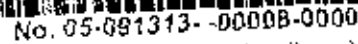


SUB-PORTFOLIO: NEG. AGR. INDUS. Y COMERCIO



№ 05-09/19-00000-19
Копия от 05.09.2019 № 15.04.12 Деп. Зап. DIR NUI VASAR
№ 11 ПАТНУЛИЯ Ли. 019 ТАСНАОИОНАЛИ
Ал. 444-8; СПУЛТАР; ҚОЛ едир. 44

1

PETITORIO



REGISTRACIÓN Y OPERACIÓN

Recepción: 2000-09-09 00000000

Folio: 4

Fecha (Año): 2000-09-09 16:43:23

Trámite: 001 MARCAS

1 REGISTRO/ 413 PRESENCIA

Dependencia: 2014 DIVISION DE SIGNOS DISTINTIVOS

FORMULARIO ÚNICO DE REGISTRO DE SIGNOS DISTINTIVOS. 2000-1

← SOLICITUD DE REGISTRO DE:

☒ Marcas de Producto o Servicio.

☐ Lemas Comerciales

☐ Marca Colectiva.

☐ Denominación de Origen

☐ Marca de Certificación.

☐ Autorización de uso de denominación de Origen

↑
SOLICITANTE

Nombre: UMARLA S.A.

Dirección: Antártica, sn, Panamericana Norte
(a 100 metros de la Tenería Alemana)

Teléfono:

Fax:

Domicilio: Ambato, Ecuador

IDENTIFICACION

C.C. ☐

NIT ☐

C.E. ☐

OTRO ☐

Número:

→
REPRESENTANTE
O APODERADO

Nombre: DANILO ROMERO RAAD

Dirección: CALLE 72 No. 10 - 03 Oficina 301
BOGOTA, COLOMBIA

Teléfono: 3490155

Fax: 2550005

E-mail: info@romerorad.com

IDENTIFICACION

C.C. ☒

NIT ☐

C.E. ☐

OTRO ☐

Número: 79.547.687

TP: 79.289

↓ Denominación del signo:

DAKKAR

Mixto

☒

Nominativo

☐

Figurativo

☐

° Marca Asociada (si es lema)

Certificado No

Solicitud No.

± Enumeración detallada de productos y servicios:

Clase 17

CAUCHO, GUTAPERCHA, GOMA, AMIANTO, MICA Y PRODUCTOS DE ESTAS MATERIAS NO

COMPRENDIDOS EN OTRAS CLASES; PRODUCTOS EN MATERIAS PLASTICAS SEMIELABORADAS;

MATERIAS QUE SIRVEN PARA CALAFATEAR, CERRAR CON ESTOPA Y AISLAR; TUBOS FLEXIBLES NO METALICOS.

" Comprobante de pago No. _____

Fecha _____

≥ PRIORIDAD : SI ☐ NO ☐

País de origen

No. de solicitud

Fecha

× PRIORIDAD DE EXPOSICIONES:
SI ☐ NO ☐

Nombre de la exposición

Lugar de celebración

Fecha

10 ANEXOS

- ☒ Comprobante de pago de la tasa de presentación de la solicitud
- ☐ Comprobante de pago de la tasa por concepto de excedente de palabras en la publicación (más de 150)
- ☐ Comprobante de pago de la tasa por concepto reivindicación de prioridad
- ☒ Poderes, si fuere el caso (Expediente No. 04-69127) *O.K.*
- ☐ Certificado de existencia y representación legal cuando el solicitante sea persona jurídica
- ☒ Artes finales 12 x 12 cm, 2 ejemplares
- ☐ Copia de la primera solicitud si se reivindica prioridad
- ☐ Traducción de la primera solicitud, si se reivindica prioridad
- ☐ Certificado de registro en el caso del artículo 6° quinquies del Convenio de París
- ☐ Autorizaciones requeridas según los artículos 135 y 136 de la decisión 486
- ☐ Reglamento de uso, si es el caso (marcas de certificación, colectivas y denominaciones de origen)
- ☐ Designación de zona geográfica, si es el caso (denominaciones de origen)
- ☐ Reseña de calidades, reputación y características de los productos, (denominaciones de origen)
- ☐ Designación de zona geográfica, si es el caso (denominaciones de origen)
- ☐ Copia de estatutos de solicitante, si es el caso (marcas colectivas, de certificación, denominaciones de origen)
- ☐ Lista de integrantes, si es el caso (marcas colectivas o de certificación)

11 REPRODUCCION DEL SIGNO A REGISTRAR



12 NOMBRE: DANILO ROMERO RAAD

FIRMA:

C.C. 79.647.687

TP: 79.289

Instrucciones para el diligenciamiento del presente formulario, ver reverso de esta página.

3

SUPERINTENDENCIA Y COMERCIO
Nominación: 0001337 00000000
Fecha (MM/AA): 2005-09-09 16:43:23
Trámite: 001 MARCAS
Registros: 2510 DIVISION DE MARCAS REGISTRADAS
Folios: 4
1 REGISTRO 411 PRESENTAR

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 05 - 69,408
FECHA : SEPTIEMBRE 9 DE 2005

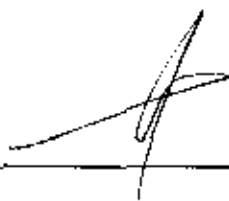
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
ROMERO RAAD ABOGADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	27551860	09/09/2005	932,000.00

***** CONCEPTO *****

CANT. REGISTRADO	CONCEPTO	TOTAL CONCEPTO
1 50005-01-01 SOLICITUDES	3 PROC. REGISTRO DE MARCAS Y LENAS	565,000.00
	TOTAL :	\$ 565,000.00

SON: QUINIENTOS SESENTA Y CINCO MIL PESOS

RESPONSABLE : 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

5

REPÚBLICA DE COLOMBIA
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RESOLUCIÓN No. 9285
(18/abril/2006)

Por la cual se concede un registro

Radiación No. 591332-0

LA JEFE DE LA DIVISION DE SIGNOS DISTINTIVOS
En ejercicio de sus facultades legales, y

CONSIDERANDO

Que la solicitud de Registro de la Marca que se tramita bajo el expediente indicado en la referencia, cumple con los requisitos previstos en las disposiciones legales vigentes.

RESUELVE

ARTICULO PRIMERO: Conceder el registro de:

LA MARCA MENTA : DAKKAR (SEGUN MODELO ADJUNTO)

PARA DESTINARLOS : CAUCHO, GUELAPERCHA, GOMA, AMIANTO, MICA Y PRODUCTOS DE ESTAS MATERIAS NO COMPRENDIDOS EN OTRAS CLASES; PRODUCTOS EN MATERIAS PLÁSTICAS SEMEJANTES LABORADAS; MATERIAS QUE SIRVEN PARA CALAFATEAR, CERRAR CON ESTOPA Y AISLAR; TUBOS FLEXIBLES NO METÁLICOS.

Productos comprendidos en la clase 17 de la Octava Edición de la Clasificación Internacional de Niza.

VIGENCIA : Diez (10) años contados a partir de la fecha de la presente resolución.

TITULAR(ES) : UMARIA S.A.
DOMICILIO : AMBATO, ECUADOR
ANTARTICA, SN. PANAMERICANA NORTE (A 100 METROS DE LA TENERIA ALEMANA)

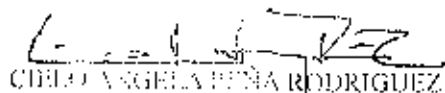
ARTICULO SEGUNDO: Asignar número de certificado al derecho concedido, previa anotación en el Registro de la Propiedad Industrial.

ARTICULO TERCERO: *Notifíquese personalmente al(a) doctor(a) ROMERO RAAD DANILLO* ----- apoderado del titular, o a quien haga sus veces, el contenido de la presente resolución, entregándole copia de la misma, advirtiéndole que contra ella proceden los recursos de reposición ante el Jefe de la División de Signos Distintivos y el de apelación para ante el Superintendente Delegado para la Propiedad Industrial, interpuestos dentro de los cinco (5) días hábiles siguientes a la fecha de su notificación.

DEQUESE Y CUMPLASE

En Bogotá D.C. a los 18 días del mes de abril de 2006

LA JEFE DE LA DIVISION DE SIGNOS DISTINTIVOS


CIELO ANGELA PEÑA RODRIGUEZ

Apoderado ROMERO RAAD DANILLO
Dirección: CARRETERA NO. 10-83 OLICENA 301
Ciudad: BOGOTÁ D.C. COLOMBIA
Teléfono:



253 250

Several successful strategies have been developed to minimize oxidation, including the use of antioxidants. There are three classes of antioxidants. The "true" antioxidants are usually phenolic compounds such as BHT, BHA, propyl gallate, and vitamin E. Reducing agents form the second class of antioxidants and include compounds such as methionine, ascorbic acid, sodium sulfite, thioglycerol, and thioglycolic acid. These act as oxygen scavengers in closed systems (where the supply of oxygen is limited) wherein they oxidize preferentially over the protein. Metabisulfite has also been used extensively, despite problems associated with inhibition by many alcohols, aldehydes and ketones, decreases in pH due to metabisulfite, and reaction with rubber closures.²⁸³ The third class of antioxidants are chelating agents such as EDTA, citric acid, and thioglycolic acid. These antioxidants act by removing trace metal ions that play a major role in initiating autooxidation reactions. EDTA may occasionally cause an increase in reaction rate by binding the EDTA-metal ion complex to the protein, resulting in site-specific generation of radicals.²¹⁴ The contribution that each of these classes of antioxidants makes to decreasing oxidation has been reviewed recently.²¹⁰

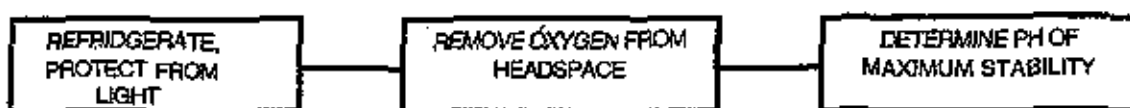
Based on the above, the first strategy for minimizing protein oxidation in a formulation is to refrigerate (as usual), protect from light, remove oxygen, and adjust the pH for minimizing oxidation (Figure 6). If these straightforward attempts do not reduce oxidation, then excipients which may have contaminants that catalyze oxidation should be removed and antioxidants added. It is sometimes necessary to further refine the formulation to stabilize the antioxidants. Finally, lyophilization or freezing should be employed if these initial strategies do not provide the shelf life extension required.

Oxidation, deamidation, and aggregation are the major pathways for protein degradation. These pathways must be understood in order to develop stable formulations. The degradation rates can be minimized either by formulating under conditions where the individual reactions are the slowest, or by adding excipients that retard selected reaction pathways. In addition, studies of these pathways provide information on the degradation products, which may or may not be biologically active. Finally, the prediction of formulation stability is necessary for drug development. The prediction of protein stability can be achieved by using accelerated kinetics at elevated reaction temperatures.

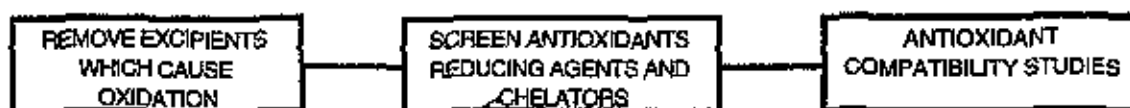
V. ACCELERATED STABILITY AND USE OF THE ARRHENIUS EQUATION

The stability of small-molecule drugs is usually determined over a wide temperature range. The rate constants determined at higher temperatures may be used to predict, via the Arrhenius equation, the shelf life at lower storage temperatures. When the kinetics clearly follow Arrhenius behavior, this approach allows

First Round Studies to Reduce Oxidation



Second Round Studies to Reduce Oxidation



Third Round Studies to Reduce Oxidation



FIGURE 6. Strategy for minimizing oxidation of protein pharmaceuticals.

for the determination of shelf life without a real time analysis.²⁹⁸ Small-molecule drugs often degrade via a single reaction mechanism, and the resulting kinetics usually follow Arrhenius behavior. However, because of the greater structural complexity of proteins, there are numerous degradation reactions that can take place in a protein drug. Many of these degradation routes and mechanisms have been summarized and discussed in review articles.^{1-3,299} Often the degradation routes at higher temperatures are not the same as those at the lower temperatures. It has become common practice to carry out accelerated stability testing for proteins, and to use the accelerated rate profiles to compare formulations and minimize the degradation at the higher temperature. This approach may give misleading results since the degradation at higher temperatures may require very different formulation conditions for maximum stability compared to the degradation occurring at the lower storage temperature.

The complexity of potential reactions in a protein is reflected not only in the type of reactions that can occur, but also in the effect of neighboring functional groups. The effect of functional groups on the rate of degradation of a nearby amino acid residue may be determined solely by the distance apart within the amino acid sequence. Examples include the kinetics of deamidation (as previously discussed in Section III) that have been shown to be highly sequence specific in small peptides¹²⁰ and hydrolysis of the peptide bond.^{300,301} In addition to these "nearest-neighbor effects", the protein conformation can dictate what reactions will dominate the degradation profile of a protein. The arrangement of the charged residues on the

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surface of the protein contribute to the overall dipole moment of the molecule as well as the local dielectric constants of solvent around potentially reactive residues. Disruption of the protein conformation can alter the spatial arrangement of charges, which in turn can affect the way the protein interacts with solvent molecules in solution.³⁰² The protein conformation can also dictate the course of a reaction if a particular structural intermediate is favored by the local geometry of the polypeptide backbone or side chains at the degradation site. Deamidation of asparagine residues, as an example, involves formation of a cyclic imide intermediate.¹⁴³ The crystallographic structure for trypsin determined by neutron diffraction revealed a common structural and hydrogen bonding motif for the three deamidated asparagine residues in this protein. This motif is consistent with the requirement for formation of the cyclic imide structure.¹³¹ The combined effects of amino acid sequence and conformation on degradation reaction rates also complicates the kinetic analysis, even when only one degradation mechanism is being investigated, because the same reaction can proceed at very different rates at different sites in the protein. As an example, photooxidation of the two methionine residues in recombinant human relaxin proceeds at very different rates.³⁰³ Altogether, these observations suggest that accelerated stability studies and, in particular, use of the Arrhenius equation to determine shelf life would be inappropriate for protein pharmaceuticals. It is therefore not surprising that some authors have suggested that real time stability data be used to meet regulatory requirements.³⁰⁴ In fact, there are enough concerns raised about the applicability of determining the shelf life of protein drugs by Arrhenius behavior that usually real time stability data is supplied to regulatory agencies as supportive data for shelf life determinations. Despite these concerns, researchers have attempted to determine protein stability using accelerated testing and evaluation of the resulting kinetics using the Arrhenius approach. The design and analysis of accelerated degradation testing for biologics has been presented by Kirkwood et al.^{305,306} A summary of accelerated stability testing for proteins is presented in Table 2. In general, the Arrhenius approach appears to be applicable to determining shelf life for proteins when only the activity is monitored. Most of these studies have been carried out over a temperature range where the protein undergoes thermal denaturation. Protein unfolding tends to be a very cooperative process as shown by the narrow range of denaturants or temperature over which the protein will unfold. If the loss of activity is only the result of a global conformational change, rather than a covalent chemical degradation, then the measurement of protein activity in effect is a result of one "reaction", namely the unfolding of the protein to a denatured state. Although the product of this reaction may involve many different unfolded forms of the protein, these different forms will be inactive and not distinguishable from each other by activity assays. If the protein, during unfolding, existed in multiple conformational forms that retained activity, then the Arrhenius plots will deviate from linearity. Such a case was observed in activity studies of the enzyme α -glucosidase isolated from the bacterial thermophile *Bacillus thermoglucosidius*.³⁰⁷ The Arrhenius plots for inactivation of this protein showed a sharp break at 73°C, indicating that the

TABLE 2**Accelerated Rate Studies in Proteins and Use of Arrhenius Kinetics**

Protein	Property monitored	Summary of study	Ref.
α_1 -Antitrypsin	Particulate formation	First order kinetics; linear Arrhenius plots where extrapolated value agrees well with real time data; activation energy = 19 kcal/mol	310
Galactosyltransferase	Enzyme activity	First order kinetics; linear Arrhenius plots	311
Cathepsin L	Enzyme activity	First order kinetics; linear Arrhenius plots	312
Lipase, α -amylase, pancreatin, diastase from different biological sources	Enzyme activity	Stability of enzymes in the solid state; linear Arrhenius plots, but over restricted temperature ranges	313
α_1 -Antitrypsin	Enzyme activity	First order kinetics; linear Arrhenius plots; activation energy = -26 kcal/mol	314
Tissue plasminogen activator	Clot lysis activity, amidolytic activity, one- to two-chain conversion	Studied over 5-60°C; linear Arrhenius plots for activity, deviation above 40°C for chain conversion	309
Recombinant human interferon- β	Bioactivity	Studied over 25-80°C; linear Arrhenius plots over entire range	315

enzyme exists in a more heat-stable form below 73°C, and in a more labile form above this temperature that nonetheless was still very active. This point was further supported by investigating the loss of activity with SDS at a specific temperature. The triphasic behavior of the Arrhenius plot showed that three conformations (which exhibited different susceptibilities to SDS denaturation) may exist. It has also been shown that the Arrhenius equation can be applied to analysis of activity loss in enzymes when the unfolding kinetics are more complex and involve more than one pathway for unfolding and inactivation of the enzyme. Incorporation of a reversible and irreversible pathway into the kinetic model yielded rate constants for each process. This analysis resulted in linear Arrhenius plots for the rate constants for each process.³⁰⁸ Loss of activity as a function of temperature could also be caused by the increased rate at the higher temperatures of a covalent chemical modification, such

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as deamidation or oxidation. Although many reactions might occur, monitoring protein activity alone will usually result in linear Arrhenius behavior since the rate of activity loss will be determined by the fastest reaction. Alternatively, only one of the modifications might cause an activity loss. Monitoring protein stability by a variety of analytical assays, rather than by the final activity loss, can complicate interpretation of accelerated rate studies. As an example, when the stability of interleukin-1 β was studied by different analytical techniques at or below 30°C the degradation pathway was deamidation, whereas at or above 39°C it was aggregation and precipitation.¹⁶⁶ That this protein degraded by two different mechanisms precluded the prediction of shelf life from simple Arrhenius behavior. Although these observations do not bode well for the use of accelerated testing to predict protein shelf life when chemical degradation reactions are investigated, it has been suggested that proper selection of temperature range and assay may allow one to generate linear Arrhenius plots.³⁰⁹ Stability studies of recombinant tissue plasminogen activator (rtPA) resulted in apparent linear Arrhenius behavior for activity and one- to two-chain conversion as measured by clot lysis and gel permeation chromatography, respectively. Unfortunately, a comparison of the rate constants determined by real time analysis at the lower temperature with rate constants determined by extrapolating the Arrhenius plot (from the higher temperatures to the lower recommended storage temperature) was not undertaken. Such an analysis would support the use of accelerated studies to predict shelf life of proteins. The linear Arrhenius behavior observed for the one- to two-chain conversion of rtPA is likely due to two important factors. First, scanning calorimetry experiments showed that the stability studies of rtPA were performed over a temperature range that was below the thermal transition of rtPA.³⁰⁹ Second, the gel permeation chromatographic assay, as well as the clot lysis assay, specifically monitors the conversion of one-chain to two-chain rtPA as a result of a proteolytic clip at one particular site in the amino acid sequence. Undoubtedly, for using accelerated studies as a predictive tool, an assay is required that specifically monitors one degradation event over a temperature range where the protein conformation is unchanged. If these conditions are met, then the complex problem of monitoring multiple reactions in a protein with different conformational states reduces to the study of a single chemical reaction of an amino acid in essentially the native conformation of the protein. The temperature behavior of the rate of this reaction will likely result in linear Arrhenius plots. In general, accelerated rate studies of protein degradation are useful tools for analysis of protein formulations if the potential difficulties are well understood.

VI. CONCLUSIONS

The formulation of protein pharmaceuticals is a more challenging task than the formulation of small-molecule drugs because proteins can undergo many diverse degradation reactions. The three major protein degradation pathways, aggregation,

deamidation, and oxidation, have been summarized in this review. These reactions can proceed at different rates depending on solution conditions such as pH, temperature, and ionic strength. Because many of these reactions occur simultaneously in a protein it is difficult to find appropriate conditions whereby all the reactions are eliminated. For example, lowering the pH of the formulation for rhGH reduces the deamidation rate, but it can also cause particulate formation.¹⁹² Thus, it may not be possible to choose a formulation that results in the lowest rate of each of the reactions. Instead, conditions are often chosen such that all reaction rates are minimized as much as possible. The effects of protein degradation such as deamidation or oxidation cannot be predicted *a priori* and have to be determined for each protein. If it can be shown that the degradation products do not reduce the safety and efficacy of the product, it may be possible to design a formulation that allows for an acceptable amount of the degradation product while minimizing all other degradation pathways. The development time for such a formulation is often longer than for small-molecule pharmaceuticals because real time stability data is often required as the result of the difficulty in analyzing the temperature dependence of the reaction rates. By choosing the appropriate assay(s) and temperature range, traditional accelerated stability studies can be performed to measure the kinetics of a particular degradation route in proteins. An understanding of the individual degradation mechanisms may allow some predictions to be made regarding protein stability, especially if each degradation pathway can be studied separately.

ACKNOWLEDGMENTS

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CONFIDENTIAL REPORT

PREFORMULATION STUDY ON THE
HUMANISED MONOCLONAL ANTIBODY
AN100226

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1.0 SUMMARY

Celltech were contracted by Athena Neurosciences, Inc. to perform a preformulation study on the humanised monoclonal antibody AN100226.

The study monitored the stability of the antibody at elevated temperatures (+23°C and +37°C) and at the intended storage temperature (+4°C) for 8 weeks in 15 formulations, comprising acetate, histidine and phosphate buffering agents covering a pH range of pH4.0 to pH7.5. The effect of salt (sodium chloride) and detergent (Tween80) additives and freeze-thawing on the stability of the antibody were included in the study. A range of stability indicating assay methods were selected for testing of samples based on Celltech's experience with this type of protein.

There were no marked changes in the samples stored at +4°C or +23°C following storage for 8 weeks. This suggested that the antibody AN100226 was relatively stable in all of the solution formulations evaluated. The results of the tests on the samples stored at +37°C gave an indication of increased fragmentation and dissociation at the lower pH values (pH4.0/5.0) and increased deamidation and aggregation at higher pH values (pH6.5 to pH7.5). The addition of salt or detergent appeared to increase the rate of these degradation processes.

From the results of the study, formulations in the pH range pH5.5 to pH6.0 were recommended for further extensive stability trials. If isotonicity is a requirement of the formulation then a pH6.0 phosphate or histidine formulation is preferable to acetate pH5.5 as the results demonstrated a more marked effect of salt on degradation in acetate (pH5.0) than in histidine (pH6.0) or phosphate (pH7.0).

2.0 INTRODUCTION

The aim of the study was to assess the stability of the humanised AN100226 IgG4 antibody over a short time period (8 weeks) using a range of 15 selected formulations based on Celltech's experience with the stability of this type of monoclonal antibody.

It is Celltech's experience that simple solution formulations can yield a shelf-life in excess of 18 months for monoclonal antibodies and these have been employed successfully in clinical trials. This study was based on such a strategy and thus evaluated parameters that can influence protein stability in solution (e.g. pH, ionic strength and buffering agent). It was assumed that the intended storage temperature for the humanised AN100226 antibody will be +2°C to +8°C and consequently the study was set up at +4°C (to represent the intended storage at +2°C to +8°C). Accelerated stability studies were performed by storing samples at elevated temperatures (+23°C and +37°C) for 8 weeks to give an indication of the longer term stability of the product in solution at the lower intended storage temperature. A range of analytical methods selected for their stability indicating properties were used to test the antibody product. Particular attention was placed on the gel permeation chromatography and isoelectric focusing methods as these have proved the most sensitive stability indicating assays in studies on other similar antibodies at Celltech.

The study was designed to generate data that could be used to select an initial formulation(s) for the final bulk product of the preclinical and first clinical lots and to identify candidate formulations for further more extensive stability studies.

3.0 MATERIALS AND METHODS

3.1 Preparation of Test Samples

3.1.1 Test antibody

Product derived from a cell culture supernatant of a preclonal cell line (3BB6), obtained during the cell line selection procedures of the AN100226 project was used for the study. The antibody was purified by Protein A affinity chromatography (Celltech Notebook Reference: 10007904 p.10).

100ml of the purified antibody at a concentration of 2.7mg/ml was concentrated using an Amicon stirred cell ultrafiltration system fitted with a TM30 membrane (30kDa cut off) to a concentration of 5.5mg/ml (determined by A_{280nm} using $E_{0.1\%} = 1.4$). This material was used to prepare the test formulations.

3.1.2 Formulation buffers

Solutions were prepared using Analar grade reagents or equivalent. The following solutions were employed as formulations in the study:

1. 50mM sodium acetate, pH4.0.
2. 50mM sodium acetate, pH5.0.
3. 50mM sodium acetate + 150mM sodium chloride, pH5.0.
4. 50mM sodium acetate + 150mM sodium chloride + 0.02% (v/v) Tween 80, pH5.0.
5. 50mM sodium acetate, pH5.5.
6. 50mM histidine, pH5.5.
7. 50mM histidine, pH6.0.
8. 50mM histidine + 150mM sodium chloride, pH6.0.
9. 50mM histidine, pH6.5.
10. 50mM sodium phosphate, pH6.0.
11. 50mM sodium phosphate + 0.02% (v/v) Tween 20, pH6.0.
12. 50mM sodium phosphate, pH6.5.
13. 50mM sodium phosphate, pH7.0.
14. 50mM sodium phosphate + 150mM sodium chloride, pH7.0.
15. 50mM sodium phosphate, pH7.5.

Buffering agents were selected to cover the pH range pH4.0 to pH7.5. In Celltech's experience the optimum pH of a formulation for this type of antibody is within the range pH5 to pH7. The pH4.0 sample was included as a condition that, in Celltech's experience, generates proteolytic and

dissociation products and the pH7.5 as a condition that promotes deamidation of this type of antibody. These two conditions were included to demonstrate the suitability of the stability indicating assays to detect degradation products.

Sodium chloride was included in selected formulations to assess the effect of ionic strength. The amount of salt added was calculated to achieve approximate isotonicity, as the route of administration was suggested as a possible limiting factor in the choice of formulations. Tween 80 was included in certain formulations to assess the effect of detergents in controlling aggregation processes.

Acetate and histidine buffers were adjusted to the appropriate pH using 5M sodium hydroxide and concentrated hydrochloric acid, respectively.

The required pH of the phosphate buffers was determined by titrating the sodium dihydrogen phosphate salt against the corresponding disodium hydrogen phosphate.

All buffers were filtered through 0.2µm filters prior to use.

3.1.3 Buffer exchange and vialling

PD10 columns (Supplier: Pharmacia, Product No: 17-0851-01) were equilibrated with the appropriate buffer.

2.5ml of the antibody (3.1.1) was loaded onto the column and eluted with 3.5ml of buffer. Fractions (0.5ml) were collected and their absorbance at 280nm recorded.

The antibody eluted predominantly in five to six 0.5 ml fractions (approximately 2.5-3.0ml). The fractions were combined and the resulting antibody concentration was approximately 4-5mg/ml (see Table 2).

Samples were passed through 0.2µm filters into sterile microcentrifuge tubes prior to aliquoting. Each test sample was split into ten aliquots (approximately 250µl) in 0.5ml sterile flip top polypropylene microcentrifuge tubes. Eight of these aliquots were required for the study at Celltech. Two were sent to Athena Neurosciences, Inc. for activity assays at the start of the study.

3.1.4 Reference standard

Ten aliquots (50 μ l) of the concentrated pre-buffer exchange antibody (3.1.1) were stored in 0.5ml sterile polypropylene flip-top tubes at +4°C in phosphate buffered saline (PBS) (pH7.0) for use as a reference standard during the study.

3.2 Sampling Plan

The sampling times were selected using Celltech's experience of degradation rates for this type of antibody. The storage of samples at -20°C was included to evaluate the effect of freeze-thawing on the product which can provide an indication of the potential for the protein to aggregate.

Temperature (°C)	Time (weeks)			
	0	2	4	8
-20		X		
+4	X			X
+23			X	X
+37		X	X	X

3.3 Analytical Methods

The following analytical methods were used to monitor the product during the course of the study. They are based on Celltech's experience of stability indicating assays for monoclonal antibodies:

3.3.1 Appearance (GMP SOP No: 60516C)

This is a visual test for particulates (precipitation) or discolouration of the sample.

3.3.2 pH (GLP SOP No: EQ0056)

The pH of each sample was determined using a calibrated micro-electrode.

3.3.3 Protein concentration and turbidity (GMP SOP No: 60284C)

Protein content of each sample was determined from the absorbance at 280nm using an extinction coefficient (EO.1%) of 1.4. The volume of test sample available for the assay was less than that required according to the SOP and as a result higher dilutions of samples had to be performed.

The absorbance at 340nm of the diluted sample was recorded as a measure of turbidity to assess particulate levels.

3.3.4 GP-HPLC (GMP SOP No: 60334C)

GP-HPLC was performed on a standardised dedicated Zorbax GF-250 column. 250µg of antibody was analysed. Results were expressed as a percent monomer and percent aggregate in each sample.

3.3.5 Isoelectric focusing (GMP SOP No: 60512C)

Isoelectric focusing (IEF) was performed using precast agarose gels over a range of pH3 to pH10. The method will detect degradation processes (e.g. oxidation, deglycosylation and deamidation) that result in a change in the net charge of the protein. Gels were stained using Coomassie Blue. 10µg of antibody was tested at each timepoint.

Densitometer scans were recorded and assessed for changes in the product profile. A visual interpretation of the gel was also recorded as required by the QC specification.

3.3.6 SDS-PAGE (GMP SOP No: 60359C)

SDS-PAGE was performed for both reduced and non-reduced samples (5-15% gels, 250V/40mA limiting per gel, until the dye front reaches 1.0cm from the bottom of the gel). 25µg of reduced samples and 10µg of non reduced samples were assayed at each test point indicated and gels were stained using Coomassie Blue. Reduced and non-reduced samples were prepared separately and run on separate gels to prevent contamination of non-reduced samples with 2-mercaptoethanol. The 25µg loads were assayed for reduced samples to increase the sensitivity of the assay for stability indicating purposes (QC release assays use 10µg load).

Purity estimates were made from densitometer scans.

Gels of reduced samples were also silver stained at the final timepoint in the study to detect the presence of minor (<0.1%, w/w) degradation products using this more sensitive staining procedure. Duplicate gels were electroblotted and probed with anti-IgG probes to determine the region of the antibody (e.g. heavy or light chain) from which the detected degradation bands were derived.

Samples were sent to Athena for activity testing at T=0 and T=8 under the shipping conditions defined by Athena (2°C-8°C).

The SDS-PAGE and activity tests were planned for Time 0 and 8 weeks only. Samples from other timepoints were stored frozen at -70°C and would be tested by these methods should the data from the 8 weeks timepoint indicate that this would provide additional information. This proved not to be necessary.

4.0 RESULTS AND DISCUSSION

4.1 Appearance and Turbidity Measurements

All samples in the study appeared as clear colourless solutions. There were no indications of precipitation or increase in particulate levels.

A340nm measurements were performed at T=2 weeks but due to the limited volume of sample available a 50-100 fold dilution had to be performed to carry out the assay. The need to dilute the samples to this extent diminished the usefulness of the measurements and therefore visual analysis was used at subsequent timepoints as the method for assessing particulate levels.

4.2 pH

The results of pH measurements of samples in the study are presented in Table 1. There was no indication of changes in pH in any of the samples. Therefore the buffering agents of each formulation were stable under the conditions of the study.

