate.

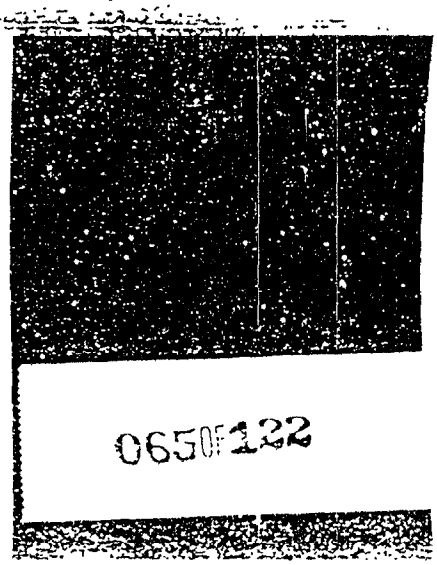
Cell survival

The cell survival test was performed on CHO-K_1-BH4 cells. Cells were grown in McCoy's 5a medium supplemented with 10% fetal calf serum and 1% antibiotics (penicillin–streptomycin). They were cultured routinely in plastic culture bottles (Nunc) at 37 °C in a water-saturated CO_2 incubator.

Preliminary investigations were carried out to determine the concentrations of EPG solution and EPG digest that would be tolerated by the CHO cells in culture. Cytotoxicity was determined as the percentage of cells surviving after exposure for 2 h to various concentrations of EPG or EPG digest. The initial concentrations were 41 mg EPG/ml for the digest and 50 mg/ml for the DMSO solution of EPG. Test solutions were prepared by dilution with culture medium. Cells were counted with a cell culture counting apparatus (Zeiss).

6-Thioguanine resistance (TG^r) test

The methodologies used were those described by Hsie et al. (1979); O'Neill et al. (1977) and Phillips et al. (1980). CHO-K_1-BH4 cells were grown in McCoy's 5a medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. They were cultured routinely in plastic culture bottles (Nunc) at 37 °C in a water-saturated



CO_2 incubator. About 5×10^5 cells/bottle were exposed for 2 h to the test solutions and then replated at 5×10^5 cells/bottle every second day. Cells (2×10^4 per bottle) were treated with 6-TG medium (2 μg/ml) for 10 days beginning with day 8. Each dose point was determined in triplicate. Methylene-blue stained mutant cell clones were counted on the 10th day. MNNG was used as the positive control in the test without S9 mix and CPA in the test with S9 mix. S9 mix was added at the level of 10%.

SCE test

The methods used were those described by Perry and Wolff (1974) and Phillips et al. (1980). CHO-K_1-BH4 cells were grown in McCoy's 5a medium supplemented with 10% fetal calf serum and 1% penicillin–streptomycin. They were cultured routinely in plastic culture bottles (Nunc) at 37 °C in a water-saturated CO_2 incubator. For the test, 1×10^6 cells were grown in 175-mm bottles for 24 h. After treatment for 2 h with the test solutions, medium containing 10 μg BUdR/ml was used. Colchicine (2 μg/ml) was added 24 h after the beginning of the BUdR exposure. 2 h later, cells were harvested, exposed to 1% citrate solution for 20 min at 37 °C, fixed in 2:1 ethanol/acetic acid, spread on slides and stained according to the standard Giemsa-fluorescent dye method. Each solution was tested with and without the addition of S9 mix. MNNG was the positive control for tests without S9 mix. A negative control with and without S9 mix was also set up. 4 slides were prepared for each dose point, and 15 metaphases were evaluated per slide.

Transformation test

EPG and EPG digest were investigated for their ability to induce transformation of cultured mouse fibroblasts of the type C3H/T10½. The method employed was that described by Mondal et al. (1976) and Reznikoff et al. (1973). C3H/T10½ mouse embryo fibroblasts were plated in plastic bottles (Nunc) and grown in Dulbecco's medium + 10% heat-inactivated fetal calf serum + 1% antibiotics (penicillin–streptomycin). Cultures were incubated in humidified incubators at 37 °C. MCA was used as positive control. 20 bottles, each containing 6000 cells, were used for each dose point. Cells were exposed to the test substance for 2 h without S9 mix and were then grown for 7 weeks, the medium being changed once or twice weekly. Cells were then stained with methylene blue, and transformed foci were identified by comparison with the background monolayer. Foci of type II and type III were identified as evidence of transforming activity. Type II foci are defined as consisting of transformed cells that, on injection into syngeneic animals, produce sarcomata in 60% of the animals treated. Type III foci are defined as consisting of transformed cells that produce sarcomata in over 90% of syngeneic animals following s.c. injection. Transformation capability was evaluated from the ratio of bottles with transformed foci to the total number of bottles used for each dose point and expressed as percentage.

Results

Salmonella reverse mutation test (Ames test)

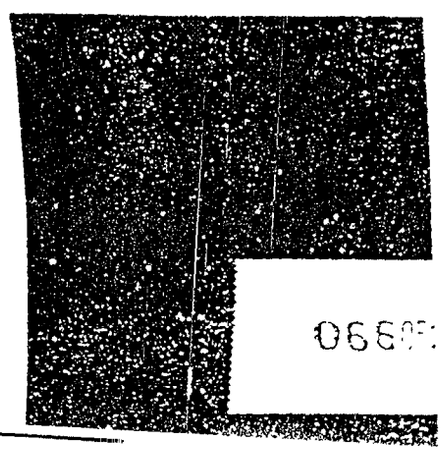
The results for EPG and EPG digest are listed in Table 1.

The data represent the means of 4 plates per dose point. They show no mutagenic activity of either EPG or EPG digest in the absence of S9 mix as metabolic activation system, even at a concentration of 2000 or 10000 μg/plate for any of the test strains used. The addition of S9 mix did not affect the results except for a doubling of the mean number of revertants when EPG was tested against TA100. It may be concluded from these results that EPG has only weak mutagenic activity in the Ames test, and then only against the tester strain TA100 in the presence of S9 mix. A positive response in this test system indicates a mutagenic activity of the base substitution type. The EPG digest showed no mutagenic activity in the Salmonella reverse mutation test against all strains, even in the presence of S9 mix.

Table 2 summarises the results of the cytotoxicity determination for the 2 samples against CHO-K_1-BH4 cells.

Table 3 shows the results of the forward mutation test in terms of 6-TG^r mutants produced in the CHO/HGPRT$^{-/+}$ test.

The surviving fractions for the concentrations used were as follows. 8% EPG digest (+S9): 0.72, 8% EPG digest (−S9): 0.59, 0.3% EPG digest



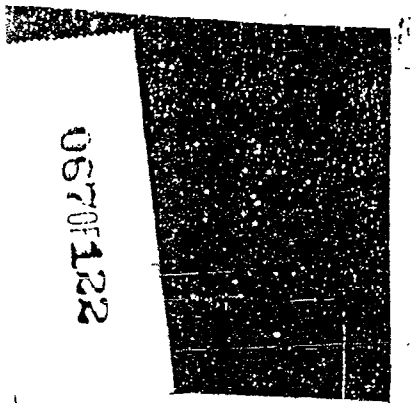


TABLE I

MEAN NUMBER OF REVERTANTS PER PLATE

Strain	S9 (%)	Concentration of EPO (μg/plate)				Concentration of EPO digest (μg/plate)			
		0	80	400	2000	0	400	2000	10000
TA1535	0	17.6 ± 3	14.0 ± 5	17.0 ± 2	16.3 ± 5.7*	18.3 ± 3.2	18.0 ± 4.6	17.7 ± 5.5	17.7 ± 3.2
TA1537	0	7.3 ± 2.5	6.3 ± 1.5	8.0 ± 3.6	5.7 ± 0.6	8.0 ± 1.0	9.0 ± 1.0	5.0 ± 2.0	7.3 ± 1.5
TA1538	0	9.7 ± 2.9	8.7 ± 1.5	8.0 ± 1.7	5.3 ± 2.9*	11.3 ± 2.1	10.0 ± 2.6	11.0 ± 4.0	7.3 ± 3.2
TA98	0	20.3 ± 5.0	20.0 ± 4.6	16.0 ± 2.0	17.0 ± 6.1*	23.0 ± 1.7	21.7 ± 2.9	22.3 ± 1.5	19.3 ± 4.0
TA100	0	81.3 ± 9.6	84.0 ± 4.6	88.7 ± 7.4	97.0 ± 13.1	65.3 ± 6.8	104.3 ± 9.0	102.3 ± 14.6	90.3 ± 3.5
TA1535	10	15.3 ± 3.2	17.3 ± 3.2	17.7 ± 4.0	15.3 ± 2.1	19.0 ± 2.0	16.7 ± 3.5	20.3 ± 1.5	19.7 ± 2.3
TA1537	10	10.7 ± 1.5	10.0 ± 2.0	10.7 ± 0.6	5.3 ± 2.1	8.7 ± 0.6	8.3 ± 0.6	9.0 ± 2.6	8.3 ± 2.1
TA1538	10	15.0 ± 3.0	13.7 ± 2.5	14.0 ± 1.0	7.3 ± 2.1	18.3 ± 1.5	19.0 ± 1.0	15.7 ± 5.1	16.0 ± 3.5
TA98	10	30.0 ± 2.6	27.7 ± 3.1	28.0 ± 3.0	15.7 ± 1.2	25.0 ± 5.3	26.3 ± 6.0	25.0 ± 2.6	25.7 ± 7.1
TA100	10	99 ± 22.6	115.7 ± 15.7	114.0 ± 3.6	109.7 ± 15.3	82.7 ± 7.6	85.3 ± 8.5	88.3 ± 4.7	106.0 ± 14.7

* Reduced background lawn.

TABLE 2

CYTOTOXICITY OF EPG AND EPG DIGEST TO CHO CELLS

Material	EPG (mg/ml)	Percentage survival without S9	Percentage survival with S9	Material	EPG (mg/ml)	Percentage survival without S9	Percentage survival with S9
Control		100	100	10% EPG	5	0	0
MNNG (0.1 μg/ml)		48	-	5% EPG	2.5	0	0
CPA		-	53				
0.3% EPG digest	0.123	82	94	1% EPG	0.5	0	0
1% EPG digest	0.41	60	91	0.5% EPG	0.25	8	7
4% EPG digest	1.64	79	78	0.25% EPG	0.125	50	58
8% EPG digest	3.28	53	69				
10% EPG digest	4.1	42	15				

(+S9): 0.87, 0.3% EPG digest (−S9): 0.89, 0.5% EPG solution (+S9): 0.042, 0.5% EPG solution (−S9): 0.084, 0.25% EPG solution (+S9): 0.56, 0.25% EPG solution (−S9): 0.6.

Results were judged to be positive if the number of induced mutants equaled or exceeded 2.5 times the control value at 50% survival. Hence for dose points without S9 mix any number of mutants exceeding 4.3 mutants/10^4 cells should be regarded as positive. Similarly, for dose points with S9 mix any number of mutants exceeding 20.8 mutants/10^4 cells represents a positive result.

Consideration of the values listed in Table 3 shows that the EPG digest is weakly positive at 8% with and without S9 mix. This corresponds to exposure to a solution containing 3.3 mg EPG/ml. In the case of undigested EPG only the results of the 0.25% solution can be considered, because the 0.5% solution was found to be too cytotoxic for the results to be interpretable. The 0.25% EPG solution was mutagenic both in the presence and absence of S9 mix but displayed higher activity in the presence of S9 mix. The 0.25% normal EPG solution corresponds to an exposure of 0.125 mg

TABLE 3

FREQUENCY OF MUTANT THIOGUANINE RESISTANT CELLS IN CHO CELLS TREATED WITH EPG AND EPG DIGEST

Material	EPG (mg/ml)	Number of mutants/bottle			Total mutants	Mean number of mutants observed	Mutants per 10^4 cells
		1	2	3			
Control − S9		0	0	1	1	0.33 ± 0.5	1.7
Control + S9		2	1	2	5	1.7 ± 0.57	8.3
MNNG (0.1 μg/ml)		8	7	11	26	8.7 ± 2.08	43.2
CPA (10 μg/ml)		12	13	9	34	11.3 ± 2.08	56.5
8% EPG digest − S9	3.28	3	1	2	6	2 ± 1.0	10
8% EPG digest + S9	3.28	6	6	6	18	6 ± 0	30
0.3% EPG digest − S9	0.123	1	1	0	2	0.7 ± 0.57	3.3
0.3% EPG digest + S9	0.123	2	1	4	7	2.3 ± 1.52	11.7
0.5% EPG solution − S9	0.205	1	3	3	7	2.3 ± 1.15	11.7
0.5% EPG solution + S9	0.205	12	9	18	39	13 ± 4.6	65
0.25% EPG solution − S9	0.103	3	3	4	12	4 ± 1.0	20
0.25% EPG solution + S9	0.103	10	11	11	32	10.7 ± 0.57	53

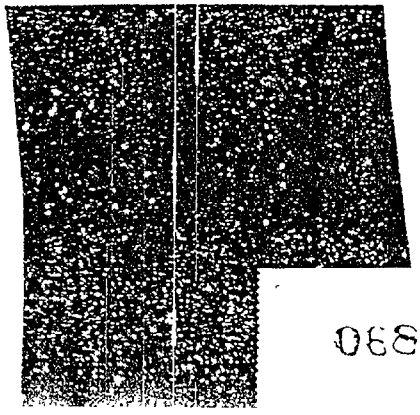


TABLE 4

IN VITRO INDUCED SCEs IN CHO CELLS

Material	EPG (mg/ml)	Mean SCEs per 60 metaphases
Negative control – S9		11.6 ± 4.3
Negative control + S9		11.3 ± 2.4
CPA (10 μg/ml)		24.3 ± 4.3
MNNG (0.1 μg/ml)		36.7 ± 4.5
8% EPG digest – S9	3.28	12.3 ± 2.1
8% EPG digest + S9	3.28	12.0 ± 1.9
0.25% EPG solution – S9	0.103	13.5 ± 2.7 *
0.25% EPG solution + S9	0.103	16.6 ± 4.6 *

* Significantly different from control value in a 2-tailed t-test at $p = 0.01$.

EPG/ml, which is the same concentration of EPG as is found in the 0.3% solution of the EPG digest. While the digest shows no mutagenic activity at this low concentration, the undigested EPG is clearly mutagenic.

Both EPG and EPG digests were examined for their ability to induce SCEs in CHO-K_1-BH4 cells. The concentrations used for this test were based on those effective in the forward mutation test, i.e., 8% for the EPG digest and 0.25% for the EPG solution. The results are listed in Table 4.

Only the undigested EPG shows clastogenic activity both with and without the addition of S9 mix. The increase in SCEs was significant for that dose point in all the slides examined.

Table 5 summarises the results of the transformation test in mouse fibroblast cell type C3HT10½. The concentrations used were the same as those found to be active in the other in vitro mutagenicity tests, i.e., 8% for the EPG digest and 0.25% for EPG.

TABLE 6

SPECIFICATION OF EPG AS SUPPLIED BY GIVAUDAN SA

Product	Ethyl phenylglycidate
No. of lot	9542-82
Density (d_4^{20})	1.1248
Index of refraction (n_D^{20})	1.5181
Acidity	1.0
Purity (ester index)	100%
Total chlor	0.1%

The results show that the solution containing 0.175 mg/ml of EPG has a capacity to transform mouse fibroblasts that is about 58% of the activity of the positive control. The EPG digest containing 3.3 mg/ml, or 26 times the concentration of the undigested EPG solution, has a capacity for transformation that is about 33% of the activity of the positive control. Although digestion does not remove the transforming potential, it reduces it by a factor of approximately 46.

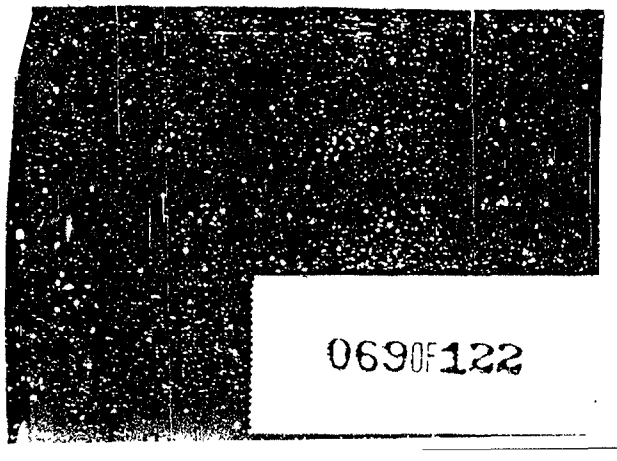
Discussion

The suspicion that EPG would possess genotoxic potential, based on structural considerations (Wade et al., 1978), has been confirmed in in vitro tests using different genetic endpoints. In a previous investigation (Wild et al., 1983), EPG was found to be mutagenic to *S. typhimurium* TA100 in a plate test with and without S9 mix, at concentrations up to 4 mg/plate. EPG was more toxic

TABLE 5

RESULTS OF THE TRANSFORMATION TEST IN MOUSE FIBROBLASTS

Material	EPG (mg/ml)	Number of bottles with transformed foci / total number of bottles	% bottles with Type II and Type III foci
Control		2/30	10
MCA (10 μg/ml)		14/20	70
8% EPG digest	3.28	6/20	30
0.25% EPG	0.103	9/20	45



when preincubation procedures were applied, and it was not mutagenic under these conditions because of its high cytotoxicity. One out of three sex-linked recessive lethal tests in *Drosophila melanogaster* showed an increased mutation frequency. No activity was noted in a micronucleus test in mice.

Under our conditions for the Salmonella/microsome reverse mutation test no significant mutagenic activity was detectable. The slight reduction in the number of revertants observed at the highest exposure level of 2000 μg EPG/plate in the absence of S9 mix, together with the reduction in the background 'lawn' on the agar plates, is evidence of a toxic effect on the test organisms. The addition of 2000 μg EPG/plate doubled the number of revertants for *S. typhimurium* TA100 compared to controls. This finding differs from that reported by Wild and coworkers (1983), who noted significant mutagenic activity in TA100 at 4000 μg/plate. The different results may be due to differences in the specification of the EPG examined, a possibility suggested by the difference in observed cytotoxicity. However, our tests may have failed to detect significant mutagenic activity in TA100 or, indeed, in any of the other strains employed because our test conditions were not optimised with regard to the concentration of S9 mix and no preincubation procedures were included. Nevertheless, our findings indicate that TA100 appears to be sensitive to EPG genotoxicity, typefying base-pair substitution activity.

In vitro digestion of a 20% EPG-water mixture, using the method of Phillips et al. (1980) abolished the mutagenic activity of EPG in TA100. It also reduced cytotoxicity by about 5-fold, so that concentrations equivalent to 10 mg EPG/plate could be tested.

EPG induced forward mutations at the HGPRT locus of CHO cells, yielding homozygous mutants resistant to 6-thioguanine. A dose-response curve could not be established because of the cytotoxicity of EPG. Although the 0.5% solution induced more mutants independent of the presence of S9 mix, the low survival rates of only 4–8% made quantitative interpretation of the results difficult. However, using a 0.25% solution increased the survival rate to 56–60% and produced positive mutagenic responses in the presence and absence of S9. In fact, the response was equivalent quantitatively to the activity of 10 μg cyclophosphamide in the presence of S9 mix or 0.05 μg MNNG in the absence of S9 mix.

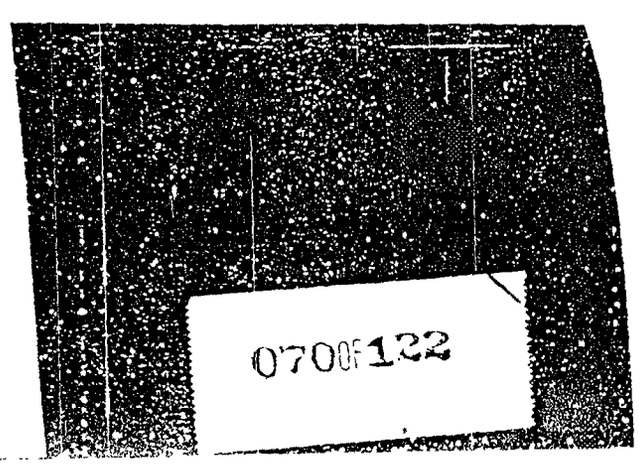
In vitro digestion again reduced the mutagenic activity of EPG in this test. The number of mutants produced by 3.3 mg EPG/ml as digest was about half that induced by 0.125 mg EPG/ml as solution, irrespective of the presence of S9. In vitro digestion therefore caused a 13-fold reduction in the mutagenic activity of EPG. If the incidence of mutants is compared for 0.123 mg EPG/ml of digest and 0.125 mg EPG/ml of solution. This reduction would be much larger for the 8% digest (3.3 mg EPG/ml), because the slope of the theoretical dose-response curve for the EPG solution would be considerably steeper than that of the dose-response curve for the EPG digest. The number of mutants produced by 0.3% of digest of the EPG solution was not significantly different from the control values, irrespective of the presence of S9 mix.