4.3 Protein Concentration

The results of protein concentration determinations for samples in the study are presented in Table 2. There were small differences between the values recorded at T=0 and the corresponding +4°C samples at T=8, however samples were diluted 50-100 fold due to the amount of sample available and this was considered to be the cause of this variation in results.

Certain samples at T=8, stored at elevated temperatures, gave large (>20%) differences compared to the corresponding values at T=0. In some cases these indicated an increase in concentration and in others a decrease. There was no particular trend to the results and therefore they were considered to be a function of assay variation. The total peak areas recorded for samples by GP-HPLC analysis were reviewed as an alternative measure of protein concentration. The total peak areas for a particular set of formulation samples were much less variable than the values determined from the absorbance at 280nm and did not follow the same trend as the estimated protein concentrations. The variation between total peak areas of the +4°C samples and the corresponding +23°C or +37°C samples, at the T=8 timepoint, was ≤ 15%. The results suggested no significant change in protein concentration during the study.

4.4 GP-HPLC

The chromatograms of all the test samples at T = 0 contained the same peaks as the reference standard consisting of one major peak (8.1-8.2 min, equivalent to approximately 150kDa), corresponding to the intact monomer, and two peaks eluting at earlier retention times (6.9-7.0 min and 7.4-7.5 min) corresponding to

aggregates. The aggregate levels recorded for all formulations at T=0 were slightly less (e.g. 2.54% in phosphate pH7) than the reference material (3.05%). Buffer exchange into alternative buffers of lower pH and/or ionic strength at slightly lower protein concentration may have led to a decrease in aggregation. The possible effect of protein concentration on aggregate levels should be further studied if a concentration of >5mg/ml is considered for the protein concentration in the final dosage form of the product.

A summary of the GP-HPLC results for the study is shown in Table 3. For the acetate and histidine formulations there was a decrease in aggregate levels following storage at +37°C (and +23°C to a lesser extent) over the course of the study that was less pronounced in the higher pH formulations (e.g. histidine pH6.5). There was a small increase ($\leq 0.8\%$) in the aggregate levels in the phosphate formulation samples stored at +37°C and there appeared to be a relationship between increase in aggregate levels and increase in pH. This might be expected as the pI of the antibody determined by IEF was recorded as pH7.3-8.2 (Figure 2).

Table 4 summarizes the results of the GP-HPLC assay at T=0 and on samples stored at +37°C for 8 weeks. The largest changes in monomer levels were recorded for the acetate formulations at pH4.0 and pH5.0 containing NaCl. As indicated from the results in Table 3, Table 4 and Figure 1 this was due to fragmentation/dissociation of the antibody at elevated temperatures and not an increase in the concentration of aggregates. Figure 1 shows two distinct additional peaks eluting at approximately 8.7 min and 9.6 min (equivalent to approximately 80kDa and 25kDa, respectively) in the acetate pH4 and acetate pH5 + NaCl formulations.

The results for the -20°C samples at T=2 (Table 3) indicated that freeze-thaw of the antibody can lead to an increase in aggregate levels in all formulations. The increases were relatively small (<1%) except in the histidine formulations pH5.5 (2%) and pH6.0 (1.4%) and particularly in the acetate formulation pH4.0 (>50%).

Overall the product appeared relatively stable by GP-HPLC analysis with only minor changes in product composition in most formulations (Table 5). According to the GP-HPLC results the optimum formulations were acetate pH5.5 and histidine pH5.5/pH6.0/pH6.5.

4.5 Isoelectric Focusing

The IEF gel profiles at T=0 for each of the formulations are shown in Figure 2. The banding pattern for all the samples comprised five distinct bands in the range pH7.35 to pH8.15 and were comparable to the reference material. Therefore the buffer exchange process had not affected the IEF profiles. For all the formulations stored at +4°C for 8 weeks no changes were detected by IEF

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(Figures 3(a), 4(a) and 5(a)). The changes observed following storage at elevated temperatures were related to the formulation and are described below.

More basic bands appeared in some of the low pH formulations (acetate pH4.0/pH5.0 and histidine pH5.5) in samples stored at +37°C during the first 4 weeks of the study and in the acetate pH5.5 at 8 weeks. These bands were most pronounced in the acetate pH4.0, acetate pH5.0 + NaCl and acetate pH5.0 + NaCl + Tween formulations (Figure 3(b)). Small amounts of the bands could also be seen in the +23°C samples of these three formulations at 8 weeks. The results inferred an increase in these additional basic bands with decrease in pH and in the presence of NaCl/Tween. The changes were concomitant with fragmentation of the antibody observed by GP-HPLC (4.4) and SDS PAGE (4.6 and 4.7).

Additional acidic bands were observed in the phosphate pH7.5 sample stored at +37°C for 8 weeks (Figure 5(b)). In the other formulations no additional bands were recorded, however there was a discernible decrease in the intensity of the most basic band of the original T=0 banding profile and increase in intensity of the acidic bands with increase in pH of the formulation in samples stored at +37°C for 8 weeks (Figure 5(b)). These changes are those normally associated with deamidation. Rates of deamidation would be expected to increase with pH over the range evaluated in the study (pH4 to pH7.5).

The relative concentration of each of the charge isoforms of the isoelectric focusing profiles was measured by densitometry scans of gels from samples stored at +37°C for 8 weeks. The relative difference in the profiles for these samples compared to the reference was then calculated to estimate the change in the isoform composition during the study (Table 7). The least change had occurred in the acetate pH5.5, histidine pH5.5, histidine pH6.0 and histidine pH6.0 + NaCl samples. The estimated changes relative to the reference material corresponded to the changes seen in the IEF banding profiles on visual inspection. The changes in the acetate formulations were due to the generation of more basic bands and in the histidine and phosphate formulations due to a general acidic shift in the profiles.

The extent of the changes in the antibody detected by IEF for the acetate formulations (+ histidine pH5.5) was similar to that recorded by GP-HPLC (Table 3) and SDS-PAGE (Tables 8 and 9). These changes were therefore most probably due to proteolysis and/or fragmentation of the antibody. Minor changes were recorded by GP-HPLC and SDS-PAGE for the phosphate formulations and therefore the changes recorded in Table 7 were concluded to be a result of deamidation.

From Celltech's experience of the stability of this type of monoclonal antibody a percentage change of <10% by IEF for samples stored at +37°C for 8 weeks infers suitable long-term stability (> 12 months) at 2°C-8°C. In this study the acetate pH5 (without salt), acetate pH5.5, histidine pH5.5, histidine pH6 (with and without salt), histidine pH6.5 and phosphate pH6 formulations fall into this

category. From the results of the GP-HPLC (section 4.4) and IEF analysis the lead formulations at this stage were identified as acetate pH5.5, histidine pH5.5, histidine pH6 and histidine pH6.5.

4.6 SDS-PAGE of Non-Reduced Samples

At Time=0 the SDS-PAGE profiles of non-reduced samples were comparable to the reference material (Figure 6(a)-(c)). Therefore the SDS-PAGE profiles of non-reduced samples were not affected by the buffer exchange process. The banding profile consisted of one intensely stained band of approximately 150kDa (intact antibody) and a number of less intense bands of lower molecular weight (80kDa, 120kDa, 140kDa and a doublet at approximately 110kDa - representing possibly dissociated/fragmented antibody). In addition a faintly stained band was present at the top of the gel (representing possibly aggregated antibody) which may correspond to the aggregation observed by GP-HPLC (4.4).

At T=8 the samples stored at +4°C and +23°C gave profiles similar to the T=0 samples. There were no marked changes to the antibody in any of the formulations stored at these temperatures (Figure 7(a) and 7(b)).

Analysis of the samples stored at +37°C for 8 weeks (Figure 7(c)) gave similar profiles for the reference material and the majority of the test formulations. However marked changes were observed for the acetate pH4.0, acetate pH5.0 + NaCl, acetate pH5.0 + NaCl + Tween and to a lesser extent acetate pH5.0 formulation. Additional bands were observed in these samples at molecular weights of approximately 100kDa, 60kDa and 20kDa. The change in profile relative to the reference material was determined by laser densitometry for the acetate formulations stored at +37°C for 8 weeks (Table 8). The results confirmed minimal change in the acetate pH5.5 sample, in agreement with the visual interpretation of the gels, a small change in the acetate pH5.0 (~5%) but marked changes in the other acetate formulations (>15%). The changes were a result of the appearance of the additional bands (particularly the 100kDa band) but there was also a noticeable decrease in the 80kDa band (e.g. from 7.5% in the reference sample to 2-4%). The results suggested dissociation or fragmentation of the antibodies in these formulations.

The changes observed in the antibody for the formulations defined above were similar in magnitude to the changes determined by IEF and GP-HPLC analysis of the same formulations. Therefore the acetate pH5.5 formulation was selected as the lead formulation for the next stage of development. The other formulations were also evaluated and the results of the analysis indicated that the acetate pH5.5 formulation was the most stable of the antibody and pointed to acetate pH5.5, histidine pH5.5, histidine pH6 and histidine pH6.5 as the lead formulations.

4.7 SDS-PAGE of Reduced Samples

4.7.1 Coomassie blue stained gels

At T=0 the Coomassie Blue stained SDS-PAGE gel profiles of the reduced samples of each of the formulations were comparable to that of

the reference material (Figure 8). The profiles consisted primarily of the H chain (50kDa) and L chain (27kDa) bands and three to four other very faintly staining bands with approximate mol. wts of 100kDa, 47kDa (doublet), 40kDa and 32kDa respectively. There was an indication from the appearance of the gels that the non H and L chain bands were more intense in the acetate formulations, particularly at pH4.0 and the purity estimates from laser densitometry (Table 9) supported this observation. From Celtech's experience with these type of antibodies low pH formulations can cause the generation of degradation products during the sample preparation process for SDS-PAGE. Therefore the increase in intensity of these bands in the acetate formulations was thought to be due to the sample preparation procedure for SDS-PAGE analysis and not to changes to the product caused by the buffer exchange process.

At T=8 the profiles for the samples stored at +4°C and +23°C (data not shown) resembled the profiles of the reference material and those seen at T=0, but the antibody in formulations at pH4.0-6.0, by visual inspection, contained increased amounts of the 47kDa doublet and 32kDa bands. The intensity of these bands appeared to increase with decrease in pH of the formulation buffer. It is known that these bands can be generated during the sample preparation procedure for SDS PAGE gels with this type of antibody in low pH ($\leq$ pH6) buffers and therefore this could have been an artefact of the method. The gels also showed evidence of a band running just below the L-chain (approx. 23kDa) which for other similar antibodies has been shown to be the partner of the 32kDa band, resulting from cleavage of the H-chain during the sample preparation procedure.

There were also a number of faintly stained bands running between the H-chain and the dye front in the acetate formulations stored at +23°C at 8 weeks.

Additional bands were more distinct in the acetate samples (pH4.0, pH5.0 + NaCl, pH5 + NaCl + Tween) stored at +37°C for 8 weeks (Figure 9). A band at approximately 30 kDa appeared between the 32kDa band and the L-chain. This was most intensely stained in the acetate pH4.0, the acetate pH5.0 + NaCl and the acetate pH5 + NaCl + Tween samples. A band was present at approximately 18kDa which was most intensely stained in the acetate pH5.0 + NaCl but also present in the acetate pH5.0 and to a lesser extent in the acetate pH4.0 formulations. The acetate pH4.0 also contained a range of low molecular weight bands between the 18kDa band and the dye front.

The histidine and phosphate formulations at +37°C for 8 weeks showed minor changes when compared to the reference sample. A range of faintly staining bands appeared between the 32kDa band and the H-chain and towards the dye front (particularly in phosphate pH7.0-7.5 formulations).

Laser densitometry was performed on the T=0 gels and the +37°C / T=8 gels to assess changes in purity of the relevant samples analysed by SDS-

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PAGE of reduced sample (Table 9). The reference material showed a 1.2% decrease but this was within the between assay variation of the method ($\pm 2\%$) and therefore was not significant. The majority of samples gave a relatively small decrease in purity ($\leq 5\%$) following storage for 8 weeks at $+37^{\circ}\text{C}$. The acetate pH5.0 gave a slightly greater decrease (7.5%) but the acetate pH4.0, acetate pH5.0 + NaCl and acetate pH5.0 + NaCl + Tween formulations showed marked changes in purity of approximately 15%. The results for the latter formulation are relatively high compared to other studies performed on this type of antibody at Celltech and indicate the antibody to be susceptible to proteolysis, either chemically at the lower pH values or perhaps as a result of the presence of a contaminating acid protease. The material was generated from a single column purification step and therefore it can be expected that material from the intended manufacturing purification process, which contains two additional column steps, would produce material of greater purity. Further stability studies on antibody purified by this process will be required.

4.7.2 Silver stained gels, Western blots and anti IgG probes

Each of the test samples at 8 weeks was analysed by SDS-PAGE (reduced samples) and the gels developed by silver staining (Figures 10 and 11). The most intensely staining bands were concomitant with those observed on the Coomassie Blue stained gels (Figures 8 and 9). However the more sensitive staining technique detected additional bands.

The gel of the samples stored at $+4^{\circ}\text{C}$ samples for 8 weeks (Figure 10) indicated that the band running at approximately 100kDa was most prevalent in the acetate pH5.0, acetate pH5 + NaCl, acetate pH5 + NaCl + Tween and phosphate pH6.0 + Tween samples and additional bands at approximately 80kDa, 38kDa and 34kDa were present in all formulations at pH4.0-pH5.5. The 34kDa band was most intense in the acetate pH4.0 sample. A range of low molecular weight ($\leq 20\text{kDa}$) bands were detected in all samples. The most intensely stained of these bands was a band at approximately 16kDa in the acetate pH4.0, acetate pH5.0 + NaCl and acetate pH5.0 + NaCl + Tween samples and the band running adjacent to the dye front in the acetate pH4.0 sample.

The gel of the samples stored at $+23^{\circ}\text{C}$ for 8 weeks (not shown) gave similar profiles to the equivalent Coomassie Blue stained gels. In formulations containing sodium chloride a number of additional bands were seen compared to the corresponding formulations without salt.

The silver stained gels of the samples stored at $+37^{\circ}\text{C}$ for 8 weeks ($T=8 / +37^{\circ}\text{C}$) gave profiles very similar to those of the corresponding Coomassie Blue stained gels (Figure 9(a)-(c)). The bands at 30kDa and 18kDa were the most predominant additional bands to the H and L chain and were present at the highest concentration in the acetate formulations at pH4.0 to pH5.0.

Four replicate gels of the T=8 / +37°C reduced samples were produced and the samples western blotted. Each membrane was separately probed with either anti human IgG (H and L chain), anti human Fab, anti human Fc or anti human L chain probes. The complement of different probes were used to define detected bands as being composed of H and/or L chain fragments and to define the H chain related bands as being composed of sections of the Fc and/or Fd regions. The developed blots are shown in Figure 12(a)-(d).

The results of the probed western blots suggested that the majority of the observed degradation products are derived from the H chain. The 30kDa and 18kDa bands in the acetate formulation were indicated to be the result of proteolysis of the H chain generating fragments composed mainly of Fc (18kDa) and Fd (32kDa) regions. The band observed at 80kDa appeared to be a conjugate of H and L chain whilst the 100kDa band seen on the silver stained gel (Figure 11) appeared as two separate bands on the blots at approximately 100kDa and 105kDa and these bands appeared to be H chain only conjugates. Details of the results for each of the blots are given in the following paragraphs.

The anti human IgG probe (Figure 12a) detected the H and L chain bands and bands at 45kDa, 40kDa, 31kDa and 17kDa (equivalent to bands of 48kDa, 45kDa, 30kDa and 18kDa on the gels). There were some differences in the assigned molecular weight values between the blots and the gels which was a function of the precision of the method. There were bands of approximately 80kDa, 100kDa and 105kDa detected in samples by the probes that were not detected by the silver stained gel. The 80kDa band was more intense in the phosphate formulations and the 100kDa in the pH5.0 to pH6.0 samples. The 105kDa band was present in the phosphate formulations and the acetate pH4.0 / pH5.0 + NaCl + Tween.

The anti Fab probe (Figure 12b) gave profiles very similar to the anti human IgG except the 18kDa band was not detected whilst a band at approximately 16kDa was visible in all samples and more predominant in the phosphate formulations. The 30kDa band was particularly prominent using these probes.

The anti Fc probe (Figure 12c) gave a strong reaction with the H chain as expected but also a slight cross reaction with the L chain. The 30kDa band was not detected with this probe whilst the 18kDa was visualised. A range of bands were visible below the L chain but these appeared to be distinct from those seen by the anti Fab probe. The 80kDa, 100kDa and 105kDa bands were detected with both the anti Fc and anti Fab probes.

The anti L chain probe (Figure 12d) gave a relatively strong reaction to the L chain but did not detect any other bands except for an indication of a band at approximately 80kDa in the phosphate and possibly the histidine formulations. This, together with the results using the other probes, suggested that the 80kDa band was a conjugate of the H and L chains.

The results of the SDS PAGE Coomassie blue and silver stained gels and western blotting analysis of reduced samples were in agreement with those from IEF, GP-HPLC and SDS PAGE of non-reduced samples in terms of the formulations that conferred most stability on the AN100226 antibody. The differences in the silver stained SDS PAGE gel profiles for the reduced samples of the lead formulations (acetate pH5.5, histidine pH5.5, histidine pH6 and histidine pH6.5) identified from the other assay results (section 4.6) were minor. However they did show less changes for the antibody formulated in histidine pH6 and therefore according to SDS PAGE analysis of reduced samples this would be the lead formulation.

4.8 Activity Assay

The activity assays were performed by Athena Neurosciences, Inc. on samples taken at T=0 and 8 weeks from the stability study. The results received from Athena are shown in Appendix 2.

The results compared activity values obtained at T=0 and those at T=8 for the +4°C and +37°C samples. There was an indication of increase in activity in the samples stored at +37°C for 8 weeks, particularly in the acetate pH4.0 and phosphate pH6.0 formulations where an increase in activity of approximately four and two fold respectively were observed. There was no obvious correlation between changes in activity and results for the other assays performed at Celltech as part of the study.

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5.0 CONCLUSIONS

Although instability at elevated temperatures was observed for the antibody in the preformulation study the overall impression was that the antibody was relatively stable in most of the formulations evaluated.

Across the evaluated pH range of pH4.0 to pH7.5, there was an indication of increased fragmentation and dissociation at low pH (pH4.0 to 5.0) and deamidation and aggregation at the higher pH values (pH6.5 to 7.5). There was a suggestion that the inclusion of 150mM sodium chloride or 0.02% (v/v) Tween 80 in the formulation had the effect of accelerating degradation processes. The stability of the antibody was similar, where evaluated, at the same pH value for different buffering agents. It is recommended that the following formulations be selected for more extensive evaluation: acetate pH5.5, histidine pH5.5, histidine pH6.0 and phosphate pH6.0. Should isotonicity be a requirement for the formulation then phosphate pH6.0 and histidine pH6.0 formulations are recommended, as the results demonstrated that addition of salt to the acetate pH5.0 formulation had a detrimental effect on stability.

The study has identified appropriate formulations for the first bulk purified lots of the antibody AN100226 and has given some pointers for further formulation studies.

Table 1 **pH Measurements of Formulations**

FORMULATION	T=0	T= 2 WEEKS 37°C	T= 8 WEEKS 37°C	T= 8 WEEKS 23°C	T=8 WEEKS 4°C
Acetate pH 4.0	4.0	4.1	4.1	4.2	4.1
Acetate pH 5.0	5.0	5.0	5.1	5.0	5.0
Acetate + NaCl pH 5.0	5.0	5.0	5.0	5.0	5.0
Acetate + NaCl + Tween pH 5.0	4.9	5.0	5.0	5.0	5.0
Acetate pH 5.5	5.5	5.5	5.5	5.5	5.5
Histidine pH 5.5	5.5	5.6	5.6	5.6	5.6
Histidine pH 6.0	6.0	6.1	6.1	6.1	6.1
Histidine + NaCl pH 6.0	6.0	6.0	6.1	6.0	6.0
Histidine pH 6.5	6.5	6.5	6.5	6.6	6.5
Phosphate pH 6.0	6.1	6.1	6.1	6.2	6.1
Phosphate + Tween pH 6.0	6.1	6.0	6.0	6.0	6.1
Phosphate pH 6.5	6.5	6.5	6.5	6.5	6.6
Phosphate pH 7.0	6.9	6.9	6.9	6.9	6.9
Phosphate + NaCl pH 7.0	6.9	6.9	6.9	6.9	6.9
Phosphate pH 7.5	7.5	7.5	7.4	7.4	7.4

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Table 2 Protein Concentrations of Formulations

FORMULATION	T=0	T= 2 WEEKS 37°C	T= 8 WEEKS 37°C	T= 8 WEEKS 23°C	T=8 WEEKS 4°C
	mg/ml	mg/ml	mg/ml	mg/ml	mg/ml
Acetate pH 4.0	4.8	5.0	4.3	5.9	4.9
Acetate pH 5.0	3.6	3.6	3.7	5.0	3.2
Acetate + NaCl pH 5.0	3.9	3.7	4.3	4.8	3.5
Acetate + NaCl + Tween pH 5.0	4.1	4.5	5.3	3.4	4.5
Acetate pH 5.5	3.6	3.6	3.7	3.9	3.2
Histidine pH 5.5	4.3	4.1	5.4	4.7	4.5
Histidine pH 6.0	3.7	3.9	4.7	4.0	3.7
Histidine + NaCl pH 6.0	3.9	3.8	3.9	3.8	4.1
Histidine pH 6.5	3.6	3.5	3.8	4.0	3.7
Phosphate pH 6.0	3.7	3.8	3.8	3.8	3.9
Phosphate + Tween pH 6.0	4.6	4.9	4.8	6.6	4.3
Phosphate pH 6.5	4.5	4.3	5.4	4.9	4.1
Phosphate pH 7.0	5.0	4.7	5.3	5.0	4.6
Phosphate + NaCl pH 7.0	4.8	5.3	5.4	5.0	4.9
Phosphate pH 7.5	4.9	4.4	5.1	4.9	4.6

Protein concentration calculated using $\epsilon_{280\text{nm}} (0.1\%) = 1.4$

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Table 3 Summary of GP-HPLC results.

Formulation		GP HPLC Result							
		T=0wks	T=2wks		T=4wks		T=8wks		
		+4°C	+37°C	-20°C	+23°C	+37°C	+4°C	+23°C	+37°C
acetate pH 4	% monomer	98.56	99.09	35.18	98.93	98.93	98.81	98.91	75.63
	% aggregate	1.44	0.00	64.82	1.07	0.00	1.19	0.88	0.05
acetate pH5	% monomer	97.89	97.96	97.78	98.03	97.73	98.02	97.84	97.52
	% aggregate	2.11	2.04	2.22	1.97	1.70	1.98	2.16	1.36
acetate pH5 + NaCl	% monomer	97.50	NA	97.14	97.38	96.30	97.50	97.46	74.50
	% aggregate	2.50		2.86	2.62	1.97	2.50	2.39	1.51
acetate pH5 + NaCl & Tween	% monomer	97.31	95.39	97.10	97.61	96.90	97.58	97.59	72.28
	% aggregate	2.69	2.12	2.90	2.39	1.70	2.42	2.14	1.35
acetate pH5.5	% monomer	97.85	97.77	97.62	97.85	97.67	97.96	97.95	98.56
	% aggregate	2.15	2.23	2.38	2.15	2.13	2.04	2.05	1.44
histidine pH5.5	% monomer	97.69	97.74	95.63	97.72	97.80	97.81	97.81	98.19
	% aggregate	2.31	2.26	4.37	2.28	2.11	2.19	2.19	1.47
histidine pH6	% monomer	97.72	97.62	96.18	97.77	97.72	97.97	97.81	97.95
	% aggregate	2.28	2.38	3.82	2.23	2.28	2.09	2.19	2.05
histidine pH6 + NaCl	% monomer	97.22	97.17	96.99	97.28	97.73	97.44	97.47	97.62
	% aggregate	2.78	2.83	3.01	2.72	2.63	2.56	2.53	2.38
histidine pH6.5	% monomer	97.87	97.81	97.70	97.93	97.84	98.09	97.98	97.95
	% aggregate	2.13	2.19	2.30	2.07	2.16	1.91	2.02	2.05

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Table 3 (cont'd) Summary of GP-HPLC results

Formulation		GP HPLC Result							
		T=0wks	T=2wks		T=4wks		T=8wks		
		+4°C	+37°C	-20°C	+23°C	+37°C	+4°C	+23°C	+37°C
phosphate pH6	% monomer	97.94	96.60	97.52	97.72	97.62	97.89	97.78	97.59
	% aggregate	2.26	2.40	2.48	2.28	2.38	2.11	2.22	2.41
phosphate pH6 + Tween	% monomer	97.49	97.24	97.22	97.41	97.33	97.70	NA	97.36
	% aggregate	2.51	2.76	2.78	2.59	2.67	2.30		2.64
phosphate pH6.5	% monomer	97.61	97.35	97.25	97.54	97.35	97.74	NA	97.30
	% aggregate	2.39	2.65	2.75	2.46	2.65	2.26		2.70
phosphate pH7	% monomer	97.46	96.98	96.99	97.37	97.07	97.60	NA	97.07
	% aggregate	2.54	3.02	3.01	2.63	2.93	2.40		2.93
phosphate pH7 + NaCl	% monomer	97.15	96.68	96.19	97.07	96.85	97.34	97.20	96.94
	% aggregate	2.85	3.32	3.81	2.93	3.15	2.66	2.80	3.06
phosphate pH7.5	% monomer	97.40	96.83	97.03	97.22	NA	97.48	96.33	96.72
	% aggregate	2.60	3.15	2.97	2.78		2.52	3.67	3.28
reference (PBS +4°C only)	% monomer	96.93	NA	NA	96.97	NA	97.10	NA	NA
	% aggregate	3.05			3.03		2.90		

Note:-

NA Not Assayed.

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Table 4 Comparison of GP-HPLC data for formulations at T = 0 with those which had been stored at +37°C for 8 weeks

Formulation	T = 0 weeks		T = 8 weeks +37°C		
	Aggregate level (% total)	Monomer IgG level (% total)	IgG Aggregate (% total)	IgG Monomer (% total)	IgG Fragment (% total)
Acetate pH4	1.44	98.56	0.05	75.63	24.32
Acetate pH5	2.11	97.89	1.36	97.52	1.12
Acetate pH5 + NaCl	2.50	97.50	1.51	74.50	23.99
Acetate pH5 + NaCl + Tween	2.69	97.31	1.35	72.28	26.37
Acetate pH5.5	2.15	97.85	1.44	98.56	ND
Histidine pH5.5	2.31	97.69	1.47	98.19	0.34
Histidine pH6	2.28	97.72	2.05	97.95	ND
Histidine pH6 + NaCl	2.78	97.22	2.38	97.62	ND
Histidine pH6.5	2.13	97.83	2.05	97.95	ND
Phosphate pH6	2.26	97.74	2.41	97.59	ND
Phosphate pH6 + Tween	2.51	97.49	2.64	97.36	ND
Phosphate pH6.5	2.39	97.61	2.70	97.30	ND
Phosphate pH7	2.54	97.46	2.93	97.07	ND
Phosphate pH7 + NaCl	2.85	97.15	3.06	96.94	ND
Phosphate pH7.5	2.60	97.40	3.28	96.72	ND

ND = None Detected

Table 5 Comparison of GP-HPLC data for formulations at T = 0 with those which had been stored at +23°C for 8 weeks

Formulation	T = 0 weeks		T = 8 weeks +23°C		
	Aggregate level (% total)	Monomer IgG level (% total)	IgG Aggregate (% total)	IgG Monomer (% total)	IgG Fragment (% total)
Acetate pH4	1.44	98.56	0.88	98.91	0.21
Acetate pH5	2.11	97.89	2.16	97.84	ND
Acetate pH5 + NaCl	2.50	97.50	2.39	97.46	0.15
Acetate pH5 + NaCl + Tween	2.69	97.31	2.14	97.59	0.27
Acetate pH5.5	2.15	97.85	2.05	97.95	ND
Histidine pH5.5	2.31	97.69	2.19	97.81	ND
Histidine pH6	2.28	97.72	2.19	97.81	ND
Histidine pH6 + NaCl	2.78	97.22	2.53	97.47	ND
Histidine pH6.5	2.13	97.83	2.02	97.98	ND
Phosphate pH6	2.26	97.74	2.22	97.78	ND
Phosphate pH6 + Tween	2.51	97.49	NA	NA	NA
Phosphate pH6.5	2.39	97.61	NA	NA	NA
Phosphate pH7	2.54	97.46	NA	NA	NA
Phosphate pH7 + NaCl	2.85	97.15	2.80	97.20	ND
Phosphate pH7.5	2.60	97.40	3.67	96.33	ND

ND = None Detected

NA = Not Assayed

Table 6 Comparison of GP-HPLC data for formulations at T = 0 with those which had been stored at +4°C for 8 weeks

Formulation	T = 0 weeks		T = 8 weeks +4°C		
	Aggregate level (% total)	Monomer IgG level (% total)	IgG Aggregate (% total)	IgG Monomer (% total)	IgG Fragment (% total)
Acetate pH4	1.44	98.56	1.19	98.81	(*)
Acetate pH5	2.11	97.89	1.98	98.02	ND
Acetate pH5 + NaCl	2.50	97.50	2.50	97.50	ND
Acetate pH5 + NaCl + Tween	2.69	97.31	2.42	97.58	ND
Acetate pH5.5	2.15	97.85	2.04	97.96	ND
Histidine pH5.5	2.31	97.69	2.19	97.81	ND
Histidine pH6	2.28	97.72	2.09	97.91	ND
Histidine pH6 + NaCl	2.78	97.22	2.56	97.44	ND
Histidine pH6.5	2.13	97.83	1.91	98.09	ND
Phosphate pH6	2.26	97.74	2.11	97.89	ND
Phosphate pH6 + Tween	2.51	97.49	2.30	97.70	ND
Phosphate pH6.5	2.39	97.61	2.26	97.74	ND
Phosphate pH7	2.54	97.46	2.40	97.60	ND
Phosphate pH7 + NaCl	2.85	97.15	2.66	97.34	ND
Phosphate pH7.5	2.60	97.40	2.52	97.48	ND
Reference Sample (PBS)	3.05	96.95	2.90	97.10	ND

(*) Fragment peak observed but below detection limit of method.

ND = None Detected.

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Table 7 Quantification of Changes Seen on IEF Analysis of Samples Stored at +37°C for 8 Weeks by Scanning Densitometry

FORMULATION	PERCENT DIFFERENCE TO REFERENCE
Acetate pH 4.0	20.4
Acetate pH 5.0	8.1
Acetate + NaCl pH 5.0	18.8
Acetate + NaCl + Tween 80 pH 5.0	22.1
Acetate pH 5.5	4.4
Histidine pH 5.5	6.0
Histidine pH 6.0	5.1
Histidine + NaCl pH 6.0	4.3
Histidine pH 6.5	8.1
Phosphate pH 6.0	9.7
Phosphate + Tween 80 pH 6.0	11.5
Phosphate pH 6.5	15.7
Phosphate pH 7.0	25.2
Phosphate + NaCl pH 7.0	26.5
Phosphate pH 7.5	52.0

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Table 2 Densitometry Results for Reduced Samples on SDS PAGE Analysis - Comparison of T=0 to +37°C Samples at T=8 weeks

Formulation	T=0	T=8 +37°C Samples	Decrease in % Purity
Acetate pH4.0	97.7	83.2	14.5
Acetate pH5.0	98.1	90.6	7.5
Acetate pH5.0 + NaCl	98.2	84.4	13.8
Acetate pH5.0 + NaCl + Tween	98.2	81.6	16.6
Acetate pH5.5	99.7	97.6	2.1
Histidine pH5.5	97.8	92.4	5.4
Histidine pH6.0	98.3	94.9	3.4
Histidine pH6.0 + NaCl	98.7	95.8	2.9
Histidine pH6.5	98.9	96.1	2.8
Phosphate pH6.0	99.0	96.5	2.5
Phosphate pH6.0 + Tween	98.7	95.7	3.0
Phosphate pH6.5	98.6	95.1	3.5
Phosphate pH7.0	99.6	95.8	3.8
Phosphate pH7.0 + NaCl	99.1	96.5	2.6
Phosphate pH7.5	99.1	97.7	1.4
Reference	100	98.8	1.2

10

112

306
205

Figure 1 Example GP-HPLC Chromatograms of AN100226 Preformulation Samples

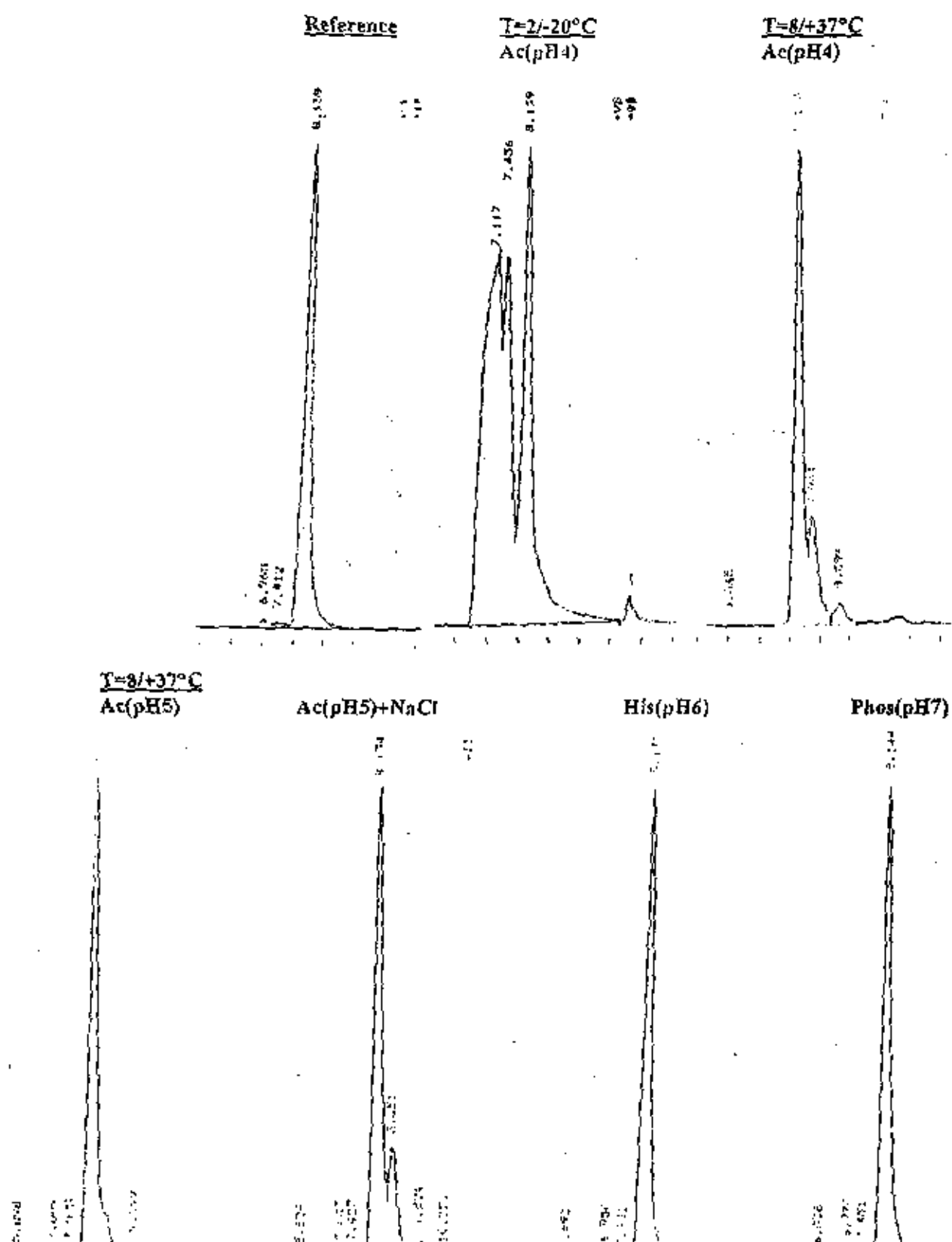
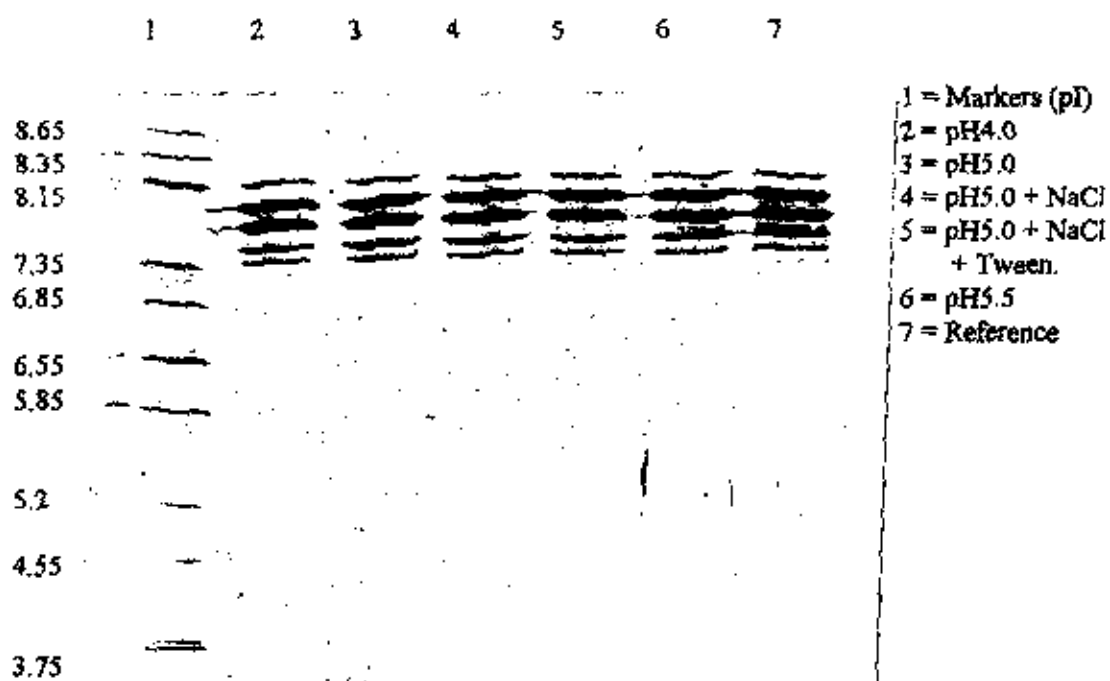


Figure 2 IEF Analysis of Samples at T=0
pH3-10 agarose IEF gels. Coomassie Blue stained.

1(a) Acetate formulations



2(a) Histidine/Phosphate formulations

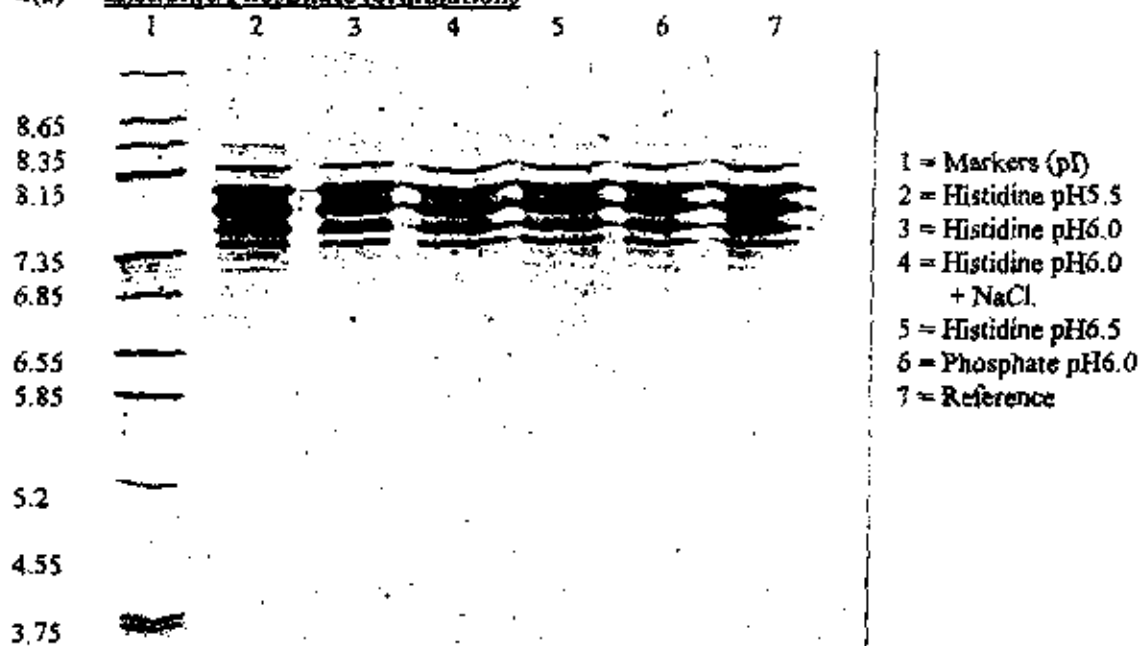
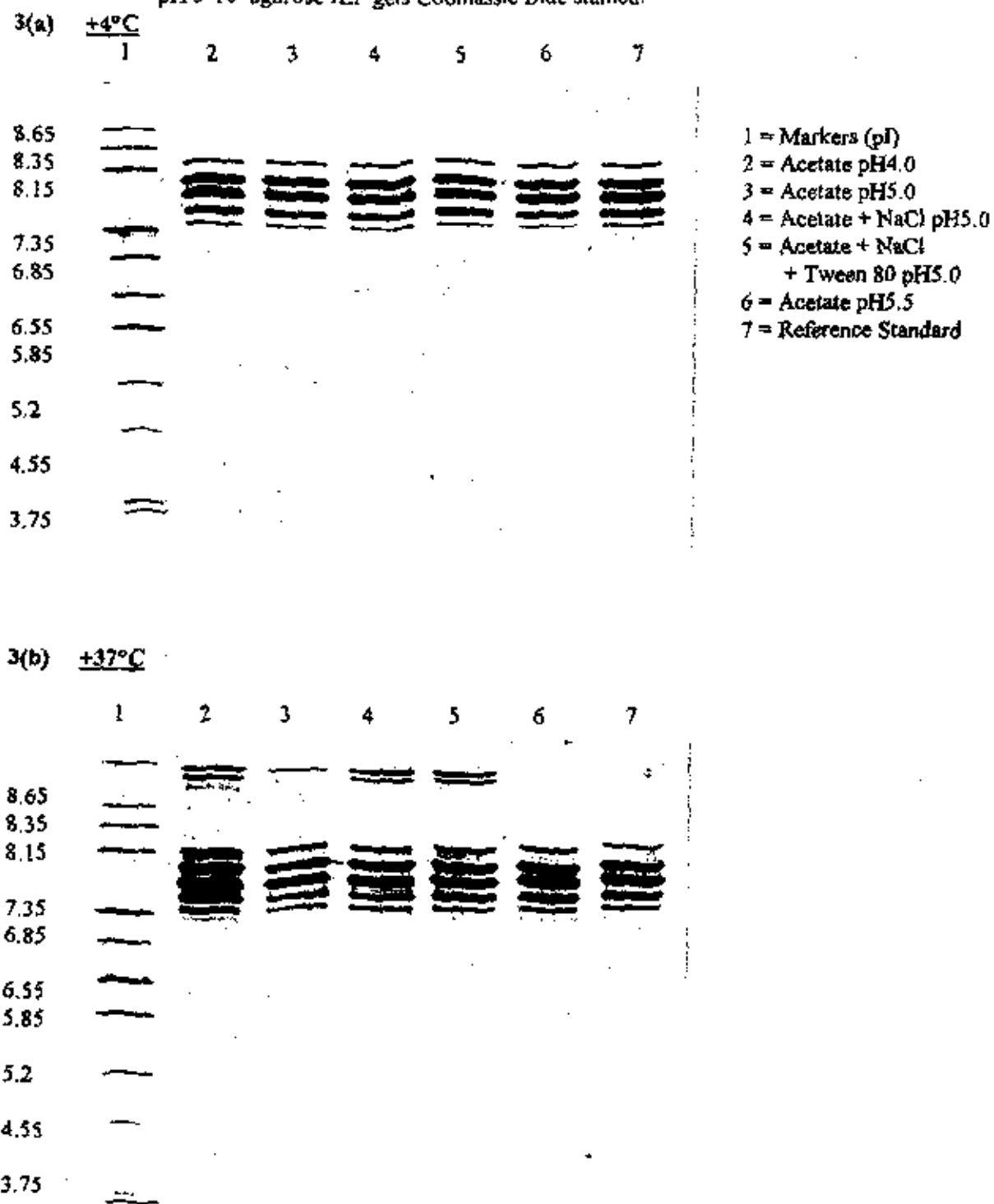


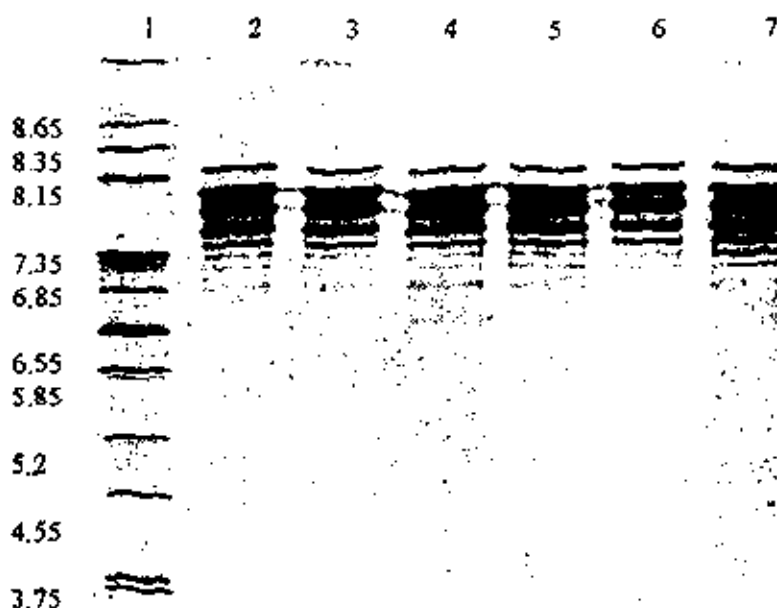
Figure 3 IEF Analysis of Samples Stored at 4°C and 37°C for 8 Weeks
pH 3-10 agarose IEF gels Coomassie Blue stained.



345
307

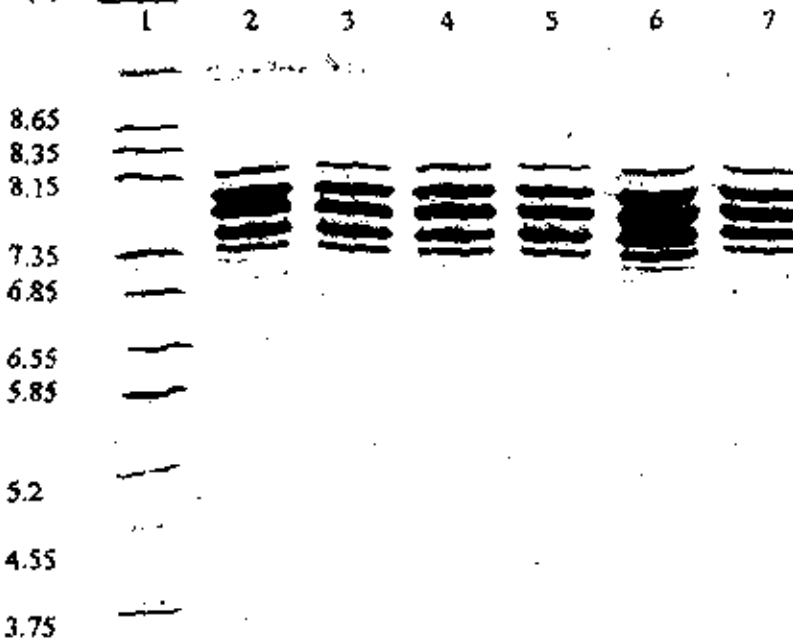
Figure 4 IEF Analysis of Samples Stored at 4°C and 37°C for 8 Weeks
pH 3-10 agarose IEF gels Coomassie Blue stained.

4(a) +4°C



1 = Markers (pI)
2 = Histidine pH5.5
3 = Histidine pH6.0
4 = Histidine + NaCl pH6.0
5 = Histidine pH6.5
6 = Phosphate pH6.0
7 = Reference Standard

4(b) +37°C

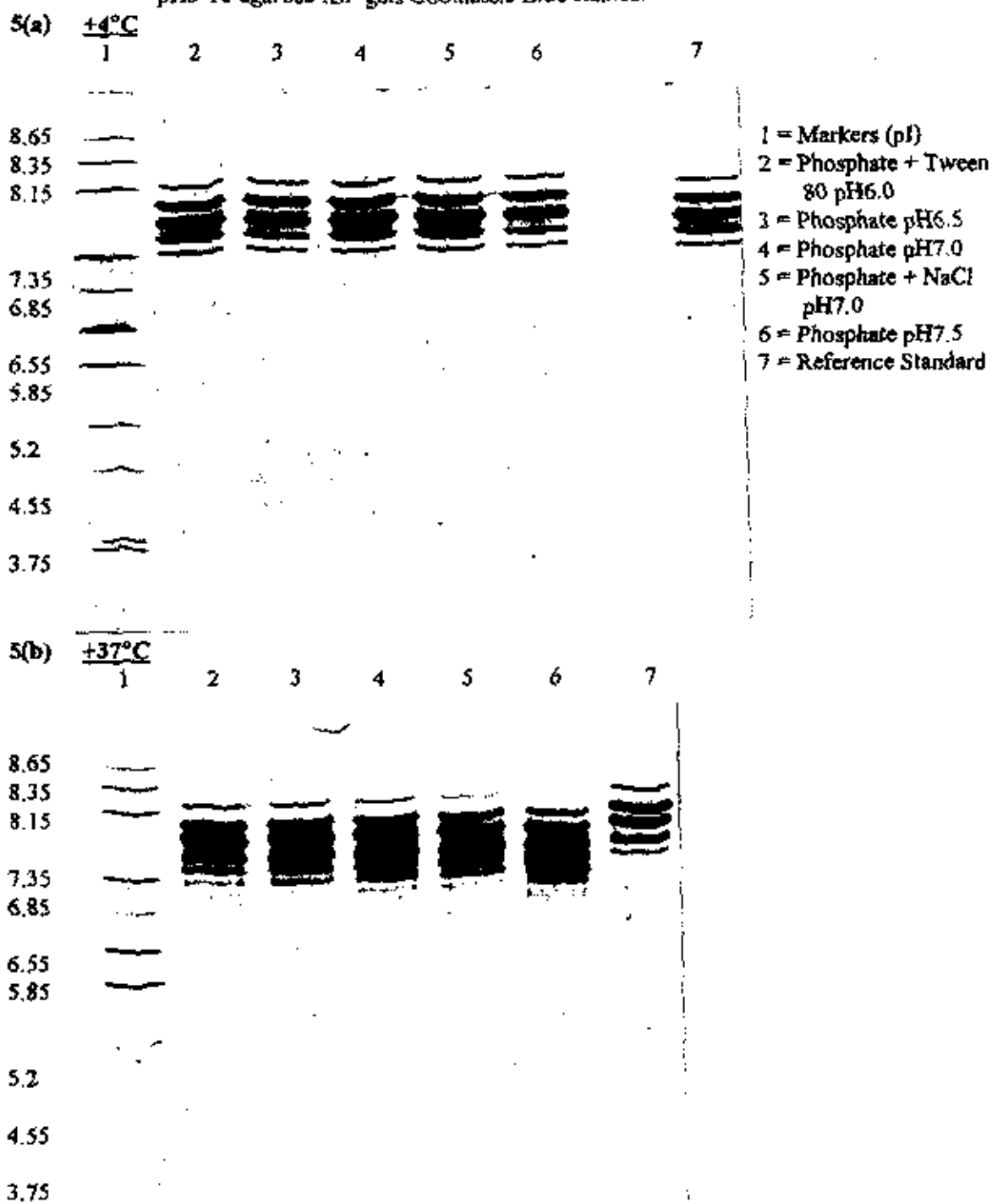


28

77

31+
305

Figure 5 IEF Analysis of Samples Stored at 4°C and 37°C for 8 Weeks
pH3-10 agarose IEF gels Coomassie Blue stained.

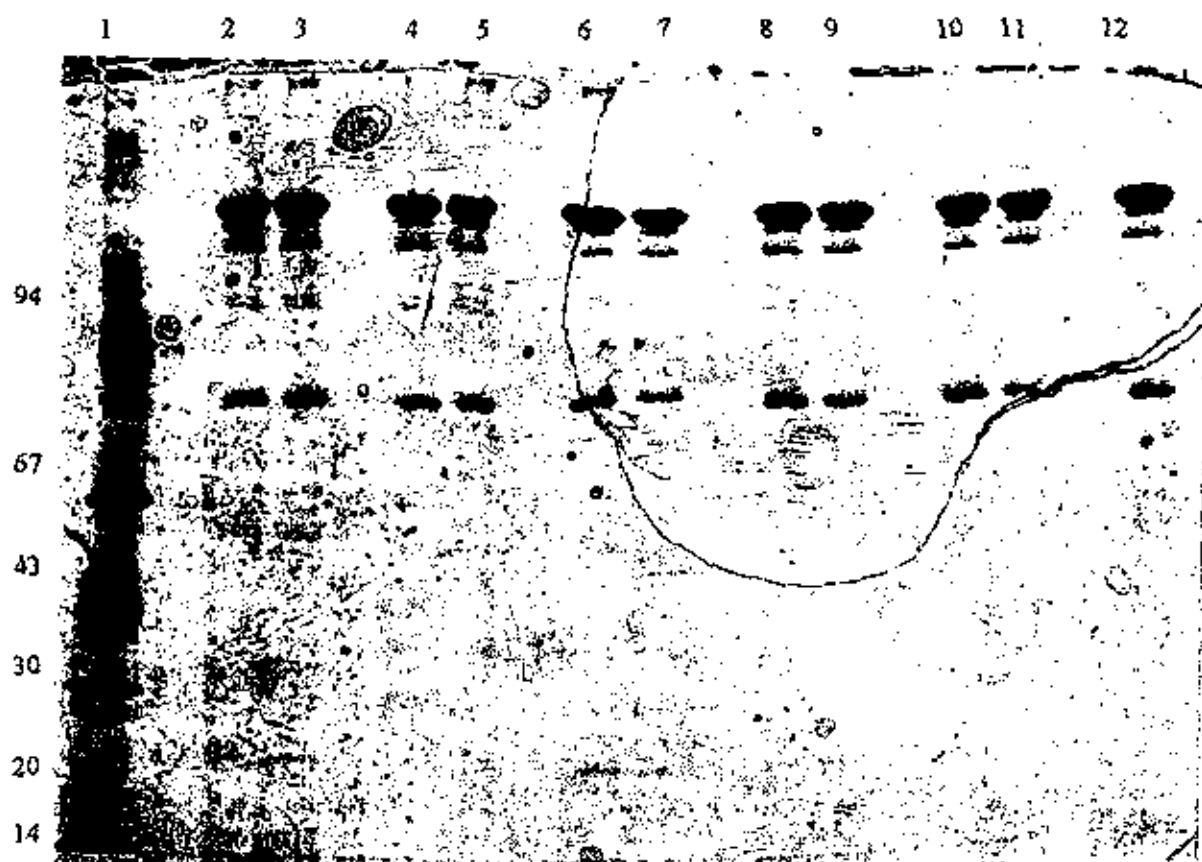


35

148

204
311

6(c)

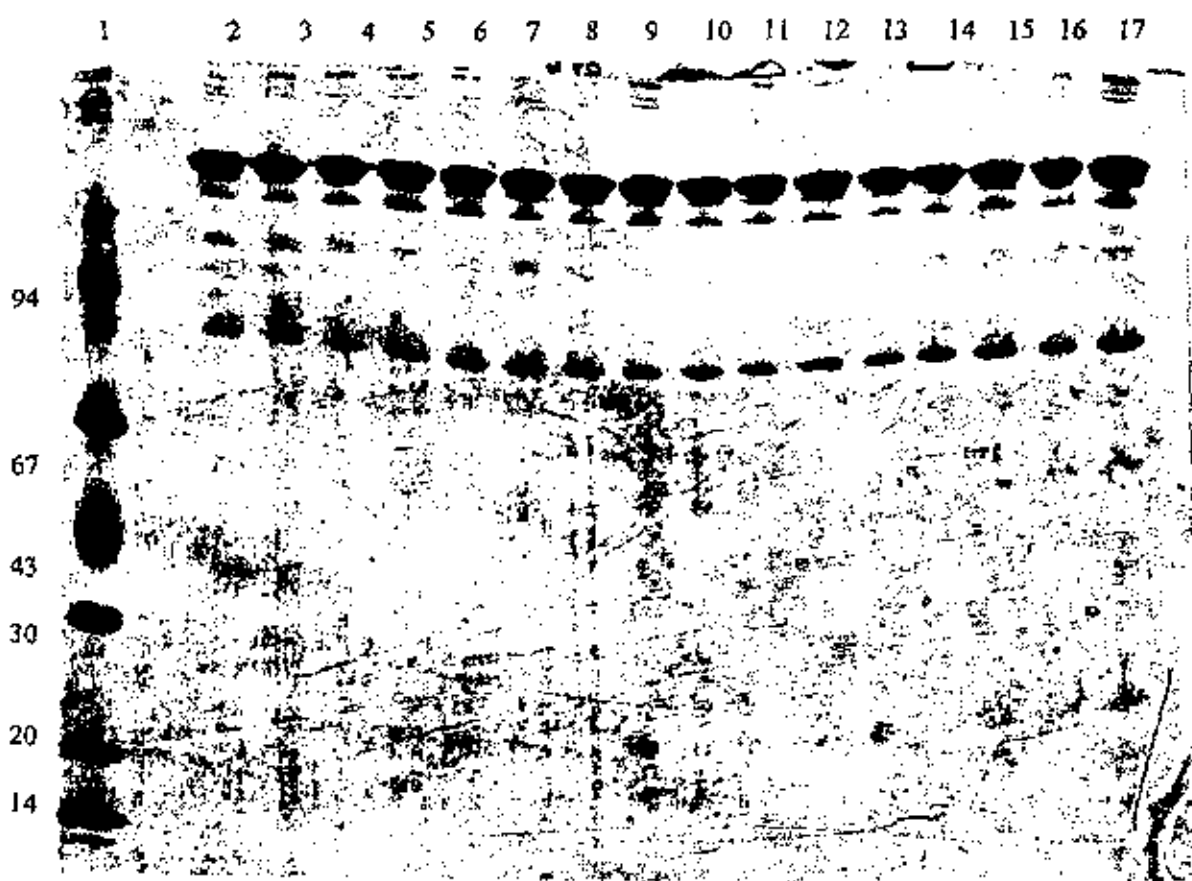


Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Phosphate pH6.0 + Tween
- 4,5 = Phosphate pH6.5
- 6,7 = Phosphate pH7.0
- 8,9 = Phosphate pH7.0 + NaCl
- 10,11 = Phosphate pH7.5
- 12 = Reference

Figure 7 SDS-PAGE Analysis of Non-Reduced Samples at T=8 weeks
 5 - 15% gradient gels. Coomassie Blue stained. 10 µg/lane.