Similar reduction in clastogenic activity was observed in the in vitro SCE test. The digest containing 3.3 mg EPG/ml, produced no significant rise in SCEs, while 0.125 mg EPG/ml a solution produced a clear clastogenic response equivalent to a 26-fold reduction in activity by in vitro digestion. It should be noted that the same concentration (0.125 mg/ml) of EPG solution gave positive results in test systems employing different genetic endpoints.

The in vitro transformation test using the mouse fibroblast cell line C3H T10} yielded positive results with both the EPG digest and the EPG solution. The solution of 0.125 mg EPG/ml has about 58% of the transforming activity of a methylcholanthrene solution containing 10 μg/ml. The digest containing 3.3 mg EPG/ml had about 33% of the transforming activity of methylcholanthrene. Thus digestion did not abolish the transforming potential of EPG but reduced it by a factor of

$$(3.3/0.125) \times (58/33) = \text{approx. } 46.$$

The positive outcome of 3 out of 4 different in vitro tests using 4 separate genetic endpoints confirms the genotoxicity of EPG under these experi



mental conditions. Such a result would be expected in view of the chemical structure containing a reactive epoxide group. However, when tested in two in vivo systems, conflicting results have been reported. Thus there is a clear need to resolve the dilemma by conducting further in vivo tests involving both germ and somatic cells. The positive transformation test is difficult to interpret in the absence of evidence for adduct formation between EPG and nuclear DNA. The interesting finding that in vitro digestion simulating the mammalian digestive process reduces the mutagenic activity of the compound in in vitro tests to some extent supports the equivocal or negative results seen so far in in vivo mutagenicity tests. From the point of view of risk to the health of the consumer this provides some assurance, because the use of this compound as a flavouring in foodstuffs is unlikely to exceed several tens ppm. However, a quantitative risk estimate cannot be made from the available data, nor can a health risk be ruled out, unless complete inactivation by in vivo mammalian digestion has been convincingly demonstrated. The work reported in this paper supports the view that digestion is likely to diminish the mutagenic potential of certain 1,2-disubstituted ethylene oxides with a molecular structure similar to EPG. It also illustrates the value of conducting a battery of in vitro mutagenicity tests if the chemical structure of the compound raises the suspicion of biological reactivity.

Acknowledgements

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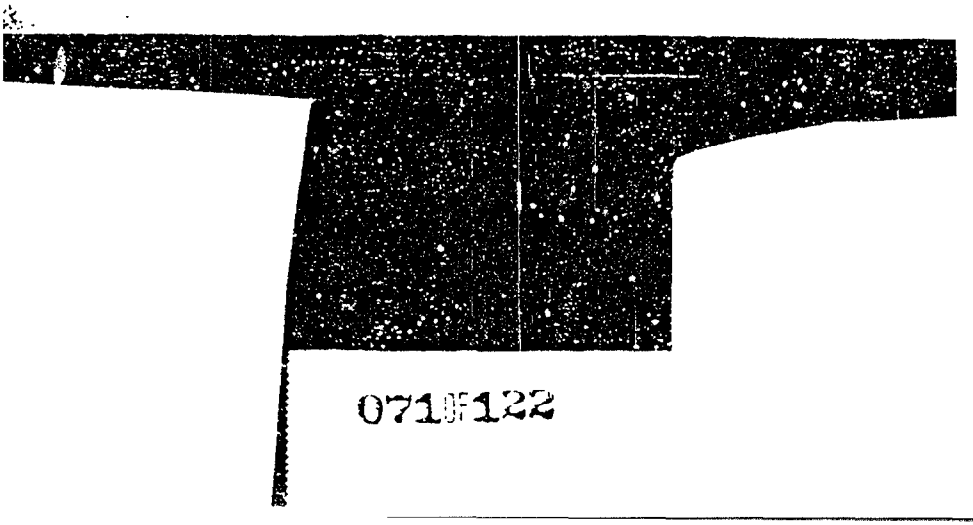

A Pepsin Pancreatin Digest Index of Protein Quality Evaluation [1]

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ABSTRACT A pepsin pancreatin digest index was devised for a rapid, accurate estimation of protein quality. The index was calculated from the amino acids released by an in vitro digestion with pepsin followed by pancreatin. The amino acids were determined by automatic amino acid analysis which allowed rapid protein quality evaluations with very small samples. Using whole egg as a standard, excellent correlation was observed between the pepsin pancreatin index values for 12 proteins and their biological values reported in the literature from feeding trials. The proteins tested were selected to cover a wide range of protein quality. The pepsin pancreatin digest index values showed better correlation with the biological values for the growing rat than did the essential amino acid index which tended to overestimate the value, or the chemical score which tended to underestimate it.

Although the nutritional quality of proteins must, in the final analysis, be established with feeding trials, in vitro methods of protein evaluation are useful in screening new protein foods and processing methods because of their rapidity. Recent reviews of laboratory methods of protein quality evaluation have been given by Grau and Carroll (1), Campbell (2), Pearson and Darby (3), Frost (4), and Mauron (5). Two procedures widely used for screening potential protein foodstuffs, the chemical score (6–8) and the essential amino acid index (9, 10) were based on the total amino acid composition. These procedures were rapid and in many cases accurate, however, no allowance was made for variations in the digestibility and availability of amino acids. Sheffner et al. (11) developed the "pepsin-digest-residue amino acid index" which combined the pattern of essential amino acids released by in vitro pepsin digestion with the amino acid pattern in the remainder of the protein. The work involved was considerable since 10 amino acids had to be determined in an acid hydrolysate as well as in a pepsin digest by the use of microbiological techniques. Therefore, this method did not appear suitable for evaluation of large numbers of food proteins. The procedure to be described in the present paper gives an amino acid index based on the release of amino acids by pepsin followed by pancreatin. The labor and sample size required for assay was reduced by the use of automatic amino acid analysis.

METHODS AND MATERIALS

Pepsin digests were prepared by incubating with shaking 500 mg dried protein (samples were dried to constant weight over P_2O_5 at room temperature) with 12.5 mg pepsin [2] in 15 ml of 0.1 N hydrochloric acid for 24 hours at 37° in a water bath. Pepsin followed by pancreatin digests were prepared by incubating 100 mg protein with 1.5 mg pepsin in 15 ml of 0.1 N hydrochloric acid at 37° for 3 hours. After neutralization with 7.5 ml of 0.2 N sodium hydroxide and addition of 4 mg pancreatin [3] in 7.5 ml of pH 8.0 phosphate buffer, the digestion mixtures were incubated for an additional 24 hours at 37°. Enzyme blanks were prepared by incubation under the described conditions with the protein sample omitted. Fifty parts per million merthiolate which were added to the digestion mixture to prevent growth of microorganisms did not interfere with the digestion and subsequent analysis. Ten milliliters of digestion mixture were added

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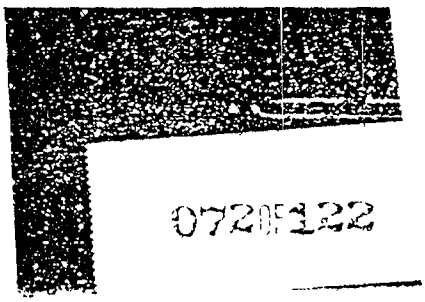
[2] B grade, California Corporation for Biochemical Research, Los Angeles.
[3] See footnote 2.



to 50 ml of one per cent picric acid solution and centrifuged for 30 minutes at 1000 × *g* to remove undigested protein and larger peptides.

Fifty milliliters of supernatant were passed through a column containing anion exchange resin [1] in chloride form into a 250 ml lyophilizing bottle. After rinsing the column with three 5-ml portions of 0.02 N hydrochloric acid, the samples were dried by lyophilization. The dried samples were dissolved and diluted to 10 ml with pH 2.2 buffer. Amino acid analysis of the samples was made by the ion exchange method of Moore et al. (12–14) with a Spinco model 120 amino acid analyzer. Basic amino acids were separated on a 10-cm column using pH 5.28 buffer. The acidic and neutral amino acids were separated on a 139-cm column using pH 3.25 buffer followed by pH 4.25 buffer after 8 hours and 30 minutes from zero time.

Chromatograms of pepsin and pepsin pancreatin hydrolysates of egg white protein are shown in figure 1. The pepsin hydrolysate represented 53 mg of protein and the pepsin pancreatin hydrolysate represented 5.3 mg of protein. Analyses of pepsin hydrolysates were unsatisfactory since large concentrations of peptides obscured peaks of some amino acids, many of which were present in small concentrations. Chromatograms of the pepsin followed by pancreatin hydrolysates gave excellent resolution of the amino acids. In those areas showing peptides, a slanting line was drawn as illustrated in figure 1 which served as a baseline for integrating the amino acid peaks. Preliminary studies showed this method of integration to be more accurate and reproducible than the usual method (14) when varying concentrations of the enzyme hydrolysate were analyzed.

The total amino acid content of the samples was determined on acid hydrolysates. Fifty-milligram samples were hydrolyzed with 2 ml of 6 N hydrochloric acid for 22 hours at 110° in a sealed tube containing a nitrogen atmosphere. After filtration and evaporation to dryness 3 times with vacuum distillation, the samples were dissolved in pH 2.2 citrate buffer and diluted to 25 ml. Tryptophan which was destroyed by acid hydrolysis was analyzed after hydrolysis with 5 N sodium hydroxide in a manner similar to that of Dreze (15).

The pepsin pancreatin digest index was calculated in essentially the same manner as the "pepsin-digest-residue amino acid index" described by Sheffner et al. (11) with slight modifications. The amino acid concentrations were expressed as grams

[1] AG 2-X 8, 200–400 mesh, California Corporation for Biochemical Research, Los Angeles.

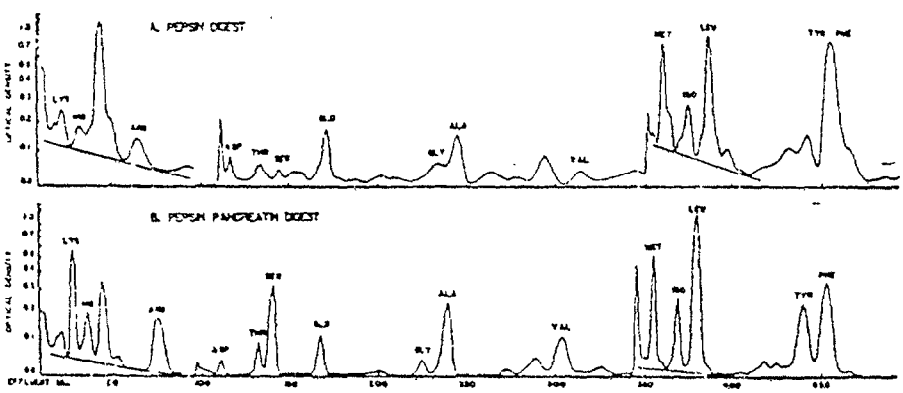


FIG. 1 Representative chromatograms from the Spinco Model 120 amino acid analyzer. A, a chromatogram of a pepsin hydrolysate of egg white; B, a chromatogram of a pepsin followed by pancreatin hydrolysate of egg white. The chromatogram of the pepsin hydrolysate shows peptides and amino acids from 10 times more protein as the chromatogram of the pepsin-pancreatin hydrolysate.

per 100 g total amino acid rather than as milligrams per gram of protein. Amino acids used in all calculations included lysine, phenylalanine plus tyrosine, methionine, threonine, valine, isoleucine, leucine, and histidine, the amino acids classified by Rose (16, 17) as essential for the growing rat. Tryptophan was destroyed during the picric acid procedure and not determined in the enzyme hydrolysates. A correction for the tryptophan content was therefore included in some calculations to see whether the tryptophan content of the proteins tested would influence the results. This correction which was the ratio of total tryptophan in the sample to total tryptophan in the reference protein was included in the geometric mean of each fraction.

The procedure was tested with 12 food proteins which covered the entire range of protein quality. Commercial preparations tested were egg white, lactalbumin, casein, soybean protein, gluten, zein,[5] wheat flour,[6] yeast[7] and gelatin.[8] Whole egg (raw), milk (pasteurized) and beef (roasted) samples were prepared by lyophilization followed by grinding and mixing with a mortar and pestle. Before lyophilization, the beef samples were homogenized with 50 ml of water with a Virtis homogenizer.

RESULTS AND DISCUSSION

The pepsin pancreatin amino acid index values calculated with and without the tryptophan correction are shown in table 1, columns 4 and 3, respectively. The biological values for growing rats as reported in the literature for the 12 proteins are shown in column 2. One or more reports of the biological values were found for

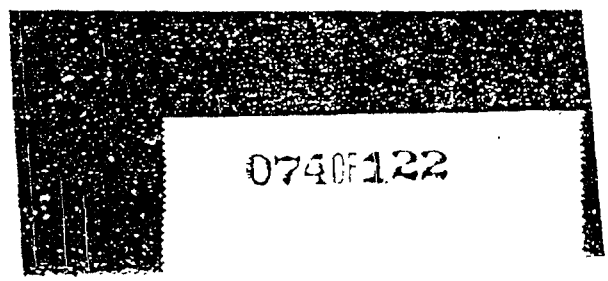
[5] All obtained from Nutritional Biochemicals Corporation, Cleveland.
[6] Pillsbury's Best, The Pillsbury Company, Minneapolis.
[7] Red Star Food Yeast, Red Star Yeast and Products Company, Milwaukee, Wisconsin.
[8] Bacto Gelatin, Difco Laboratories, Detroit, Michigan.

TABLE 1

Comparison of pepsin pancreatin digest index (calculated with and without the tryptophan correction) with biological values reported in the literature for the growing rat, the chemical score[1] and the essential amino acid index[2]

Food protein	Literature biological value for growing rats	Pepsin pancreatin digest index: Excluding tryptophan correction	Pepsin pancreatin digest index: Including tryptophan correction	Chemical score	Essential amino acid index
Whole egg	96,[3] 97 [4]	100	100	100	100
Egg white	83,[3] 82,[3] 97 [4]	89	90	100	97
Milk	90,[3] [4] 84 [3] (dried), 94 [4]	87	86	79	89
Lactalbumin	85,[3] 84 [3]	85	87	79	88
Beef	76 [3,4]	80	78	81	83
Casein	73,[3] 69 [3] 78 [5]	78	79	66	89
Soybean	raw 57,[3] 59 [5] heated 75,[3,5] 74 [5]	68	67	51	82
Yeast	63,[3] 69 [4]	74	74	55	81
Wheat flour	52 [3,5]	54	52	42	61
Gluten	40,[4] 61 [5]	49	47	40	55
Zein	—	42	27	17	33
Gelatin	25,[3] 0 [4]	29	18	17	23
Correlation coefficient, r =		0.982 (0.995) [6]	0.990 (0.994) [6]	0.940	0.979
Regression equation,[10] Y =		1.097X − 11.00 (0.954X + 0.65) [6]	0.970X − 0.54 (0.940X + 1.98) [6]	0.831X + 14.48	1.006X − 9.46
SE of estimate =		1.45 (0.92) [6]	1.05 (1.03) [6]	2.58	1.65

[1] Bender (8).
[2] Oser (20).
[3] Block and Mitchell (7).
[4] Sommer (24).
[5] Mitchell and Block (6).
[6] Mitchell and Beadles (25).
[7] Rippon (26).
[8] Bender, Miller and Tannan (27).
[9] Values in parentheses exclude gelatin.
[10] Y = predicted biological value, X = pepsin pancreatin digest index.



each protein except zein which does not support growth or maintain body weight without supplementation (18).

Whole egg was selected as the standard and set equal to 100 since it had the highest biological value of the proteins tested. A better correlation was observed between the pepsin pancreatin digest index and the biological value when whole egg was used as the standard ($r = 0.990$) than when egg white was used as the standard ($r = 0.976$).

Arginine which has been classified as a semi-indispensable amino acid (19) and is required only in small amounts (16) was not included in the calculations of the pepsin pancreatin digest index shown in table 1. A better correlation was observed between the pepsin pancreatin digest index and the biological values when arginine was excluded ($r = 0.990$) than when it was included ($r = 0.972$) in the calculations

Index values calculated with the tryptophan correction showed better correlation with biological values from the literature ($r = 0.990$) than did index values calculated without the tryptophan correction ($r = 0.982$). The index values were virtually the same with both methods of calculation for all proteins tested except for zein and gelatin which were devoid of tryptophan. When indexes for zein and gelatin were excluded from the correlation, the correlation coefficients were the same whether the tryptophan correction was included ($r = 0.994$) or excluded ($r = 0.995$). Rose (16) showed the minimal level of tryptophan required for growth was lower (0.2%) than for the other essential amino acids. Therefore, good estimations can be made of the biological value without including the tryptophan correction except for proteins low in or devoid of tryptophan. This would eliminate the need for a separate basic hydrolysis and short column run.

Table 1 shows a comparison of the pepsin pancreatin digest index values with the chemical score of Bender (8) and the essential amino acid index of Oser (20). The latter 2 methods were calculated from the total amino acid content of the food proteins tested. The pepsin pancreatin digest index showed better correlation with the literature biological values ($r = 0.990$) than did the chemical score ($r = 0.940$) or the essential amino acid index ($r = 0.979$). Similarly the standard error of estimate was smaller for the pepsin pancreatin digest index (1.05) than for the chemical score (2.58) and the essential amino acid index (1.65). In general, the chemical score underestimated the biological value of the protein, whereas the essential amino acid index overestimated the biological value. There were cases where the agreement between the essential amino acid index or the chemical score and the biological value was poor. For example, the essential amino acid indexes of casein, milk, and lactalbumin were equal, but the biological value of casein was actually lower than milk and lactalbumin.

Because a value of 100 rather than 96 or 97 was assigned to the egg standard, the pepsin pancreatin digest index slightly overestimated the biological values. More precise values may be obtained by multiplying the index by the correction factor (biological value of whole egg/100). This correction agreed closely with the regression line ($Y = 0.970X - 0.54$). This correction could not be accurately applied to the essential amino acid index or the chemical score because their corresponding regression lines ($Y = 1.006X - 9.46$ and $Y = 0.831X - 14.48$) did not correspond to the correction factor.

The essential amino acid index and the chemical score are useful in screening large numbers of potential foods. However, many potential food sources such as seed and leaf proteins require extensive processing which often may have a deleterious effect on the nutritive value of the protein (21). For example, carbohydrates may react with the side chain of some amino acids like lysine (22). Other processes may improve the nutritive value by inactivating digestive enzyme inhibitors. Procedures based on the amino acid composition using acid hydrolysis may not reveal these changes in the nutritive value induced by the processing. However, they would be detected by the pepsin pancreatin digestion. Hence, the pepsin pancreatin digest index would be useful not only in estimating the nutritive value of processed proteins but also in evaluating processing procedures.

A disadvantage of this procedure is the calculation required for the index. Furthermore, any evaluation based on amino acid analysis does not take into account the contribution to the welfare of the animal of factors other than amino acids. Mitchell (23) suggested that some protein foods may contain factors other than amino acids which add to their nutritive value. Although processing procedures and processed foodstuffs must, in the final analysis, be tested with feeding trials, a procedure such as the one described would be useful in preliminary work because of its rapidity and accuracy. The procedure's greatest advantage was the small sample size required. Only 150 mg of protein were required for estimation of the biological value. Thus many variations of a process could be readily tested on a small laboratory scale.

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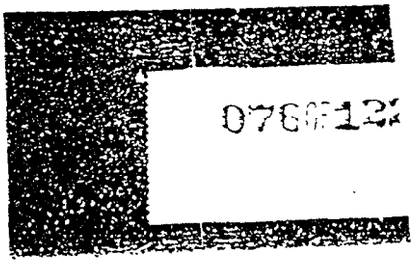

In Vitro Digestion for Pigs and Poultry

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New Inhibitors of Human Renin That Contain Novel Replacements at the P_3 Site[†]

Annette M. Doherty,*[‡] James S. Kaltenbronn,[‡] James P. Hudspeth,[‡] Joseph T. Repine,[‡] William H. Roark,[‡] Ila Sircar,[‡] Frank J. Tinney,[‡] Cleo J. Connelly,[‡] John C. Hodges,[‡] Michael D. Taylor,[‡] Brian L. Hatley,[‡] Michael J. Ryan,[‡] Arnold D. Essenburg,[‡] Stephen T. Rapundalo,[‡] Ronald E. Wendsaar,[‡] Christine Humblet,[‡] and Elizabeth A. Lunney[‡]

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A series of renin inhibitors with novel modifications at the P_3 site has been prepared. Structure–activity relationships reveal that for a particular P_3 fragment the in vitro potency is highly dependent on the nature of the P_2 portion in addition to the P_1–P_1' group. The length of the P_2 side chain and choice of a N,N-disubstitution have been found to be important for in vitro potency, although the degree of unsaturation in the P_2 side chain is not particularly significant. Molecular modeling studies have shown that it is possible for the P_2 side chain to interact unfavorably with the P_3 binding site. It has been possible to control the specificity for renin over cathepsin D by correct modification at the P_2 and P_1–P_1' sites. Variations at the P_3 site have been utilized to lower the log P values of these renin inhibitors while maintaining high potency. Compound 42, which exhibited an IC_{50} of 1.0 nM, log P of 2.3, and showed high specificity for renin, was selected for further studies. It was found to be very stable under neutral, acidic, and basic conditions. In simulated intestinal juice, compound 42 had a half-life of 15 min while it was virtually unaffected by simulated gastric juice after 4 h. Compound 42 produced a significant hypotensive response upon intravenous administration to the salt-depleted normotensive cynomolgus monkey.

Introduction

Renin is an aspartic proteinase synthesized and stored in the epithelioid cells of the juxtaglomerular apparatus of the kidney. It is a highly specific proteolytic enzyme that cleaves the polypeptide angiotensinogen to produce the decapeptide angiotensin I.[2] Angiotensin I, which has no known biological activity, is subsequently cleaved by angiotensin converting enzyme (ACE) to afford the vasoactive peptide angiotensin II. The renin–angiotensin system plays a central physiological role in the regulation of blood pressure and electrolyte homeostasis.[3] Recent evidence indicates that renin and angiotensinogen genes and their products are expressed at many local tissue sites.[4] The existence of a local renin–angiotensin system (RAS) is thought to contribute to the control of vascular tone.[5] The activity of the tissue system under different conditions may influence the pharmacologic response to inhibitors of the RAS.[6]

The design of ACE inhibitors as useful drugs in the treatment of hypertension and heart failure has been reviewed.[7] Renin however possesses much greater substrate specificity than ACE, and this property has led to intense interest in the search for effective inhibitors of renin.[8]

Most of the potent renin inhibitors reported are based upon the natural substrate angiotensinogen sequence incorporating nonhydrolyzable transition-state moieties at the active site.[9-15] Thus these compounds remain partly peptidic in nature and their therapeutic efficacy is limited due to low oral activity and short duration of action. Several studies of renin inhibitors in animals[16-20] and more recently in humans have been reported.[21,22]

In a search for novel and effective renin inhibitors[23] we became interested in variation at the P_3 site. Nomenclature for renin inhibitors illustrated for compound 17 in Scheme I[24] where a histidine residue is present in the substrate angiotensinogen and similarly in many of the potent inhibitors synthesized to date.[9-15] It has been reported that the presence of histidine at P_3 is important for potency[11,15] as well as high selectivity[13,14,25] toward renin over related aspartic proteinases. It has been suggested that the protonated state of the imidazole group at P_3 is a contributor to the inhibitor specificity.[26] However Rosenberg et al. have reported that replacements of the P_3 imidazole group with other heterocycles of varying basicity did not ad-

[†] Abbreviations follow the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature for amino acids and peptides. *Eur. J. Biochem.* **1984**, *138*, 9–11. Additional abbreviations are as follows: ACE, angiotensin converting enzyme; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HOBT, 1-hydroxybenzotriazole; Z, benzyloxycarbonyl; TFA, trifluoroacetic acid; TEA, triethylamine; BOPT, 1-hydroxybenzotriazolyl-tris(dimethylamino)phosphonium hexafluorophosphate; Sta, (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid; ACHPA, (3*S*,4*S*)-4-amino-3-hydroxy-5-cyclohexylpentanoic acid; DiSta, (3*R*,4*S*)-4-amino-3,5-dihydroxy-6-methylheptanoic acid; Di(3,4)Sta, (3*S*,4*S*)-4-amino-3,5-dihydroxy-6-methylheptanoic acid; Di(3,4)ACHPA, (3*S*,4*S*)-4-amino-3,5-dihydroxy-6-cyclohexylpentanoic acid; DiSta(3,4), (3*S*,4*S*)-4-amino-3,5-dihydroxy-6-methylheptanoic acid; AHM, 4-(2-amino-ethyl)-1-methylimidazole.

[‡] Department of Chemistry.

[§] Department of Pharmacology.

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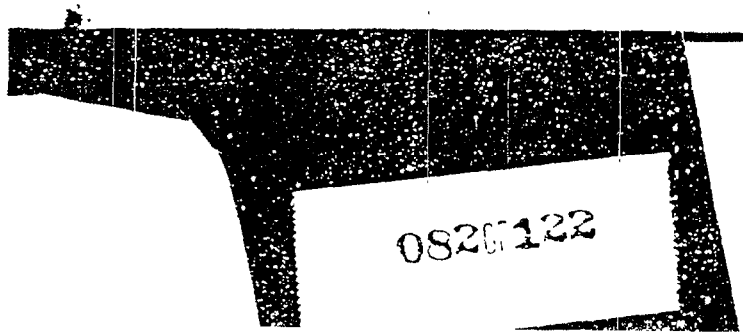
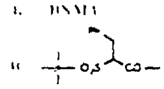
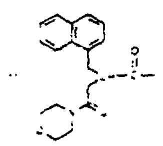
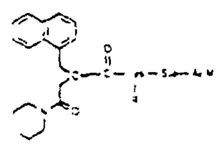
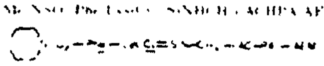
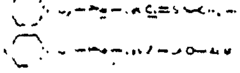
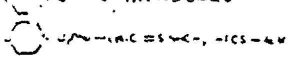
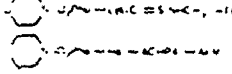


Table I. [illegible]

No.	compd	[illegible]	[illegible]	[illegible]	molecular formula
1	[illegible]	[illegible]	[illegible]		[illegible]
	BNMA [illegible]				
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
12	[illegible] SNHCH		[illegible]	[illegible]	[illegible]
18	[illegible] ONHCH	[illegible]	[illegible]	[illegible]	[illegible]
19	[illegible] SNHPh	[illegible]	[illegible]	[illegible]	[illegible]
20	[illegible]	[illegible] M	[illegible]	[illegible]	[illegible]
21	[illegible] NCN SCH	[illegible]	[illegible]	[illegible]	[illegible]
22	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
23	[illegible] ONH	[illegible]	[illegible]	[illegible]	[illegible]
24	[illegible]	[illegible] M	[illegible]	[illegible]	[illegible]
25	[illegible] COCH	[illegible]	[illegible]	[illegible]	[illegible]
	BNMA [illegible] AEM				
26	[illegible] SNHCH	[illegible]	[illegible]	[illegible]	[illegible] HCl
27	[illegible] NHCNHNO	[illegible]	[illegible]	[illegible]	[illegible] HCl
28	[illegible] NCNSCH	[illegible]		[illegible]	[illegible] HCl
	BNMA [illegible] NHCH Ph				
29	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
30	[illegible] SNHCH	[illegible]	[illegible]	[illegible]	[illegible] HCl
31	[illegible] NHCNHNO	[illegible]	[illegible]	[illegible]	[illegible] HCl
	[illegible] SNHCH [illegible] ACHPA AEM				
32	[illegible] BNMA	[illegible]		[illegible]	[illegible]
33		[illegible]		[illegible]	[illegible]
34		[illegible]	[illegible]	[illegible]	[illegible] HCl



No.	compd	[illegible]	[illegible]	[illegible]	molecular formula
35	[illegible] SNHCH	[illegible]	[illegible]	[illegible]	[illegible] HCl
36	[illegible] NCNSCH	[illegible]		[illegible]	[illegible] HCl
37	[illegible] NHCNHNO	[illegible]		[illegible]	[illegible]
38	BNMA [illegible] SNHCH [illegible] ACHPA NHCH [illegible]	[illegible]		[illegible]	[illegible]
39	BNMA [illegible] SNHCH [illegible]	[illegible]		[illegible]	[illegible] HCl
40	BNMA [illegible] SNHCH [illegible] AEM	[illegible]		[illegible]	[illegible] HCl
41	[illegible] Phe [illegible] SNHCH [illegible] ACHPA AEM	[illegible]	[illegible]	[illegible]	[illegible] HCl
42				[illegible]	[illegible] HCl
43				[illegible]	[illegible] HCl [illegible]
44		[illegible]		[illegible]	[illegible] HCl
45		[illegible]	[illegible]		[illegible]

[illegible] Not measured. [illegible]

[illegible]versely affect the potency of a series of statine derived renin inhibitors.[illegible] Moreover, potent renin inhibitors with a [illegible]aspartyl residue at the P[illegible] site have recently been reported.[illegible]

[illegible] J. Med. Chem. 1987, [illegible]

[illegible] Hoo[illegible], K. Y.; Carlson, W. D.; Bernatowicz, M. S.; Haber, E. J. Med. Chem. 1987, [illegible]

Scheme I

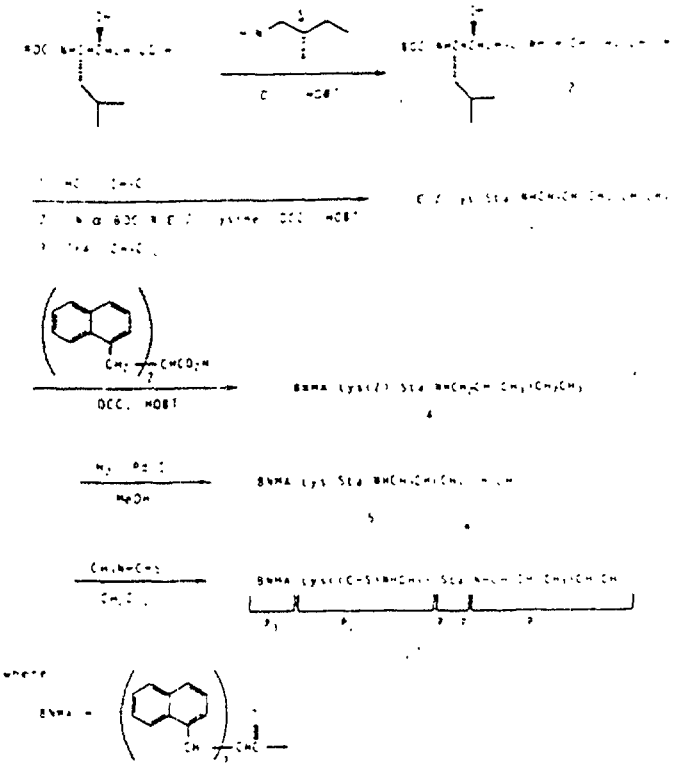


Table II. Analogs with Variation

no.[a]	structure	mp, °C (dec)	solvent system[b]	R_f	molecular formula[c]
BNMA-NH-CH(CH$_2$CH$_2$CH$_3$)-CO-NH-CH(CH$_3$)-CO-NH$_2$					
16					
17					
18					
19					
20					
21					

[a] Compounds tested at pH 7.4. [b] Abbreviations: BuOH, butanol; AcOH, acetic acid; EtOAc, ethyl acetate. [c] Analyses were within ±0.4% of the calculated values.

Our study into the effect of variation at the P$_1$ site indicates that replacement of histidine with modified lysine derivatives maintains high potency but often reduces selectivity for renin. However, suitable modifications, par-

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de Gasparo, M.; Cumin, F.; Sunderlage, J.; Catanese, T. T.; Wood, J. M. *Monatsh. Chem. Suppl.* **1989**, *27*, 67.

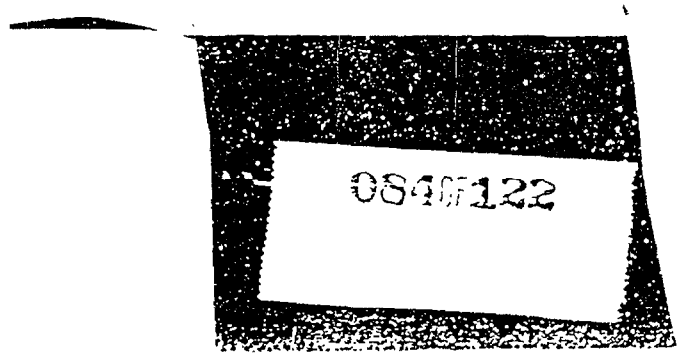


Table III. [illegible]

compd	structure	[illegible]	[illegible]	[illegible]	molecular formula
52	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
53	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
54	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
55a	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
55b	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
56	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
57	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
58	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
59	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
60	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
61a	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
61b	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
62	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
63	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible]

ticularly at the P site in our lysine type P substituted renin inhibitors restores high selectivity.

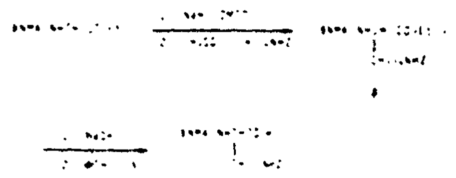
Thus we have prepared a large range of novel compounds (Tables I–III) many of which are highly potent and selective for renin.

Chemistry

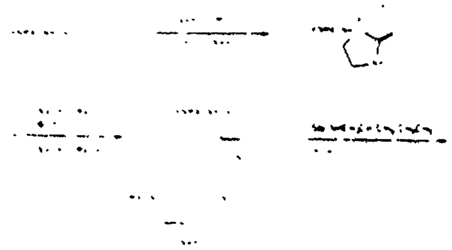
Table I lists compounds 17–45 with a lysine P residue, where the ε-amine is substituted with a variety of groups. These compounds were prepared by using known synthetic methods. Scheme I illustrates the synthesis of 17, exemplary of this group. In addition the corresponding histidine analogue 1 is included in Table I for comparison (BNMA-His-Sta-NHCH CH(CH)CH CH).

Thus BOC-statine was coupled with (S)-methylbutylamine (MBA) by using the standard DCC/HOBT procedure to afford 2 which was followed by hydrochloric acid deprotection and subsequent coupling with Nα-BOC-Nε-Z-lysine. Trifluoroacetic acid treatment and neutralization gave 3. Coupling with bis(1-naphthylmethyl)acetic acid gave intermediate 4. Hydrogenolysis to remove the Z protecting group gave 5, which was treated

Scheme II



Scheme III



with methyl isothiocyanate in dichloromethane affording renin inhibitor 17.

The syntheses of renin inhibitors 39, 43, and 44 in Table I began by coupling of Nα-BOC-difluorostatine (DFSta) with 1-(2-aminoethyl)morpholine (AEM), deprotection and subsequent coupling with Nα-BOC-Nε-Z-lysine. Acidic deprotection was followed by standard coupling procedures with either bis(1-naphthylmethyl)acetic acid (BNMA) or

Kaltenbronn, J. S.; Hudspeth, J. P.; Lunney, E. A.; Michniewicz, B. M.; Nicolaides, E. D.; Repine, J. T.; Roark, W. H.; [illegible] M. A.; Tinney, F. J.; Woo, P. K. W.; Essenburg, A. D. J. Med. Chem. 1990, [illegible]

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[illegible] Pals, D. T.; Kati, W. M.; Turner, S. R.; [illegible] J. M.; Watt, W. J. Med. Chem. 1986, 29, 2080. [illegible] M. H.; Seaton, J. P.; Maher, R. H. Biochemistry 1985, [illegible] Hopkins, P. B.; Gelb, M. H. J. Med. Chem. 1987, [illegible]

Table IV. Structure-Activity Data

compd	structure	K_i, nM, of inhibition at pH 7.4 and 37 °C: renin (human plasma)	renin (monkey plasma)	IC_{50}, nM
17	BNMA-Leu-Leu-Val-Sta-NHCH$_2$CH$_2$NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_3$	0.5	1	18
26	BNMA-Leu-Leu-Val-Sta-AEM	0.6	1	10
32	BNMA-Leu-Leu-Val-Sta-NHCH$_2$CH$_2$CH$_2$-AEM	1.0 × 10^{-3} M	50	
35	BNMA-Leu-Leu-Val-Sta-AHPA-AEM	0.05	1	60
42		0.05	1	0.5

BNMA-NHCH(R^1)CO-Sta-Val-Sta-NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_3$

20	R^1 = CH$_2$-NHCO-NH-NHNO	0.3	50	70
22	R^1 = CH$_2$CH$_2$CH$_2$CH$_2$NHCO-Val-NHCH$_3$	0.4	10	17
23a	R^1 = CH$_2$CH$_2$CH$_2$CH$_2$NHCO-Leu-H	0.4	10	17

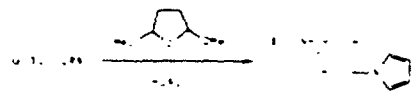
BNMA-NHCH(R^1)CO-Leu-Val-Sta-NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_2$NHCO-Val-AHPA-AEM

24	R^1 = CH$_2$-Val-NHCH$_3$	0.005	10	1000
25	R^1 = CH$_2$-NH-NHNO	1.0 × 10^{-7} M	10	>100
29	R^1 = CH$_2$CH$_2$CH$_2$CH$_2$NH$_2$	1.0 × 10^{-7} M	50	>100

morpholinosulfonylphenylalanine.[5] Compound 43 was obtained by Swern oxidation of the precursor difluorostatine compound.

Renin inhibitors 32, 33, 34, 41, and 42 were synthesized by using standard chemistry from intermediate Leu-Z-AC$_5$HPA-AEM by coupling with the requisite P$_1$-P$_2$ group, hydrogenolysis, and acylation.

The inhibitors in Table II, 46–51 illustrate the variation in chain length at the P$_1$ position. The pyrrole analogue 48 (Table II) was prepared from aminoacid 6.[6]



The other modified lysine chain length compounds shown in Table II (46, 47, 49–51) were synthesized in racemic form via alkylation of the diester 7 with the appropriate halide or mesylate (Scheme II).

It should be noted that during the analogous synthesis of the two-carbon homologue 46 from 7 that cyclization to the five-membered ring 11 occurred (Scheme III). However, hydrolysis and decarboxylation to afford 12 followed by coupling with Sta-NHCH$_2$CH$_2$CH$_2$CH$_2$CH$_3$ occurred under the usual conditions to afford 13 which was successfully converted to renin inhibitors 46 and 47.

Compounds in Table III were synthesized in order to investigate the effect of unsaturation in the P$_1$ lysine side chain. The analogues 52–63 were prepared by using the alkylation chemistry described for some of the compounds in Table II.

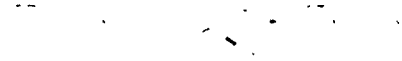
Thus renin inhibitor 52 was prepared via alkylation of BOC-NHCH(R)CO-Et (10) with 4-chloro-2-butynyl-7-but-2-enylamine.[8] Due to problems experienced in the selective removal of the Z protecting group, compounds 53 and 53a–60 were prepared by using the alkylation chemistry with BOC-NHCH(R)CH=CH-CH$_2$-Br[9] and BOC-NHCH(R)-C≡C-CH$_2$-Br[9] as the electrophiles, respectively. Compound 54 was obtained by Lindlar reduction of analogue 53a.

Table V. Physical Data for Renin Inhibiting Compounds

compd	log P*	solubility,[a] mg/mL
17	1.25	<0.01
26	1.31	<0.01
30	0.70	<0.01
32	1.11	2.75
41	2.41	2.83
42	2.26	3.0

[a] pH 7.4 phosphate buffer.

Acetylene analogues 61–63 were prepared via alkylation of diester 14 with 1-[(tert-butoxycarbonyl)amino]-4-chloro-2-butyne (15), affording intermediate 16.



Results and Discussion

1. Structure Activity Relationships. Most of the compounds in Tables I–V have been tested for inhibition of monkey plasma renin activity (PRA). For comparative purposes some of the compounds have also been evaluated for inhibition of human plasma renin activity. Since PRA values are considerably higher in the salt-depleted furosemide treated monkey than the human, the IC$_{50}$ values for the former are considered to be more accurate. Clearly the observed trends within the monkey plasma IC$_{50}$ values are predictive for results in human plasma.

From Table I it is interesting to note that changes in substitution at the P$_1$ site center as a lysine-substituted side chain. For example, direct comparison of 17 with 26 and 21 with 28 illustrates that when P$_1$-P$_2$ is statine, it is preferable for the P$_1$ to be substituted with the (2S)-methylbutylamine group rather than the (aminomethyl)-morpholine group. However, this same trend is not observed when P$_1$-P$_2$ is AC$_5$HPA (comparison of 38 with 22). The inactivity of the N,N-dimethylamide P$_1$-substituted analogue 39 was somewhat surprising in view of the high potency of many of the other thiourea analogues and the good potency of the corresponding analogue with His at P$_1$ (BNMA-His-Sta-NHCH$_2$CH$_2$CH$_2$CH$_3$, IC$_{50}$ = 62 nM). These results indicate that potency is affected by the combination of P$_1$ and P$_2$ substitutions chosen. Molecular modeling

[5] W. Zoller, H. Rebenstorf, K. [illegible], Ger. Offen. 1959 [illegible].
[6] Evans, A. D. Hunter, J. L. [illegible], 1962, [illegible].
[7] Pedersen, B. Rüttinger, J. Oberste, J. Grove, H. [illegible], 1969, [illegible].
[8] The synthesis of these electrophiles was carried out according to the procedure described by Marszak-Fleury, Marszak, [illegible], 1958, [illegible].

studies with the inactive *N,N*-dimethylamide analogue 39 and the active compound 17 are described in section I and provide some explanation for the potencies observed.

At the P_1-P_1' site the expected increase in potency by replacement of statine with ACHPA[9] is illustrated by comparison of 26 with 32 and 35 with 34.

The statine-containing inhibitor 26 is slightly more potent than the corresponding fluorine-containing analogue 30, a trend reported previously.[10] However this same trend is not seen when comparing the ACHPA and corresponding difluoro-substituted ACHEA group in compounds 42 and 41, where the potencies are very similar.