7(a) +4°C Samples

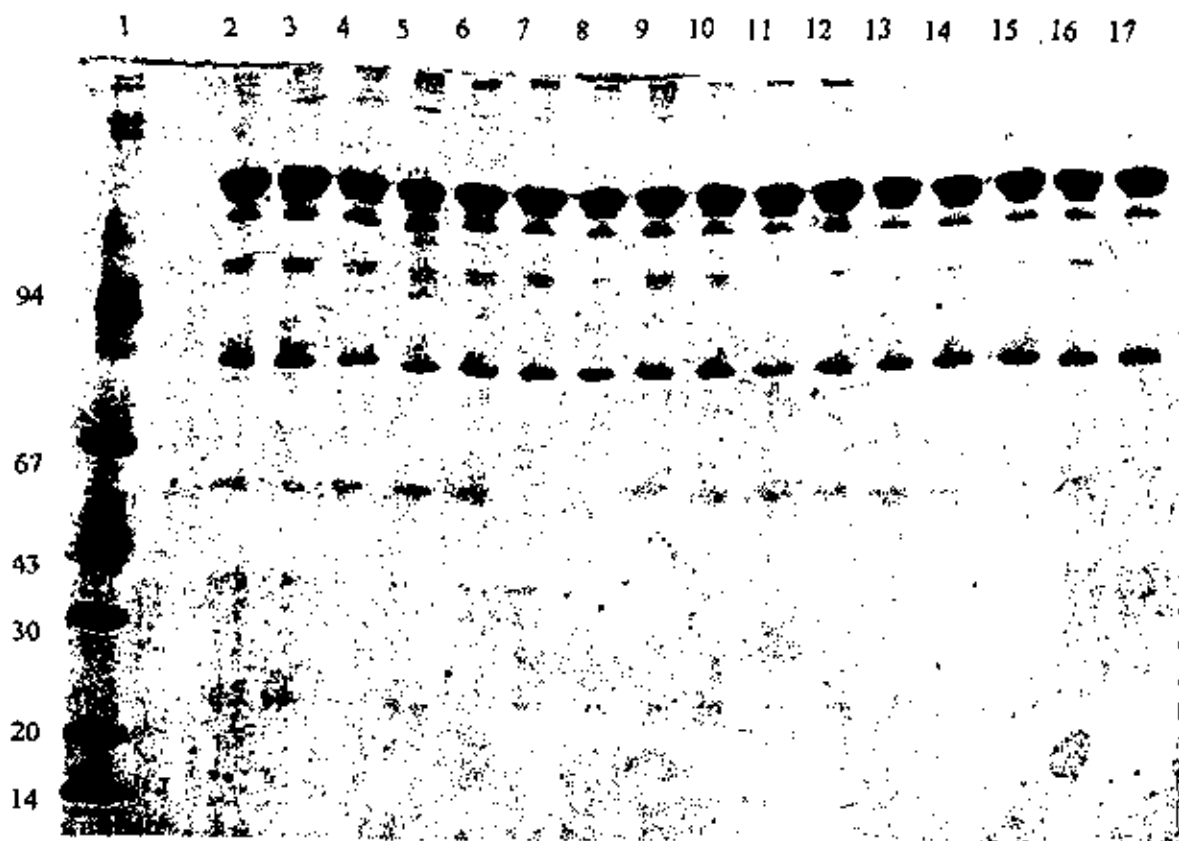


Key:

1 = Molecular weight markers (kDa)
 2 = Acetate pH4.0
 3 = Acetate pH5.0
 4 = Acetate pH5.0 + NaCl
 5 = Acetate pH5.0 + NaCl + Tween
 6 = Acetate pH5.5
 7 = Histidine pH5.5
 8 = Histidine pH6.0
 9 = Histidine pH6.0 + NaCl

10 = Histidine pH6.5
 11 = Phosphate pH6.0
 12 = Phosphate pH6.0 + Tween
 13 = Phosphate pH6.5
 14 = Phosphate pH7.0
 15 = Phosphate pH7.0 + NaCl
 16 = Phosphate pH7.5
 17 = Reference

7(b) +23°C Samples

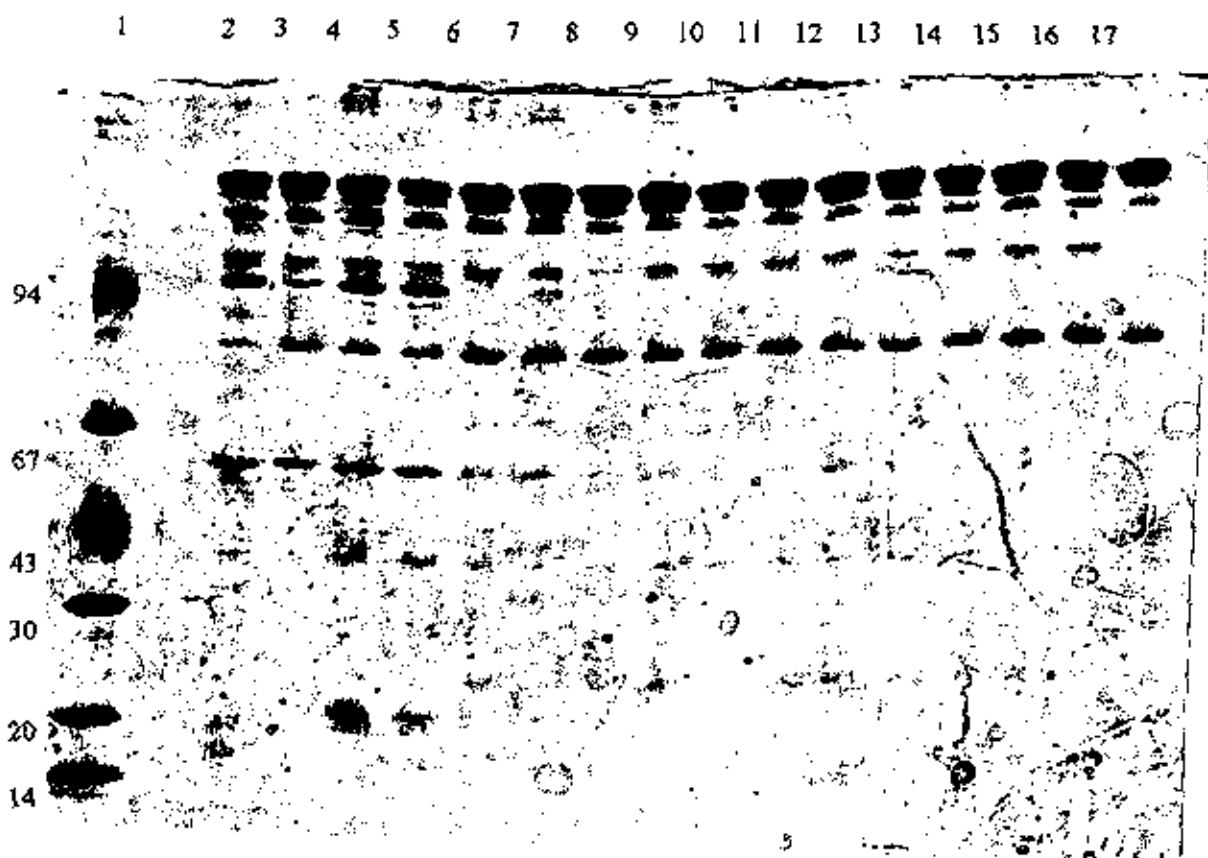


Key:

- 1 = Molecular weight markers (kDa)
- 2 = Acetate pH4.0
- 3 = Acetate pH5.0
- 4 = Acetate pH5.0 + NaCl
- 5 = Acetate pH5.0 + NaCl + Tween
- 6 = Acetate pH5.5
- 7 = Histidine pH5.5
- 8 = Histidine pH6.0
- 9 = Histidine pH6.0 + NaCl

- 10 = Histidine pH6.5
- 11 = Phosphate pH6.0
- 12 = Phosphate pH6.0 + Tween
- 13 = Phosphate pH6.5
- 14 = Phosphate pH7.0
- 15 = Phosphate pH7.0 + NaCl
- 16 = Phosphate pH7.5
- 17 = Reference

7(c) +37°C Samples



Key:

1 = Molecular weight markers (kDa)
 2 = Acetate pH4.0
 3 = Acetate pH5.0
 4 = Acetate pH5.0 + NaCl
 5 = Acetate pH5.0 + NaCl + Tween
 6 = Acetate pH5.5
 7 = Histidine pH5.5
 8 = Histidine pH6.0
 9 = Histidine pH6.0 + NaCl

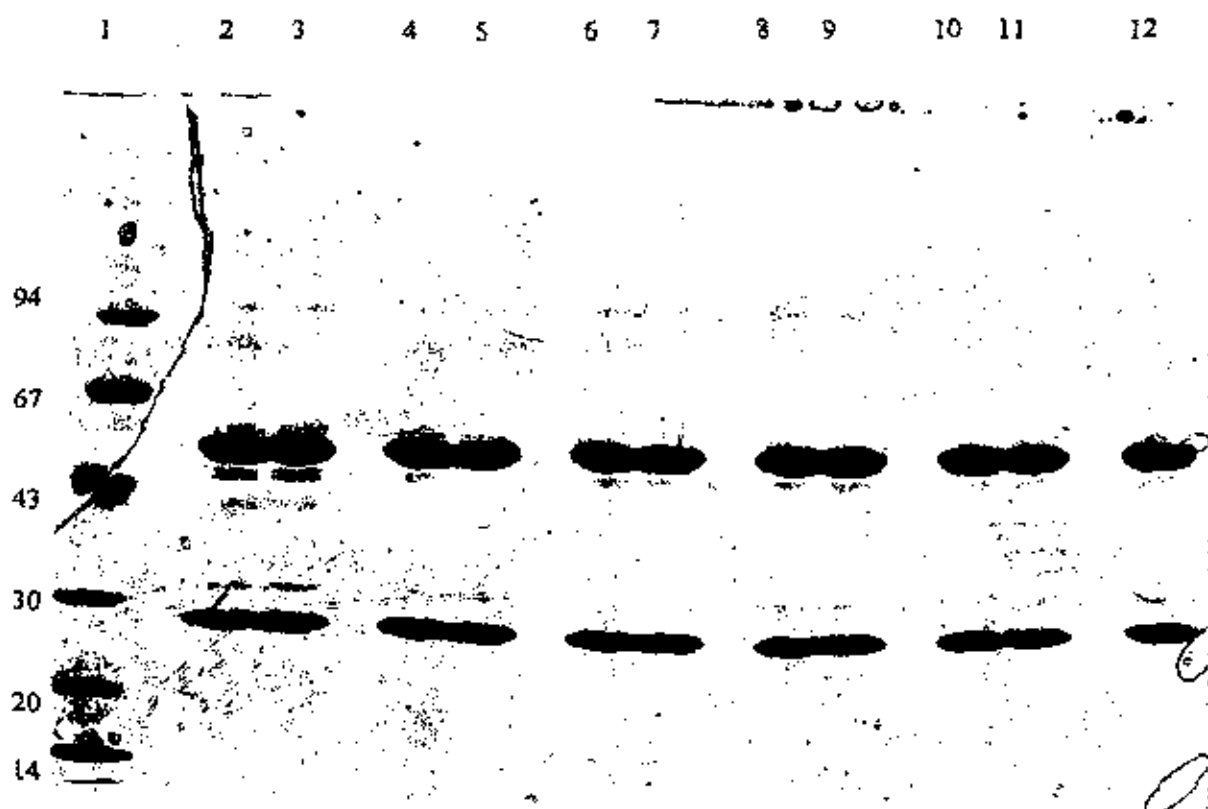
10 = Histidine pH6.5
 11 = Phosphate pH6.0
 12 = Phosphate pH6.0 + Tween
 13 = Phosphate pH6.5
 14 = Phosphate pH7.0
 15 = Phosphate pH7.0 + NaCl
 16 = Phosphate pH7.5
 17 = Reference

318
375

Figure 8 SDS-PAGE Analysis of Reduced Samples at T=0

5-15% gradient gels. Coomassie Blue stained. 25 μ g/lane

8(a)



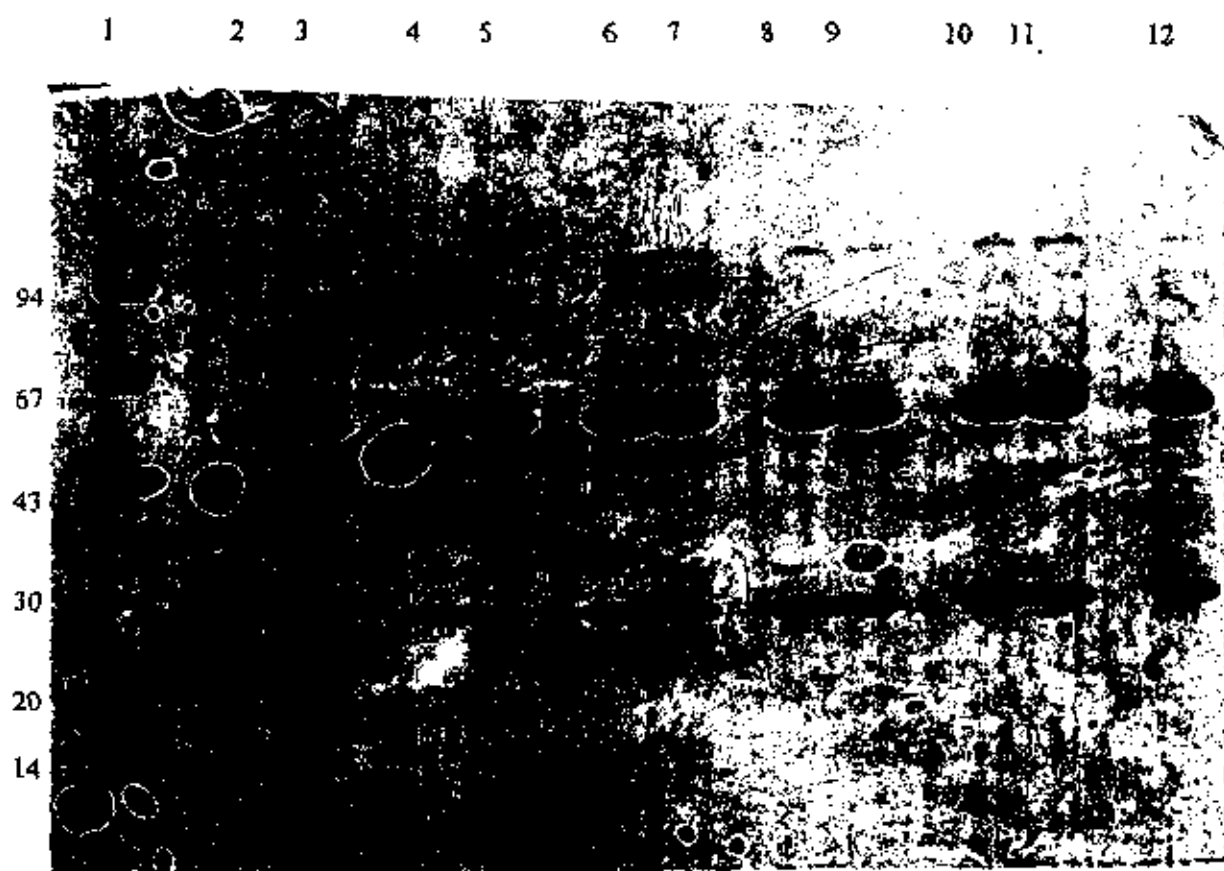
Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Acetate pH4.0
- 4,5 = Acetate pH5.0
- 6,7 = Acetate pH5.0 + NaCl
- 8,9 = Acetate pH5.0 + NaCl + Tween
- 10,11 = Acetate pH5.5
- 12 = Reference

125

376

8(b)



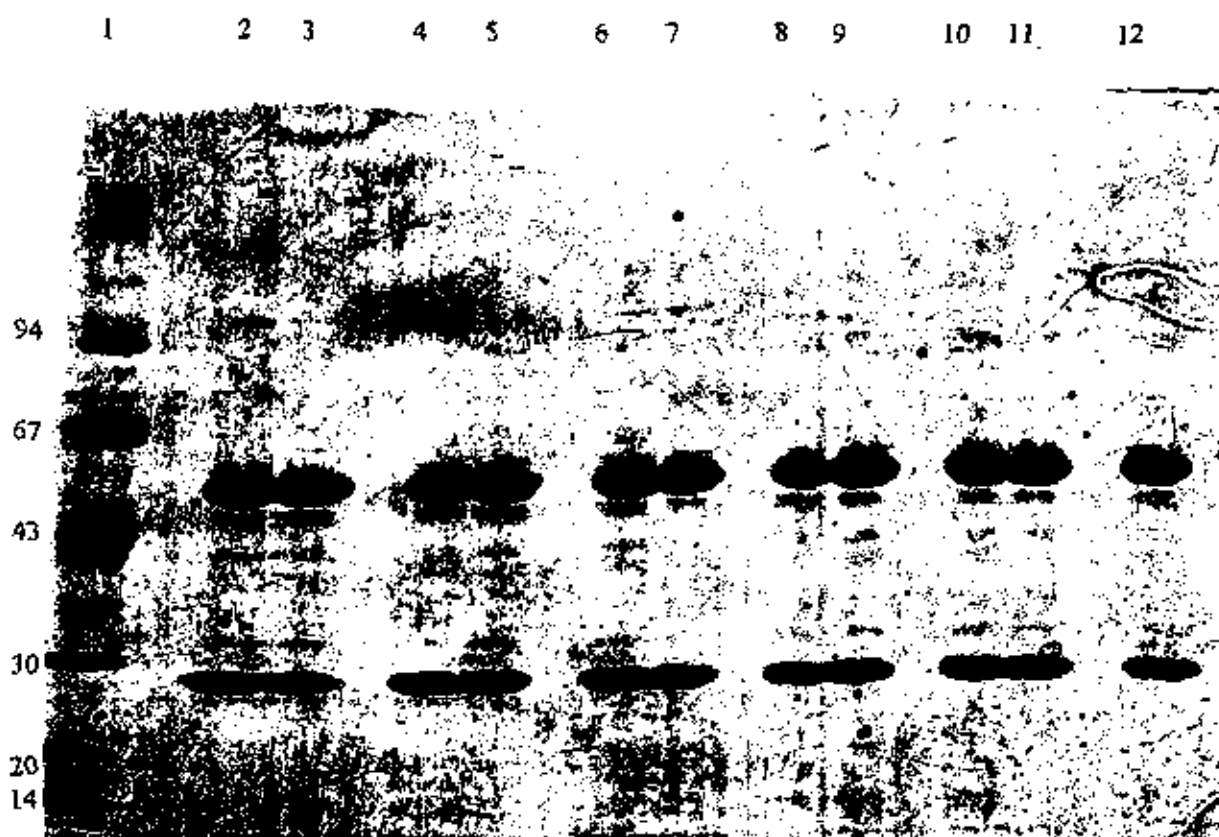
Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Histidine pH5.5
- 4,5 = Histidine pH6.0
- 6,7 = Histidine pH6.0 + NaCl
- 8,9 = Histidine pH6.5
- 10,11 = Phosphate pH6.0
- 12 = Reference

42

125

8(c)



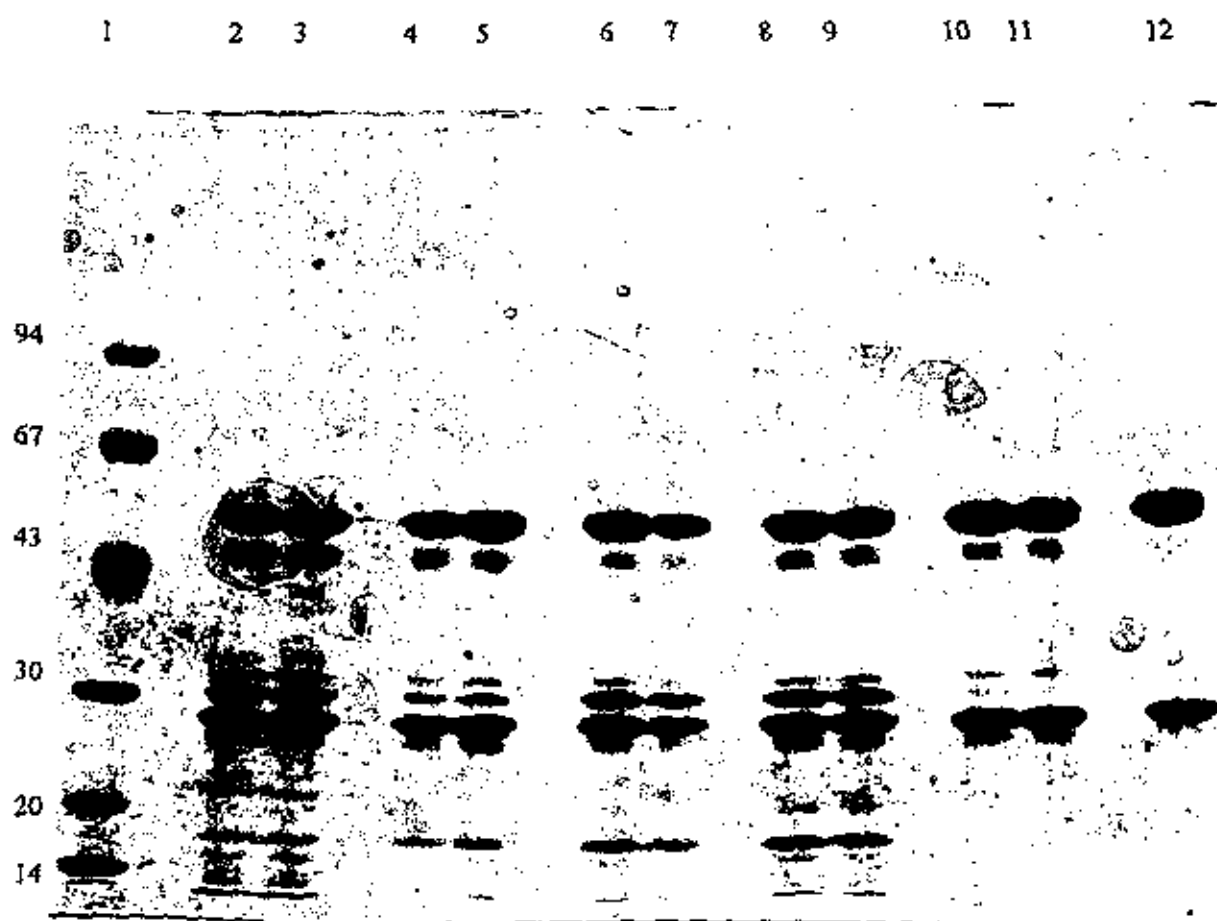
Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Phosphate pH6.0 + Tween
- 4,5 = Phosphate pH6.5
- 6,7 = Phosphate pH7.0
- 8,9 = Phosphate pH7.0 + NaCl
- 10,11 = Phosphate pH7.5
- 12 = Reference

227
318

Figure 9 SDS-PAGE Analysis of Reduced Samples after 8 weeks storage at 37°C

5-15% gradient gel. Coomassie Blue stained. 25µg/Lane



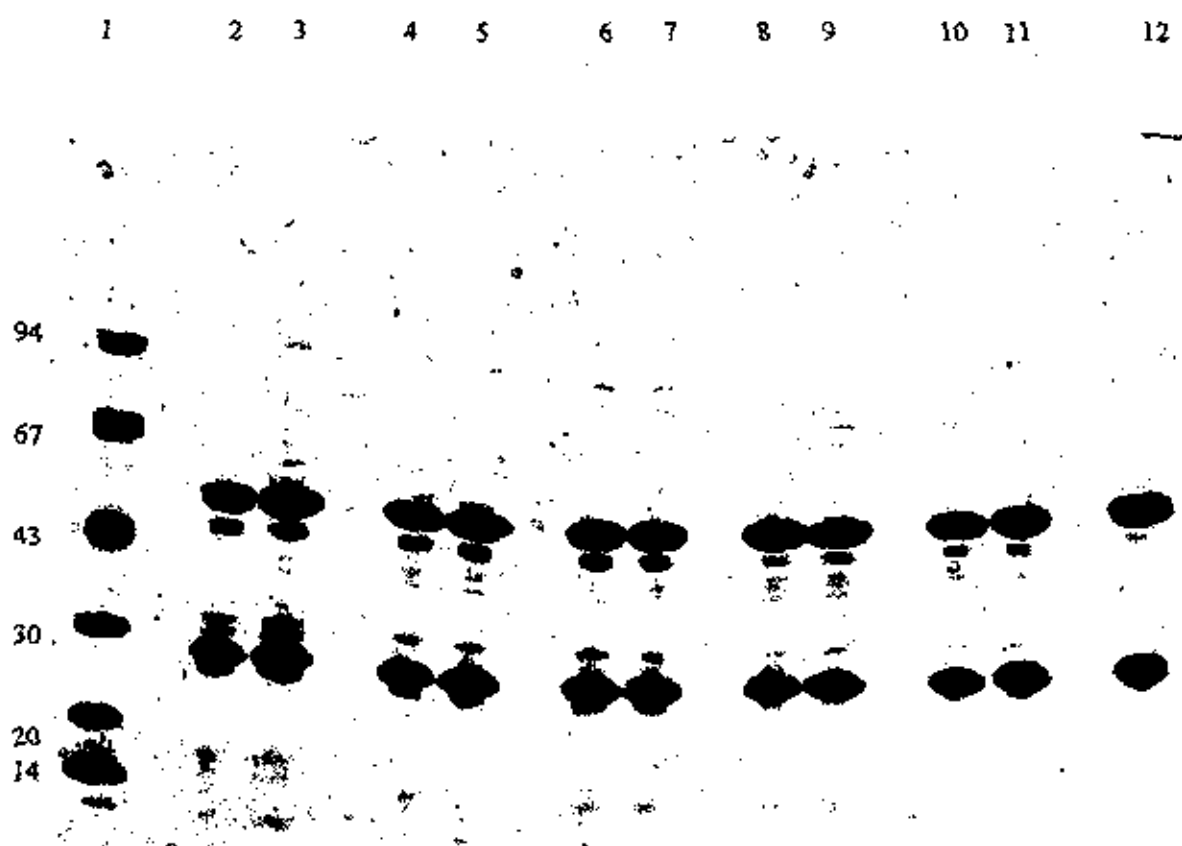
Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Acetate pH 4.0
- 4,5 = Acetate pH 5.0
- 6,7 = Acetate + NaCl pH 5.0
- 8,9 = Acetate + NaCl + Tween 80 pH 5.0
- 10,11 = Acetate pH 5.5
- 12 = Reference Standard

128

374

9(b)

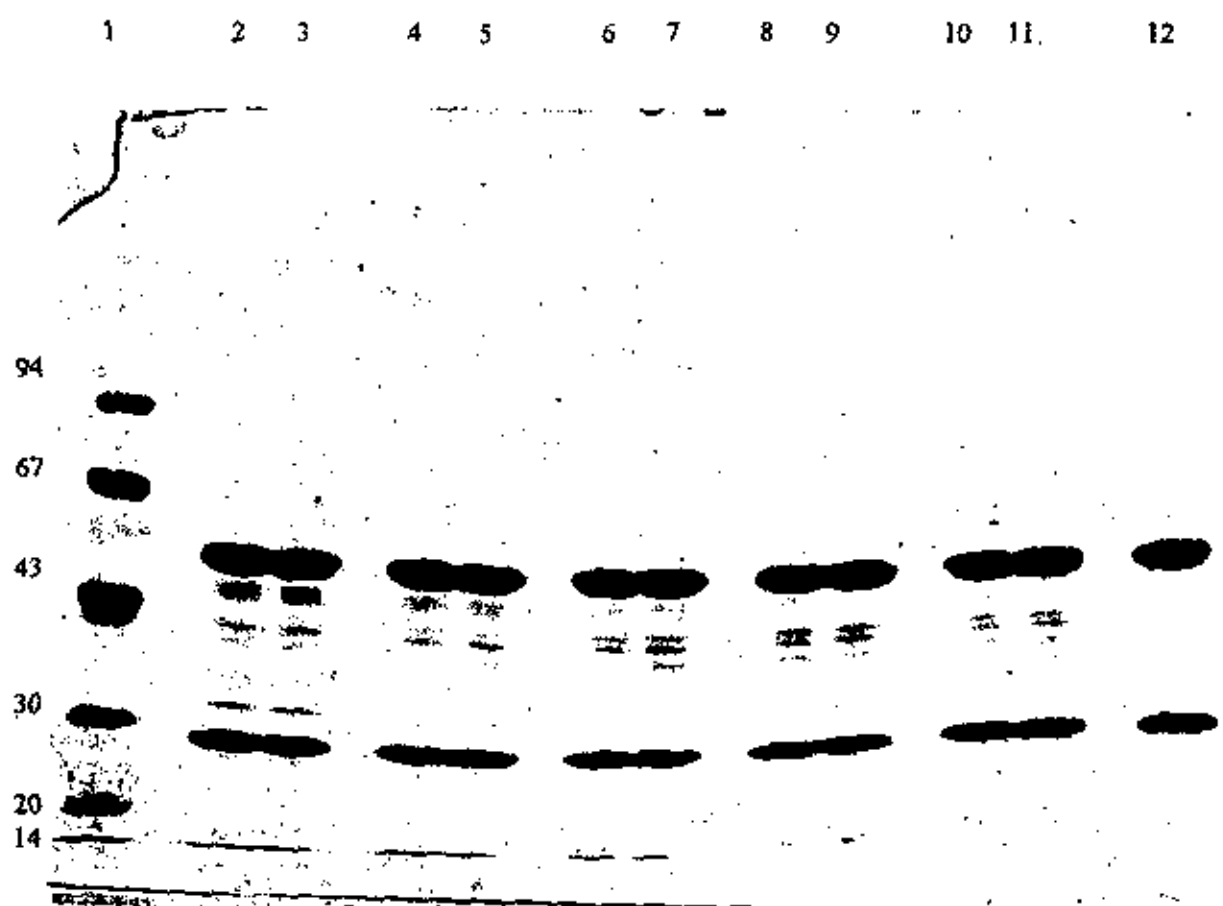


Key:

- 1 = Molecular weight markers (kDa)
- 2,3= Histidine pH 5.0
- 4,5= Histidine pH 6.0
- 6,7= Histidine + NaCl pH 6.0
- 8,9= Histidine pH 6.5
- 10,11= Phosphate pH 6.0
- 12 = Reference Standard

325
370

9(c)



Key:

- 1 = Molecular weight markers (kDa)
- 2,3 = Phosphate + Tween 80 pH 6.0
- 4,5 = Phosphate pH 6.5
- 6,7 = Phosphate + pH 7.0
- 8,9 = Phosphate + NaCl pH 7.0
- 10,11 = Phosphate pH 7.5
- 12 = Reference Standard

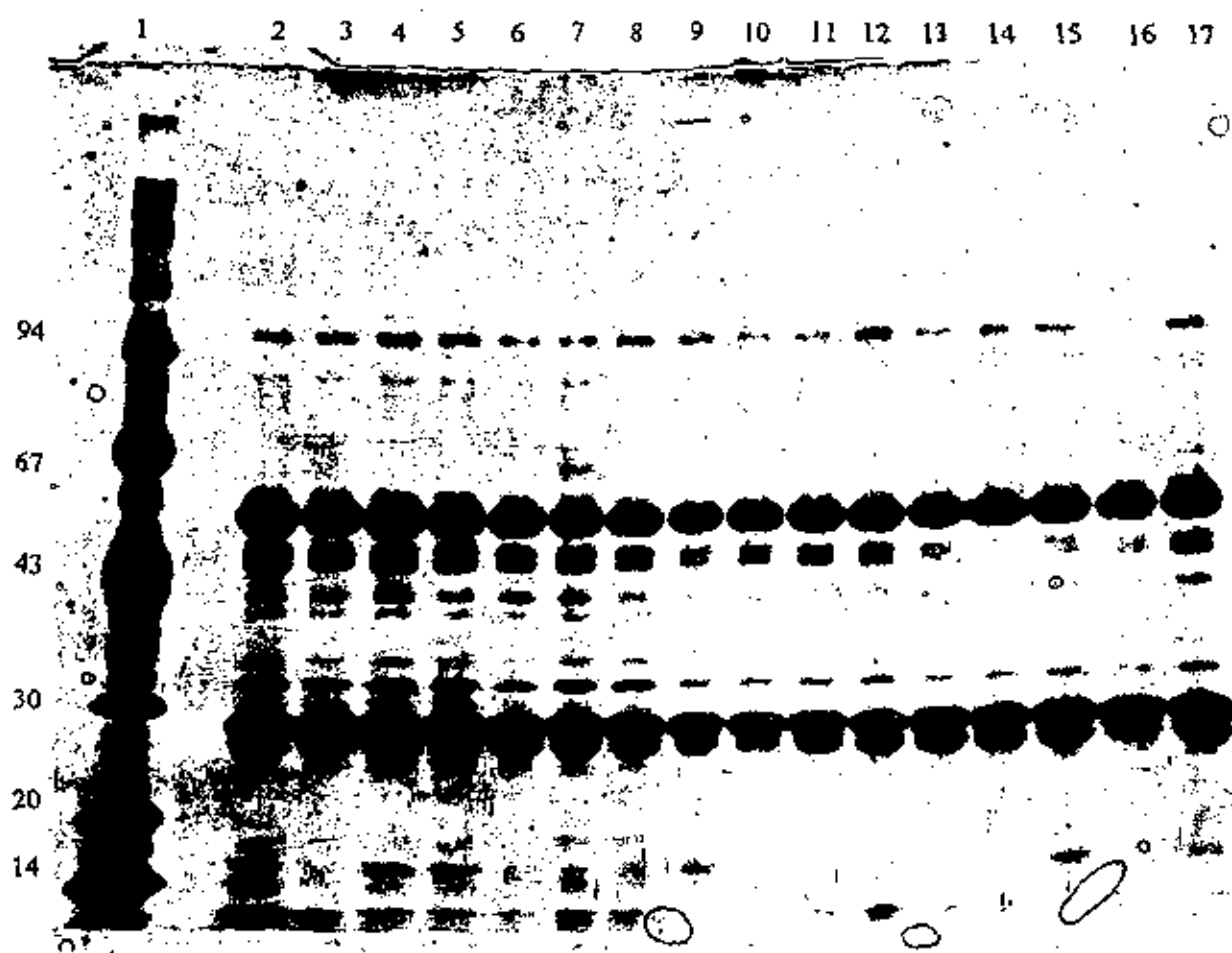
89

120

324
327

Figure 10 SDS-PAGE Analysis of Reduced Samples After 8 Weeks Storage at 42C

5-15 gradient gel. Silver stained. 10µg/Lane



Key:

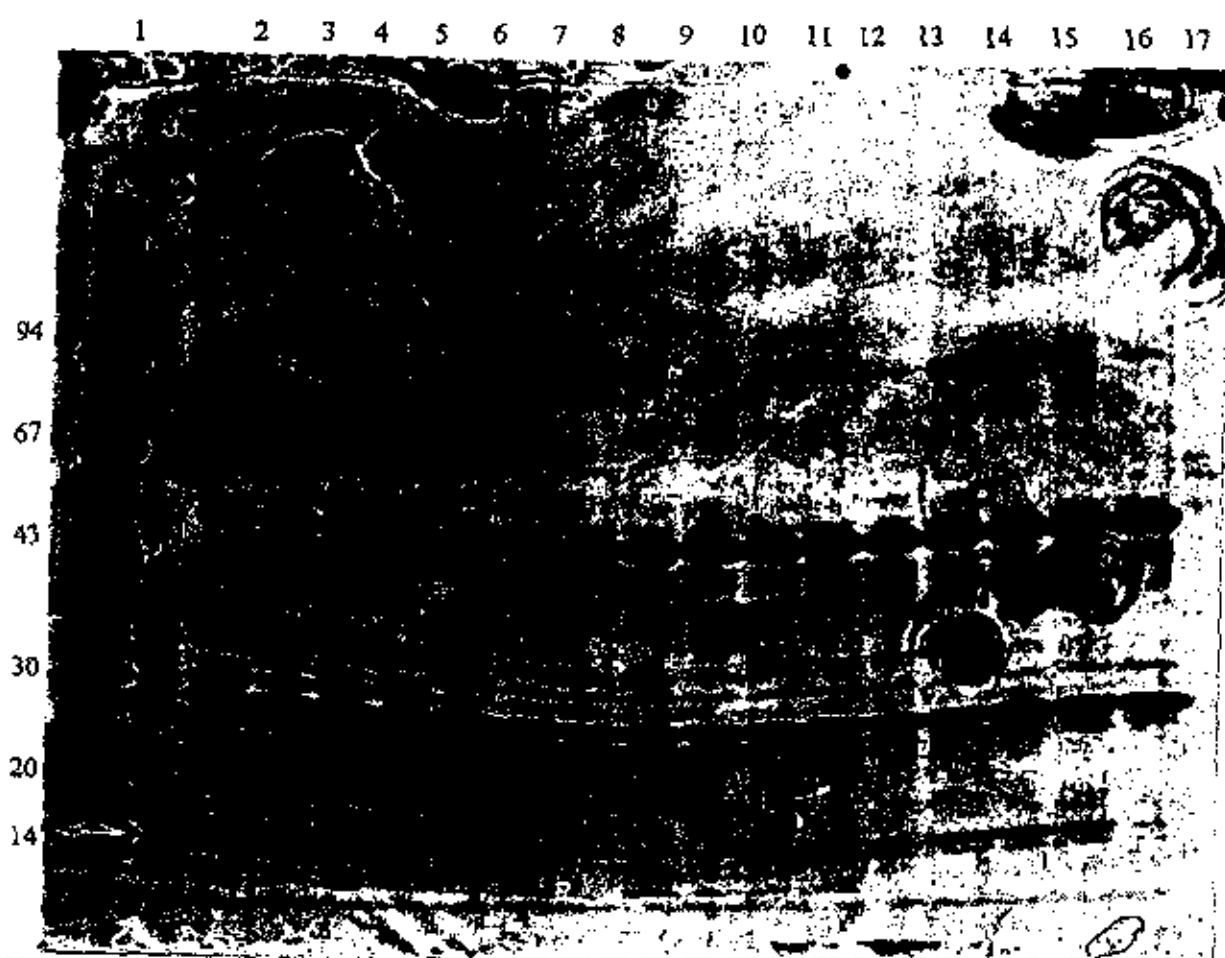
1 = Molecular weight markers (kDa)
 2 = Acetate pH 4.0
 3 = Acetate pH 5.0
 4 = Acetate + NaCl pH 5.0
 5 = Acetate + NaCl + Tween 80 pH 5.0
 6 = Acetate pH 5.5
 7 = Histidine pH 5.5
 8 = Histidine pH 6.0
 9 = Histidine + NaCl pH 6.0

10 = Histidine pH 6.5
 11 = Phosphate pH 6.0
 12 = Phosphate + Tween 80 pH 6.0
 13 = Phosphate pH 6.5
 14 = Phosphate pH 7.0
 15 = Phosphate + NaCl pH 7.0
 16 = Phosphate pH 7.5
 17 = Reference Standard

325
322

Figure 11 SDS-PAGE Analysis of Reduced Samples After 8 Weeks Storage at 37°C

5-15% gradient gel. Silver stained. 10µg/Lane



Key:

1 = Molecular weight markers (kDa)
2 = Acetate pH 4.0
3 = Acetate pH 5.0
4 = Acetate + NaCl pH 5.0
5 = Acetate + NaCl + Tween 80 pH 5.0
6 = Acetate pH 5.5
7 = Histidine pH 5.5
8 = Histidine pH 6.0
9 = Histidine + NaCl pH 6.0

10 = Histidine pH 6.5
11 = Phosphate pH 6.0
12 = Phosphate + Tween 80 pH 6.0
13 = Phosphate pH 6.5
14 = Phosphate pH 7.0
15 = Phosphate + NaCl pH 7.0
16 = Phosphate pH 7.5
17 = Reference Standard

132

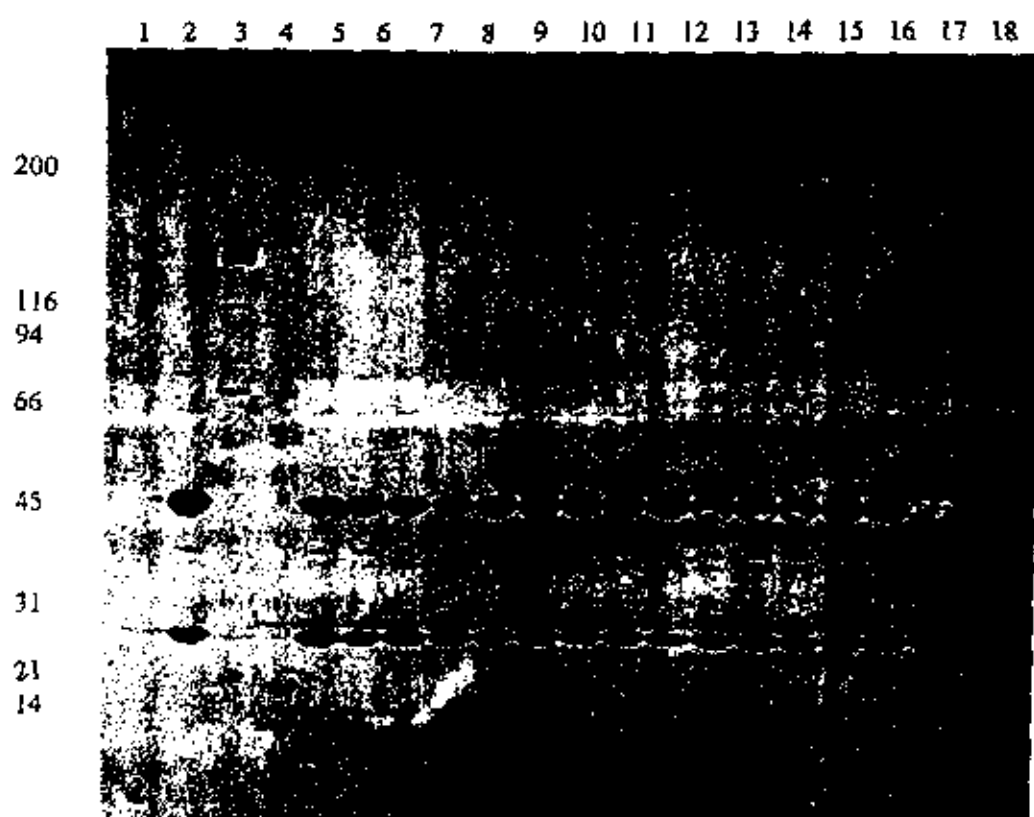
Figure 12 Anti IgG Probed Western Blots of Reduced SDS-PAGE Analysis of T=8 weeks +37°C Samples

Key to blots:

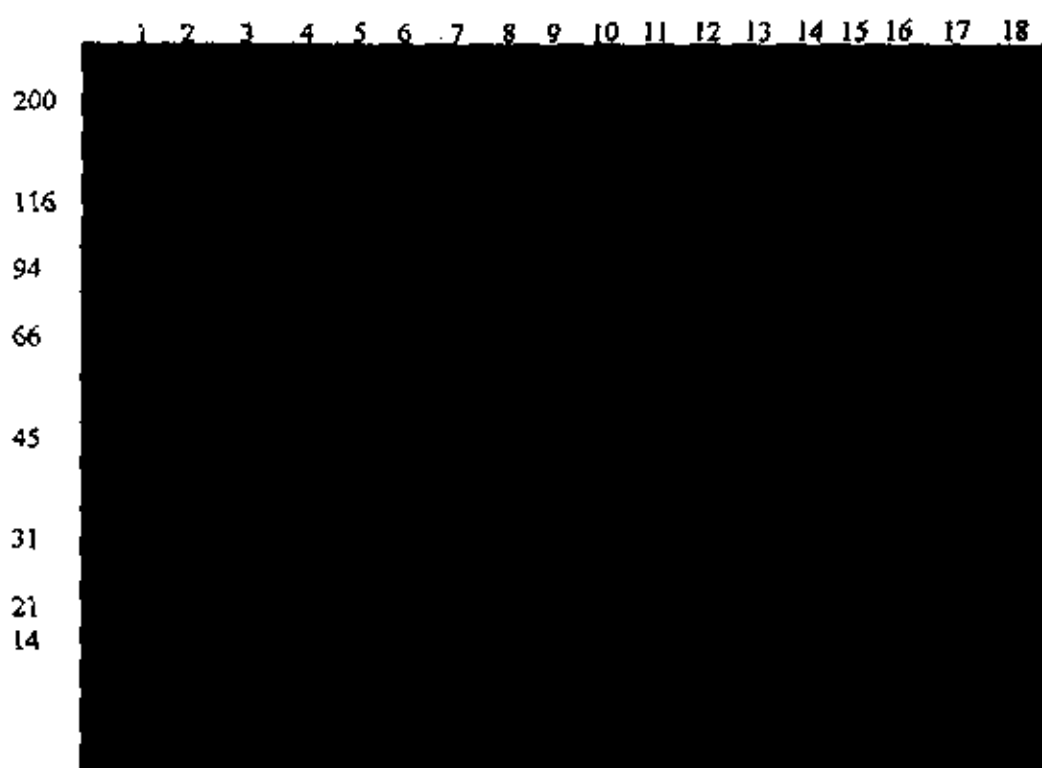
- 1 = Reference
- 2 = High molecular weight markers (kDa)
- 3 = Low molecular weight markers (kDa)
- 4 = Acetate pH4.0
- 5 = Acetate pH5.0
- 6 = Acetate pH5.0 + NaCl
- 7 = Acetate pH5.0 + NaCl + Tween
- 8 = Acetate pH5.5
- 9 = Histidine pH5.5
- 10 = Histidine pH6.0
- 11 = Histidine pH6.0 + NaCl
- 12 = Histidine pH6.5
- 13 = Phosphate pH6.0
- 14 = Phosphate pH6.0 + Tween
- 15 = Phosphate pH6.5
- 16 = Phosphate pH7.0
- 17 = Phosphate pH7.0 + NaCl
- 18 = Phosphate pH7.5

505
324

12(a) Probe 1 Anti Human IgG



12(b) Probe 2 Anti Fc



51

+34

327
Enc

Formulation Study for Stability of AN100226

Purpose: This study examines the stability of AN100226 in various buffers over XX period of time. The antibody has been prepared in 16 different buffers at concentrations ranging from 3.8 to 5.5 mg antibody / ml test buffer. At XX time intervals the samples will be compared for antibody activity according to a standardized assay. AN100226 is an antibody that binds to $\alpha 5$ integrin on the surface of mononuclear white blood cells, and this binding interaction will serve as the basis of activity measurement assay. The samples will be diluted 1:1000 in a standard diluent (PBS, 5% human serum) and then titrated for their ability to compete attachment of fluorescently labeled AN100226 to the surface of $\alpha 5$ integrin positive cells. This method will provide the IC_{50} of the test antibodies according to their ability to diminish cell fluorescence as measured by FACS analysis.

Test and Control Articles

Identification of Test articles: AN100226 was prepared by Celltech Biologics, 216 Bath Road Slough SL1 4EN, Berkshire, UK (lot # 7904/10), and was shipped to Athena Neurosciences on January 31, 1994. All samples are stored at Athena at 4°C. The material is supplied in the following test buffers at the indicated concentration. Sample size ranges between 50 and 100 μ l:

Formul- size number	Code	Buffer Description	Protein Concentr -ation (mg/ml)
1	1B	50mM Acetate, pH 4.0	4.7
2	2B	50mM Acetate, pH 5.0	3.9
3	3B	50mM Acetate + 150mM NaCl, pH 5.0	3.9
4	4B	50mM Acetate + 150mM NaCl + 0.02% TWEEEN 80, pH 5.0	4.0
5	5B	50mM Acetate pH 5.5	3.9
6	6B	50mM Histidine, pH 5.5	4.3
7	7B	50mM Histidine, pH 6.0	3.9
8	8B	50mM Histidine + 150mM NaCl, pH 6.0	3.9
9	9B	50mM Histidine, pH 6.5	3.9
10	10B	50mM Phosphate, pH 6.0	3.8
11	11B	50mM Phosphate + 0.02% TWEEEN 80, pH 6.0	4.6
12	12B	50mM Phosphate, pH 6.5	4.5
13	13B	50mM Phosphate, pH 7.0	5.0
14	14B	50mM Phosphate + 150mM NaCl, pH 7.0	4.8
15	15B	50mM Phosphate, pH 7.5	4.8

Identification of control articles: AN100226 (lot number 7904/10) diluted in phosphate buffered saline was supplied by Celltech Biologics, 216 Bath Road Slough SL1 4EN, Berkshire, UK (lot # 7904/10) at a concentration of 5.5 mg/ml. This sample was included as part of the test samples described above. A previous shipment of AN100226 (lot number XX) was provided by Celltech Biologics and received at Athena on XX. This sample is also in PBS buffered saline and is at a concentration of XX. The earlier shipment of antibody has already been characterized in the competition assay and will be used to verify the activity of the current shipment.

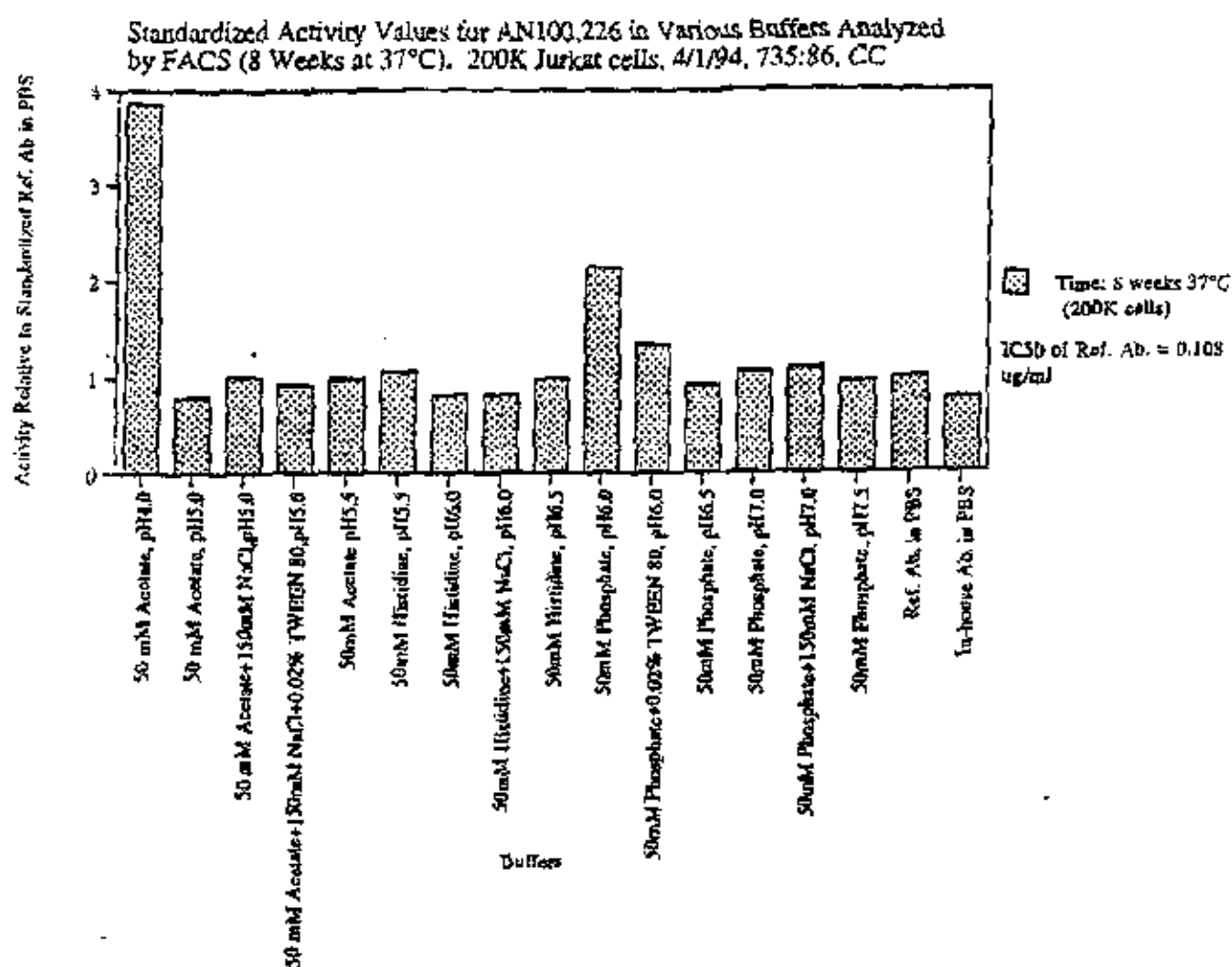
Preparation of test article: Samples will be diluted 1:1000 in PBS, 5% human serum (lot #XX, Gibco Cal.#XX) by adding 10 μ l of sample to 990 μ l of buffer (1:100) and then adding 10 μ l of one-step diluted sample to 90 μ l of buffer (1:1000 final). Concentration of the various samples and standards will now be between 3.8 and 5.5 μ g/ml.

Experimental design: Each of the test and standard samples (already diluted 1:1000) will be diluted in a two fold dilution series (with 6 steps). 50 μ l of 1:1000 material be mixed with 50 μ l of PBS, 5% hu serum. 50 μ l of this mixture will be added to another 50 μ l of PBS, 5% hu serum; and the process will be repeated a total of six times:

Dilution Series	Origin	Antibody Concentration
First	1:1000 of original samples	5.5 - 3.8 μ g/ml
Second	1:2 of First	2.75 - 1.9 μ g/ml
Third	1:2 of Second	1.38 - 0.95 μ g/ml
Fourth	1:2 of Third	0.69 - 0.48 μ g/ml
Fifth	1:2 of Fourth	0.34 - 0.24 μ g/ml
Sixth	1:2 of Fifth	0.17 - 0.12 μ g/ml

25 μ l of each of the six test samples (First - Sixth) will be mixed with an equal volume of buffer containing 2×10^5 Jurkat cells in wells of a round bottom microtiter plate (final concentration of test antibody ranges from 2.75 - 1.9 in the first dilution to 0.085 - 0.06 in the sixth dilution). The cells and test antibody will be incubated on ice for 30 minutes at 4°C. 50 μ l of FITC-conjugated AN100226 will then be added to the test samples for a final concentration of 1 μ g / ml and incubated with the cells for 30 minutes on ice. The samples will then be washed twice with PBS, 2% FBS (fetal bovine serum), and resuspended in 400 μ l of PBS, 2% FBS. The samples will then be examined by FACS analysis. It is known that the IC_{50} for this assay is 0.2 μ g/ml (C698/44); therefore, all of the test samples will be at this level concentration.

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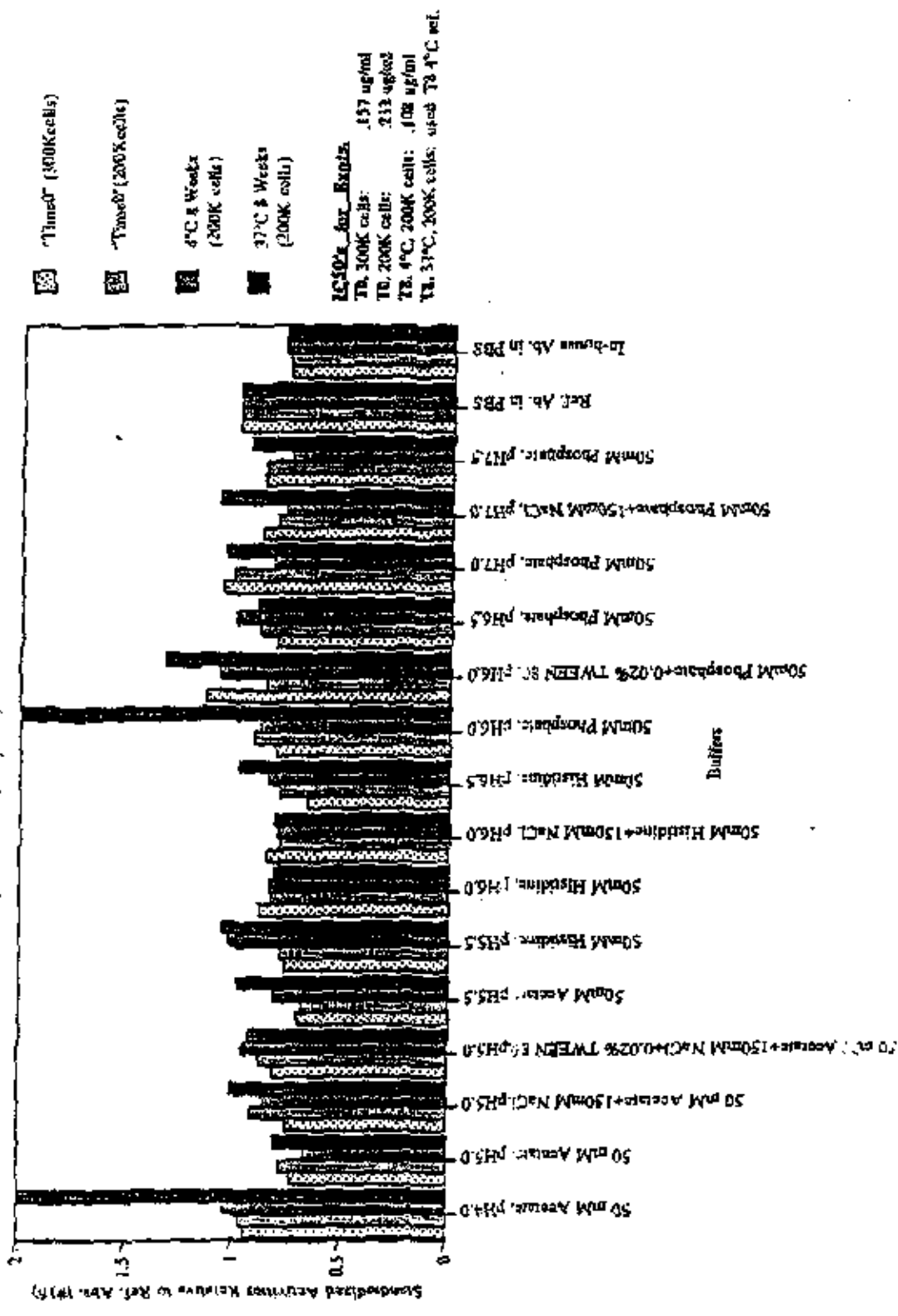


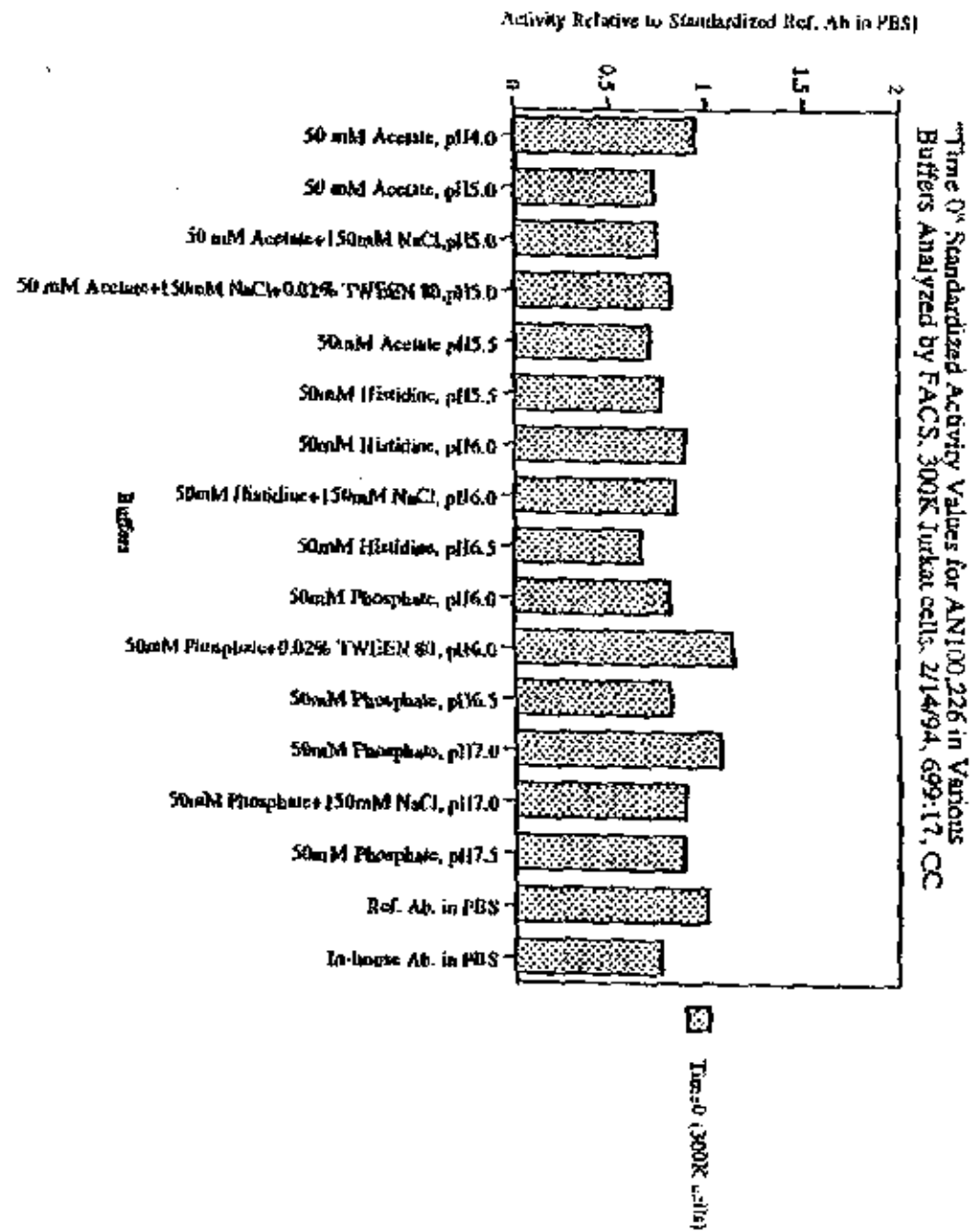
48044 100,228 KC50 (mpe)

UNSC Comparison for A4100,228 Stability Timepoints			
Staphylococcal Activity is KC50 of Heat, A4100,228 of samples			
Conc'd Buffer Name Final A4100,228 AB was stored in	TIME 0 (2/14/94) 300,000 cells/ml IC50 Standardized Activity	TIME 0 (2/21/94) 200,000 cells/ml IC50 Standardized Activity	TIME 0 (2/21/94) 200,000 cells/ml IC50 Standardized Activity
1 50 mM Acetate, pH 4.0	0.185	0.241	0.97
2 50 mM Acetate, pH 5.0	0.214	0.299	0.78
3 50 mM Acetate, pH 6.0	0.208	0.254	0.82
4 50 mM Acetate + 150mM NaCl, pH 5.0	0.192	0.265	0.88
5 50mM Acetate, pH 5.5	0.221	0.339	0.69
6 50mM Histidine, pH 5.5	0.203	0.285	0.78
7 50mM Histidine, pH 6.0	0.178	0.278	0.83
8 50mM Histidine + 50mM NaCl, pH 6.0	0.185	0.282	0.80
9 50mM Histidine, pH 6.5	0.236	0.291	0.80
10 50mM Phosphate, pH 6.0	0.182	0.252	0.92
11 50mM Phosphate + 100mM NaCl, pH 6.0	0.136	0.272	0.86
12 50mM Phosphate, pH 6.5	0.187	0.28	0.80
13 50mM Phosphate, pH 7.0	0.147	0.264	0.82
14 50mM Phosphate, 150mM NaCl, pH 7.0	0.178	0.264	0.82
15 50mM Phosphate, pH 7.5	0.178	0.268	0.87
16 Heat AB in PBS	0.167	0.293	1.00
17 100mM AB in PBS	0.203	0.302	0.77
47°C storage			
TIME 8 WEEKS (4/19/94)			
1 50 mM Acetate + 150mM NaCl, pH 4.0	0.112	0.096	1.01
2 50mM Acetate, pH 5.5	0.131	0.086	0.94
3 50mM Acetate, pH 5.5	0.105	0.082	0.98
4 50mM Acetate, pH 5.5	0.105	0.082	0.98
5 50mM Acetate, pH 5.5	0.105	0.082	0.98
6 50mM Acetate, pH 5.5	0.105	0.082	0.98
7 50mM Histidine, pH 5.5	0.128	0.084	0.82
8 50mM Histidine + 150mM NaCl, pH 6.0	0.134	0.081	0.82
9 50mM Histidine, pH 6.5	0.127	0.085	0.89
10 50mM Phosphate, pH 6.0	0.121	0.088	2.12
11 50mM Phosphate + 100mM NaCl, pH 6.0	0.1	0.081	1.33
12 50mM Phosphate, pH 6.5	0.107	0.101	0.81
13 50mM Phosphate, pH 7.0	0.128	0.084	1.06
14 50mM Phosphate + 150mM NaCl, pH 7.0	0.136	0.099	1.08
15 50mM Phosphate, pH 7.5	0.144	0.075	0.85
16 Heat AB in PBS	0.108	1.00	1.00
17 100mM AB in PBS	0.136	0.136	0.78

Note: Number of cells used in testing reflects 100,000 cells/ml. A4100,228 is not present in all samples; amounts shown are relative to A4100,228.