Comparison of the different acyl groups on the P_2 side chain indicates that the phosphonoacetate, isovalate, and Z groups tend to be detrimental to in vitro potency, i.e., compounds 20–24, and 29. This may be related to the orientation of the lysine side chain in the flap region of the enzyme cleft. However it should be noted that all of the lysine-derivatized compounds 17–25 (with the exception of 20 and 24) are more potent than 5 which contains a free lysine group at P_2. It can be seen that substitution of histidine at P_2 (compound 14) does not appear to adversely affect the in vitro potency with many of the compounds studied.

The activity of 43 is somewhat surprising in view of the fact that other N-Z-lysine compounds showed poor activity, i.e., 29 and 32. Presumably this is partly a result of the presence of the difluorostatone moiety at P_1-P_1' which is believed to bind in the active site as the hydrated form of the difluoroketone group. Unfortunately, other analogues could not be prepared since attempts to remove this Z group were unsuccessful in the presence of the difluoroketone moiety.

Turning to the effect of the P_1' group in our renin inhibitors, comparison of 32–37 and 34 indicates little change in in vitro potency. However, substitution with dimethylsulfonylphenylalanine and particularly the morpholinosulfonylphenylalanine P_1-P_1' groups, i.e., 11 and 12, caused a substantial increase in in vitro potency.

Comparison of 26 with 35, 27 with 37, and 28 with 36 indicates that the bicyclic naphthylmethylbutanoyl group is preferable to the substituted succinic acid P_2' group in these examples.

More recently, efforts have been directed toward incorporation of polar P_1' groups in an attempt to lower the log *P* values and increase aqueous solubility of our renin inhibitors. Thus use of the sulfonylphenylalanine P_1-P_1' fragment has given rise to several highly active compounds (41–45) with lower log *P* values (see Table I). The aqueous solubility of selected inhibitors over a range of log *P* values is shown in Table V. As the log *P* value is lowered, aqueous solubility is indeed increased.

In general the activity results from the P_2 chain length study indicate that a four-carbon chain, as for lysine, appears to be optimal. However some caution must be exercised when comparing compounds in Table I with those in Table II since those with the unnatural amino acid chain length (i.e., 4) are all diastereomeric mixtures at the P_2 site (except 18 which was synthesized from L-ornithine) and hence the true IC$_{50}$ values may be lower than those determined for the mixtures.

However some comparisons may still be made with certainty. Thus the fall off in potency observed on shortening the length of the side chain from 4 through 2 carbons is illustrated by comparison of 17 with 47 and 25 with 46. Comparison of 22 with 48 indicates a slight loss of potency of the three-carbon analogue. Five- and six-carbon chain compounds show little difference in potency

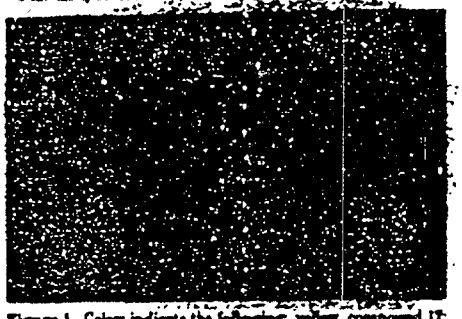


Figure 1. Colors indicate the following: yellow, compound 17; blue, C terminus of compound 30.

relative to the natural amino acid four-carbon chain length illustrated by comparison of 17 with 49 and 51 (note 49 and 51 are diastereomeric mixtures).

Table III includes compounds with unsaturation in the lysine side chain. Most of these are mixtures, diastereomeric at the P_2 site (although in a few cases separation has been possible i.e., 55a,b, 61a,b, 62, and 63). The *E* and *Z* olefinic analogues are of comparable potency to the saturated compounds, i.e., comparison of 53 with 17 and 54 with 25 (note 53 and 54 are diastereomeric mixtures). In general the acetylenic analogues have shown comparable or slightly higher potency than the corresponding saturated analogues: for example, comparison of 55a with 25, 57 with 32, and 62 with 42.

Many highly potent compounds have been synthesized within this acetylenic series with variation of the N-terminal acyl group, i.e., 55a, 58, 59, 60, 61a, 62, and 63.

2. Selectivity. Table IV illustrates the specificity of some selected inhibitors of renin (from Tables I–III) over the closely related aspartic proteinase cathepsin D (bovine). When statine is present at P_1-P_1', selectivity for renin over cathepsin D is less than 100 (compounds 17, 26, 30–32, and 35a). Different chain lengths or degree of unsaturation at the P_2 site do not appear to affect the selectivity significantly, illustrated by compounds 50, 52, and 55a. However when statine is replaced by ACHPA and MBA with AEM (simultaneously) there is a substantial increase in selectivity (cf. compound 32 with compounds 17, 26, and 38). Thus compounds 42, 56, 58, and 59 are all highly specific inhibitors of renin.

3. Molecular Modeling. In an attempt to rationalize some of the structure activity relationships described above we studied possible orientations for P_2 side chains in the active site of renin. This molecular modeling study involved docking analyses of several renin inhibitors into a model of human renin derived from the crystal structures of various fungal aspartic proteases.[9] The thiourea derivative of lysine, found in compound 17, (Figure 1), was among the substituents studied. Particular attention was given to the relationship upon binding, between the Lys-(CH$_2$)$_4$-NH-C(=S)-NH-CH$_3$ side chain and the P_2' substituent which can occupy a vacant S_2' binding site (with statine-type moieties at P_1 and P_1' there is no P_1' side chain). Results indicate that P_2' side chains having a certain length and flexibility, are able to extend into the S_2' binding pocket thus reducing the P_2 binding site availability. In the

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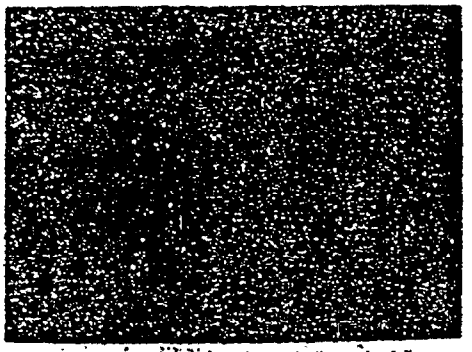


Figure 2. Dot VdW surfaces. Colors indicate the following: yellow, thiazole side chain of compound 17; blue, *N,N*-dimethylamide of compound 39.

example of the inactive analogue **39**, the lack of potency might be due to the interaction of the long P_2 side chain with the *N,N*-dimethylamide at P_3' upon docking into the enzyme (Figures 1 and 2).

Moreover, further modeling analyses supported the premise that the *N,N*-dimethylamide functionality was compatible with the cleft. This was substantiated when the histidine derivative of **39** (BNMA-His-Sta-NCH(CH$_3$)$_2$) was prepared and found to be active (monkey renin, IC_{50} = 62 nM). With the shorter substituent at P_3, there is no interference between the P_2 and P_3 residues upon binding.

Thus, molecular modeling studies involving docking experiments indicate that a potential unfavorable interaction between the P_2 and P_3 substituents may occur in some cases, resulting in lower than expected potency.

4. Evaluation of Compound 42. Compound **42** was chosen for further evaluation due to its high potency and selectivity for renin. In simulated intestinal juice at 37 °C, **42** possessed a half-life of 37 min. It was virtually unaffected in simulated gastric juice after 4 h. Compound **42** was also found to be very stable in neutral, acidic, and basic aqueous solutions, with more than 95% inhibitor remaining after 24 h. Renin inhibitor **42** was selective for renin versus other closely related aspartic proteinases, inhibiting bovine cathepsin D with an IC_{50} = 3.7 μM and porcine pepsin with an IC_{50} > 100 μM.

Renin inhibitor **42** (IC_{50} = 1.1 nM) was selected for in vivo evaluation. Figure 3 illustrates the blood pressure response to an intravenous infusion of 1 mg/kg of **42** over 1 h to salt-depleted normotensive cynomolgus monkeys (*N* = 2). Mean arterial pressure fell by a maximum of 22 mmHg. However, compound **42** showed no oral activity upon po administration (>30 mg/kg).[17]

Figure 3. Effect of compound **42** (1 mg/kg iv infusion, 1 h) on blood pressure in salt depleted normotensive cynomolgus monkeys (*n* = 2).

Conclusion

Renin inhibitors with novel modifications at the P_2 site were prepared and tested in vitro. A number of conclusions can be drawn from the structure–activity relationships.

First, it is clear that the cleft of the renin enzyme tolerates a wide range of acyl-substituted lysine side chains at the P_2 site. Side chains smaller than the natural lysine C4 chain appear to adversely affect in vitro potency. Unsaturation in the lysine chain or longer carbon side chains showed little effect on potency.

Second, the activity of the P_2-modified renin inhibitors is largely dependent on the nature of the P_3 site. We have shown by modeling that it is possible for the P_2 side chain to interact unfavorably with the P_3 binding site and this may provide some explanation for the low activity of analogue **39**.

Third, variation at the P_3 site has been utilized to lower the log *P* values of representative renin inhibitors effectively. The stability of the P_2 lysine modified renin inhibitors in gastric and intestinal juices has been found to be fairly high. However, these factors are clearly not the only requirements for oral efficacy and duration of action. Hence, although a reasonable and prolonged drop in blood pressure was noted upon intravenous administration of compound **42**, oral administration showed little effect on blood pressure. Poor oral absorption and/or extensive elimination are possible reasons for this observation.[17,18]

Finally we have shown that it is possible to control the specificity of our inhibitors between renin and cathepsin D by simple modification at the P_2 and P_3, P_3 sites.

Experimental Section

The NMR spectra were recorded on a Varian EM-390, Varian XL-300, or an IBM WP-200SY instrument. The FAB MS was determined on a VG analytical "70-0F" HF mass spectrometer in the positive ion mode with xenon used as the target gas. Rotations were recorded on a Perkin Elmer Model 141 polarimeter. TLC was carried out on pressured sheets (silica gel 60F-254, Merck). Silica gel chromatography was done with Kieselgel 60 (70–230 mesh) or Kieselgel 60 (230–400 mesh for flash).

All compounds were purified by chromatography on silica gel and were usually obtained as solid foams that often retained solvent even on prolonged drying under vacuum. Intermediates and the compounds in Tables I–IV all showed the correct molecular ion in the FAB mass spectrum. The NMR was consistent with the assigned structures. The k_w' values of the compounds in Tables I–III were determined by using a standard HPLC correlation method.[9]

Modeling. Studies were carried out with the VAX version of the QUEST molecular modeling program[11] with use of an Evans and Sutherland ES-350.

A model of human renin derived from the crystal structures of various fungal aspartic proteases including endothiapepsin, penicillopepsin, and rhizopuspepsin[12] were used in this study.

(17) Tawa, R. U. *Pharm. Int.* **1986**, 179.
(18) Humphries, M. J.; Ringrose, P. S. *Drug. Metab. Rev.* **1986**, *17*, 283.
(19) Hicks, J. F.; Young, A. M. *J. Chromatogr.* **1984**, *316*, 675.
(20) Version 3.0. Tripos Associates, Inc., Unpublished. Evans and Sutherland, 580 Arapeen Drive, Salt Lake City, UT 84108.

The dosing syringes were positioned within the confines of the water jacket containing three needles in contact with the paws. Solenoid pinch valves were activated, and solution delivery was initiated with a short delay. Pressure and heart rate were monitored with standard Gould Biotach units and the signals routed to the P and P peak detector modules. Final determinations were obtained through molecular mechanics treatment by using the Hyperchem software available with the unit.

Biological Methods. Inhibition of renin activity in vitro was inhibitor dose was determined by a radioimmunoassay for angiotensin I based on the method of Haber et al.[43] The in vitro angiotensin I generation was performed in a total of 0.5 mL of monkey plasma containing excess renin and angiotensinogen. The plasma was buffered with 0.1 M sodium phosphate, pH 7.4, containing 0.1% EDTA and 0.1% 8-hydroxyquinoline. Inhibitors were added at a single concentration or a dilution series in ethanol, methanol, DMSO, or water. Incubation was for 30 min at 37 °C. Following incubation, each mixture was analyzed for angiotensin I via radioimmunoassay in quintuplicate. Unlabeled angiotensin I and counting were carried out in tubes coated with rabbit anti-angiotensin I antibody (Gamma Coat RIA kit, Diagnostic Systems Laboratories, Webster, TX). Human plasma renin activity ranged from 1.0 ng/mL per h. Values for inhibitor tubes were compared to vehicle (DMSO)-control tubes to estimate percent inhibition. At the concentration used, DMSO inhibits the generation of angiotensin I by <10%. The inhibition results were expressed as IC_{50} values, which were obtained by plotting % inhibition vs concentration and estimating the concentration producing 50% inhibition by using nonlinear regression analysis. IC_{50} values for inhibition of human renin were determined by the method reported previously.[44]

Inhibition of bovine cathepsin D enzyme activity was assessed in duplicate by the hydrolysis of bovine hemoglobin (3× crystallized Sigma) at pH 3.2 and 37 °C (modified from Kobayashi et al. and Antonov et al.[45,46]). Net absorbance at 280 nm was measured in acid precipitated supernatant fractions of inhibited vs uninhibited control assays. The IC_{50} values were determined as described above.

Intravenous Administration of 42. Male cynomolgus monkeys weighing between 3.9 and 5.7 kg were instrumented with vascular access ports (Norfolk Medical Products, Skokie, IL) for intravenous blood pressure monitoring and drug administration. The animals had previously been trained to sit quietly in primate restrainer chairs (Primate Products, Woodstock, CT). For normotensive studies, animals were placed in their usual cages. Doses for intravenous (iv) bolus were prepared in physiological saline (Abbott Laboratories, North Chicago, IL) for administration of compound 42.

Each monkey was pretreated with furosemide (Lasix, IVAX SP 50, Hialeah Research) 2 mg/kg per day, im, for four consecutive days before testing. Blood pressure was determined by using a stathom pressure transducer (Gould) polygraph and computer data acquisition system. Compound 42 was given as a solution in a vehicle of ethanol, alcohol and 5% dextrose (EtOH:PG:5% D/W). The compound was dissolved in ethanol (5% by volume) and propylene glycol (10% by volume) to which 5% dextrose in water was added. The concentration was adjusted such that the total dose was given over 1 h in a volume of 1 mL/kg.

Oral Dosing. Oral studies were carried out in room dependent hypertensive cynomolgus monkeys. These were presented a partial occlusion (≈50%) reduction in renal blood flow via the left renal artery by utilizing an adjustable clamp. Twelve more weeks later monkeys were challenged with an intravenous infusion (20 ng/kg per min for 30 min) of the angiotensin II receptor antagonist saralasin (Sar^1 Val^5 Ala^8 All Roche Inc. Torrance, CA). Monkeys were included in renal dose studies if the renal occlusion raised blood pressure by at least 15 mmHg above pre-occlusion blood pressure and if blood pressure fell by at least 15 mmHg during the saralasin infusion. Blood pressure and heart rate were measured by using a computer data acquisition system. The vehicle consisted of a mixture of 75% dimethylacetamide (DMA), Tween 80, and 95% distilled water (added in this order). The

(43) Blundell, T. L.; Cooper, J.; Foundling, S. I.; Jones, D. M.; Atrash, B.; Szelke, M. *Biochemistry* **1987**, *26*, 5585.

(44) Haber, E.; Koerner, T.; Page, L. B.; Kliman, B.; Purnode, A. *J. Clin. Endocrinol. Metab.* **1969**, *29*, 1349.

(45) Aoyagi, T.; Morishima, H.; Nishizawa, R.; Kunimoto, S.; Takeuchi, T.; Umezawa, H. *Biochem. Biophys. Res. Commun.* **1972**, *...*

concentration was adjusted for a total dose administered in a volume of 1 mL/kg. Compound 42 was given by oral gavage by using a French catheter (Jorgensen Labs, Loveland, CO).

Stability Studies. Simulated gastric juice was prepared according to USP XXI (without pepsin) by dissolving 2.0 g of sodium chloride and 3.2 g of purified porcine stomach mucosal pepsin in 7.0 mL of concentrated HCl and diluting to 1000 mL with water. The solution was placed in a water bath at 37 °C. Samples were withdrawn periodically and analyzed by HPLC on a Bondapak phenyl column (Waters Associates, Milford, MA) using a mobile phase of 20% acetonitrile–ammonium phosphate monobasic buffer (0.05 M, pH adjusted to pH 3 with H$_3$PO$_4$) flow rate of 1.0 mL/min and a wavelength detection at 214 nm.

Stability testing in simulated intestinal juice of SPXXI (1985) was carried out by using the above experimental conditions.

General Synthetic Procedures. General Procedure A. Coupling Reactions Using Dicyclohexylcarbodiimide and 1-Hydroxybenzotriazole. The amine·HCl component (1.0 mmol), the carboxy component (1.0 mmol), and HOBT (1.0 mmol) were dissolved in DMF (5 mL) and cooled to 0 °C. The solution was treated with DCC (1.0 mmol) in DMF (5 mL) and stirred for 5–10 min. The amine component (1.0 mmol) was then added. After stirring at 0 °C for 1 h and at 25 °C for 24 h, the suspension was filtered and the filtrate evaporated to dryness. The residue was taken up in EtOAc (30 mL) and washed with saturated NaHCO$_3$ solution (10 mL) and brine (10 mL). The organic layer was dried (Na$_2$SO$_4$), evaporated to dryness, and the residue chromatographed on silica gel.

General Procedure B. Coupling Reactions Involving Dicyclohexylcarbodiimide and 1-Hydroxybenzotriazole. The carboxy component (1.0 mmol) and HOBT (1.0 mmol) were dissolved in DMF (5 mL) and cooled to 0 °C. The solution was treated with DCC (1.0 mmol) in DMF (5 mL) and stirred for 5–10 min. The amine component (1.0 mmol) was then added. After stirring at 0 °C for 1 h and at 25 °C for 24 h, the suspension was filtered and the filtrate evaporated to dryness. The residue was taken up in EtOAc (30 mL) and washed with saturated NaHCO$_3$ solution (10 mL) and brine (10 mL). The organic layer was dried (Na$_2$SO$_4$), evaporated to dryness, and the residue chromatographed on silica gel.

General Procedure C. Coupling Reactions Involving Dicyclohexylcarbodiimide and 1-Hydroxybenzotriazole. This procedure is similar to general procedure B with the modified workup described. After filtration and evaporation under reduced pressure, the residue was taken up in EtOAc (30 mL) and washed sequentially with 10 mL portions of 1 N citric acid, saturated NaCl, saturated NaHCO$_3$ solution, and saturated NaCl solution. Any precipitate was filtered from the organic layer which was then dried (Na$_2$SO$_4$) and evaporated and the residue chromatographed.

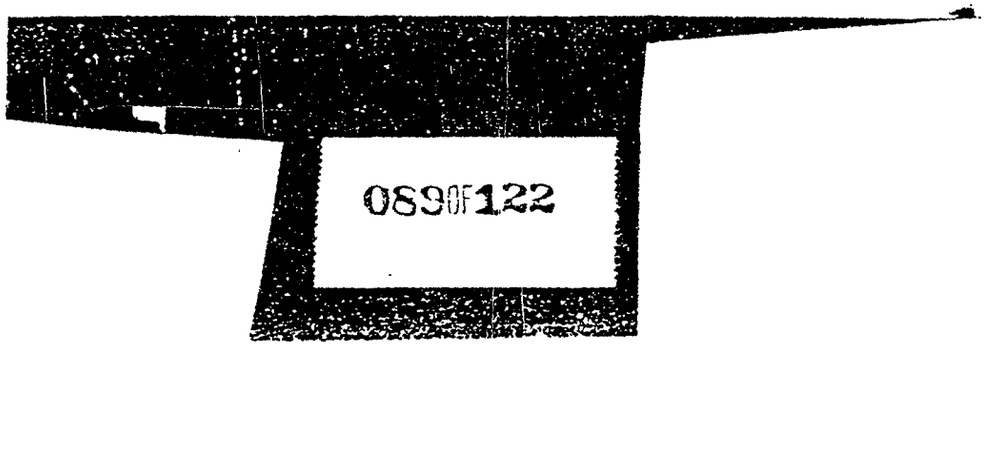
General Procedure D. Removal of *tert*-Butoxycarbonyl Group. A solution of the BOC-protected amine (1.0 mmol) in CH$_2$Cl$_2$ (5 mL) was treated with TFA (2 mL) and stirred for 2 h at room temperature. Reaction completion was assessed by TLC. The solvent was removed under reduced pressure and the residue diluted with EtOAc (30 mL) followed by washing with NaHCO$_3$ solution. The organic layer was washed with brine and dried (Na$_2$SO$_4$). The solvent was evaporated and the residue dried under high vacuum for several hours.

Alternative Procedure D. A solution of the BOC-protected amine component (1.0 mmol) in CH$_2$Cl$_2$ (5 mL) and CH$_3$I (MeOH (1.1) was cooled to 0 °C and dry HCl gas bubbled into the mixture for 15 min. After the solution was stirred for a further 30 min, the solvent was evaporated under reduced pressure. The residue was taken up in CHCl$_3$ and the solvent removed under reduced pressure. This procedure was repeated twice more. The residue was then dried under high vacuum for several hours and the resulting amine hydrochloride used directly in ensuing reactions.

General Procedure E. Removal of Benzyloxycarbonyl Groups by Catalytic Hydrogenation. A solution of the Z-protected amine component (1.0 mmol) in methanol (15 mL) was treated with 10% palladium on carbon (0.1 mmol) and stirred under a hydrogen atmosphere for 4–8 h. Reaction completion was assessed by TLC. The mixture was filtered and evaporated under reduced pressure.

BNMA-His-Sta-NHCH$_2$CH(CH$_3$)CH$_2$CH$_3$ (1). Title compound 1 was prepared as previously reported.[38]

Z-Leu-Sta-NHCH$_2$CH(CH$_3$)CH$_2$CH$_3$ (3). ...N-BOC...Z-...(1.0 mmol) and Sta-NHCH$_2$CH(CH$_3$)CH$_2$CH$_3$·HCl



(21–9 °C (1.50 g, 5.34 mmol) were coupled according to general procedure A with HOBT (0.72 g), TEA (0.74 mL, 5.34 mmol), and DCC (1.10 g, 5.30 mmol) in DMF (20 mL). The product was purified by column chromatography on silica gel eluting with 1:1 EtOAc–hexane to afford the coupled product (2.20 g, 85.7%) as a white foam. Deprotection of BOC-Lys(Z)-Ala-NHCH$_2$CH(CH$_2$CH$_2$CH$_3$)$_2$ was carried out according to general procedure D (a) to afford the product 3 (2.10 g, 88.3%) as a white foam, utilized directly in the next reaction.

BNMA-Lys(Z)-Ala-NHCH$_2$CH(CH$_2$CH$_2$CH$_3$)$_2$ (4). Compound 3 (2.10 g, 4.14 mmol) and beta-naphthylmethylbromic acid (1.31 g, 4.21 mmol) were coupled according to general procedure B. The crude product was recrystallized from CHCl$_3$ to afford a white foam (1.56 g, 57.9%). The residue from the mother liquor was purified by column chromatography on silica gel eluting with CHCl$_3$–EtOAc (3:1) to afford additional product (0.50 g, 18.6%). Anal. ($C_{39}H_{46}N_4O_6$) C, H, N; C: calcd, 72.88; found, 72.34. FAB MS: [M + H]$^+$ m/z 829.3 (R29).

BNMA-Lys-Ala-NHCH$_2$CH(CH$_2$CH$_2$CH$_3$)$_2$ (5). Compound 5 was prepared in 95.9% yield from 4 (1.95 g, 2.40 mmol) by general procedure E. Anal. ($C_{31}H_{40}N_4O_4 \cdot 1.5H_2O$) C, H, N. FAB MS: [M + H]$^+$ m/z 695.5 (695).

***N*-[(1,1-Dimethylethoxy)carbonyl]-5-(1*H*-pyrrol-1-yl)-L-norvaline (6).** A solution of α-BOC-ornithine (1.20 g, 5.20 mmol) in AcOH (20 mL) was treated with 2,5-dimethoxytetrahydrofuran (0.80 g, 6.10 mmol) and heated to reflux while allowing AcOH (~15 mL) to distill off. The remainder of the acid was removed under reduced pressure. The residue was taken up in dilute NaOH, acidified with 1 N citric acid, and extracted twice with CHCl$_3$. The organic extracts were dried (Na$_2$SO$_4$), filtered, and evaporated. The crude product was purified by chromatography on silica gel with EtOAc to afford the product 6 as a yellow oil (0.70 g, 47.7%).[39,40]

Diethyl [[3-(1-Naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]amino]propanedioate (7). A suspension of (diethylaminomalonate hydrochloride (4.13 g, 0.0195 mol) in CH$_2$Cl$_2$ (100 mL) was treated with beta-naphthylmethylacetyl chloride (7.0 g, 0.0195 mol) at 0 °C. TEA (5.50 mL, 0.039 mol) was added dropwise and the whole stirred at 0 °C for 30 min and at room temperature overnight. The suspension was filtered and evaporated under reduced pressure.

The residue was taken up in EtOAc and washed with 1 N HCl, H$_2$O, saturated NaHCO$_3$ solution, and brine sequentially. After the residue was dried (Na$_2$SO$_4$) and evaporated, the product 7 was obtained as a white solid (9.26 g, 95.4%), mp 118–120 °C. MS (EI) [M]$^+$ m/z 497 (495). ^{1}H NMR (CDCl$_3$) δ 1.10 (6 H, t, J = 7.1 Hz), 2.95–3.20 (5 H, m), 4.05 (2 H, q, J = 7.1 Hz), 5.95 (1 H, d, J = 4.4 Hz), 7.15–7.85 (14 H, m). Anal. ($C_{31}H_{31}NO_5$) C, H, N.

Diethyl [[3-(1-Naphthalenyl)-2-(1-naphthalenyl)-1-oxopropyl]amino]-2-[5-[[(phenylmethoxy)carbonyl]amino]pentyl]propanedioate (8). Sodium hydride (50% in oil, washed free of oil with dry petroleum ether, 0.48 g, 9.50 mmol) was suspended in DMSO (10 mL). The malonate 7 (4.74 g, 9.50 mmol) was added in portions and the suspension stirred until evolution of hydrogen had ceased. The solution was then treated with 1-Z-amino-5-[(methylsulfonyl)oxy]pentane (3.10 g, 9.50 mmol) and KI (1.50 g) in DMSO (10 mL) and the mixture stirred for 5 days. The solution was then diluted with EtOAc and washed twice with water, saturated NaHCO$_3$ solution, and saturated NaCl, sequentially. Drying (Na$_2$SO$_4$) and removal of the solvent under reduced pressure gave the crude product 8. Purification by silica gel chromatography (1% MeOH–CHCl$_3$) afforded pure material 8 (1.70 g) and a mixture of starting material 7 and product 8 (2.40 g, ~1:1 ratio by NMR) and additional 7 (3.00 g). FAB MS (M + H)$^+$ m/z 717.3 (717).

1-Z-Amino-5-[(methylsulfonyl)oxy]pentane. To 5-aminopentan-1-ol (20 g, 0.19 mol) in dioxane (100 mL) and water (50 mL) at 0 °C was added benzyl chloroformate (27.7 mL, 0.19 mol) in dioxane. After half of this reagent had been added NaOH (7.7 M, 50 mL, 0.19 mol) was added. After complete addition the reaction was warmed to room temperature and stirred for 60 h. Extraction into EtOAc and washing with H$_2$O, NaHCO$_3$, and brine gave, after drying (Na$_2$SO$_4$) and evaporation, the crude product 5-Z-aminopentan-1-ol (45 g, 98.5%). This material (10.0 g, 0.042 mol) was dissolved in CHCl$_3$ (150 mL) and cooled to −20 °C. TEA (5.90 mL, 0.042 mol) followed by methanesulfonyl chloride (3.30 mL, 0.042 mol) were added. After stirring for 2 h the solution was washed with saturated NaHCO$_3$ and then saturated NaCl. The organic layer was dried (Na$_2$SO$_4$) and evaporated. The crude material was filtered through a small plug of silica gel to afford the product 1-Z-amino-5-(methylsulfonyloxy)pentane (12.40 g, 99.5%) as an oil. ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.40–1.90 (6 H, m), 3.00 (3 H, s), 4.30 (2 H, dd, J = 6.4, 12.8 Hz), 4.20 (2 H, m), 4.90 (1 H, br s), 5.10 (2 H, s), 7.36 (5 H, s). MS (CI + CH$_4$) (M + H)$^+$ m/z 301.9 (316).

(±)-2-[[3-(1-Naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]amino]-7-[[(phenylmethoxy)carbonyl]amino]-heptanoic Acid (9). A solution of the diester 8 (1.20 g, 1.8 mmol) in dioxane (10 mL) and MeOH (20 mL) was treated with NaOH solution (0.20 g in 5 mL H$_2$O, 5.0 mmol) and the resulting mixture stirred for 3 h at room temperature. The solution was diluted with H$_2$O and washed with Et$_2$O. Acidification to congo red with dilute HCl was followed by extraction with ethyl acetate. The combined organic extracts were washed with water and then brine. After drying (Na$_2$SO$_4$) and evaporation under reduced pressure the residue was dissolved in toluene (50 mL) and dioxane (10 mL) and heated under reflux for 4 h to effect decarboxylation. The solvent was evaporated under reduced pressure and the crude product 9 (0.70 g) utilized directly in the next reaction: FAB MS (M + H)$^+$ m/z 617.2 (617).

2-Iodo-1-[(*tert*-butyloxycarbonyl)amino]ethane (10). To 2-bromoethylamine hydrobromide (10.0 g, 48.8 mmol) suspended in CH$_2$Cl$_2$ (150 mL) at room temperature was added TEA (7.11 mL, 51.2 mmol) followed by di-BOC dicarbonate (10.64 g, 48.8 mmol) in CH$_2$Cl$_2$ (40 mL). After stirring for 16 h the solvent was evaporated under reduced pressure and the residue taken up in EtOAc and washed sequentially with H$_2$O and brine. Drying (MgSO$_4$) and concentration gave a colorless oil which was purified by column chromatography on silica gel eluting with CH$_2$Cl$_2$. The product 2-bromo-1-[(*tert*-butyloxycarbonyl)amino]ethane was obtained as a white solid (5.80 g, 54%). ^{1}H NMR (90 MHz, CDCl$_3$) δ 1.45 (9 H, s), 3.40 (4 H, m). Anal. ($C_7H_{14}NO_2Br$) C, H, N.

The bromide (5.20 g, 23.0 mmol) was taken up in acetone (20 mL) and treated with excess anhydrous sodium iodide (5.0 g). After stirring for 24 h at room temperature the suspension was diluted with Et$_2$O and filtered through a small pad of neutral alumina. Evaporation of the solvent gave a crude yellow oil which was purified by column chromatography on silica gel eluting with 10% Et$_2$O in hexane to afford a colorless oil (4.42 g, 70.2%) identified as the title iodide. ^{1}H NMR (90 MHz, CDCl$_3$) δ 1.48 (9 H, s), 3.20 (2 H, m), 3.80 (2 H, m).

Ethyl 1-[(1,1-Dimethylethoxy)carbonyl]-3-[[3-(1-naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]-amino]-2-oxo-3-pyrrolidinecarboxylate (11). To sodium hydride (50% in oil, washed free of oil with dry petroleum ether, 0.70 g, 15.5 mmol) in DMSO (40 mL) was added beta-naphthylmethyl acetimidomalonate (6.88 g, 13.8 mmol) in DMSO (40 mL) portionwise. The mixture was stirred for 1.5 h after which 2-iodo-1-[(*tert*-butyloxycarbonyl)amino]ethane (10) (4.42 g, 15.8 mmol) in DMSO (30 mL) was added. After 72 h in the dark the reaction was diluted with EtOAc (1 L), and the layers were separated. The aqueous layer was extracted with EtOAc twice, and the combined organic extracts were washed with H$_2$O, saturated NaHCO$_3$, and brine. Drying (MgSO$_4$), concentration, and chromatography on silica gel employing 25% EtOAc in hexane afforded recovered malonate (1.25 g, 47%), iodide (0.64 g, 10.2%), and the title compound 11 (2.75 g, 11%). ^{1}H NMR (200 MHz, CDCl$_3$) δ 1.17 (3 H, t, J = 7 Hz), 1.50 (9 H, s), 1.95 (1 H, m), 2.38 (1 H, ddd, J = 7, 2.5 Hz), 3.05 (1 H, m), 3.30 (2 H, m), 3.47 (2 H, m), 4.25 (2 H, m), 4.30 (2 H, m), 5.14 (1 H, s), 7.41–8.08 (14 H, m), 7.90 (2 H, m). FAB MS (M + NBA)$^+$ m/z 696.1 (695). Anal. ($C_{41}H_{42}N_2O_6$) C, H, N.

(±)-4-[[(1,1-Dimethylethoxy)carbonyl]amino]-2-[[3-(1-naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]-amino]butanoic Acid (12). Treatment of ester 11 (2.25 g, 3.02

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mmol) in MeOH (30 mL) with NaOH solution (0.47 g in 15 mL 4 M H_2O). The mixture was heated for 4 h, resulting in ester hydrolysis. After dilution with H_2O (150 mL), the pH was adjusted to 2-3 with 1 N HCl. The mixture was extracted with EtOAc (three times), and the organic layer was washed with water and dried ($MgSO_4$). After evaporation of the solvent in vacuo, the resulting white foam (2.81 g) was taken up in toluene (50 mL) and refluxed for 2 h. The solvent was evaporated, and the residue was taken up in MeOH (80 mL). NaOH solution (0.75 g in 5 mL of H_2O, 4.75 mmol) was added, and the reaction was stirred for 1 h. The pH was once again brought to 2-3 with 1 N HCl, and the mixture extracted with several portions of EtOAc. After the mixture was washed with H_2O, the combined organic extracts were dried ($MgSO_4$) and evaporated under reduced pressure. The title amino acid (12) was obtained as a white foam (2.90 g, 81.3%): ^1H NMR (90 MHz, $CDCl_3$) δ 1.17 (9 H, s), 1.55 (1 H, m), 1.90 (1 H, m), 2.90 (2 H, m), 3.12 (3 H, m), 3.90 (3 H, m), 4.19 (1 H, dd, J = 14, 8 Hz), 6.65 (1 H, br s), 7.29 (2 H, m), 7.42 (6 H, m), 7.65 (2 H, dd, J = 7.8, 3.4 Hz), 7.77 (2 H, d, J = 8.6 Hz), 7.90 (2 H, d, J = 7.6 Hz), 12.42 (1 H, br s). FAB MS (M + H$^+$ BOC) m/z = 484.2 (484).

1,1-Dimethylethyl [4-[[2-Hydroxy-4-[(2-methylbutyl)-amino]-1-(2-methylpropyl)-4-oxobutyl]amino]-3-[[3-(1-naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]-amino]-4-oxobutyl]carbamate (13). The title compound 13 was obtained by coupling of previous acid 12 (0.50 g, 0.83 mmol) and Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$ (257 mg, 0.83 mmol), according to general procedure B. The product was purified by column chromatography on silica gel eluting with 2-4% MeOH in $CHCl_3$ (0.60 g, 64.1%) obtained as a white foam: ^1H NMR (90 MHz, $CDCl_3$) δ 0.80 (12 H, m), 1.0-2.20 (12 H, m), 1.40 (9 H, s), 3.10 (2 H, m), 3.35-4.35 (6 H, m), 6.45 (1 H, d, J = 9.8 Hz), 7.25-7.55 (8 H, m), 7.80-8.00 (6 H, m). FAB MS (M + H) m/z = 767.6 (767).

Diethyl (*S*)-[[2-[[(4-Morpholinylsulfonyl)amino]-3-phenyl-1-oxopropyl]amino]propanedioate (14). A solution of (morpholinosulfonyl)phenylalanine (2.57 g, 0.68 mol), HOBT (1.80 g, 0.68 mol), and diethylaminomalonate hydrochloride (1.47 g, 0.68 mol) in DMF (20 mL) was cooled in ice, and TEA (0.96 mL, 0.68 mol) was added, followed by a solution of DCC (1.40 g, 0.68 mol) in DMF (25 mL). After stirring at 0 °C for 30 min the mixture was allowed to warm to room temperature and stirred overnight. Filtration and evaporation gave the crude residue which was taken up in EtOAc and washed with 1 N HCl, H_2O, saturated $NaHCO_3$ solution, and brine, respectively. Drying ($MgSO_4$) and removal of the solvent under reduced pressure gave 14 as a viscous oil (3.10 g, 96.1%): ^1H NMR (90 MHz, $CDCl_3$) δ 1.00 (6 H, t, J = 7 Hz), 2.90-3.30 (6 H, m), 3.80 (4 H, t, J = 4.8 Hz), 4.10 (1 H, m), 4.29 (4 H, dd, J = 7, 14 Hz), 4.80 (1 H, d, J = 8.4 Hz), 5.12 (1 H, d, J = 8.6 Hz), 7.02 (1 H, d, J = 6.4 Hz), 7.21-7.41 (5 H, m). MS (CI + CH_4) (M + H)$^+$ m/z = 472 (472).

Diethyl (*S*)-2-[4-[[(1,1-Dimethylethoxy)carbonyl]-amino]-2-butynyl]-2-[[2-[(4-morpholinylsulfonyl)amino]-1-oxo-3-phenylpropyl]amino]propanedioate (16). A suspension of sodium hydride (0.48 g, 0.072 mol, 60% in oil, washed free of oil) was suspended in DMSO (75 mL) under nitrogen and treated in portions with a solution of the malonate 14 (17.0 g, 0.036 mol) in DMSO (50 mL). The mixture was stirred for 4 h and then treated with 1-[(tert-butoxycarbonyl)amino]-4-chloro-2-butyne (15)[9] (7.47 g, 0.036 mol) and KI (1.0 g). After stirring for 30 h the mixture was diluted with EtOAc and washed with 1 N citric acid, H_2O, and saturated NaCl solution, respectively. After drying ($MgSO_4$), the solvent was evaporated under reduced pressure and the residue chromatographed on silica gel, with 1% MeOH in $CHCl_3$, as eluent to afford 15 (14.2 g, 61.9%) as a viscous golden oil. FAB MS (M + H - BOC)$^+$ m/z = 556.9 (556).

BNMA-Lys(C=SNHCH$_2$)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (17). Compound 5 (0.50 g, 0.74 mmol) was dissolved in CH_2Cl_2 (15 mL) and cooled to 0 °C. Methyl isothiocyanate (0.054 g) was added, and the mixture was allowed to warm to 25 °C and stir for 6 h. The solvent was evaporated and the crude product purified by column chromatography on silica gel with a gradient of EtOAc-hexane (1:1) to eluent to pure EtOAc. The appropriate fractions were combined to afford the product 17 as a white foam (0.40 g, 70.0%): FAB MS (M + H)$^+$ m/z = 766.2 (766). Anal. (C, H, N).

BNMA-Lys(C=O)NHCH$_2$-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (18). To a cooled (0 °C) mixture of 5 (0.50 g, 0.74 mmol) in CH_2Cl_2 (15 mL) and TEA (0.21 mL, 1.5 mmol) was added methyl isocyanate (42 μL, 0.71 mmol) and the mixture allowed to warm to room temperature. After 4 h of stirring, EtOAc was added to the white suspension and the mixture filtered. The solid was dried to afford the product 18 (0.38 g, 35%): Anal. (C, H, N).

BNMA-Lys(C=S(NHPh))-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (19). By using the procedure of example 17 phenyl isothiocyanate gave the title compound 19. Anal. (C, H, N).

BNMA-Lys(P=O(OPh)$_2$)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (20). To a solution of 5 (0.50 g, 0.72 mmol) in CH_2Cl_2-THF (4:1, 15 mL) at 0 °C was added TEA, followed by diphenyl phosphorochloridate (0.15 mL, 0.72 mmol). After stirring for 3 h, the solution was evaporated and diluted with EtOAc. Washing with saturated $NaHCO_3$ solution and brine, drying (Na_2SO_4), and evaporation under reduced pressure afforded the crude product 20. Chromatography on silica gel, eluting with EtOAc/hexane (1:1 → 5:1) afforded the pure product 20 (0.66 g, 70.0%) as a white foam. Anal. (C, H, N).

BNMA-Lys(C=NCN(BCH$_3$))-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (21). A solution of 5 (0.31 g, 0.44 mmol) in $CHCl_3$ (10 mL) was treated with dimethyl cyanoimidodithiocarbonate[4] (70 mg, 0.57 mmol) and stirred at room temperature overnight. After evaporation under reduced pressure, the residue was chromatographed on silica gel with EtOAc-hexane (1:1) → EtOAc as eluent to afford the product 21 (0.30 g, 47.7%) as a white foam. Anal. (C, H, N).

***N*-[2-Hydroxy-4-[(2-methylbutyl)amino]-1-(1-methylpropyl)-4-oxobutyl]-α-[[3-(1-naphthalenyl)-2-(1-naphthalenylmethyl)-1-oxopropyl]amino]-1*H*-pyrrole-1-hexanamide (22).** Compound 22 was prepared from bis(1-naphthylmethyl)succinic acid and (6-pyrrol-2-aminohexanoyl)-Sta-2,5-methylhexylamine in 71% yield by the methods described for example 18. Anal. (C, H, N).

BNMA-Lys(C(O)NH$_2$)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (23). A solution of 5 (0.80 g, 0.81 mmol) in dioxane (15 mL) was treated with AcOH (1.0 mL) followed by sodium cyanate (200 mg, 0.46 mmol) in H_2O (1 mL). The reaction mixture was warmed to 45 °C for 4 h, then cooled, and partitioned between EtOAc and saturated $NaHCO_3$ solution. The EtOAc layer was washed with brine, dried (Na_2SO_4), filtered, and evaporated. Column chromatography on silica gel eluting with EtOAc, then EtOAc/MeOH (9:1) afforded 23 (0.28 g, 69.2%) as a white foam. Anal. (C, H, N).