Activities of AN101,226 Relative to Internal Standard Ref. Abs. in PBS
4 FACs Assays on Jurkat Cells (2/14/94-4/1/94, CC)

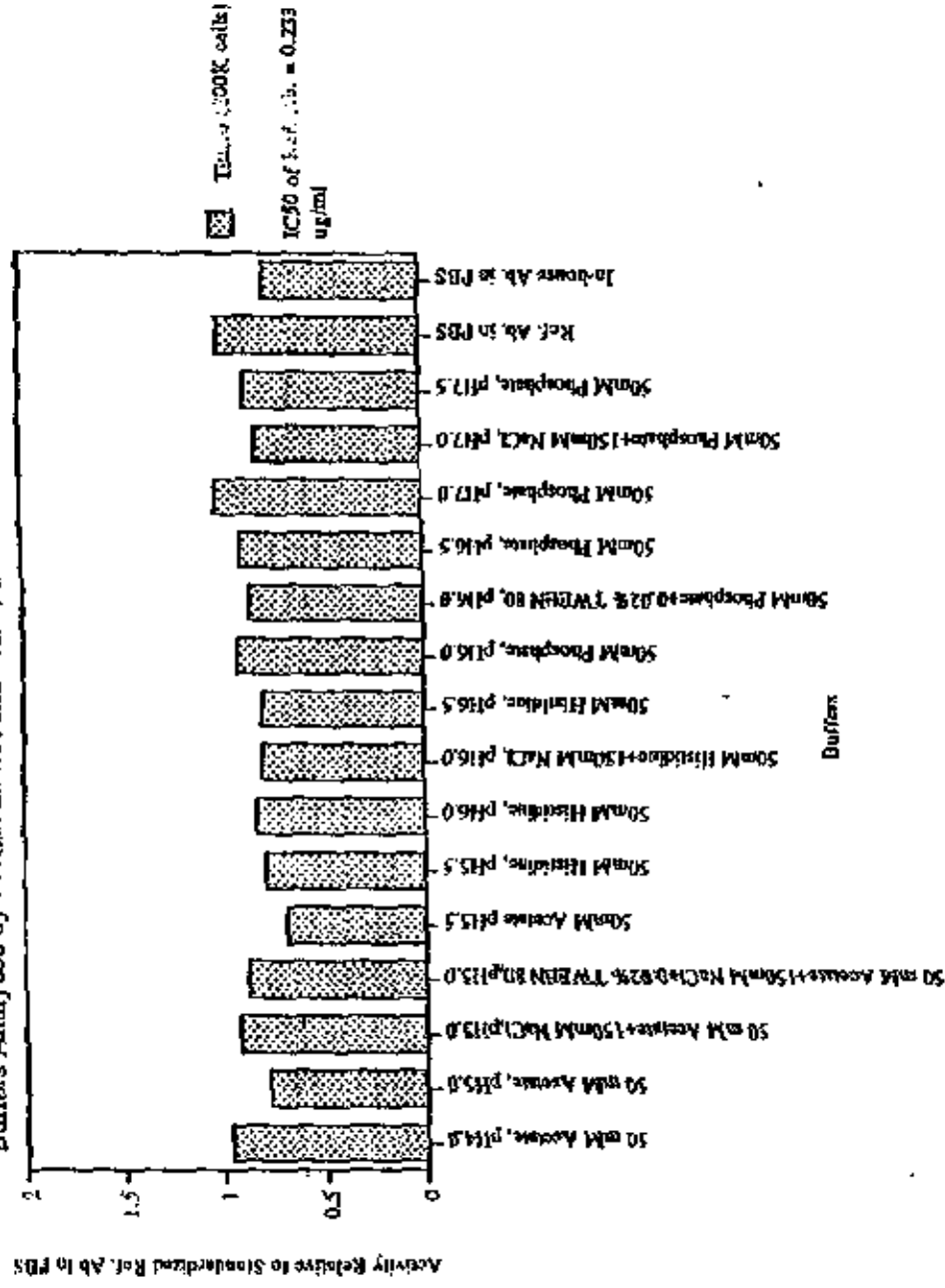


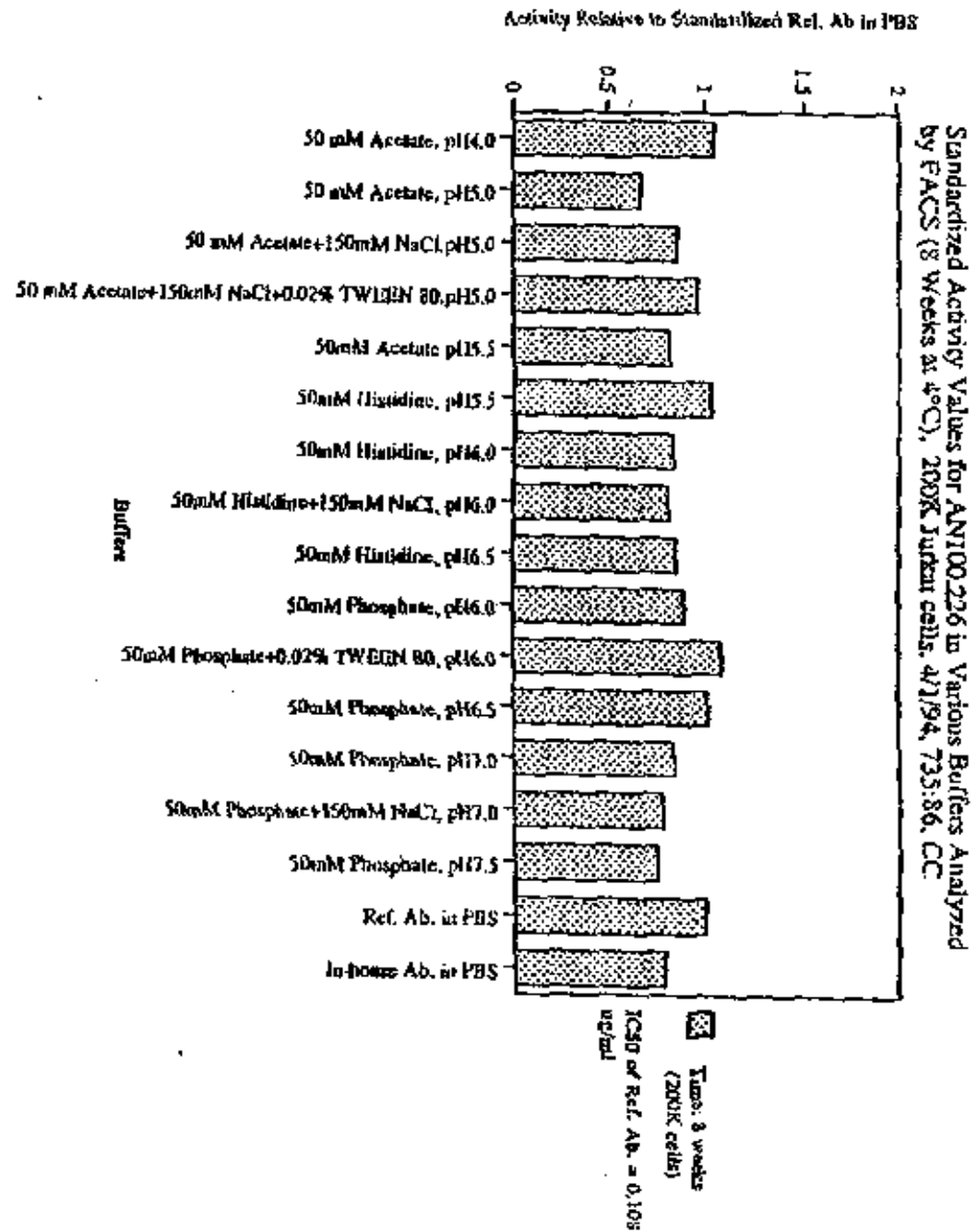


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"Time 0" Standardized Activity Values for AN100.226 in Various Buffers Analyzed by FACS. 200K Jurkat cells, 2/21/94, 699:45, CC





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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogada
Dilia Rodríguez
Apoderada

ASUNTO	Radicación	05 91313
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	 1 2 4 5 7
	Folios	003

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Via Nacional	☐		
Via PCT (fase nacional)	☒	Cap. I	☒
		Cap. II	☐
No. Sol. Internacional	PCT/US2004/003873		
Fecha de Presentación Interna.	09/02/2004		

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

1.1.	<u>Descripción:</u>	Folios 37 – 76	09/Septiembre/2005
1.2.	<u>Rta. Solicitante:</u>	Folios 194 – 337	08/Junio/2010
1.3.	<u>Reivindicaciones:</u>	Folios 77 – 86	09/Septiembre/2005
		Folios 183 – 184	29/Abril/2009
1.4.	<u>Prioridad:</u>	US 60/455818	10/Febrero/2003

2. Objeto de la invención

- Título de la solicitud: "Formulación de inmunoglobulina y método de preparación de la misma".

Según las reivindicaciones 1-17 se refiere a una formulación que comprende natalizumab, un amortiguador de fosfato, un polisorbato y cloruro de sodio.

El problema planteado en esta solicitud es la necesidad de composiciones estables de inmunoglobulina.

Para solucionar este problema técnico la solicitud en estudio proporciona la composición de la presente solicitud

- Clasificación Internacional de Patentes A 61 K 39/00

3. Determinación del Estado de la Técnica

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es 10.02.03, se buscaron documentos del estado de la técnica relacionados, con fecha de publicación anterior a esta.

Nº	Documento	Título	Fecha de Publicación
D7	WO 0056772	"Anticuerpos humanos que se enlazan a IL-12 humana y sus métodos para producirlos"	25.05.00
D8	Gastroenterology 2001; 121: 268-274	"Prueba randomizada controlada por placebo de un anticuerpo monoclonal humanizado a la integrina alfa-4 en enfermedad de Crohn activa"	2001

D7 <http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&adjacent=true&locale=es-p&FT=D&date=20000928&CC=WO&NR=0056772A1&KC=A1> enseña composiciones de anticuerpos que contienen 0.1-250 mg/ml de un anticuerpo humano, 1-50 mM de fosfato de sodio, 150 mM de cloruro de sodio a pH 6 y 0.005-0.1% de polisorbato y se dice que los auxiliares usados son para aumentar la estabilidad del producto (ver p. 104-105)

D8 <http://www.gastrojournal.org/article/PIIS001658501996568/abstract> pagina 269, enseña composiciones que comprenden natalizumab en un búfer de histidina en infusión intravenosa.

4. Análisis de patentabilidad (artículo 14 D486)

4.1. Nivel Inventivo (artículo 18 D486)

Características esenciales	D7	D8
	Formulaciones	Formulaciones acuosas

Formulación acuosa estable	acuosa estable	
Natalizumab	---	Natalizumab
Amortiguador de fosfato	Amortiguador de fosfato	Amortiguador
Polisorbato	Polisorbato	---
Cloruro de sodio	Cloruro de sodio	---

Se aprecia que la diferencia con D7 radica en que dicho documento no se menciona de manera específica el natalizumab.

Teniendo en cuenta el objeto de la solicitud, el cual es proveer composiciones estables de natalizumab, es una derivación del estado de la técnica, ya que una persona versada en la materia tomaría las composiciones de D7 y mediante sus conocimientos usuales en formulación farmacéutica y usaría el natalizumab de D8 con la expectativa de que también fuesen composiciones estables, debido a que D7 ya usaba composiciones de anticuerpos con los mismos auxiliares que tienen una estabilidad aumentada.

Finalmente, no se demuestra un efecto mejorado o sorprendente de las composiciones reivindicadas en comparación con las conocidas en el estado de la técnica.

En respuesta a los argumentos del solicitante, en donde se adjunta un estudio de estabilidad de natalizumab, concluyendo que la composición reivindicada es inventiva; se le aclara que, tal como lo dice el solicitante, la combinación de cloruro de sodio-polisorbato aceleraba la degradación del activo y recomienda el fosfato, si esto lo comparamos con D7 que revela composiciones estables de un anticuerpo humano que contienen polisorbato-cloruro de sodio-fosfato, se demuestra que el problema a solucionar con la presente solicitud ya fue solucionado por D7, ya que dicho documento ya estabilizaba anticuerpos humanos con búfer de fosfatos, por lo tanto sería obvio incorporar las enseñanzas de D8 a D7 y obtener las composiciones reivindicadas con la expectativa de que fueran estables gracias a la utilización de búfer de fosfatos.

5. Conclusión:

Los requisitos de patentabilidad de la presente solicitud se encuentran afectados así.

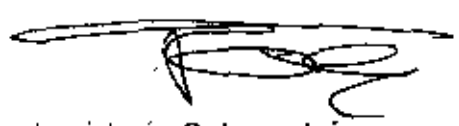
Nº	Documento	Reivindicaciones Afectadas	Requisito que Afecta
D7	WO 0056772	1-17	Nivel Inventivo

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		242	
	Gastroenterology	1-17	Nivel Inventivo
D8	2001; 121: 268-274		

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo 5 del título primero de la Circular Única N° 10 de 2001.



José Luis Salazar López
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 31 / 08 / 2011

CONCEPTO TECNICO No. 2490	EXAMINADOR TECNICO Código No. 202024
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Revisó: Consuelo Leguizamón 

I. INTRODUCTION

The major goal in designing a suitable drug formulation is to provide a *compatible environment that ensures the maximum stability of the drug*. This stability needs to be supported for a time period measured in years, in order to provide an adequate shelf life for the drug. For traditional, low molecular weight drugs, rational design of the formulation is well understood. It is based on an elucidation of the principal degradation routes and their kinetics, as well as an understanding of the mechanism of degradation. A significant body of literature exists in this field. For more complex protein and peptide drugs, no such literature is readily available on the pharmaceutical aspects of stability. The purpose of this review is to survey the biochemical literature in order to present an overview of the degradation mechanisms and major causes of instability in peptide and protein drugs. Some excellent reviews related to protein formulation have been published recently addressing the general stability of protein pharmaceuticals,¹ effect of formulation stabilizers,² protein degradation pathways,³ and analytical methods to study protein degradation.⁴ The stability of protein pharmaceuticals has also been reviewed recently as part of an in-depth series on pharmaceutical biotechnology.^{5,6}

II. PROTEIN AGGREGATION AND FORMULATION STABILITY

With the advent of biotechnology, greater amounts of protein have been made available to study various degradation reactions including aggregation. Early studies of proteins provided insight into the mechanisms of protein aggregation, but these studies failed to spark the interest of researchers in biotechnology.⁷ After the first successful expression of recombinant proteins, the formation of insoluble aggregates was observed in the host bacteria.⁸ These insoluble aggregates, referred to as inclusion bodies, reduced the yield of soluble active protein and required dissociation to recover bioactivity of the expressed protein. For successful commercialization of recombinant proteins, methods were developed to recover native protein from intracellular protein aggregates (for reviews, see References 9 and 10). Many recent studies have been performed to determine the underlying mechanisms of both *in vivo* and *in vitro* protein aggregation (for reviews, see References 11 and 12).

In contrast, there have been few detailed reviews of protein aggregation in formulations. The existing literature on protein formulations has usually focused on the chemical mechanisms for degradation and this literature is often directed toward research on therapeutic proteins. Usually, the therapeutic protein formulations described in the literature were designed to minimize aggregation since aggregation can have several detrimental effects including loss of activity, altered half-life, and increased immunogenicity. However, these studies are rarely designed to investigate the mechanisms of protein aggregation in the formulation. To minimize or eliminate aggregation, excipients such as sugars or surfactants are

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often empirically added to the formulation. A different mechanism of inhibiting aggregation may exist for each excipient. In addition, the physical state of the formulation (liquid or solid) affects protein stability. For solid formulation, the components of the formulation (such as buffer, excipients, etc.), the method of achieving the dry state (such as lyophilization), and the conditions of the dry state affect protein stability. To assure protein stability for both liquid and solid formulations, the processes that cause protein aggregation must be understood.

A. Mechanisms of Protein Aggregation

To determine the factors influencing protein aggregation, the major causes of protein aggregation should be investigated. The distinction between association and aggregation must first be defined. Protein association is often considered a reversible process involving the interaction of two or more native protein molecules and association may often result in reversible precipitation of the protein (see Section II.A.2). Protein aggregation usually involves the interaction of two or more denatured protein molecules and this process is often not reversible without dramatic changes in the solvent environment (see Section II.A.1). Therefore, protein denaturation must occur prior to the formation of aggregates according to this definition. Protein denaturation in solution is primarily caused by the solvent environment (pH, salt, cosolvents, etc.), temperature, or surface interactions. For lyophilized proteins, denaturation is dependent upon the final state of the protein as well as the changes in the protein solution during freeze-drying (see Section II.D). The solvent environment may affect the physical state of the protein. The protein assumes the most thermodynamically favorable state in a given solvent environment. For example, in the presence of denaturants, the protein can assume a compact nonnative state with exposed hydrophobic residues that are normally buried in the protein core or it can form a random coil structure characteristic of a fully unfolded protein. When a protein is heated, it may also form different denatured states. At elevated temperatures, proteins often become more flexible and their collision frequency increases, resulting in a propensity to form aggregates. In addition, many surfaces used in pharmaceutical applications are hydrophobic and protein adsorption to these surfaces can occur. Protein adsorption at hydrophobic surfaces may result in the accumulation of denatured protein at the surface, which results in an increased local concentration of denatured protein and subsequent aggregation. An air interface is a hydrophobic surface and, therefore, agitation of protein solutions with an air interface can also cause aggregation. The physical state of a protein and its stability depend on several environmental factors. Detrimental changes in the protein environment can result in protein denaturation. In general, the denatured state, which can be defined as any nonnative conformation, often has exposed hydrophobic surfaces that may interact with other proteins both in solution and on surfaces.

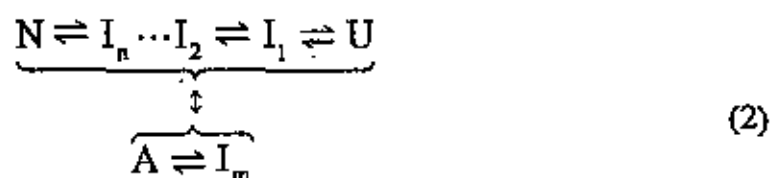
1. Denaturation

Many proteins are only marginally stable in solution; therefore, they are easily denatured and this results in aggregation. Nature has evolved methods to eliminate denatured proteins from the body and thereby reduce aggregation and prevent autoimmunity (see Section II.B). These methods usually involve proteolytic degradation or glomerular filtration. In addition, intracellular processes remove denatured proteins and solubilize protein aggregates. One class of intracellular proteins, referred to as chaperonins, have the ability to bind denatured or aggregated proteins and facilitate their renaturation to the native state (for reviews, see References 13 and 14). When the chaperonins become overwhelmed by the high expression level of a recombinant protein, intracellular aggregates (inclusion bodies) may form.¹⁵⁻¹⁷ Inclusion body formation of many recombinant therapeutic proteins (e.g., human insulin,⁸ human growth hormone (hGH),^{18,19} and human interferon-gamma²⁰) required extensive protein folding studies to recover the native protein. These studies, in turn, have provided a basic understanding of the processes of denaturation and aggregation.

Two basic pathways have been observed for proteins during denaturation and folding. The simplest pathway involves a two-state model. In this model, the native state, N, exists in equilibrium with the denatured state, D:



The native state is not a rigid structure in solution and it can fluctuate to form several stable, active states that are difficult to detect by many methods.²¹ The denatured state is an ensemble average of several nonnative protein conformations. The characteristics and stability of the denatured state are dependent upon the environment (e.g., the solvent conditions). The presence of denaturants, cosolvents (see Section II.C), or extremes of pH can destabilize the native protein. As the protein denatures, the internal hydrophobic core residues are often exposed to the solvent.²² Interactions between hydrophobic residues in the interior of the protein determine the physical stability of the protein.²³⁻²⁶ Stability of proteins has been successfully altered by making single residue substitutions in the hydrophobic core.²³⁻²⁵ Thus, through protein engineering, it is possible to determine the critical hydrophobic residues and increase protein stability. Once the hydrophobic core residues are exposed to the solvent, they can interact with other hydrophobic surfaces such as container surfaces, air interfaces, or even with other denatured proteins. Exposure of these hydrophobic residues may proceed through a sequential series of definable thermodynamic states referred to as intermediates. The formation of stable intermediate conformations that self-associate during folding has been observed for a number of proteins.^{12,27-31} The second general pathway for denaturation and folding would then include these stable intermediate species:



As shown in Equation 2, the native protein unfolds to form a native-like intermediate, I_n , and this intermediate further unfolds to form other intermediates. Often, the protein will unfold to form an off-pathway or misfolded intermediate, I_m . These misfolded intermediates are often thermodynamically stable and their rate of folding to the native state can be significantly slower than the other intermediates. These misfolded intermediates usually contain nonnative disulfide bonds that must be reduced to allow folding to the native state.³² Misfolded intermediates and hydrophobic intermediates on the folding pathway may form aggregates, A. Although the misfolded intermediates and aggregates may eventually refold to the native state, the renaturation of these species is usually performed through the addition of denaturing agents such as sodium dodecyl sulfate (SDS), guanidine hydrochloride, or urea. If the misfolded intermediates or aggregates form nonnative disulfide bonds, the dissociation and folding to the native state require reducing agents as well as a denaturant.

Although many physicochemical interactions can lead to protein aggregation, several researchers have postulated that noncovalent interactions, specifically hydrophobic interactions, are the major cause of aggregation from the denatured state.^{9,33-37} Many studies of protein folding indicate that stable hydrophobic intermediates self-associate.²⁷⁻³¹ For example, the hydrophobic molten globule intermediates of carbonic anhydrase and bovine growth hormone self-associate during refolding.^{28,30,38,39} If a thermodynamically stable intermediate state does not exist as suggested in the two-state model, hydrophobic interactions may still drive the aggregation of the protein since the gradual exposure of a hydrophobic surface will also increase the propensity of the protein to aggregate. For refolding of recombinant hGH, association of the protein was observed at high protein concentrations, but an intermediate species responsible for the aggregation was not identified.²⁷ Hydrophobic interactions are also the strongest nonpolar forces and may be the driving force for refolding.²⁶ The endothermic kinetics^{30,40,41} and loss of entropy^{40,42} observed during protein aggregation are further evidence of these nonpolar forces. Exposure of hydrophobic regions can occur during denaturation by chaotropic agents^{29,34,37,40,43} or by increased temperature.^{41,44-49} Both denaturants and temperature have been used to study the stability of proteins. If the two-state model as shown in Equation 1 is assumed, the concentration of denaturant required to achieve an equal concentration of native and denatured protein will provide an indication of the thermodynamic stability of the protein. This approach can also be used to calculate the free energy of unfolding of the protein, which defines the thermodynamic stability of the protein.⁵⁰ First, the fraction of denatured protein, f_d , is calculated for a given condition (denaturant concentration, temperature, etc.) by

using a method that measures the extent of denaturation. Spectroscopic methods such as absorbance, circular dichroism, or fluorescence are commonly used for these studies. The fraction of denatured protein is then defined by the equilibrium between the denatured, D, and native, N, states:

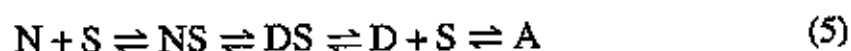
$$f_d = \frac{[D]}{[N] + [D]} = \frac{K_{ND}}{1 + K_{ND}} \quad (3)$$

where K_{ND} is the equilibrium constant. The equilibrium constant for denaturation is also related to the free energy of denaturation, ΔG_{ND}^0 , as a function of the denaturant concentration, $[C]$:

$$K_{ND} = \exp\left(\frac{-\Delta G_{ND}^0}{RT}\right) = \exp\left(\frac{-(\Delta G_{H_2O}^0 + A[C])}{RT}\right) \quad (4)$$

where $\Delta G_{H_2O}^0$ is the free energy of denaturation without denaturant and A corresponds to a linear relationship between denaturant concentration and change in free energy. By nonlinear least squares methods, experimental data can be used in Equations 3 and 4 with the denatured fraction as the dependent variable and the denaturant concentration as the independent variable. In addition, an equal concentration of both native and denatured protein is often achieved through heating to the appropriate temperature, usually referred to as the melt temperature, T_m . Both the heating and denaturation profiles of a protein are measured at equilibrium, and the results are used to calculate the thermodynamic stability of the protein as defined by Equations 3 and 4. These analyses are based on the assumptions that the protein does not form a stable intermediate state and does not aggregate.

Another factor that can cause protein denaturation is surface interaction. Adsorption of proteins to interfaces has been extensively reviewed.⁵¹⁻⁵³ Adsorption studies have shown that proteins will adhere strongly to hydrophobic surfaces, but protein adsorption to hydrophilic surfaces is very weak.⁵³ After adsorbing to a hydrophobic surface, a gradual denaturation of the protein may occur with the denatured protein remaining bound to the surface.⁵¹⁻⁵⁴ Once the buried hydrophobic residues in the protein are exposed and adsorbed to the surface, dissociation of the protein from the surface can result in aggregation of the hydrophobic denatured protein. This process can be described by a series of reactions:



where N is the native state, S is the surface, D is the denatured protein, and A is aggregate of denatured protein. Detailed models of protein adsorption for several

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proteins have been described previously.⁵³ Protein adsorption to surfaces can also decrease the thermostability of the protein.⁵⁴ For example, denaturation and inactivation of β -galactosidase occurred upon interaction with wall surfaces at low temperatures and the inactivation was independent of the air-liquid interfacial area.⁵⁵ Aggregation of denatured insulin on hydrophobic surfaces has been well studied. Monomeric insulin adsorbs to a hydrophobic surface or interface and is denatured.⁵⁶⁻⁶⁰ Unlike many native proteins, native monomeric insulin has exposed hydrophobic surfaces that may initiate the surface adsorption. Subsequent denaturation and accumulation at the surface results in the formation of nonnative protein aggregates.⁵⁶⁻⁵⁸ Surface adsorption that results in denaturation of the protein can be reduced or eliminated by either utilizing a more hydrophilic container or adding components to the formulation. Typically, glass vials are used for protein formulations, but silica, a slightly charged hydrophobic surface, can readily adsorb proteins. The use of more hydrophilic surfaces such as polyethylene oxide reduces protein adsorption and, thereby, prevents surface denaturation.⁵³ Unfortunately, these materials are not widely used in protein pharmaceuticals. For therapeutic proteins, components are usually added to the protein formulation to prevent adsorption to the glass surface. These additives may inhibit surface adsorption by coating the surface and/or binding to the protein. Serum albumin is often added to formulations because it competes for surface sites to prevent adsorption of the therapeutic protein. However, the addition of serum albumin may not be as effective as the addition of surfactants.⁶¹ Surfactants may bind to either the protein or the hydrophobic surfaces and inhibit protein adsorption. Copolymers of ethylene oxide and propyl oxide block copolymers called Poloxamers (e.g., Pluronic and Genapol series), and other polyethers such as the polysorbate series (e.g., Tween®) have been used to prevent protein adsorption. In particular, Genapol PF-10,⁵⁸ Pluronic F-68,⁶⁰ Tween,⁵⁷ and Triton X-100⁵⁷ have been used to prevent surface adsorption and aggregation of insulin. Nonpolar solvents such as alcohols⁵⁷ and urea⁵⁹ also provided a significant reduction in surface adsorption and denaturation of insulin. When adding surfactants and other stabilizers to the protein formulation, the effects of the additive on the pharmacological properties of the drug must be considered and additional toxicology studies may be required for materials that are not generally regarded as safe (GRAS). The additives may also cause leaching of the container components and stability studies must also address this possibility.⁶⁰

Proteins can also be denatured by other interfacial phenomena. Gas interfaces and shear forces from pumping or filtration can both cause significant denaturation of the protein. Like many solid surfaces, gas interfaces are nonpolar and can cause protein denaturation. The rate of denaturation at a gas-liquid interface is dependent upon the residence time of the protein at the interface.⁶² When shaking of protein solutions occurs in the presence of a gas-liquid interface, the rate of protein denaturation is increased resulting in the formation of both soluble and insoluble protein aggregates.⁶³ Protein denaturation caused by agitation is the result of both the interfacial and shear forces on the protein. Shear denaturation of proteins can

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occur as the result of agitation,^{63,64} pumping,^{65,66} or filtration.⁶⁴ As in the case of protein adsorption, surfactants have been added to formulations to reduce interfacial⁶² and shear-induced⁶⁶ denaturation. The stability of many therapeutic proteins is often studied by subjecting the final configuration (e.g., stoppered vial) to shaking for several hours. If the protein in the final configuration retains its native state and does not aggregate, the formulation is considered stable against surface or shear-induced denaturation. These vigorous testing procedures are performed to assure that the patient is not administered aggregated material as the result of storage or handling. Often, these formulations will require a stabilizing agent (e.g., surfactant) to prevent protein denaturation.

2. Charge Neutralization and Solubility

Denaturation usually occurs prior to protein aggregation in solution, but the formation of native protein oligomers can also lead to aggregates of denatured protein. These phenomena often involve the association of the native protein as the result of changes in the solvent environment. The resulting protein oligomers may undergo subsequent denaturation over time as the result of the solvent conditions and protein-protein interactions. When a protein is placed in a high concentration of salt, the surface charges on the protein can become masked such that charge-charge repulsion does not occur. This surface charge neutralization can result in the association of neutral protein molecules. A similar phenomenon of charge neutralization can occur when the pH of the solution approaches the isoelectric point of the protein. As the isoelectric point is reached, the number of neutral protein molecules increases and association often occurs. Both salt and pH changes may lead to subtle changes in protein conformation in the associated state, resulting in the formation of irreversible aggregates. If the association process and salt or pH changes do not irreversibly alter the conformation of the protein, dissociation can occur by adjusting the pH or reducing the salt concentration. In the development of a stable formulation, the pH of the formulation is selected by taking into account factors such as the isoelectric point of the protein, solubility, and chemical degradation (e.g., deamidation). To reduce chemical degradation, the pH of many protein formulations is approximately 5 to 7. The isoelectric point of many hormones and blood proteins is also in the range of pH 5 to 7.⁶⁷ Thus, the formulation pH must then be adjusted to avoid both chemical degradation and isoelectric precipitation. To ensure adequate solubility, the protein formulation is usually maintained at least 0.5 pH units above or below the protein's isoelectric point.

The solubility limit for native proteins in solution is dependent upon the solvent environment as well as the physical characteristics of the system (e.g., temperature). Many therapeutic proteins can be maintained in a soluble state at concentrations ($\mu\text{g/mL}$ to mg/mL) that are adequate for efficacy. Each protein must be studied for its solubility in a given solvent environment even though the

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insoluble protein may not cause any adverse side effects such as increased immunogenicity (see Section II.B.2). Factors such as pH, excipients (see Section II.C), and buffer components influence the protein solubility and the chemical and physical stability. These factors also have an impact on protein lyophilization (see Section II.D). The protein in solution can occupy a specific volume of the solution as the result of its tertiary structure. The sum of the specific volumes of each protein molecule in solution will dictate the number of molecules that can be packed into a specific volume of water containing buffer components and excipients. Therefore, as the protein concentration is increased, the packing density of the protein molecules increases, resulting in increased collisions and eventually the formation of a second phase (solid protein). This process usually occurs by a nucleation and growth mechanism where the critical nuclei are often soluble associated protein that rapidly grow to form insoluble protein precipitates or crystals.⁶⁸ Several methods can be used to assess the solubility of a protein in a given solution. These methods often include adding solid protein to a solution and concentrating a solution by ultrafiltration. In most cases, the solubility limit of native proteins is not observed for therapeutic applications.

B. Pharmaceutical Consequences of Protein Aggregation

When developing stable formulations for a therapeutic protein, one must consider the effect of the formulation on the pharmacology of the drug. In particular, if the formulation does not prevent denaturation and aggregation of the protein, the pharmacology, immunogenicity, and toxicology of the denatured or aggregated protein must be studied to determine its safety and efficacy. The Food and Drug Administration requires rigorous testing procedures to determine the stability of a pharmaceutical preparation over time. As the protein degrades chemically or physically, the activity, half-life, and immunogenicity of the protein can be altered. Therefore, several studies must be performed to determine the extent of degradation that is acceptable for administration. The formation of soluble aggregates in a protein formulation can have a significant effect on the pharmacokinetics and immunogenicity of the protein. In addition, insoluble aggregates decrease the product quality and are generally considered unacceptable. The end user, physician or patient, is usually instructed not to administer a solution containing precipitates. An understanding of both the pharmacokinetics and immunogenicity of aggregated proteins is required for the development of a safe and efficacious product.