BNMA-Lys(Tos)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (24). Bis(1-naphthylmethyl)succinic acid (0.44 g, 1.3 mmol) and Lys(Tos)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (0.61 g, 1.22 mmol) (prepared by the method described for 3) were coupled according to general procedure C to afford the product 24 (0.99 g, 97.0%) as a white foam after chromatography on silica gel (eluting with 0 → 3% MeOH in $CHCl_3$). Anal. (C, H, N, Cl).

BNMA-Lys(COCH$_3$)-Sta-NHCH$_2$CH(OH)CH$_2$CH$_2$CH$_3$ (25). A solution of *N*-acetylimidazole (0.124 g, 0.72 mmol) and 5 (0.50 g, 0.72 mmol) in DMF (2 mL) was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue taken up in EtOAc. The solution was washed with 1 N citric acid, saturated $NaHCO_3$, and brine, respectively. After solution was dried (Na_2SO_4) and column chromatographed on silica gel with 0 → 1% MeOH in $CHCl_3$ as eluent, the product 25 was obtained (0.24 g, 46.2%) as a white foam. Anal. (C, H, N).

BNMA-Lys(C=S(NHCH$_3$))-Sta-AEM (26). By procedures similar to those used for the synthesis of compound 17, but using (aminoethyl)morpholine instead of 2(*S*)-methylbutylamine, 26 was obtained as a white foam from BNMA-Lys-Sta-AEM in 67% yield. Anal. (C, H, N, S). (HPLC: 70:30 → 0:100 H_2O, CH_3CN, over 30 min, Vydac column, 1.5 mL/min flow rate; 90% purity @ 220 nm).

BNMA-Lys(C=NH(NHNO$_2$))-Sta-AEM (27). To a solution of BNMA-Lys-Sta-AEM (synthesized by procedures similar to those for compound 5) (1.20 g, 2.0 mmol) in EtOH (50 mL) was added 2-methyl-1-nitro-2-thiopseudourea (385 mg, 2.20 mmol) and the mixture stirred at room temperature for 72 h. The solvent

[9] Iizuka, K.; Kamir, I.; Kubota, T.; Akahane, K.; Imamura, H. *Bull. Chem. Soc. Jpn.* **1985**, *58*, 2301.

was evaporated and the residue chromatographed on silica gel by using a gradient elution of 5 → 12% MeOH in $CHCl_3$. The product **27** was obtained as a white foam (0.52 g, 85%): Anal. (C, H, N, Cl).

BNMA Lys(C=NCN)SCH$_2$(1-Sta-AEM (28). By procedures similar to those used in the synthesis of compound **21**, **28** was obtained as a white foam (0.26 g, 89% final step): Anal. (C, H, N).

BNMA Lys(Z)-Sta-Leu-NHCH$_2$Ph (29). By procedures similar to those used for the synthesis of compound **1** but substituting Leu-NHCH$_2$Ph instead of 2(*S*)-methylbutylamine, the title compound **29** was obtained as a white foam (0.70 g, 65% final step): Anal. (C, H, N).

BNMA Lys(C=S)NHCH$_2$(1-Sta-Leu-NHCH$_2$Ph (30). By procedures similar to those for the synthesis of **17**, the title compound **30** was obtained as a white foam (1.20 g, 70% final step): Anal. (C, H, N).

BNMA Lys(C=NH)NHNHSO$_2$(1-Sta-Leu-NHCH$_2$Ph (31). By procedures similar to those used for the synthesis of **27**, the title compound **31** was obtained as a white foam (0.24 g, 43% final step): Anal. (C, H, N).

Preparation of (*S*,*S*)-ε-Amino-α-hydroxy-*N*-[2-(4-morpholinyl)ethyl]cyclohexanepentanamide (ACHPA-AEM). The title compound was prepared according to the literature procedure recently reported[47].

BNMA Lys(C=S)NHCH$_2$(1-ACHPA-AEM (32). Preparation for BNMA-Lys-ACHPA-AEM as described for **17** and purification by chromatography on silica gel eluting with 15% MeOH in $CHCl_3$ gave the title compound **32** as a white foam (360 mg, 80% final step): FAB MS (M + H)$^+$ = 543 (calcd for $C_{29}H_{51}N_6O_3S_2$ + HBPLA analysis indicated 88% purity).

BBPS Lys(C=S)NHCH$_2$(1-ACHPA-AEM (33). Substituting (+)-2-benzyl-3-(*tert*-butylsulfonyl)propionic acid[48] for (*R*)-1-naphthylmethylsuccinic acid and ACHPA for Sta, title compound **33** was prepared by methods similar to those described for compound **17**. After purification by chromatography on silica gel eluting with 2 → 8% gradient of MeOH in $CHCl_3$, the product **33** was obtained as a white foam (1.05 g, 61% final step). The compound was converted to the citrate salt for analysis: Anal. (C, H, N).

Compound 34. By using the procedures for the synthesis of **17** substituting 2-(1-naphthylmethyl)-3-morpholinone-(ethylsulfonyl)propionic acid[49] for (*R*)-1-naphthylmethylsuccinic acid and ACHPA for Sta, title compound **34** was obtained: FAB MS (M + H)$^+$ = 808 (calcd for $C_{42}H_{69}N_7O_6S_2$ + H, 808.4728). Anal. (C, H, N, S).

Compound 35. Title compound **35** was prepared (0.62 g, 80%) as a white foam according to procedures similar to those for **17** substituting ACHPA-AEM for Sta-NBU-B$_2$-HOCH$_2$CH$_2$OH: Anal. (C, H, N).

Compound 36. Title compound **36** (0.52 g, 77%, final step) as a white foam was prepared according to procedures described for **17**: Anal. (C, H, N, S).

Compound 37. Title compound **37** (0.30 g, 90%, final step) as a white foam was prepared according to procedures described for **27**: Anal. (C, H, N, S) calcd 11.6%, found 11.6%.

BNMA Lys(C=S)NHCH$_2$(1-ACHPA-NHCH$_2$CH$_2$OCH$_2$CH$_2$OH (38). Title compound **38** was prepared as a white foam (1.30 g, 90%) by using methods described previously for **17** starting with the (*S*,*S*)-dimethylamide of BOC-Sta (prepared by reaction of (*S*,*S*)-dimethylamine hydrochloride salt and BOC-Sta in the presence of carbonyldiimidazole (1 equiv) and DBU (1 equiv) at 0 °C): FAB MS (M + H)$^+$ = 742 (calcd for $C_{36}H_{67}N_7O_5S_2$ + H, 742.4728). Anal. (C, H, N, Cl, S) calcd 4.2% found 4.2%. HPLA: 20 min, 1 mL/min, 0.1% TFA in H_2O (A), 0.1% TFA in 90% MeCN/10% H_2O (B), 0% B → 100% B linear gradient, Vydac column, 1.5 mL/min, t_R = 9.01 min.

BNMA Lys(C=S)NHCH$_2$(1-DFS-Sta-AEM (40). Compound **40** was obtained in 55% yield by procedures described previously for (*S*,*S*)-ε-amino-α-hydroxy-*N*-[2-(4-morpholinyl)ethyl]-γ-phenylalaninamide (ACHPA-AEM)[47]: FAB MS (M + H)$^+$ = 847 (calcd for $C_{43}H_{67}N_6O_5S_2$ + H, 847.4670). Anal. (C, H, N, S).

Preparation of *N*-[(Dimethylamino)sulfonyl]-(1-phenyl-alanine. A solution of phenylalanine (1.81 g, 0.01 mol) in 1 *N* NaOH (20 mL) was treated with a solution of *N*,*N*-dimethylsulfamoyl chloride (2.86 mL, 0.02 mol) in THF (20 mL) and stirred vigorously at 25 °C for 1 h. The reaction mixture was then treated with additional 1 *N* NaOH (20 mL) and *N*,*N*-dimethylsulfamoyl chloride (2.86 mL, 0.02 mol) and stirred 1 h further at 25 °C. Finally 1 *N* NaOH (20 mL) and Et_2O (40 mL) were added. The mixture was shaken and the aqueous layer separated and acidified to pH 1 by addition of 1 *N* HCl (35 mL). The product was extracted into EtOAc and the solution was dried ($MgSO_4$) and evaporated to a gum which slowly solidified (4.0 g, 69.9%): ^{1}H NMR (90 MHz, $CDCl_3$) δ 2.7 (s, 6 H, $N(CH_3)_2$), 3.0–3.5 (m, 2 H, CH_2), 4.2 (m, 1 H, CH), 6.8–7.4 (m, 10 H, ArH).

***N*-[(Dimethylamino)sulfonyl]-(1-Phe-Lys(C=S)-(NHCH$_2$)-ACHPA-AEM (41).** By methods described previously for **17**, **41** was obtained as a white foam (0.58 g, 25%) after column chromatography on silica gel eluting with 0 → 6% MeOH in $CHCl_3$: FAB MS (M + H)$^+$ = 780.6 (780.4). Anal. (C, H, N, S). HPLA: 20 min → 60% B, 1 mL/min, 100% purity, t_R = 12.43 (98% I and 2% II), 13.22 (II).

***N*-(1-Morpholinylsulfonyl)-(1-Phe-Lys(C=S)(NHCH$_2$)-ACHPA-AEM (42).** By methods described previously for **17**, **42** was obtained as a white foam (0.82 g, 61%) after purification by column chromatography on silica gel eluting with 5% MeOH in $CHCl_3$: Anal. (C, H, N, S).

***N*-(1-Morpholinylsulfonyl)-Phe-Lys(Z)-DFO-AEM (43).** Isovaleryl chloride (0.4 mL, 0.74 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C was added DMSO (0.6 mL, 1.50 mmol) and the solution stirred for 10 min. The difluoroketatine analogue (0.50 g, 0.57 mmol) in CH_2Cl_2 (5 mL) was added and the solution stirred at 0 °C for 30 min following by warming to 20 °C for a further 45 min. After the solution was recooled to −60 °C, dry Et_3N (0.6 mL, 2.5 mmol) was added and the reaction warmed to room temperature slowly. Dilution with CH_2Cl_2 and washing with saturated $NaHCO_3$ solution was followed by extraction of the aqueous layer with further portions of CH_2Cl_2. The combined organic extracts were washed with brine and dried ($MgSO_4$). Filtration, concentration under reduced pressure and chromatography on silica gel (5% → 10% MeOH in $CHCl_3$) gave the product **43** as a mixture of diastereomers (378 mg, 75.6%): FAB MS of the deprotected (M + H)$^+$ = 808.3 (808.4) [M + H]$^+$ 988.3. Anal. (C, H, N, F, Cl).

***N*-(1-Morpholinylsulfonyl)-Phe-His-ACHPA-AEM (44).** A mixture of Boc-His-ACHPA-AEM (1.13 g, 1.6 mmol), HOBT·H$_2$O (0.30 g, 2.0 mmol) and DCC (0.74 g, 3.6 mmol) was dissolved in DMF (15 mL) and stirred at room temperature for 24 h. After 10 min a precipitate appeared. A solution of *N*-[(4-morpholinylsulfonyl)-(1-phenylalanine (2.47 g, 7.5 mmol) in DMF (15 mL) was then added and the reaction was stirred for 24 h at room temperature. The resulting suspension was concentrated *in vacuo* (1 mmHg) and the concentrate was filtered, rinsing the solid with EtOAc (10 mL). The filtrate and washings were evaporated *in vacuo*. Flash chromatography of the residue on silica gel eluting with 1 → 10% MeOH in $CHCl_3$ gave the product which was dissolved in $CHCl_3$, evaporated to a foam, and dried to constant weight at 0.5 mmHg, room temperature to afford 2.5 g (72%) of *N*-(1-morpholinylsulfonyl)-Phe-His(trityl)-ACHPA-AEM: TLC ($CHCl_3$–MeOH 9:1) R_f 0.35; MS (FAB) [M + H]$^+$ = 1094 (1094.6). This material was used without further purification.

A solution of the previous intermediate (2.38 g, 2.35 mmol) in 80% HOAc (50 mL) was heated on a steam bath until the solution temperature was 90–95 °C for 5 min. Distilled H_2O (100 mL) was then added and the mixture was cooled to room temperature. Solid was removed by filtration, rinsing with 10% HOAc. The combined filtrate and washings were evaporated to a syrup which was redissolved in distilled H_2O (75 mL), filtered, concentrated to 50 mL, frozen and lyophilized to give the title compound **44** (1.56 g, 87.4%) as a colorless powder: MS (FAB) [M + H]$^+$ = 760.4 (760.4). Anal. (C, H, N). HPLC 99.4% purity).

***N*-(1-Morpholinylsulfonyl)-Phe-Lys(C=S)NHCH$_2$)-PCS-AEM (45).** Title compound **45** was prepared by methods similar to those used in the preparation of **40** and **44** but with ethyl 1-(5-isocyanato-2-fluorophenyl)-cyclobutyl-1,2-difluoro-

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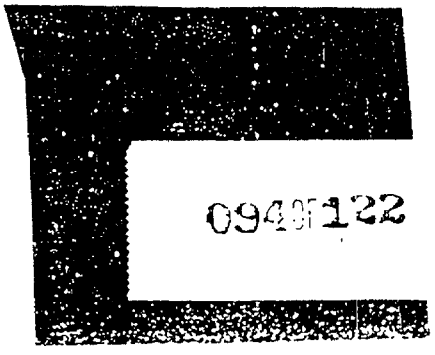


For use in staining endocrine tissue, dilute this test solution with an equal volume of water

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 30 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, and 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 13 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 20 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).

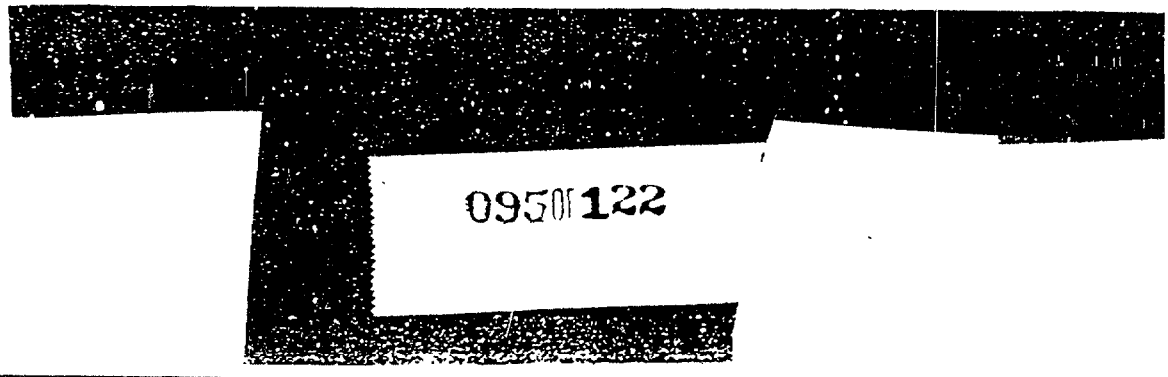
Hydrogen Sulfide TS—A saturated solution of hydrogen sulfide, made by passing H_2S into cold water. Store it in small, dark amber-colored bottles, filled nearly to the top. It is unsuitable unless it possesses a strong odor of H_2S, and unless it produces at once a copious precipitate of sulfur when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



(see in the section, *Volumetric Solutions*) add water to make 250 mL. Add to the resulting solution an equal volume of sodium acetate solution freshly prepared by dissolving 13.6 g of anhydrous sodium acetate in water to make 500 mL and adjusting with 0.5 *N* acetic acid to a pH of 7. Store in a refrigerator, and use within 2 weeks.

Intestinal Fluid, Simulated, TS—Dissolve 6.8 g of monobasic potassium phosphate in 250 mL of water, mix, and add 190 mL of 0.2 *N* sodium hydroxide and 400 mL of water. Add 10.0 g of pancreatin, mix, and adjust the resulting solution with 0.2 *N* sodium hydroxide to a pH of 7.5 ± 0.1. Dilute with water to 1000 mL.

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

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

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

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

$$800B - 5C$$

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

Iodochloride TS—Dissolve 16.5 g of iodine monochloride in 1000 mL of glacial acetic acid.

Iodoplatinate TS—Dissolve 300 mg of platinic chloride in 95 mL of water. Immediately prior to use, add 3.5 mL of potassium iodide TS, and mix.

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

Iron Salicylate TS—Dissolve 500 mg of ferric ammonium sulfate in 250 mL of water containing 10 mL of diluted sulfuric acid, and add water to make 500 mL. To 100 mL of the resulting solution add 50 mL of a 1.15% solution of sodium salicylate, 20 mL of diluted acetic acid, and 80 mL of a 11.6% 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°.

2.12 Pepsins, Gastricsins and Their Zymogens

Pepsin A

EC 3.4.23.1

Gastricsin (pepsin C)

EC 3.4.23.3

Andrew P. Ryle

2.12.1 General

Acid proteinases are found in the gastric juice of mammals and have been reported in the juices of birds, amphibia and fishes. The major enzyme from the pig, pepsin A, is a single polypeptide of 327 residues [1] and is formed by cleavage of 44 residues from the amino terminus of pepsinogen A [2]; one or more of the peptide fragments removed inhibit the activity of pepsin A and other acid proteinases at pH values above 5 [3]. The other zymogens investigated show a similar decrease in molecular weight from about 39000 to about 35000 on activation and this activation is at least partly autocatalytic.

Besides having proteinase and peptidase activity, pepsin can catalyze the hydrolysis of suitable depsipeptides (ester analogues of peptides) and even of sulphite esters.

Application of methods: in clinical chemistry, biochemistry and pharmacy.

Enzyme properties relevant in analysis: in most of the species examined the gastric acid proteinases show considerable heterogeneity. In young mammals the zymogen of chymosin (EC 3.4.24.4, formerly called rennin), which has a high ratio of milk-clotting to proteolytic activity, is secreted. It is not considered further in this chapter. As the animal develops chymosin is replaced by gastricsin and by pepsin (secreted as their zymogens). These zymogens, and their enzymes, are immunologically distinct [4]. The enzymes have somewhat different substrate specificities [5, 6] and also occur in multiple molecular forms, both as zymogens and as enzymes [7, 8], though how these differ is not entirely clear. The pepsins and gastricsins are further distinguished by the differing distribution of their zymogens in the gastric mucosa and in their occurrence in low concentrations in plasma, urine and semen [9, 10].

A characteristic feature of the gastric acid proteinases, also shared by similar proteinases of microbial origin, is their sensitivity to inactivation by diazoketones [11] and by 1,2-epoxy-3-(4-nitrophenoxy)-propane [12]. They are also inhibited by pepstatins, pentapeptides produced by strains of *Actinomycetes*, which bind extremely tightly (K_i ca. 10^{-9} mol/l) to pepsins but 10 to 100 times less tightly to gastricsins [13].

Most pepsins share another unusual property: great lability at pH values above 6. The gastricsins are rather less sensitive to this alkaline denaturation, and chicken pepsin is stable up to pH 10.5 at 25° [14].

Pepsins and gastricsins can catalyze transpeptidations of both the amino-transfer and the carboxyl-transfer types with suitable substrates [15]. These reactions not only have important implications for the mechanism of action of the enzymes; they may also interfere in assays using peptide substrates with unprotected amino or carboxyl groups, or assays using any peptide substrate if other peptides are also present.

Methods of determination: the rather broad specificity of the acid proteinases of the stomach has allowed the development of numerous methods of assay; some differ only trivially, but some have been devised to be especially useful for particular purposes. The methods fall into two broad groups: those which use a protein substrate and those which use a synthetic substrate, usually a peptide. The assays using protein substrates are useful when one wishes to measure total acid proteinase, although it must be recognized that the sensitivity and the linearity of the response may differ for different enzymes.

Assays with protein substrates

The extent of hydrolysis of protein substrates is usually determined by measuring the concentration of the products that are not precipitated by trichloroacetic acid; this measurement may depend on the ultraviolet absorbance of the supernatant or on its reaction with *Folin-Ciocalteu* reagent. Use of a radio-iodinated protein as substrate increases the sensitivity of the assay about 250-fold [16]. Another sensitive method employs *N,N*-dimethylated globin as substrate and measures the actual peptide bonds hydrolyzed by the reaction of the liberated amino groups with trinitrobenzene sulphonate [17]. A third sensitive method [18] uses fluorescamine to detect amino groups liberated from succinylated albumin and gives a linear response with 1 to 18 ng of pig pepsin. These methods should be valuable for measuring the total acid proteinases when the high sensitivity is important.

The milk-clotting activities of pepsin and gastricsin can also be used for their estimation. This turbidimetric assay [3, 19] is particularly useful because it provides a means of estimating pepsins and gastricsins in the presence of their zymogens.

Assays based on the diffusion of the enzyme into a gel containing an insoluble substrate have been used, but have been criticized [20] as being inaccurate even over a limited range of enzyme concentration.

Assays with synthetic substrates

Assays using peptides or other synthetic substrates offer the possibility of distinguishing between the different enzymes. Many substrates have been described, with detection of the reaction by various means, including reaction with ninhydrin, reaction with fluorescamine, direct absorption spectrophotometry and fluorimetry. Table 1 lists some useful synthetic substrates together with their kinetic data and the mode of detection used. It is obvious that some of the newer substrates are hydrolyzed very much faster than the earlier ones. Use of such sensitive substrates, especially when coupled with the use of fluorimetry for detection, provides an extremely sensitive assay for suitable enzymes. Such substrates would no doubt be more widely used if they were more readily available.

Caution regarding heterogeneity and specificity

Since the gastric acid proteinases are so heterogeneous and since little is known of the substrate specificities or specific activities of the minor components, there must be some uncertainty regarding what is being measured with any assay of catalytic activity when it is applied to a mixture such as an activated mucosal extract, or to whole gastric juice.

Other means of assay

Pepsins and gastricsins can also be estimated by active-site titration with radioactive diazoacetyl peptides [11] though how all the minor enzymes respond is not known. Cathepsin D can be estimated by active-site titration with pepstatin [21] and the same technique could be applied to the pepsins. The weaker binding of the inhibitor by the gastricsins would make their estimation by this method more difficult.

Radioimmunoassay [4] can be used to measure, separately, pepsins and gastricsins (enzymes corresponding to the pepsinogens of groups I and II, respectively) without distinguishing between the members within each group.

Selected assays

The assays with haemoglobin and acetyl-L-phenylalanyl-L-diiodotyrosine as substrates are described here. The assay with haemoglobin is still useful for estimating total proteinase activity (although it fails to detect pig pepsin B [22], an enzyme for which no human homologue has been described).

Although acetyl-L-phenylalanyl-L-diiodotyrosine is not a very sensitive substrate, it is commercially available and not prohibitively expensive. The method used for this substrate is readily adaptable to use with any other peptide substrate for which the ninhydrin method is the best available.

Table 1. Some small substrates for pepsins and gastricsins

Substrate*	Method**	pH	k_{cat} (s^{-1})	K_m (μmol l)	k_{cat}/K_m ($l \times mmol^{-1} \times s^{-1}$)	Ref***
Ac-Phe-Dit*	N	2.0	0.20	75	2.67	a
Z-His-Phe-Tro-*OEt*	N	4.0	0.51	230	2.22	b
Z-His-Nph-Phe-*OMe*	S	4.0	0.29	460	0.63	c
Z-His-Nph-Pla-*OMe*	S	4.0	0.[illegible]	400	1.93	c
Z-Gly-Gly-Phe-Phe-*OP4P*	N	3.5	71.8	420	171	d
Z-Ala-Ala-Phe-Phe-*OP4P*	N	3.5	282	40	7050	e
Phe-Gly-His-Nph-Phe-Ala-Phe-*OMe*	S	4.0	20	40	500	f
Z-Gly-Ala-Nph-Trp-$NHCH_2CH_2OH$	S	4.0	41.2	266	155	g
Dns-Ala-Ala-Phe-Phe-*OP4PMe*+	F	3.1	288	39	7510	h
Pro-Thr-Glu-Phe-Nph-Arg-Leu	S	3.1	90	77	1170	13
ditto – pig gastricsin	S	3.1	54	95	568	13
ditto – human gastricsin	S	3.1	23	370	62	13
Ac-Tyr-Leu-Val-His-NH_2	a poor substrate for pig and human pepsins					
ditto – pig gastricsin	N	2.1	1.06	280	3.45	36
ditto – human gastricsin	N	2.1	n.d	n.d	1.2	36
HCO-Tyr-Phe	N	no kinetic data available – a better substrate for human gastricsin than human pepsin			–	37
bisphenyl sulphite (25 °C)	S	3.7	0.022	100	0.22	i

* Data are for pig pepsin and 37 °C unless stated otherwise

** N ninhydrin; S spectrophotometry, F fluorimetry

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\+ Abbreviations

Z-	benzyloxycarbonyl-
Dns-	1-dimethylaminonaphthalene-5-sulphonyl
-*OP4P*	-3-(4-pyridyl)-propyl ester
-*OP4PMe*+	-*OP4P* methylated on the pyridine nitrogen
Dit	3,5-diiodotyrosine or its residue
Pla	3-phenyllactic acid or its residue
Nph	4-nitrophenylalanine or its residue

Phenyllactic acid and all amino acids mentioned (except glycine) have the L-configuration.

Determination of pepsin and gastricsin in mixtures

Methods depending on two different principles have been described. Use can be made of the greater sensitivity of pepsin to alkaline denaturation to permit the determination of the proteinase activity of gastricsin alone after suitable treatment with alkali. The difference between this activity and that found without alkali treatment is the activity due to pepsin [6, 36].

The second group of methods relies on the differing specificities of pepsin and gastricsin. Total activity can be measured with a protein substrate, and pepsin alone with Ac-Phe-Dit; after suitable conversion of the units in which the activities are expressed the difference gives the activity due to gastricsin [6]. This method has been found to correlate well with that using differential inactivation when applied to human gastric juice [36].

Alternatively, pepsin can be assayed with Ac-Phe-Dit and gastricsin with Ac-Tyr-Leu-Val-His-NH_2 [37]. The pH optimum for human gastricsin acting on Ac-Tyr-Leu-Val-His-NH_2, 25 mmol/l, is 3.8 [38]. The peptide formyl-Tyr-Phe [39] can also be used for estimation of gastricsin although it seems to be more slowly hydrolyzed. Kinetic parameters for the formyl peptide have not been published.

Determination of enzymes and zymogens in mixtures

Two principles can be applied here. The total activity determined, whether with a protein or a synthetic substrate, after activation near pH 2 is the sum of the activities of the enzyme and zymogen. Alkali treatment of the unactivated mixture will destroy the enzyme so that the zymogen alone will now be measured after activation at pH 2. Inactivation of gastricsin will require a higher pH than that which is adequate for the destruction of pepsin.

Some substrates are hydrolyzed at a significant rate at pH values which are high enough not to allow the activation of the zymogen, so that by their use the enzyme can be assayed in the presence of the zymogen. The milk-clotting assay has been used for this purpose [3, 19] and the release of dye from dyed casein or collagen (azocasein, azocoll) can also be used [40].

International reference methods and standards: the *International Commission for the Standardization of Pharmaceutical Enzymes* published a report [23] in 1966 describing an assay for pepsin very similar to *Anson*'s original method [24], except that the temperature used was 25 °C and the unit corresponded to the liberation in one minute of material behaving like one micromole of tyrosine in the *Folin-Ciocalteu* reaction, rather than the millimole of *Anson*'s unit.

A committee of the *Japanese Society of Gastroenterology* has published a report [25] describing an assay also based on the method of *Anson* [24].

An international reference substance for pepsin is available ([23] cf. Vol. II, chapter 2.3).

Enzyme effectors: alcohols are weakly inhibitory to pepsin [26]. Some peptides may act as activators or inhibitors in the hydrolysis of others [27, 28]. These effects of secondary peptides are less marked at pH 2 than at higher pH values.

2.12.2 Method with Haemoglobin as Substrate

Assay

Method Design

Principle: the method described by *Anson* [24] depends on the estimation of those products of hydrolysis of haemoglobin (which is denatured at the pH of the assay) which are soluble in trichloroacetic acid solution. The estimation originally depended on the use of *Folin-Ciocalteu* reagent but was modified [29] to use the ultraviolet absorbance of the products instead. The response with this modification is linear for pig pepsin over a wider range and, since UV spectrophotometers are now generally available, only the UV method will be described. The method given below has been scaled down and modified to eliminate a dilution step. *Tang* [6] describes a similar assay for human pepsin and gastricsin conducted at pH 3.1, and the method using the *Folin-Ciocalteu* reagent has been adapted to the *Technicon* AutoAnalyzer [30]

Optimized conditions for measurement: the behaviour of haemoglobin as substrate for pig pepsin has been critically studied using the *Folin-Ciocalteu* method [31]. The conclusion of this study is that the conditions used by *Anson* are close to optimal except that the concentration of haemoglobin should be somewhat higher. However so many workers have used the same concentration as *Anson* that it seems best to retain it. The pH of the incubation mixture is 1.7, achieved by inclusion of HCl to give 0.05 mol/l. If the enzyme samples are buffered (as with eluates from a chromatographic column) it is necessary to include additional, compensating HCl.

Temperature conversion factors: the temperature is altered from the 35.5°C of *Anson*'s assay to 37°C for conformity with widespread practice. *Jardillier et al.* [31] state that the extent of hydrolysis found at 37°C is 25% higher than that at 25°C. This is not in accord with the statement of *Anson* [24] that the rate at 35.5°C is 1.82 times that at 25°C. In any case the temperature coefficient may differ for different enzymes.

Equipment

Constant-temperature bath, UV spectrophotometer.

Reagents and Solutions

Purity of reagents: all reagents of highest purity available. Commercial standard HCl may contain mercuric chloride. It should not be used.

Preparation of solutions (for about 50 determinations): make all solutions in re-purified water (cf. Vol. II, chapter 2.1.3.2).

1. HCl (0.30 mol l).

   dilute 30 ml HCl, 1 mol/l, with 70 ml water.

2. Trichloroacetic acid (40 g/l w/v):

   dissolve 20 g TCA in water, make up to 500 ml with water.

3. Haemoglobin solution approx. neutral (25 g/l):

   stir 2.5 g bovine haemoglobin enzyme substrate powder (*Armour Pharmaceutical Co.* Ltd., England), with about 90 ml water, dialyze against two batches of about 1000 ml water each to remove small peptides and adjust to 100 ml. 2.5 mg Thiomersal may be added as preservative.

4. Haemoglobin solution, acid (20 g/l):

   acidify the haemoglobin solution (3) with 1 quarter of its volume of HCl (1) or other appropriate concentration, and filter or centrifuge to remove any insoluble material remaining.

Stability of solutions: acid (1) is stable at room temperature for a long period of time; TCA solution (2) may decompose during one year to a minor degree to chloroform and CO_2 which does not impair the determination. Solution (3) may be kept at 0 – 4 °C for 1 or 2 weeks; solution (4) should be used within one day.

Procedure

Collection and treatment of specimen: collect gastric juice by the usual gastroenterological procedures, with or without stimulation as appropriate for the investigation, and assay without delay. Dilute the juice with water (a 25-fold dilution may be suitable). Mince mucosa finely and extract with phosphate buffer, pH 6.9 (Na_2HPO_4 and NaH_2PO_4 both 10 mmol/l) for estimation of the zymogens.

Stability of the enzyme in the sample: since the acid proteinases are themselves proteins, solutions which are acidic should be assayed without delay.

Details for measurement in tissue: assay mucosal extracts in phosphate buffer, 20 mmol/l, pH 6.9 (see above) using HCl, 0.315 mol/l, for acidification of the haemoglobin.

Assay conditions: wavelength 280 nm; light path 10 mm; incubation volume 1.2 ml; 37 °C. All measurements in duplicate. Run blank measurements by adding the trichloroacetic acid (2) to the haemoglobin (4) before the enzyme is added. When the enzyme is partly purified (as with fractions from chromatography columns) so that the absorbance of the solution at 280 nm could contribute only negligibly to the final absorbance, a single pair of blank determinations which contain water (or the appropriate buffer) in place of the enzyme may be used. Equilibrate all solutions to 37 °C before use.

Standard curve: assay suitable dilutions of solutions of pepsin A and C that have been previously assayed by the method of *Anson* [24], cf. chapter 2.15.2, p 270 (Fig. 1)

Measurement

Pipette successively into 15 ml centrifuge tubes*:		concentration in assay mixture
sample	0.2 ml	volume fraction 0.167 pepsin 2.5 – 13 mg/l gastricsin 2.5 – 8 mg/l
equilibrate to 37 °C		
haemoglobin solution (4)	1.0 ml	haemoglobin 16.7 g/l HCl 50 mmol/l
mix and incubate for exactly 10 min		
trichloroacetic acid (2)	5.0 ml	
shake; centrifuge 10 min at 1000 *g*, or filter (*Whatman* No. 3, 7 cm circles) to remove precipitate; read absorbance against water.		

* If a centrifuge capable of taking many small tubes and a self-filling attachment for the spectrophotometer is available the volumes used in the assay may be halved. For the most accurate work a new standard curve should be prepared for each new batch of haemoglobin substrate.

Determination of gastricsin and zymogens

Gastricsin: Fig. 1 also shows a curve prepared with pig pepsin C (pig gastricsin). With this enzyme the digestion proceeds much more slowly when the increase in absorbance

has exceeded about 0.3, so that the plot is far from linear. The abscissa values for pepsin C were arbitrarily adjusted to make the two curves coincide at 0.45/0.29 (abscissa/ordinate). The curve for pepsin C underlines the necessity of preparing a standard curve for each different enzyme assayed.

Tang [6] describes a similar assay conducted at pH 3.1, close to the pH optimum for human gastricsin.

Zymogens: pig pepsinogens A and C may both be assayed in exactly the same way as the enzymes (with HCl of the appropriate concentration). Inclusion of a pre-incubation at pH 2 before addition of the substrate makes no difference to the activity found, indicating that the activation is very rapid under the conditions of the assay. This is not necessarily true for other zymogens or other pH values in the assay mixture.

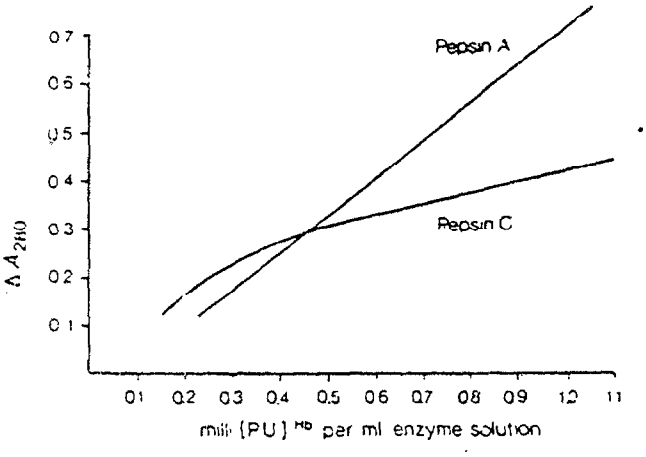


Fig. 1. Standard curves for assay of pig pepsins A and C with haemoglobin as substrate

Calculation: the differences ΔA_{280} between the mean sample and mean blank absorbances are converted into *Anson*'s units by reference to a standard curve prepared with a sample of pig pepsin previously assayed by *Anson*'s method [24]. Such a curve is shown in Fig. 1. The unit ([P.U.]^{Hb}) used is the activity that liberates soluble products equivalent to 1 millimole of tyrosine, as estimated by the *Folin-Ciocalteu* method, per 6 ml of incubation mixture per min.

It is not possible to convert these units into international units as defined by the Enzyme Commission because neither the colour in the *Folin-Ciocalteu* reaction nor the absorbance at 280 nm is caused solely by tyrosine residues. Furthermore, cleavage of any single peptide bond in the substrate could result in the production of a soluble

peptide containing no, one, or more than one residue of aromatic amino acid or even no soluble peptide at all. An attempt to convert $[P.U.]^{Hb}$ to units is likely to be more misleading than helpful.

Validation of Method

Precision, accuracy, detection limit and sensitivity: at about $\Delta A_{280} = 0.6$ the coefficient of variation for pepsin is about 0.05. For pepsin C, $\Delta A_{280} \equiv 0.3$, it must be about 0.07 (not measured).

Sources of error: incorrect pH in the assay mixture due to incorrect compensation for buffers in the enzyme sample; possible inhibitors or activators in the sample.

Specificity: the method detects most acid proteases, with varying sensitivity; pepsin B of the pig is not detected.

Reference ranges: in human gastric juice 6 to 123 (mean 54) $[P.U.]^{Hb}/l$ is found The values are corrected from the erroneous ones in the second edition of this work [32]

2.12.3 Method with Ac-Phe-Dit as Substrate

Assay

Method Design

Principle

$$\text{Ac-Phe-Dit} + H_2O \xrightarrow{\text{pepsin}} \text{Ac-Phe} + \text{Dit}\,.$$

The peptide is hydrolyzed at the peptide bond; the liberated amino groups are estimated by the colorimetric reaction with ninhydrin.

Optimized conditions for measurement: the assay described is conducted at pH 2.0, the optimum for pig pepsin A at the substrate concentration used which is at the limit

of its solubility. *Tang* [6] describes a similar assay at a slightly higher pH but in the unbuffered solution the pH control must be less certain. For automated method cf. [34, 35].

Temperature conversion factors: not known.

Equipment

Constant-temperature bath, boiling water-bath, spectrophotometer.

Reagents and Solutions

Purity of reagents: HCl, NaOH, acetic acid, sodium acetate are analytical grade reagents; 2-methoxyethanol, ninhydrin, hydrindantin must be of good quality with low blank reaction; ethanol is a standard laboratory reagent.

Preparation of solutions (for about 100 determinations): make all solutions in repurified water (cf. Vol. II, chapter 2.1.3.2).

1. Ac-Phe-Dit (1 mmol/l; NaOH, 10 mmol/l):

   dissolve 62.2 mg Ac-Phe-Dit in 10 ml NaOH, 0.1 mol/l. Wash the solution quantitatively into a 100 ml volumetric flask and make up the volume with water. The peptide dissolves only very slowly in NaOH, 10 mmol/l, but remains in solution if dissolved in this way.

2. HCl (50 mmol/l):

   dilute 5 ml HCl, 1 mol/l, with 95 ml water. This concentration is suitable for assay of enzymes dissolved in water, giving a final concentration of HCl, 10 mmol/l (pH 2). If the enzyme is in a buffered solution the HCl concentration must be increased so that the final incubation mixture is at pH 2.

3. Ninhydrin reagent:

   the reagent described is the modified reagent of *Moore & Stein* [33]; other quantitative ninhydrin reagents would also be suitable. Dissolve 20 g ninhydrin (indane trione hydrate) and 3 g hydrindantin in 750 ml peroxide-free 2-methoxyethanol, taking care not to include air bubbles. The solvent is sufficiently free from peroxides if, when mixed with half its volume of 4% KI solution, only a light straw

colour is obtained. To this solution add 250 ml acetate buffer (544 g $CH_3COONa \cdot 3H_2O$ and 100 ml glacial acetic acid in 1 litre), mix and store the deep red solution under nitrogen in a dark bottle.

4. Diiodotyrosine (0.25 mmol/l):

dissolve 21.7 mg diiodotyrosine in HCl, 50 mmol/l, and make up to 200 ml with the same.

5. Ethanol (60% v/v):

dilute 1200 ml absolute ethanol with 800 ml water.

Stability of solutions: the substrate solution (1) is stable for weeks at 0–4°C. The HCl solution (2) is practically indefinitely stable at room temperature. The ninhydrin reagent (3) can be stored for about a month at room temperature. Diiodotyrosine (4) is stable for several weeks at room temperature.

Procedure

Collection and treatment of specimens: cf. p. 229. Gastric juice and mucosal extracts should be dialyzed to lower the blank values.

Stability of the enzyme in the sample: cf. p. 229.

Details for measurement in tissue: the high ninhydrin blank prohibits assay of undialyzed tissue extracts.

Assay conditions: wavelength 570 nm; light path 10 mm; incubation volume 1.0 ml; 37°C; measuring volume 7 ml. All measurements in duplicate.

Since the ninhydrin reagent reacts with primary and secondary amines and ammonium salts the tubes used for the reaction must be free from such contaminations. This is achieved by reserving a set of tubes for this reaction and cleaning them only by soaking in detergent, followed by thorough rinsing and drying.

Blank reactions must be prepared for each solution assayed by adding the ninhydrin to the enzyme before the substrate and omitting the incubation at 37°C. The concentration of enzyme is proportional to the difference between the mean sample absorbance and the mean blank if this difference does not exceed 0.6.

Standard curve for diiodotyrosine: treat 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml diiodotyrosine solution (4) made up to 1.0 ml with water, with 1.0 ml ninhydrin reagent (3) each, boil and dilute in the same way as the reaction mixtures. Plot absorbance, corrected for blank, ΔA_{570}, *versus* diiodotyrosine amount (µmol). The slope of the plot should exceed 2.8 μmol^{-1} (equivalent to an absorption coefficient of 1.96 $l \times mmol^{-1} \times mm^{-1}$). If a smaller slope is observed the ninhydrin solution should be replaced.

Measurement

Pipette into clean Pyrex tubes:	blank	sample	concentration in assay mixture:
sample	0.50 ml	0.50 ml	pepsin A 15 – 80 mg/l
equilibrate to 37 °C			
HCl solution (2)	–	0.25 ml	HCl 0.01 mol/l
substrate solution (1)	–	0.25 ml	Ac-Phe-Dit 0.25 mmol/l
mix and incubate for exactly 20 min			
ninhydrin reagent (3)	–	1.0 ml	
at any convenient time:			
HCl solution (2)	0.25 ml	–	
ninhydrin reagent (3)	1.0 ml	–	
substrate solution (1)	0.25 ml	–	
mix, cover tubes and place in boiling water-bath for exactly 15 min; cool;			
ethanol (5)	5.0 ml	5.0 ml	
read absorbance against water.			