1. Activity and Biological Half-Life

Some proteins such as human insulin reversibly self-associate to form oligomers of the native protein. Recombinant human insulin was the first recombinant

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therapeutic protein approved for human use. Many of the early formulation stability studies were performed on recombinant human insulin. For insulin, it was critical to understand both association of native protein and aggregation of denatured protein, both for production from *Escherichia coli*⁶⁹ and for development of a successful product formulation. Insulin exists in several associated states that can reversibly dissociate to the fully active monomer. The zinc hexamer of insulin is a complex of insulin and zinc that results in the formation of insulin crystals.⁵⁷ This form of insulin has long-acting properties (e.g., extended biological half-life) when administered subcutaneously because it slowly dissociates into dimers and, eventually, monomers that diffuse through the capillary membrane and into the blood.⁷⁰ Many recent studies have been performed to alter the self-association properties of insulin to produce stable dimeric or monomeric forms that are absorbed more rapidly and have a reduced biological half-life for treatment of hyperglycemia onset after meals.⁷¹⁻⁷³ These studies include engineering novel insulin mutants that do not associate⁷⁰⁻⁷² and using surfactants to alter the distribution of associated and monomeric insulin.⁷³ An understanding of insulin association has been required for successful treatment of diabetes. The study of insulin aggregation in solution has also been important for the development of insulin delivery devices.^{56-60,74,75} Light scattering studies of insulin aggregation revealed the formation of large aggregates (100 to 200 nm).^{56,74,75} Insulin aggregates larger than the native hexamer are deleterious since the amount of active insulin is unknown. These aggregates may also invoke an unwanted and, perhaps, dangerous immune response.⁷⁶

2. Immunogenicity

Before the development of recombinant human insulin, insulin was obtained from animals (e.g., cattle and pigs). These animal-derived forms were immunogenic even after extensive purification.⁷⁷ However, the cause of this immunogenicity has never been clearly determined and it is likely that both the differences in the primary sequence of the human and animal-derived insulin and the integrity, degradation, and aggregation of the extracted animal forms invoked an immune response. Investigations of the relationship between aggregation and immune response have been performed with bovine serum albumin. These studies demonstrated that the administration of aggregated protein resulted in an increased immune response and the production of antibodies with a reduced affinity for the native protein.⁷⁸ Another recombinant therapeutic protein, hGH, has been studied for its immunogenicity as a function of aggregation. Initial therapies for hypopituitary dwarfism utilized hGH extracted from the pituitaries of cadavers. The extracted protein often contained aggregated or degraded forms and these preparations produced measurable amounts of antibodies to hGH after a single administration. To assess the effect of hGH aggregation on immune response, a study was conducted with hGH in both the aggregated and monomeric forms. This study

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indicated that aggregates of hGH were the primary cause of immunogenicity in patients.⁷⁹ Another study of hGH aggregation involved the administration of either the monomer or dimer of hGH. Both the dimer and monomer yielded equally low incidence of immunogenicity and the growth response was reported as equivalent.⁸⁰ *In vivo* monomeric growth hormone binds and causes the dimerization of two growth hormone receptors.⁸¹ Therefore, the dimer of hGH must dissociate to have a biological effect. In addition, the presence of antibodies to hGH may not alter the effectiveness of the protein.⁸² These results suggest that immunogenicity, although undesirable, does not always diminish the efficacy of a protein therapeutic. The major concern in developing an immunogenic response to the therapeutic protein is the effect of this response on the patient's own protein (e.g., autoimmunity). Further research on the aggregation of other therapeutic proteins continues to provide a better understanding of the role of aggregation in the immune response.

C. Effects of Excipients on Protein Aggregation

To prevent the formation of aggregates that can cause undesirable immunogenicity or altered half-life, the protein formulation usually contains buffer components and excipients. Because the solvent environment dictates the ability of the protein to maintain its native state and avoid denaturation from physical forces such as heat or agitation, different excipients are empirically tested for their ability to stabilize the protein. These excipients can range from salts to surfactants. Excipients and buffer components added to solutions to increase the stability or solubility of the protein are often referred to as cosolvents because they affect the solvation of the protein.

1. Cosolvent Effects

The thermodynamics of solvent interactions with proteins has been studied for a variety of different proteins and cosolvents.⁸³⁻⁸⁵ These studies have shown that cosolvents may affect the physical stability of proteins by two different mechanisms. One mechanism is the preferential hydration of the protein, which is defined as the exclusion of the cosolvent from the protein surface. The cosolvent is excluded from the protein surface as the result of either inert steric exclusion effects (e.g., polyethylene glycols), an increase in the surface tension of water (e.g., sugars, some salts, and amino acids), or chemical incompatibility with the protein surface (e.g., charge repulsion, 2-methyl-2,4-pentanediol, MPD).⁸³ In each case, the protein does not interact with the cosolvent and becomes fully hydrated, surrounded by a cosolvent-free layer of water. The application of cosolvents that cause preferential hydration of the protein stabilizes compact protein conforma-

tions because cosolvent addition results in an increase in the free energy of the system (increased surface tension). To reduce the free energy of the system, the solvent-accessible surface area of the protein is minimized. These effects are described by a thermodynamic analysis of the system. The chemical potential of the protein ($\delta\mu_p/\delta m_p$) changes as the result of cosolvent addition and this change is defined by the product of the change in the chemical potential of the cosolvent ($\delta\mu_s/\delta m_s$) and the preferential interaction of the cosolvent with the protein ($\delta m_s/\delta m_p$):

$$\left[\frac{\delta\mu_p}{\delta m_s} \right]_{T,P,m_p} = - \left[\frac{\delta m_s}{\delta m_p} \right]_{T,\mu_w,\mu_s} \left[\frac{\delta\mu_s}{\delta m_s} \right]_{T,P,m_p} \quad (6)$$

where μ is the chemical potential, m is the molality, T is the system temperature, P is the system pressure, and the subscripts denote protein (p), cosolvent (s), and water (w). The preferential interaction term ($\delta m_s/\delta m_p$) has been measured and is used to describe interactions between several cosolvents and proteins.⁸³⁻⁸⁹ If the preferential interaction term is negative, the cosolvent causes preferential hydration of the protein or, in other words, the binding interaction is negative. A positive preferential interaction is the other major mechanism of cosolvent action where the cosolvent binds to the protein surface.⁸⁴ Cosolvent binding is either nonspecific or specific to sites on the protein surface.⁸³ Denaturation studies have shown that many denaturants do not bind specific sites, but instead cause global changes in the protein conformation.^{90,91}

Either cosolvent binding or preferential hydration have been observed for several cosolvents and proteins and a summary of each cosolvent effect is shown in Figure 1. The classification of cosolvents into these two distinct categories, binding and hydration, is often difficult because several additional factors influence their behavior.⁹² Concentration effects can alter the solution behavior of many cosolvents. For example, polyethylene glycol (PEG) at high concentrations (>10% w/v) exerts a nonspecific negative preferential interaction with proteins causing precipitation.⁸⁷ On the other hand, PEG at low concentrations (<1% w/v) has been observed to bind to hydrophobic surfaces on denatured proteins.⁹² The major difficulty in analyzing the stabilizing effect of these solvents is the inability to separate denaturation events from physical changes in the solvent environment. The interplay between thermodynamic changes in the solvent environment and alterations in the protein conformation determines the stability of a protein in a given solvent. The two-state equilibrium denaturation model described in Equation 1 is combined with Equation 6 to describe the thermodynamic contribution of the solvent environment to denaturation of the protein:

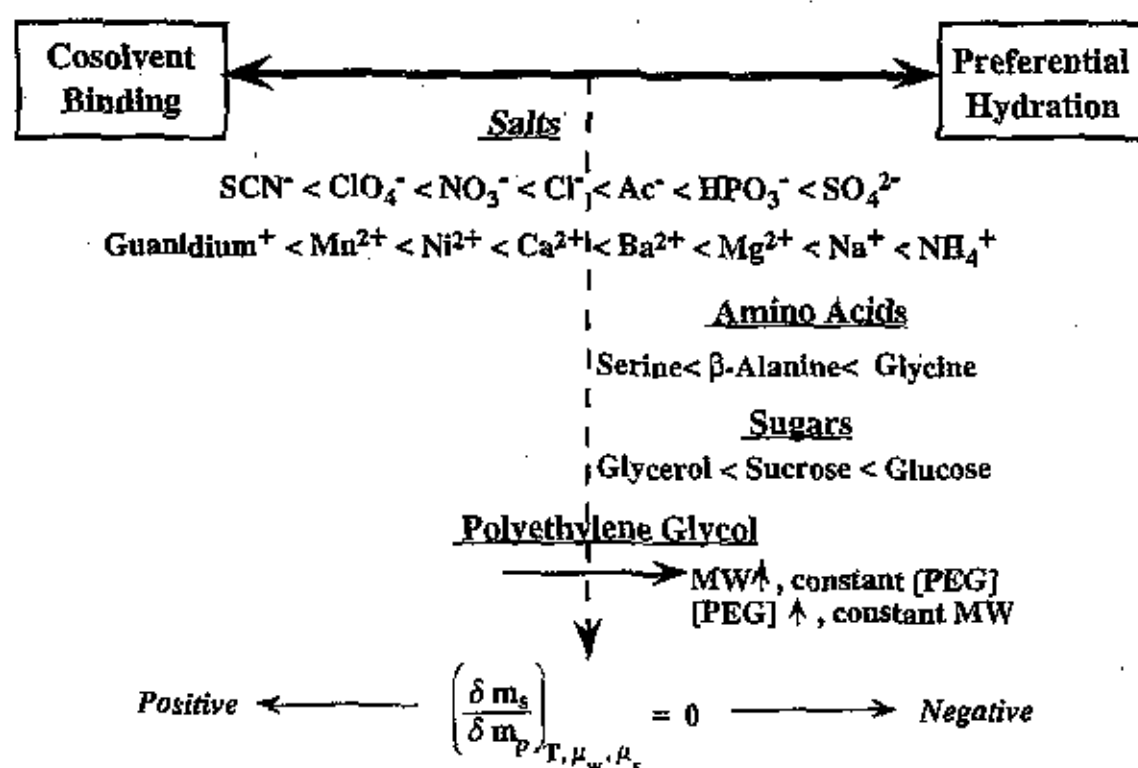


FIGURE 1. Cosolvent interaction diagram for native proteins.^{84,86-88,92} The cosolvents to the left of the middle arrow have been shown to bind nonspecifically to several proteins (positive preferential cosolvent interactions). The cosolvents to the right of the middle arrow have been observed to act by preferential hydration of proteins resulting in a negative preferential interaction. The mechanism of interaction for PEG is dependent upon the PEG concentration and molecular weight as discussed in the text.

$$\left[\frac{d \ln K_{ND}}{d \ln a_s} \right]_{T, P, m_p} = \left[\frac{\delta m_s}{\delta m_p} \right]_{T, \mu_w, \mu_s}^D - \left[\frac{\delta m_s}{\delta m_p} \right]_{T, \mu_w, \mu_s}^N \quad (7)$$

The denaturation equilibrium constant, K_{ND} , as defined in Equations 3 and 4, is related to the preferential interaction of the cosolvents with each state: the denatured, D, or native, N, conformation. The nonideality of the cosolvent in the aqueous solution is expressed as the activity coefficient of the cosolvent, a_s , which is related to the change in chemical potential of the solution upon addition of cosolvent ($\delta\mu_s/\delta m_p$). If the cosolvent interacts with the native state by preferential exclusion from the protein surface, it will reduce the solubility of the protein, assuming that the protein maintains its native conformation in the presence of the cosolvent. In contrast, Equation 7 describes mathematically the delicate balance between binding and exclusion that must be applied to reduce or eliminate denaturation. Thus, cosolvents that provide the greatest stability

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against denaturation and aggregation should bind weakly to proteins without perturbing their tertiary structure and should provide a lower free energy state for the native protein than its denatured forms. Several examples of cosolvents that balance these interactions have been described for protein folding^{92,93} and stability studies.^{1,83} These results indicate that each protein will behave differently in the presence of cosolvents, but the general scheme shown in Figure 1 is applicable for many proteins.

2. The Role of Surfactants

Although surfactants are generally considered cosolvents, they display several unique features that are not observed for other cosolvents. For example, many ionic surfactants, such as SDS, cause denaturation of proteins at relatively low concentrations (<0.1% w/v). Nonionic surfactants do not usually cause denaturation even at moderate concentrations (1% w/v). The most common nonionic surfactants originate from either the polysorbate or polyether families. The polysorbate compounds (e.g., Tweens) consist of a sorbitol-polyethylene oxide head group and a hydrocarbon tail. The typical polyethers are PEG and polyethylene oxide-polypropylene oxide block copolymers (e.g., Pluronic[®]). With the exception of PEG, these polymers have the distinct surfactant characteristics of both hydrophilic and hydrophobic domains.

The pharmaceutical industry utilizes a wide variety of surfactants. Nonionic and ionic surfactants have been approved for use in parenteral pharmaceutical formulations. Surfactants were originally added to many formulations to increase protein solubility or stability (see Section II.A.1). Surfactants can assemble at hydrophobic interfaces such as air-liquid or solid-liquid interfaces. In addition, surfactants reduce the surface tension of the solution and thereby decrease the free energy of the system. As the free energy of the system is reduced, the protein-protein and protein-surface interactions are reduced. Proteins are not preferentially hydrated by low concentrations of surfactants (<1% w/v). As discussed in Section II.C.1, this effect is dependent upon the surfactant concentration as observed for PEG interactions. The major rationale for using surfactants in protein formulations is their ability to bind to hydrophobic interfaces that are potential denaturation sites. Recent studies show that surfactants act at hydrophobic container surfaces,⁵⁷⁻⁶¹ air-liquid interfaces,⁶²⁻⁶⁶ and hydrophobic surfaces on the protein.^{29,92,94} By binding to the protein's exposed hydrophobic surface, surfactants reduce the hydrophobic interactions between solvated protein molecules, thus reducing aggregation.^{29,94} The dual action of surfactants provides a mechanism for preventing or reducing surface-induced denaturation, as well as protein-protein interactions.

The choice of surfactant is dependent upon the physical characteristics of the protein, the system (e.g., container, head space, etc.), and the presence of other cosolvents. Several studies have investigated the ability of surfactants to prevent

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denaturation and subsequent nonspecific aggregation of insulin.⁵⁷⁻⁶¹ These studies show that the type and concentration of surfactant necessary to inhibit aggregation can vary with test conditions and the system studied. In addition, the presence of other cosolvents may alter the physical characteristics of the surfactants. For example, addition of cosolvents that cause preferential hydration of the protein (e.g., sugars) may also cause hydration of the surfactant, which results in a phase separation (aqueous/surfactant or aqueous/solid). The concentration of surfactant used in the formulation may also alter its interactions with the protein. Most nonionic surfactants, especially polysorbates, have a relatively low critical micelle concentration (<0.1% w/v) and, therefore, exist in micellar form in many formulations. These micelles exist in equilibrium with the monomeric surfactant molecules. The protein can interact with either the micellar structure or the monomeric surfactant molecules. The system becomes extremely complex when the formulations contain multiple components or surfactants at concentrations above their critical micelle concentration. Future studies should provide additional evidence that surfactants bind to native⁵⁷⁻⁶¹ as well as denatured proteins^{29,34} and this research, combined with an understanding of the solution properties of surfactants, should provide some fundamental rules for the use of surfactants in protein formulations.

3. Recent Discoveries

Formulation research has lead to the discovery of new excipients that can provide increased solubility and stability for pharmaceuticals. These excipients must meet the requirements of aqueous solubility, nontoxicity, and nonreactivity. In addition, the excipient should stabilize the native protein conformation and prevent nonspecific adsorption and aggregation. Rapid clearance from the body and lack of biological effects are also required for suitable excipients. Advances in the development of new excipients would include improvements in ease of use, increased stability at room temperature, and unrestricted administration (delivery route or dosage). Although many of the cosolvents discussed in the previous section and listed in Figure 1 are used as excipients in approved pharmaceutical applications, these excipients usually do not meet all of these criteria when used alone. Therefore, a combination of excipients is often used to take advantage of the characteristics of each excipient. The characteristics of these complex mixtures are often difficult to measure and describe in a theoretical context. Thus, many protein formulations contain excipient combinations that are chosen on the basis of the formulation scientist's experience.

Recently, cyclodextrins have been extensively studied in protein formulations because they have many desirable physicochemical properties. Development of toxicology data and industrial processes have increased the potential use of cyclodextrins. Cyclodextrins are unique molecules that have been studied for their

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ability to microencapsulate small molecules. Cyclodextrins have a "donut-like" structure of six (alpha), seven (beta), or eight (gamma) glucopyranose units (for reviews, see References 95-97). These compounds have been used in diagnostic kits for several years and have only recently been studied for use as pharmaceutical excipients.⁹⁵ Cyclodextrins have been postulated to decrease aggregation and nonspecific adsorption by specifically interacting with the hydrophobic sites on proteins.⁹⁶ If the cyclodextrins bind to hydrophobic residues exposed on the protein surface, they should inhibit association reactions that are driven by hydrophobic interactions. These excipients have a particular affinity for aromatic amino acids and may encapsulate them in the inner core of the cyclodextrin.⁹⁵ It seems unlikely that cyclodextrin binding of aromatic residues would increase protein solubility and stability because few native proteins have solvent exposed aromatic groups. Several proteins have been studied in formulations containing 2-hydroxypropyl- β -cyclodextrin (HPCD). In each case, HPCD increased protein solubility and stability. When HPCD was used in elevated temperature stability studies, protein aggregation was inhibited and the proteins (interleukin-2, tumor necrosis factor, macrophage colony stimulating factor, insulin, and growth hormone) were maintained in their native conformation.^{95,96} For lyophilization, HPCD also provided improved reconstitution characteristics and prevented aggregation of tumor necrosis factor⁹⁸ and interleukin-2.⁹⁹ The nontoxicity and stability properties of cyclodextrins justify further studies of these excipients to understand the mechanisms of their interaction with proteins. One drawback of their use, however, is the relatively high concentrations (10 to 20%) needed to effect stabilization.

Trehalose also has many desirable physicochemical properties. It is similar in composition to the other disaccharides (e.g., maltose) commonly used in protein formulations, but it has a unique structure: α -D-glucopyranosyl- α -D-glucopyranoside. This structure results in a physical orientation of hydroxyl groups that is distinct from other disaccharides of glucose (α , β or 1,4 linkages). This difference in structure may cause trehalose to have distinct physical properties. The fundamental basis for stabilization of proteins by trehalose has been recently debated.^{100,101} The two hypotheses for trehalose stabilization of proteins are the "water-substitute" and "glassy state" models. Both of these models describe the effect of trehalose on the stabilization of dried protein. The water-substitute hypothesis postulates that trehalose stabilizes dried proteins by hydrating the protein at sites previously occupied by water.¹⁰¹ On the other hand, the glassy state hypothesis suggests that trehalose stabilization is the result of an increased glass transition temperature ($T_g = 77^\circ\text{C}$, see Section II.D). To date, the mechanism for enhanced stability of dried proteins in trehalose formulations is not well understood and there have not been any published studies on the use of trehalose in aqueous formulations. However, trehalose has been shown to provide enhanced stability of proteins when compared to several other excipients. In particular, trehalose prevents aggregation of proteins in the dried or glassy state at elevated temperatures ($>25^\circ\text{C}$).^{100,101}

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For example, restriction endonucleases, commonly used for genetic engineering, have been dried in trehalose and have maintained activity after storage at elevated temperatures (55°C) for several months. In contrast, formulations with other saccharides (e.g., sucrose, mannitol, and lactose) did not preserve the proteins under these conditions.¹⁰⁰ The application of trehalose to formulations has primarily been for stabilization during drying.^{100,101} More recent studies have shown that trehalose does not provide adequate protection against denaturation caused by freezing, but trehalose can prevent dehydration-induced denaturation.¹⁰² These studies indicate that trehalose is a good lyoprotectant that preserves protein during drying, but not a good cryoprotectant (e.g., PEG).^{102,103} Most commercial applications of trehalose have focused on ambient temperature drying and, thus, have avoided the freezing process.¹⁰⁰ Since trehalose appears to function as a water-substitute, causing hydration of proteins, it should also find utility in aqueous formulations where additional hydration of the protein may prevent denaturation (see Section II.C.1). Further investigations of this excipient in both aqueous and lyophilized formulations should yield insight into its ability to enhance protein stability. These studies, along with toxicology data, could result in the use of this excipient in pharmaceuticals.

D. Lyophilization Induced Aggregation

The formulation scientist often has to choose between the development of either a solid or liquid formulation. The most common method for producing a dry final protein product is lyophilization. Often, the preferred protein formulation is a lyophilized solid because many of the chemical degradation reactions are retarded (see chemical degradation sections) and the potential for denaturation during handling and storage is eliminated. Lyophilization is a complex process and usually involves three main steps: freezing, primary drying, and secondary drying. The thermodynamics and process variables of the lyophilization process determine the rate of final product formation (dry solid) and the characteristics of the final product.¹⁰⁴⁻¹⁰⁶ Unfortunately, lyophilization can often result in the denaturation of the protein. Recent studies utilized Fourier-transform infrared (FTIR) spectroscopy to assess the changes in conformations of proteins in the dry state.^{103,107} These experiments suggest that the dehydration of a protein can cause a measurable conformational change.¹⁰⁷ In addition to having an impact on the final state of the protein, lyophilization can affect the protein stability. For example, when hGH was lyophilized with a rapid freezing cycle, insoluble precipitates were observed upon reconstitution and the extent of precipitation was dependent upon the pH of the formulation.¹⁰⁸ Thus, the major factors affecting the state of the lyophilized protein are the residual moisture content, formulation components (e.g., excipients), and the lyophilization process.

1. Residual Moisture Effects

Initial studies of cryobiology revealed that the residual moisture and head space gases in a vial have an impact on the structure and stability of the dried protein. These studies demonstrated that there was an optimum residual moisture required to maintain the stability of the formulation of several proteins.¹⁰⁹ More recent studies have applied first principles to determine the theoretical amount of water required to maintain a protein in the fully hydrated state — a complete monolayer of water molecules. These studies focused on the fundamentals developed in gas adsorption for determining the monolayer at the solid surface in heterogeneous systems (e.g., gas-solid). Removal of the hydration monolayer in the absence of excipients resulted in aggregation of the protein, but maintaining an excess amount of water resulted in a loss of biological activity.¹¹⁰ Additional studies have investigated the moisture-dependent aggregation of several lyophilized proteins (see, e.g., References 111 and 112). The effects of residual moisture were studied for human growth hormone formulations. The most stable conditions were glycine/mannitol formulations with a low water content to minimize aggregation. In contrast, excipient-free formulations were more stable at higher water contents where a hydration monolayer exists around the protein.¹¹² These results further support the hypothesis that the protein must remain hydrated either by having increased residual moisture levels or adding water-substituting excipients. Therefore, every formulation and protein will have an optimum moisture content to minimize aggregation. After determining the optimum residual moisture, the maintenance of this level becomes critical to the stability of the dried formulation. The most critical variable is the stopper configuration and composition. The ability of different stoppers to contribute moisture to the final product is dependent upon the composition of the stopper. A number of elastomeric stopper components (e.g., 9310/50 Gray) have been designed to have low moisture absorption.¹¹³ Finally, by determining the appropriate residual moisture and vial configuration, one major component of the lyophilized final product is defined.

2. Excipient Stabilization During Lyophilization

Another important variable in the development of a stable lyophilized formulation is the amount and type of excipients used in the formulation (for reviews, see References 114–116). The role of excipients in lyophilization has been attributed to two different potential mechanisms. One potential mechanism of excipient stabilization of lyophilized protein is the change in the glass transition temperature (T_g) of the protein mixture upon addition of excipients.¹⁰⁵ The ability of an excipient to increase the T_g of a lyophilized protein formulation has been hypothesized to prevent denaturation and aggregation of proteins in the solid state by maintaining the solid in an amorphous state at high temperatures.^{100,105} Another

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mechanism postulated for the stabilizing effect of excipients is commonly referred to as water-substitution.^{101,114-117} The excipients that can act as water-substitutes often allow for a reduction in the residual moisture and provide increased stability of the protein. In addition, many of these excipients (e.g., mannitol) are required to produce a dry cake of high quality and excipients are often added to produce a uniform powder that is easily reconstituted. To assess the effect of additives on stabilization during drying, a model protein, poly-L-lysine, was studied by FTIR after lyophilization from different formulations.¹⁰⁷ These experiments, along with similar studies of lactate dehydrogenase and phosphofructokinase lyophilization, revealed a correlation between denaturation and formulation composition.^{103,107} The formulations containing both a lyoprotectant (e.g., sugars) and a cryoprotectant (e.g., PEG) yielded the least denaturation of the proteins. Any reduction in unfolding during drying resulted in improved reconstitution of the proteins. The addition of a hydrating cosolvent (e.g., mannitol) or the surfactant (PEG) alone did not prevent denaturation during drying.¹⁰³ Thus, to minimize denaturation during lyophilization, the formulation must contain both cryoprotectants and lyoprotectants. The detailed mechanism of interaction for these excipients is not known. Since the PEG concentration used in this study was 1% w/w, it may either act as a nonspecific exclusion agent or bind to hydrophobic surface residues. If a nonspecific exclusion mechanism is assumed for PEG, then the sugars must act as water-substitutes to stabilize the protein structure. However, to distinguish between these mechanisms, lower concentrations of PEG should be used to assess the possibility of surfactant-protein interactions. Other studies have focused on the effects of different excipients on therapeutic proteins. In many formulations, salts are added to control the osmolality of the solution. Lyophilization in the presence of sodium chloride has caused several proteins to precipitate.^{114,115} The effect of sodium chloride on the freezing process (freezing point depression) may cause the observed aggregation¹¹⁶ or an increased binding of the salt to the protein may occur during lyophilization.^{83,117} To circumvent these difficulties, mannitol or other sugars are often used as isotonicifiers. Further examples of other excipients have been presented in an extensive study of several excipients. This study revealed a correlation between stability and preferential interaction mechanisms of cosolvents that was analogous to the relationships observed in aqueous solutions (see Section II.C.1 and Figure 1).^{103,107,113,115,118} However, similar analyses of other protein systems should be performed to validate this correlation and assess the potential relationship between changes in the T_g of a protein formulation and its stability.

3. Changes in Local Environments During Freezing

In addition to excipient effects, local changes in the solvent environment during freezing may also affect the stability of proteins. One major change is the crystallization of excipients or buffer components with subsequent changes in pH.

Crystallization of excipients can result in a partitioning of the protein and excipient into separate crystalline states and thereby reduce any interaction between them.¹¹⁴ During freezing, buffer components may also crystallize, which results in a dramatic decrease in pH.^{105,114} In particular, disodium phosphate crystallizes during freezing and this results in a large decrease in pH whereas potassium phosphate crystallization does not cause a significant change in pH. For solutions that contain the buffer as a minor component, the buffer may not crystallize and alter the pH.¹¹⁴ If the solution pH changes during freezing, the protein may become denatured and aggregate upon reconstitution. A correlation between freeze-thaw stability and pH was determined for human growth hormone where a slight change in pH (0.4 pH units) significantly reduced aggregation.¹⁰⁸ Because the lyophilization process occurs in the solid state at equilibrium conditions (i.e., stepwise changes over long times), the protein can achieve a denatured state that is thermodynamically stable. However, the rapid reconstitution of the dried protein does not allow the protein to proceed through the same series of equilibrium states to return to the native conformation. Therefore, upon reconstitution, the denatured protein will often self-associate before it can refold to the native state. In addition to buffer components, local changes in excipient concentrations can also occur. Studies of lyophilized human growth hormone formulations have shown that the relative amounts of excipient and protein vary with location within the dry cake.¹¹⁹ The lyophilization process and choice of formulation components can dramatically affect the stability of the protein. Conditions for lyophilization must be based upon the buffer composition and concentration to prevent crystallization and pH changes that can denature the protein. The lyophilization conditions must also account for potential crystallization of excipients and the subsequent changes in the excipient-protein interactions.

Protein aggregation in solution or during lyophilization has a significant impact on product quality, efficacy, and immunogenicity. Changes in protein conformation can result in protein aggregation. Alteration of protein conformation and subsequent aggregation can also cause chemical degradation of the protein. Chemical degradation can involve covalent modifications of amino acid residues. Of these covalent modifications, deamidation and oxidation are the most common types of chemical modifications that occur in proteins. In the following sections, the nonenzymatic deamidation of glutamine (Gln) and asparagine (Asn) residues and the oxidation of methionine (Met), cysteine (Cys), histidine (His), and tryptophan (Trp) residues in proteins are described. The impact of this degradation on the development of protein pharmaceuticals is also discussed.

III. PROTEIN DEAMIDATION AND FORMULATION STABILITY

Nonenzymatic deamidation of Asn and Gln can occur in proteins. The deamidation of Asn and Gln is a hydrolysis reaction requiring water, and can often occur at rapid rates under physiological conditions. This reaction has always been

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considered somewhat unique when compared to other chemical modifications that occur in proteins because the resulting products are the naturally occurring amino acid residues, aspartic (Asp) and glutamic acid (Glu).^{120,121} It was assumed that the deamidation reactions observed in proteins were a consequence of the extraction and detection procedures used in the isolation of proteins. The first careful studies of protein deamidation, using cytochrome C, showed that protein deamidation could occur *in vivo*.¹²² In addition to undergoing a simple deamidation to give the carboxylate, Asn can deamidate and undergo an isomerization that yields an isoaspartic acid residue.^{123,124} Although this is a nonnatural amino acid replacement, an effective repair mechanism may have evolved that utilizes carboxyl methyltransferase.¹²⁵ This enzyme methylates isoaspartyl residues specifically, and it has been proposed that this methylation can reverse the isomerization to yield the normal alpha-Asp residue.¹²⁶ That this enzyme exists and may function as a repair mechanism, along with the observation that proteins can deamidate *in vivo*, suggests that protein deamidation may have an important biological role. This degradation mechanism may have profound effects on protein clearance rates and effective delivery of the protein pharmaceutical. This section discusses some of the mechanisms involved in deamidation, the consequences of this interesting reaction, and some of the strategies used to minimize this degradation in the formulated protein.

A. Mechanisms of Protein Deamidation

1. General Rules

Some of the first careful studies of deamidation on peptides were performed by Robinson and co-workers and presented in a series of articles that are summarized in a 1974 review.¹²⁰ Deamidation rates were measured for a number of model tetra- and pentapeptides of different amino acid sequences. At 37°C, a broad range of deamidation half-times ($t_{1/2}$) was observed, ranging from 6 to 3407 days. Generally, the Gln peptides are less prone to deamidation than the Asn peptides as reflected in the greater $t_{1/2}$ values for the Gln peptides, although there is some overlap in the measured $t_{1/2}$ values of Asn- and Gln-containing peptides.

The determined variation in rates as a function of sequence enabled Robinson et al.^{120,127} to make a number of important observations regarding the role of amino acid sequence on deamidation. Polar residues preceding Asn and Gln residues (attached to the amino terminus of Asn or Gln) resulted in enhanced rates of deamidation, whereas bulky hydrophobic residues resulted in decreased rates. Interestingly, neighboring serine (Ser) and threonine (Thr) residues also increased the rate of deamidation. As already mentioned, deamidation may serve as a biological regulator of clearance mechanisms. Glycosylation of proteins at putative Asn sites requires the sequence Asn-X-Thr or Ser (where X is any amino acid), and this may be a possible control element to prevent deamidation at particular sites in

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the protein.¹²¹ It has not been determined whether further control of the regulatory process is mediated by loss of carbohydrate from the N-linked Asn sites.

Robinson et al.¹²⁰ attempted to relate amino acid sequence to rates of deamidation in an effort to predict which sites are susceptible to deamidation. Although the general rules proposed can be interpreted by the mechanisms elucidated for hydrolysis in amides of peptides, the ability to predict deamidation "hot spots" in a protein is somewhat limited because protein conformation affects deamidation kinetics. The mechanisms involved have been reviewed by Wright¹²¹ and are summarized below.

2. General Acid-Base Catalysis

Deamidation of Asn and Gln residues is an acid- and base-catalyzed hydrolysis reaction. The acid-catalyzed step involves protonation of the amide leaving group. The base-catalyzed step involves nucleophilic attack of the amide carbonyl carbon by hydroxide or conjugate base (the general base not only may generate hydroxide from a water molecule but also may activate another nucleophile by abstraction of a proton). The order of the acid- and base-catalyzed steps is determined by reaction conditions such as the pH of the solution. The transition state of this reaction is believed to be an oxyanion tetrahedral intermediate that can be stabilized by proton donors, leading to an enhanced rate of deamidation (Figure 2). Because deamidation is both acid- and base-catalyzed, there is a particular pH where the reaction rate is minimized. This conclusion is supported by studies of deamidation in Gln-containing pentapeptides¹²⁸ and cytochrome C¹²⁹ where the pH of maximum stability for the Asn and Gln residues was approximately pH 6.