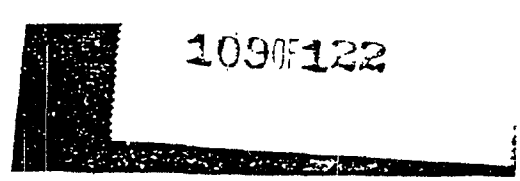
Determination of zymogens: incubation for 10 min with the HCl solution (2) before addition of the substrate is more than adequate to allow complete activation of the zymogens. The blanks should be similarly pre-incubated since the initial activation peptides and their digestion products will produce some colour with ninhydrin

Calculation: subtract the mean absorbance of the blank from the mean absorbance of the sample. Read the corresponding µmoles of diiodotyrosine from the standard curve. The activity concentration of the enzyme in the sample is

$$b = \frac{n \times 1000}{t \times v} \quad \text{U/l}$$

where

n	amount of diiodotyrosine liberated in µmol
1000	conversion factor from ml to l
t	incubation time in min
v	sample volume in ml.



Determination of inhibitors of pepsin

Apart from such chemicals as diazoketones [11, 41] and 1,2-epoxy-3-(4-nitrophenoxy)-propane [12] which irreversibly inactivate pepsins by covalent modification, the enzymes can be inhibited by three classes of agents: proteins from *Ascaris lumbricoides*, pepstatins and peptides liberated, on activation, from the amino terminus of the zymogens. All these inhibitors have been reviewed [13]. The activity of pepsins on protein substrates can also be lowered by acidic polysaccharides, but this effect appears to be due more to the binding of the polysaccharide to the substrate than to an interaction with the enzyme [42, 43].

All the reversible inhibitors of pepsins could be assayed by their effect on the enzymes, but the choice of enzyme and of substrate is important; furthermore some of the enzyme-inhibitor interactions may be time-dependent [44, 45]. The *Ascaris* inhibitors and pepstatins are potent inhibitors of pig pepsin at pH 3 (K_i ca. 10^{-9} mol/l), but bind much less tightly to pig and human gastricsin (pepsin 5). At the concentration of enzyme (ca. 10^{-7} mol/l) used in the assay with haemoglobin as substrate, at 50% inhibition the inhibitor will be present (at equilibrium) almost entirely as the enzyme-inhibitor complex, so that the inhibition is essentially stoichiometric. At higher degrees of inhibition, however, or with more sensitive substrates (and so less enzyme) significant proportions of the inhibitor will still be free at equilibrium.

The inhibitory activation zymogen-derived peptides dissociate from the enzymes at low pH values, so that they must be assayed at pH 5, at which the milk-clotting assay [19] can be used [45]. The association of enzyme and inhibitor is time-dependent and pre-incubation of 30 min or more may be necessary; furthermore K_i values may be considerably larger than those of the gastricsins [13] so that no set formula can be provided and design of an assay will require some care.

Validation of Method

Precision, accuracy, detection limit and sensitivity: with an activity of about 5 nmol diiodotyrosine per min in the assay tube the coefficient of variation is about 0.06. Detection limit is 0.004 U.

Sources of error: the presence of small peptides other than the substrate could decrease or increase the apparent activity of the enzyme [27, 28]. It is not known how serious such possible interference is in practice.

Specificity: pig pepsin C [5] and human gastricsin [6] have little or no activity against Ac-Phe-Dit.

Reference ranges: in human gastric juice 23 – 81 (mean 53) U/l [32].

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Third Edition

Volume V

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Casein, resorufin-labeled

15 mg, Cat. No. 1080733

Preparation: Casein from cow milk reacts with activated resorufin [N-(resorufin-4-carbonyl)piperidine-4-carbonic acid-N'-hydroxysuccinimide ester*] with the following purification by chromatography. Ca. 90 µg resorufin are bound to 1 mg casein (control by total hydrolysis using pronase*).

Stability: Stable at −20 °C, stored dry and protected from light. An aqueous solution is stable for several months at −20 °C and for 2–3 days at +4 °C. It is recommended to store aqueous solutions in aliquots at −20 °C.

Spectral properties: Absorption (excitation) maximum in the neutral and alkaline range λ = 574 nm, ε = 66000 [l x mol^{-1} x cm^{-1}], in the acidic range λ = 467 nm.

Emission maximum in the neutral and alkaline range λ = 584 nm, in the acidic range λ = 559 nm.

Application: The preparation is used as an unspecific protease substrate. It is especially well suited for the detection of traces of protease activities.

Principle: By treatment with proteases, peptides, resorufin labeled, which cannot be precipitated by trichloroacetic acid, are released from casein, resorufin labeled. These peptides are equivalent to the proteolytic activity present.

Application example for the determination of proteolytic activity according to Twining (1).

Solutions/Reagents

I Substrate solution
Casein, resorufin-labeled, 0.4% (w/v) in redist. water
II Incubation buffer
Tris-HCl, 0.2 mol/l, pH 7.8, $CaCl_2$, 0.02 mol/l
III Sample solution
IV Stop reagent
Trichloroacetic acid, 5% (w/v), in redist. water
V Assay buffer
Tris-HCl, 0.5 mol/l, pH 8.8

Assay procedure:

Wave-length:	574 nm
Cuvettes:	Sarstedt cuvettes (10 x 4 x 45 mm)
Light path:	1 cm
Temperature:	37 °C

Pipette into reaction vessels		sample blank	sample
substrate solution	(I)	50 µl	50 µl
incubation buffer	(II)	50 µl	50 µl
redist. water		100 µl	—
sample solution	(III)	—	100 µl
Incubate at 37 °C for a suitable space of time (15 min till overnight). Stop reaction by addition of			
stop reagent	(IV)	480 µl	480 µl
Incubate for 10 min at 37 °C, subsequently centrifuge for 5 min and pipette into Sarstedt cuvettes			
supernatant		400 µl	400 µl
assay buffer	(V)	600 µl	600 µl
mix and immediately read absorbance of the sample against blank at room temperature (= ΔA sample)			

On application of 0.1 µg trypsin *sequencing grade** in the sample volume (activity 110 U/mg, Chromozym®** TRY as substrate) the absorbance difference (ΔA_{sample}) amounts to 0.07, when incubating for 15 min under the aforementioned conditions. Using 0.1 µg endoproteinase Asp-N *sequencing grade** ΔA_{sample} is 0.09.

The assay will be more sensitive (by factor 10) if the determination is carried out fluorimetrically.
The detection limit can be lowered by a further factor ca. 10 by prolongation of the incubation time to 16 h (overnight).

Total hydrolysis with pronase in the standard assay (e.g. 1 mg pronase overnight at 37 °C) results in ΔA_{sample} = 1.9.

Reference

1 Twining, S. S. (1984) Anal. Biochem. 143, 30–34

* available from Boehringer Mannheim GmbH
** registered trademark of Pentapharm AG, Basel, Switzerland

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PURIFICATION AND SPECTROPHOTOMETRIC ASSAY OF NEOMYCIN PHOSPHOTRANSFERASE II[1]

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Received November 29,1975

SUMMARY: Neomycin phosphotronnferase II is maximally released by osmatic shocking of R+ E. coli between late log and early stationary phase. A 300-400-fold purification of the enzyme protein is accomplished by streptomycin sulfate and ammonium sulfate precipitations of osmotic shockates, followed by affinity and ion-exchange chromatography. The recovered enzyme preparation is electrophoretically 90% pure, is free of ATP-ase activity, and can be conveniently assayed spectrophotometrically by linking the production of ADP to pyruvate kinase and lactate dehydrogenase. The purified enzyme, however, is not stable.

INTRODUCTION

A new family of enzymes, found in gram negative bacteria carrying R-factors, inactivate aminoglycoside antibiotics and act as the principle agents of clinical resistance to antibiotic therapy (1). Investigations of the characteristics of these enzymes have been hindered by a lack of highly purified preparations and reliable assay methods. ATP-dependent phosphorylation is a common enzymatic mechanism of inactivation by R+ bacteria. Consequently, some of the inactivating enzymes resemble the kinase enzymes and, once purified, should be subject to similar methods of assay and study.

Neomycin phosphotransferase II catalyzes an ATP- dependent phosphorylation of the 3'hydroxy group of the aminohexose

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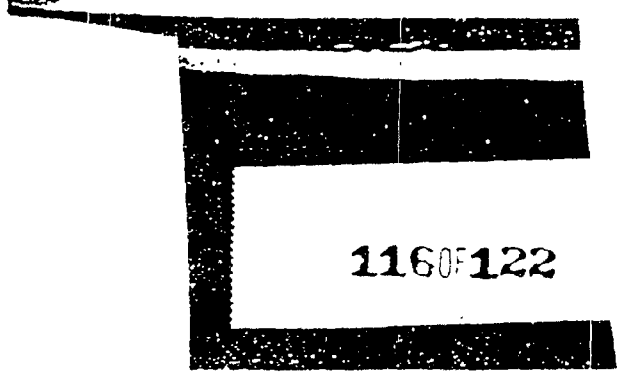
[2]To whom inquireis should be adressed.



moiety of kanamycins, neomycins, paromomycins, ribostamycin, and butirosins (2-4). The initial discovery, partial characterization, and partial purification (< 10% purity) relied upon fixed time assays employing radioactivity (2) or microbiological activity (4) measurements. This report describes a highly purified preparation conveniently assayed by the continuous spectrophotometric method employed for other kinase enzymes.

EXPERIMENTAL

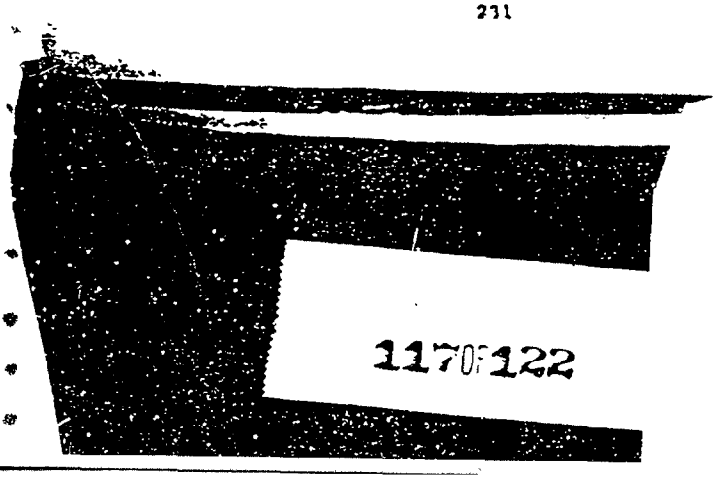
Kanamycin sulfate (potency 750 meq/mg) and E. coli JR76.2/W677[1] were gifts from Dr. Julian Davies. The organism produces gentamicin adenylyl transferase, described previously (5), as well as neomycin phosphotransferase II. The procedures for growing the bacteria, extracting enzymes by osmotic shocking, precipitating proteins with ammonium sulfate, and chromotographing the recovered protein on DEAE agorose were identical for both enzymes. Column fractions containing neomycin phosphotransferase II were desalted on a column of Bio-Gel P-2, equilibrated with 10 mM tris-HCl buffer pH 7.8 containing 0.125 mM EDTA, 0.3 mM dithiothreitol, and 1 mM Mg^{++} (standard buffer). The desalted enzyme was applied to a gentamicin C_{1a}-Affi-Gel 10 affinity column described previously (6), and eluted with 2M ammonium sulfate in standard buffer. Peak fractions were again desalted on Bio-Gel P-2 and applied to a second column of DEAE agarose (Bio Rad, DEAE Bio-Gel A) equilibrated with standard buffer. The purified enzyme was eluted from DEAE agarose with a 0-0.3 M potassium acetate gradient in standard buffer.

Column elution gradients were determined from conductivity measurements using a Lab-Line Biometer. Disc gel electrophoresis was conducted in a Bio-Rad Model 300A cell driven by a model 400 power supply. Gel scanning was performed on a Gilford 2410-S Linear Transport. Enzymatic activity in crude fractions was determined by the radioactivity assay method of Ozanne et al (7). Purified fractions were assayed spectrophotometrically at 340 nm and 25°C using a Gilford model 240 spectrophotometer and a Leeds and Northrup XL-610 recorder, by linking ADP production to pyruvate kinase and lactate dehydrogenase. Reaction mixtures contained 1.9 units pyruvate kinase, 1.6 units lactate dehydrogenase, 0.1 umole DPNH, 0.4 umoles phosphoenol pyruvate, 5 umoles magnesium acetate, 10 umoles potassium acetate, 0.2 umoles ATP, 0.066 umoles kanamycin, 25 umoles tris-HCl pH 7.8, and 0-0.1 units of neomycin phosphotransferase in a total volume of 0.5 ml. Control assays were conducted in the absence of kanamycin. Units of enzyme were defined as umoles product formed per minute. Assay chemicals were obtained from Sigma.

RESULTS AND DISCUSSION

The yield of neomycin phosphotransferase obtained by osmotic shock varies considerably as a function of the growth curve of

[1] E. coli JR66/W677 (2-4) has the same parental origin as E. coli JR76.2/W677 (J. Davies, personal communication).



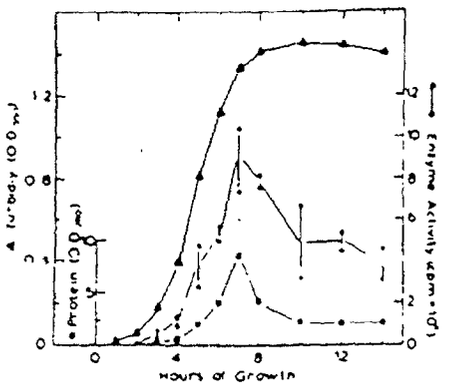


FIGURE 1: Release of neomycin phosphotransferase and protein by osmatic shock as a function of bacterial growth. After addition of 1% overnight inoculum, protein and enzymatic activity were determined on osmatic shockates prepared from 40 ml culture samples, harvested at the times indicated. The open and closed circles represent neomycin phosphotransferase activity obtained in the presence and absence of 0.5 mM magnesium, respectively.

E. coli, reaching a peak between log and stationary phase, then dropping to half the peak level in stationary phase (Figure 1). These results are similar to gentamicin adenylyl transferase (5) which peaks in early stationary phase but drops to a quarter of peak level, and similar to gentamicin acetyl transferase (6) which peaks in late log phase but drops to undectable levels. Differences between the activity profiles of the three enzymes suggest small differences in their periplasmic location. In contrast, Nossal and Heppel (8) observed no decrease in the amount of periplasmic enzymes catalyring hydrolytic reactions, released by osmotic shock from E. coli in stationary versus logarithmic phase, except under conditions incorporating magnesium in the shock medium. As shown in Figure 1, magnesium had only a small effect on the release of the phosphorylating enzyme, occuring throughout the growth curve. The magnesium-dependent differences between activity profiles of hydrolytic and aminoglycoside inactivating enzymes suggest large differences in their periplasmic location.

The first chromatographic step in the purification of neomycin phosphotransferase employs DEAE agarose. This is also the key

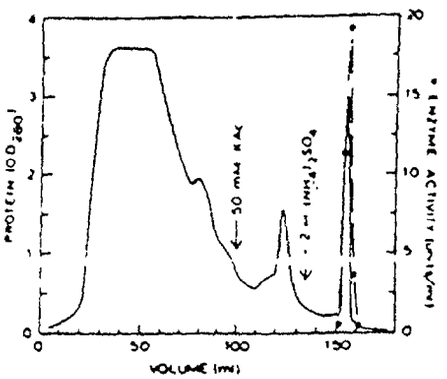


FIGURE 2: Affinity chromatography of neomycin phosphotransferase II on gentamicin C_{1a}-Affi-Gel 10. 250 mg of protein obtained from a first DEAE agarose column were added to a 1 x 30 cm column and eluted at a flow rate of 0.5 ml/min.

step in the purification of gentamicin adenylyl transferase (5), and serves to separate the two enzymes in extracts from bacteria containing R-factors which direct the synthesis of both. Separation is necessary at this stage since it has not yet been possible to elute the adenylylation enzyme from gentamicin affinity columns. However, the step is neither necessary nor significantly advantageous in the purification of the phosphorylating enzyme.

The key step in the purification of neomycin phosphotransferase is affinity chromatography on gentamicin C_{1a}-Affi Gel 10. A 20-fold purification is achieved by this step, estimated from the protein profile of Figure 2. Specific activities of peak fractions varied from 2-13 units/mg in four separate preparations. These activities are considerably higher than those obtained by Matsuhashi et al (4) from affinity columns. The difference is probably dependent upon the inclusion of a spacer between the antibiotic and agarose during preparation of affinity resins. Umezawa and his associates (4,9) and others (10) have shown that in the absence of a spacer, aminoglycoside inactivating enzymes elute from antibiotic affinity columns within salt gradients (which is atypical of affinity chromatography (11)) of moderate ionic strength in similar fashion to the elution of

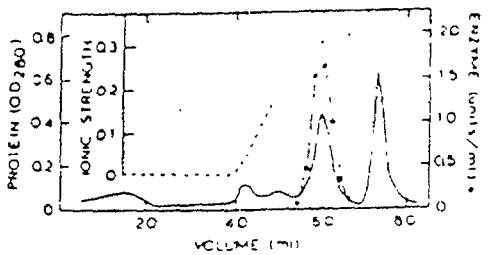


FIGURE 3: Ion-exchange chromotography of neomycin phosphotransferase II on DEAE agarose. 5.6 mg of protein obtained from the affinity column were added to a 1.5 x 30 cm column and eluted at a flow rate of 0.5 ml/min. The salt gradient was generated with equal volumes (27 ml) of standard buffer and standard buffer plus 0.3 M potassium acetate. The final protein peak was eluted with 0.3 M ammonium sulfate.

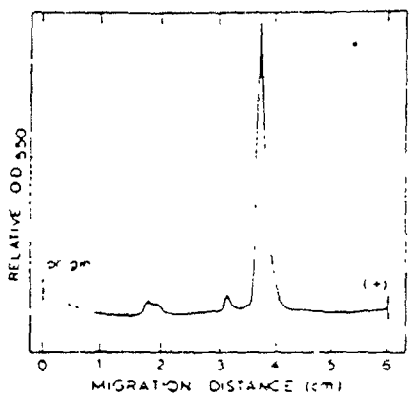


FIGURE 4: Disc gel electrophoresis scan of purified neomycin phosphotransferase II. Approximately 50 µg of the peak fraction from DEAE agarose chromotography were placed on 7.5% polyacrylamide gels and run at pH 8.9 and 3 milliamps/tube. Gels were stained for protein in a solution containing 0.1 g coomassie blue, 45 ml methonol, 45 ml H_2O and diluted 3-fold with water just before use. Destaining was carried out in 10% TCA for 12 hours.

these enzymes from DEAE ion-exchange resins (3,5,6). In contrast, we have found that inclusion of a spacer renders elution by high ionic strength extremely difficult. Once bound to a gentamicin C_1a-Affi-Gel 10 column, gentamicin adenylyl transferase cannot be eluted by high concentrations of salt, gentamicin acetyl transfer-

ase elutes as a very broad dilute peak (6), and neomycin phosphotransferase elutes in a full column volume using a concentration of ammonium sulfate which approaches the limits of sulubility of the protein (Figure 2). It therefore appears that a spacer is necessary to accomplish the firm and specific binding of aminoglycoside enzymes, normally attributed to affinity chromotography.

The final step in the purification is ion-exchange chromotography on a second DEAE-agarose column, shown in Figure 3. Gel electrophoresis of active fractions produces three protein bands, two minor components and a major band comprising 90% of the added protein, as shown in Figure 4. Assays of gel slices from parallel gels show all the phosphotransferase activity migrating with the major band. A 3-fold purification is achieved by the final step, estimated from the protein profile of Figure 3.

Specific activities of peak fractions were nearly constant within a single preparation, but varied from 3-10 units/mg between different preparations. In only one preparation was the specific activity increased by the final purification step reaching a value of 10 units/mg, and this occurred with the preparation having the lowest specific activity after affinity chromotography. It appears that contaminant proteins exert a stabilizing effect on neomycin phosphotransferase II. Once the enzyme has been eluted from the affinity column, it becomes highly unstable, losing as much as 50% of its activity in less than a week. Attempts to stabilize the enzyme have not been successful.

Calculations of intermediate specific activities, recoveries, and overall fold of purification were complicated by the instability of purified fractions and also by the inaccuracy of the radioactivity assay. However, the overall purification can be estimated at 300-400-fold on the basis of protein fractionation. In our hands the radioactivity assay lacks linearity with time and enzyme concentration. Similar results were obtained with the radioactivity assay for adenylylation (5). Nevertheless, the radioactivity assay is necessary for semi-quantitative detection of the enzyme in early stages of purification. Substantial ATPase activity dominates the spectrophotometric assay in these initial fractions, but passes through gentamicin affinity

columns. Following this step, background rates in control assays were small (~0.002 ΔOD/min) and independent of the protein concentration in assayed fractions. Spectrophotometric assays of neomycin phosphotransferase II activity following affinity chromotography were linear with time and enzyme concentrations. Use of this assay should greatly facilitate the characterization of this and other aminoglycoside antibiotic phorphorylating enzymes.

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