The observations discussed previously have been interpreted using this general acid/base-catalyzed mechanism.¹³⁰ Amino acid residues adjacent to the Gln or Asn residue can function as general acid groups (Thr or Ser) by providing a proton in the acid-catalyzed step. Other amino acids may act as nucleophiles (Asp, Glu, and His at neutral pH), by attacking the amide carbonyl carbon. Proton donors such as Thr, Ser, and protonated lysine (Lys) and arginine (Arg), stabilize the oxyanion transition state, which accelerates the deamidation reaction.

Sequence plays a major role in governing deamidation rates in peptides. However, the conformation and flexibility of polypeptide chains in a folded protein may bring potential catalytic residues from distant regions in the sequence into close proximity of the deamidating amide side chain.¹³⁰ Alternatively, constraints on the main-chain conformation by hydrogen bonding may inhibit the deamidation of particular Asn residues in proteins.¹³¹ It has also been suggested that potential catalytic side groups may be prevented from participating in the deamidation reaction because of hydrogen bonding or interactions with cofactors or ligands.¹²¹

As discussed previously, Asn residues tend to be more prone to deamidation than Gln. However, Gln reacts faster than Asn when Gln is at the N-terminus of

the polypeptide chain. In this case, the amide carbonyl carbon undergoes nucleophilic attack by the N-terminal amino group, which results in the cyclical pyrrolidone carboxylic acid¹³² (Figure 3). Such a reaction takes place in human relaxin A chain, where the N-terminal amino acid is a Gln residue.¹³³ Asn is unable to undergo such a cyclization-deamidation process because the shorter side chain would cause an unfavorably strained ring structure. Recently, however, Lura and Schirch¹³⁴ found that Asn removed one residue from the N-terminus can form a cyclic deamidation product where again the N-terminal amine functions as the nucleophile. The deamidation of C-terminal Asn may also form a cyclic structure due to nucleophilic attack of the carbonyl carbon of the amide by the C-terminal carboxylate.¹³⁵⁻¹³⁷ However, unlike the deamidation reaction at the N-terminus, this cyclic anhydride structure is unstable and rapidly breaks down to aspartate.

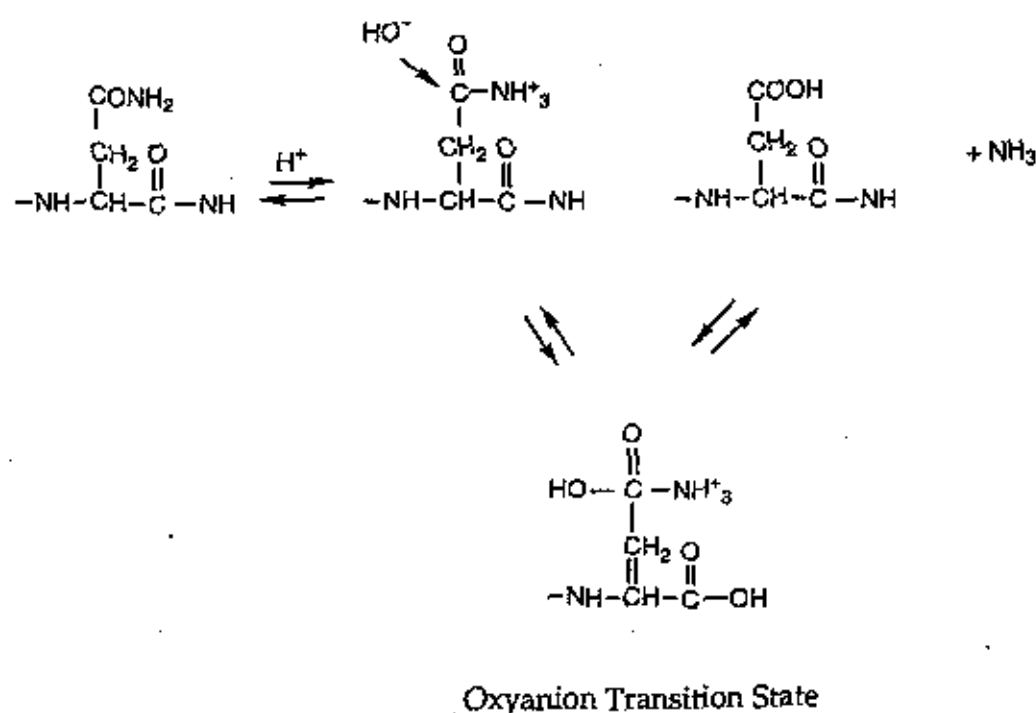


FIGURE 2. General acid and base catalysis of deamidation. The tetrahedral oxyanion intermediate is inferred to be the transition state. Stabilization of this structure by protein donors will increase the rate of the deamidation reaction.

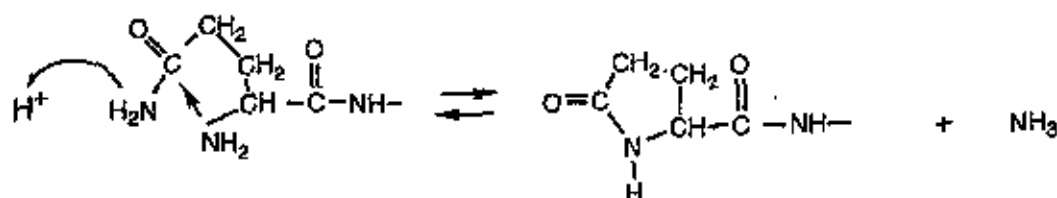


FIGURE 3. Deamidation of N-terminal Gln to form pyrrolidone carboxylic acid. The ring structure is formed after nucleophilic attack of the amide carboxyl by the N-terminal amine.

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Overall, in terms of the general acid/base-catalyzed mechanism, these interpretations provide a firm understanding of how adjacent amino acid residues modulate the deamidation reaction. What is not clear, however, is whether these interactions actually increase the rate of deamidation above its uncatalyzed value. Wright and Robinson¹³⁸ showed that the rates of deamidation of Asn and Gln in peptides were greatly enhanced over those determined for acetamide. They concluded that if Asn and Gln were incorporated into proteins, the result would be increased rates of deamidation, probably due to the stabilized oxyanion transition state and proximity to potential nucleophilic nitrogens in adjacent residues. In particular, main-chain amide -NH- groups function as hydrogen bond donors, and stabilize the tetrahedral oxyanion transition state (Figure 2). Wright and Robinson¹³⁸ proposed that the greater length of the Gln side chain compared to Asn makes the distance from the main-chain -NH- group to the negatively charged oxygen of the transition state too long to form good hydrogen bonds. Therefore, the formation of the oxyanion transition state from Asn is favored over Gln, which results in a faster deamidation rate for Asn. It has also been suggested that Gln residues are capable of immobilizing water molecules through hydrogen bonding to the side-chain amide and to peptide amide backbone proton donors or acceptors.¹³⁹ Asn residues are not as easily involved in such hydrogen bonding due to the shorter chain length of the side chain. It has been hypothesized that this ability to trap and immobilize water prevents interaction with bulk mobile water, which is required for the hydrolytic deamidation process.¹²¹

3. Deamidation and Isomerization via a Cyclic Imide Intermediate

In their original work, Robinson et al. demonstrated that large bulky hydrophobic groups on the N-terminal side of the amide residue resulted in low rates of deamidation.¹²⁰ These original studies did not investigate peptides that had glycine (Gly) residues next to the Asn. More recent measurements of peptides containing Asn-Gly sequences have extended the lower range for deamidation half-life to 1.4 days at 37°C.¹⁴⁰ Additional studies of Asn-Gly, Asn-Ser, and Asn-Ala sequences in peptide and proteins have shown that the Asn residue deamidates by formation of a cyclic imide intermediate. The cyclic imide is formed when the carbonyl carbon of the Asn side-chain amide undergoes nucleophilic attack by the main-chain peptide nitrogen from the adjacent C-terminal residue (Figure 4). The cyclic imide forms most readily when the C-terminal residue is Gly, and less so if it is Ser or Ala, presumably because the small size of Gly does not interfere with the formation of the cyclic structure. The deamidation of Gln residues is not expected to proceed by this mechanism because the extra carbon in the Gln side chain makes formation of a cyclic imide intermediate very unfavorable. The formation of cyclic imides from Asn-containing peptides has been demonstrated.^{124,141} The breakdown of the imide

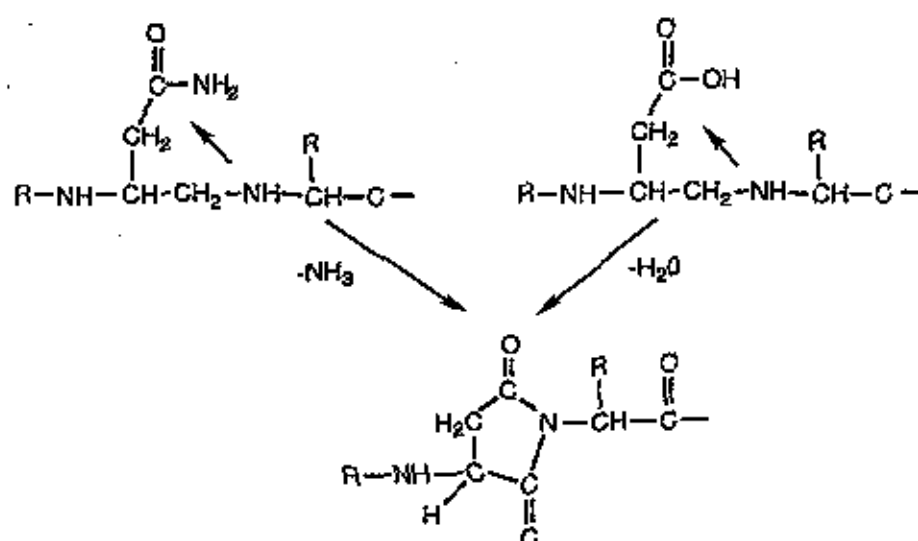


FIGURE 4. Formation of a cyclic imide as a result of nucleophilic attack by main-chain amide nitrogen on the carboxyl carbon of either the side-chain amide or carboxylic acid groups.

is itself base-catalyzed, which involves hydrolysis of either one of the nitrogen-carbonyl bonds of the imide. Depending on which bond is hydrolyzed, the imide yields either the normal α -linked or isomerized β -linked Asp peptide^{121,142} (Figure 5). The hydrolysis reactions are completely reversible and therefore the final deamidated products are interconvertible through the cyclic imide intermediate.¹⁴⁰ Therefore, given the complete reversibility of the hydrolysis of the cyclic imide bonds, Asp-Gly sequences may also form imide intermediates that subsequently hydrolyze to iso-Asp-Gly. It has been shown that isopeptide formation is thermodynamically favored over the α -linked Asp peptide at equilibrium, usually in a 3:1 ratio^{121,142} and therefore isomerization of Asp-Gly sequences proceeds through the same mechanism as deamidation of Asp-Gly sequences.¹⁴⁰ Clark extended this mechanism to proteins¹⁴³ and showed that many of the Asx-Gly, Asx-Ser, or Asx-Ala sequences (Asx = Asp or Asn) that favor imide formation in peptides are unable to do so in proteins because in native proteins these residues exist in conformations where the peptide bond amide cannot approach the carbonyl carbon of the side-chain amide to form the imide. Conformation undoubtedly plays a major role in this mechanism, but it appears that flexibility of the polypeptide chain may be also very important, as shown by deamidation studies of ribonuclease^{144,145} where an Asn that ordinarily does not deamidate in the native structure deamidates in the denatured protein. Further support for the importance of the peptide chain flexibility is the deamidation of Asn¹⁴⁹ in the sequence Asp-Thr-Asn-Ser-His in recombinant hGH.¹⁴⁶ This deamidation appears to proceed through formation of the cyclic imide rather than nearest-neighbor catalysis. The crystal structure of hGH shows poorly ordered polypeptide conformation in the region of residues 147 to 153.¹⁴⁷

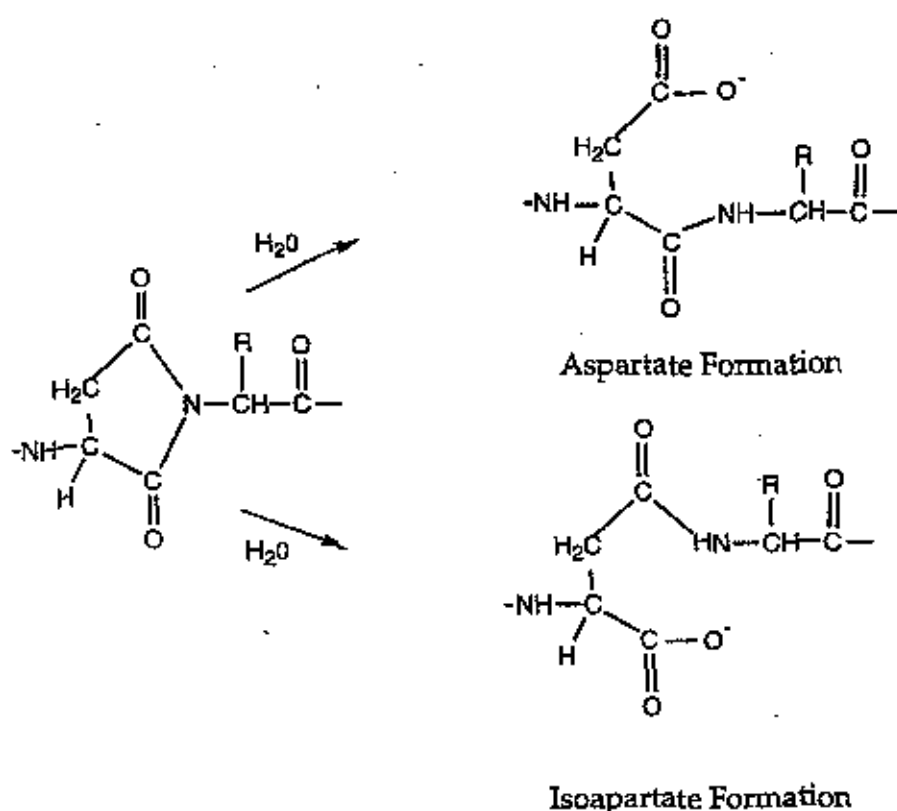


FIGURE 5. Formation of either aspartate or isoaspartate as a result of hydrolysis of either of the carbonyl nitrogen bonds in the cyclic imide.

In a recent study using the hexapeptide Val-Tyr-Pro-Asn-Gly-Ala, a fragment of adrenocorticotrophic hormone (ACTH), deamidation was strongly base-catalyzed above pH 5.¹⁴⁸ In fact, the pH of maximum stability was significantly lower (pH 3 to 4) than that observed for other amide-containing peptides and proteins including cytochrome C.¹²⁹ Deprotonation of the peptide amide nitrogen results in a more potent nucleophile, and therefore this reaction is expected to be highly base-catalyzed rather than acid/base-catalyzed, as in the general mechanism discussed previously. Below pH 4, the rate of deamidation of this peptide was much lower and probably proceeds through the general acid-catalyzed reaction, without isopeptide formation. Kinetic studies of an ACTH hexapeptide with Asp substituted for Asn¹⁴⁹ showed that the alpha-Asp hexapeptide is in equilibrium with the iso-Asp hexapeptide from pH 6 to 10, consistent with the imide intermediate mechanism. At highly acidic pH (<3), the predominant degradation products were the Val-Tyr-Pro-Asp and Gly-Ala peptides from hydrolysis of the Asp-Gly peptide bond. Significantly, the cyclic imide peptide was stable for up to 1400 h and constituted approximately 7% of the degradation products. As the pH increased, the formation of the cyclic imide-containing peptide became more predominant relative to the hydrolysis of the Asp-Gly bond. Although the cyclic imide-containing peptide was less stable at higher pH values, as much as 40% of the peptide contained the cyclic imide after 8000 h at

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pH 4. The rest of the peptide was distributed between the peptide bond hydrolysis products and the iso-Asp- and Asp-containing hexapeptides. These observations are completely consistent with recent protein studies showing that, under certain conditions, the cyclic imide intermediate forms as a degradation product. The first demonstration that a cyclic imide could be formed during storage was in preparations of freeze-dried recombinant methionyl growth hormone (r-met hGH) that were stored at 45°C for 4 months.¹⁵⁰ In r-met hGH, Asn¹²⁹ and Asp¹³⁰ residues have been shown to be the major sites for deamidation and isomerization, respectively.¹⁴⁶ Ion exchange chromatography and mass spectral analysis of tryptic peptides showed that Asp¹³⁰ formed a stable cyclic imide. The isolation of the imide was possible using ion exchange techniques because the negative charge of Asp¹³⁰ was lost upon formation of the cyclic imide. A clear demonstration that Asn¹²⁹ deamidated through the same route was not possible using ion exchange chromatography because formation of a cyclic imide from Asn does not result in a charge change. This, apparently, is not an isolated case because similar behavior was found in liquid formulations of recombinant porcine somatotropin (PST) at pH 5.0.¹⁵¹ After storage at 37°C for 72 h, approximately 30% of PST had an Asp-Gly sequence on the protein converted to the stable cyclic imide. The optimal pH for cyclic imide formation for PST was pH 5. Incubation of PST at pH 9 did not result in cyclic imide or iso-Asp formation. However, once the cyclic imide was formed, further incubation at pH 9 resulted in the disappearance of the cyclic imide. Although the formation of iso-Asp was not demonstrated in this particular experiment, incubation at pH 9 of a PST tryptic peptide that contained the cyclic imide yielded peptides with either Asp or iso-Asp. The tryptic peptides were generated by trypsin digestion of PST at pH 6, rather than at pH > 8, in order to prevent hydrolysis of the cyclic imide that formed during the storage of PST. The formation of a stable cyclic imide apparently is not restricted to Asp-Gly sequences in proteins, as shown by the recent work with recombinant hirudin, a potent inhibitor of human- α -thrombin.¹⁵² Isolation of two cyclic imide proteins, comprising ~1% of the total protein, was accomplished by using reversed phase chromatography. Mass spectral analysis of V8 protease digests showed that these cyclic imides formed from Asn-Gly sequences in hirudin.¹⁵³ The cyclic imide was present in the initial product, presumably before formulation, although the purified protein was not exposed to extremes of pH or temperature. Although the cyclic imide variants were not generated upon long-term storage, this work shows that the cyclic imide intermediates of Asn-Gly deamidation sequences are stable enough to be detected and isolated. This may have further consequences for formulation of proteins.

B. Consequences of Deamidation in Proteins

Although deamidation and aspartyl isomerization occurs in proteins, the protein formulator must determine the impact this has on the safety and efficacy

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of the protein drug. We refer the reader to Wright's review on the deamidation of specific proteins.¹²¹ Robinson and Rudd¹²⁰ also have summarized research until 1974 on the occurrence and some effects (especially biological specific activity) of deamidation of proteins. This section will draw from this body of knowledge in discussing specific issues of interest to researchers involved in formulation of protein drugs.

1. Activity

As with most degradation routes in proteins, it is impossible to generalize the effect of deamidation or cyclic imide formation on protein activity. There are many examples where deamidation has no effect on the activity of the protein, such as in the case of interleukin-1 α ,^{154,155} insulin,¹⁵⁶ aspartate aminotransferase,¹⁵⁷⁻¹⁶³ hGH,¹⁶⁴ bovine seminal ribonuclease,¹⁶⁵ and hirudin.¹⁵² On the other hand, proteins such as cytochrome C,¹²² interleukin-1 β ,^{166,167} lysozyme,¹⁶⁸ triosephosphate isomerase,¹⁶⁹⁻¹⁷¹ calmodulin,¹⁷² adrenocorticotropin,¹⁷³ soluble CD4,¹⁷⁴ OKT-3 monoclonal antibody,¹⁷⁵ and hGH releasing factor¹⁷⁶ exhibit decreased activity after deamidation. Thus, the effect on protein activity needs to be ascertained on a case-by-case basis. To further complicate matters, different activities of a protein may be affected differently by deamidation. For example, deamidation of murine epidermal growth factor (EGF) at a single site does not affect its binding to the EGF receptor or its mitogenic activity,¹⁷⁷ whereas deamidation eliminates EGF's immunosuppressive biological activity.¹⁷⁸ Thus, it is important to determine if all activities related to the therapeutic use of the protein are unaffected by the deamidation reaction.

2. Metabolic Half-Life

Deamidation may serve as the "molecular clock" that determines the metabolic *in-vivo* turnover time of proteins.¹⁷⁹ Recently, the metabolic half-lives in HeLa cells of 35 structurally characterized proteins were correlated with a number of physical and chemical properties.¹⁸⁰ Of all the properties examined, only Asn and Gln content showed a significant inverse correlation with the *in-vivo* half-life. Wright¹²¹ has discussed how deamidation at Gln and Asn might affect the clearance times of proteins by making the deamidated protein more susceptible to protease digestion, one of the first clearance mechanisms in the body. The main argument is that the enhanced deamidation rates in proteins relative to those in the free amino acids are greatly suppressed because of higher order structure (the enhancing effects of neighboring residues is discussed in Section III.A).¹³⁸ Increased flexibility or disruption of

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the polypeptide chain that includes the putative deamidation site may result in greatly increased rates of deamidation. Deamidation in proteins can be a cooperative process whereby the initial deamidation increases the rate of deamidation at additional sites. This cooperative process may involve a minor structural change that moves Asn closer to residues that catalyze the deamidation (as in bovine RNase),¹²¹ promote flexibility, or alter conformation in such a manner as to favor deamidation at a different Asn site that may be a substantial distance from the first site (as in cytochrome C).¹²¹ If this process ends up being highly cooperative, then further deamidation may lead to a more flexible polypeptide and ultimately to unfolding of the protein. The end result of this may be to shunt slightly misfolded forms of the protein into completely unfolded polypeptide chains that are more susceptible to proteolytic degradation. The inhibition of refolding of denatured proteins by deamidation makes unfolding irreversible and allows the degradation machinery to effectively clear the damaged proteins. If deamidation is involved in such clearance mechanisms, which may not be the case for all proteins, then controlling deamidation may be critical because clearance after administration may be much faster than for nondeamidated forms of the drug. Thus, even for proteins where activity is unaltered by deamidation, faster clearance of deamidated protein may result in loss in efficacy because of a more rapid removal of the protein from the body.

3. Protein Conformation

Deamidation of Asn and Gln results in the thermal denaturation of ribonuclease, lysozyme, and α -amylase becoming irreversible. In the case of ribonuclease, deamidation affects the folding kinetics.^{168,181,182} This is not surprising when one considers that a major charge change occurs at the site of modification. Increasing the net negative charge on the protein may alter the delicate balance of different forces that are responsible for maintaining the native conformation of a protein.¹⁸³ In addition to altering surface charge, protein conformation can be radically altered, especially if the reaction proceeds through the cyclic imide intermediate. Viscosity measurements of deamidated variants of carbonic anhydrase suggested that deamidation is responsible for changes in structure and hydration.¹⁸⁴ It is also possible that, even though gross changes in conformation are not detected, severe local perturbations in conformation can occur due to iso-Asp formation. The global conformation of recombinant bovine calbindin, as determined by 2D-NMR spectroscopy, is unperturbed after deamidation at Asn⁵⁶ and all elements of secondary structure are intact.¹⁸⁵ However, although the α -Asp form has identical conformation compared to nondeamidated protein, the iso-Asp form is significantly perturbed in the vicinity of the deamidation site, predominantly in the polypeptide segment Lys⁵⁵-Asp⁵⁸.

4. Self-Association and Aggregation

Denaturation of proteins and exposure of hydrophobic interiors is a major cause of aggregation as discussed previously. If irreversible unfolding of proteins occurs due to deamidation, it makes them more susceptible to irreversible aggregation. It is also possible that if deamidation does not result in unfolding of the protein, the tendency to aggregate and become insoluble may in fact be reduced because of the greater repulsion between molecules due to the increased net negative charge on the protein. Chemical modifications such as succinylation have been used to increase net negative charge and disrupt the association of multisubunit proteins.¹⁸⁶ Multisubunit proteins are highly susceptible to disruption of the quaternary structure as shown in the case of triosephosphate isomerase (TPI).^{169,187} Deamidation of Asn¹⁵ and Asn⁷¹ in both subunits results in subunit dissociation, presumably because of the close proximity of the deamidation sites at the subunit interface and the strong repulsion between the newly created negative charges. The resulting monomers are rapidly degraded by trypsin whereas the dimeric TPI is resistant to proteolysis.

5. Immunogenicity

Very little is known about the effects of deamidation on the immunogenicity of proteins. Deamidation to the α -Asp may have little effect on the immunogenicity of proteins, as in the case of hGH,¹⁶⁴ unless deamidation results in a conformational change and/or aggregate formation. It is possible that a charge alteration at a surface residue could result in a sequence that is recognized as a foreign epitope, which would render the protein immunogenic. The more drastic substitution of an isoaspartate may have an even greater impact on the immunogenicity of the protein. The inclusion of an extra methylene group in the carbon main-chain backbone of the protein may result in a local conformational change. The generation of newly exposed antigenic determinants as a result of deamidation could elicit immune responses.¹⁸⁸ It has been found that deamidated serum albumin is immunogenic, and it was suggested that the altered protein could induce autoimmune reactions.¹⁸⁹ On the other hand, although insulin deamidates by way of the cyclic imide pathway to form iso-Asp, the immunogenicity in rabbits is similar to that of the unmodified protein.¹⁹⁰ Altogether, these data suggest that the effects of deamidation on protein immunogenicity, as in the case of activity, needs to be addressed on a case-by-case basis. As more protein drugs are introduced into clinical trials, a greater understanding of the relationship between deamidation and immunogenicity will evolve. The use of transgenic animal models that express the human protein will allow researchers to test the immunogenicity of deamidated human recombinant proteins.¹⁹¹

C. Parameters that Affect and Strategies to Inhibit Protein Deamidation

It has been argued that many proteins deamidate *in-vivo*, giving both deamidated and cyclic imide products.¹³² Deamidated forms are also often generated by extraction and purification procedures used to isolate the protein from the organism. The impact on function, pharmacokinetics, and immunogenicity may be critical in determining whether a protein can be effectively delivered to the target tissues. Therefore, it is prudent to minimize the deamidation reaction as much as possible in the protein formulation. Some of the strategies that can be considered are now discussed.

1. Effect of pH

The mechanism of deamidation often dictates the best choice of pH. If deamidation proceeds by a general acid/base-catalyzed mechanism, then it may be possible to formulate at an optimum pH where both rates are minimized (for peptides ~pH 6). Deamidation that proceeds through the cyclic imide intermediate is base-catalyzed, and therefore lowering the pH should greatly reduce the rate of deamidation. This may be very difficult because other routes of degradation may predominate at lower pH. For example, lowering the pH in r-met hGH reduced the rate of deamidation but induced particulate formation.¹³² The presence of Asp-Gly sequences also make controlling deamidation by lowering pH more difficult. The rate of degradation of Asp-containing hexapeptide increases near pH 3 to 5.¹⁴⁸ If this also occurs for Asn-Gly sequences in proteins, then the key is to balance the formation of iso-Asp from Asn and Asp residues as a function of pH. In the neutral to basic pH range, where the formation of iso-Asp hexapeptide by the cyclic imide hexapeptide predominantly occurs, there was a fundamental difference in the degradation of the Asn-hexapeptide when compared to the Asp-hexapeptide.¹⁴⁸ The Asn-hexapeptide degraded by base and buffer catalysis, whereas the degradation rate of the Asp-hexapeptide was independent of pH and buffer ion. Oliya and Borchardt have suggested that this difference is due to the nature of the leaving group.¹⁴⁸ The carboxylate anion increases the electron density surrounding the carbonyl carbon center via resonance, making the carbonyl carbon less electrophilic and less susceptible to nucleophilic attack by the peptide amide nitrogen that is required for formation of the cyclic imide. As the pH of the solution increases, the equilibrium concentration of the deprotonated nucleophilic peptide amide increases, whereas the concentration of the more electrophilic protonated carboxyl group decreases. These effects appear to offset each other, resulting in a rate for the Asp-hexapeptide that is pH independent.

2. Effect of Temperature

The rate of deamidation in Gln-¹²⁸ and Asn-¹⁹³ containing pentapeptides increased at higher temperatures. The variation of rate constant with temperature for the Gln pentapeptides was dependent on the sequence of the pentapeptide. Scotchler and Robinson concluded that any useful generalizations for quantitative prediction of temperature dependence would require additional experiments.¹²⁸ More recent experiments with the hexapeptide Val-Thr-Pro-Asn-Gly-Ala showed that, at pH 5.0 and 7.5 and at 25 to 70°C, the rates of deamidation obeyed the Arrhenius equation ($E_A \approx 22$ kcal/mol at both pHs).¹⁹⁴ Determination of activation energies for protein deamidation is difficult because most of the proteins undergo a change in conformation at higher temperatures. Such was the case for formulation studies of interleukin-1 β where deamidation was the major route of degradation below 30°C and aggregation above 39°C.¹⁶⁶

3. Effect of Excipients

As early as 1942, researchers observed that the deamidation was markedly dependent on buffer anion.¹⁹⁵ Gilbert et al.¹⁹⁶ investigated the deamidation of Gln to Glu and pyrrolidone carboxylic acid in the presence of different sodium buffer salts at pH 8 and 37°C. The rates of deamidation were much faster in phosphate, arsenate, and bicarbonate, and slightly faster in phosphate and borate. There was no observed rate enhancement by sulfate, nitrate, acetate, chloride, or pyruvate anions. Determination of deamidation rates of the pentapeptides Gly-Thr-Asn-Glu-Gly and Gly-Arg-Asn-Arg-Gly as a function of buffer anion also showed that phosphate, borate, and other anions accelerated the rate of deamidation. In particular, phosphate anion appeared to accelerate the deamidation rate the most in comparison with the other anions studied. Buffer catalysis of the Asn-hexapeptide was also observed in the pH range of 7 to 11.¹⁹⁴ Recently, the deamidation reaction of N-terminal-blocked Boc-Asn-Gly-Gly was studied as a function of different salts, buffer anions, and organic solvents.¹⁹⁷ The addition of aprotic salts NaCl, MgSO₄, and Na₂SO₄ had little effect on the observed rate constants, whereas (NH₄)₂SO₄ and K₂HPO₄/KH₂PO₄ greatly enhanced the rate constants. The latter effect was assumed to be due to catalytic effect of these buffers, but an explanation for this effect based on mechanism was not provided. The catalytic effect at 5°C of the buffers Tris, H₂PO₄⁻, NH₃, CO₃⁼, and imidazole was tested with variable buffer anion concentration at constant 1 M ionic strength made up with NaCl. The rank ordering of enhancement of the deamidation rate was H₂PO₄⁻ >> CO₃⁼ $\approx$ imidazole > NH₃ > Tris. The deamidation rate decreased with the addition of organic solvents. The reduction in rate constant was directly proportional to concentration of the organic solvent, as well as the type of solvent. Many of the

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solvents explored were those customarily used in protein isolation and crystallization, and are unlikely to be used in formulation work. However, solvents such as ethanol reduced the rate of deamidation and so it may be possible to find other organic solvents that reduce the deamidation rate and still be pharmaceutically compatible. Similar experiments with polyethylene glycols (PEG 4000 and 8000) showed only a slight retardation of deamidation rate in these peptides as a function of PEG concentration. Also noted was that glycerol retarded the deamidation rate in gamma globulin and serum albumin preparations that had been thermally denatured and then stored at pH 7 at 37°C for up to 90 days.¹⁹⁸ Recently, it was shown that the main effect on deamidation rate in the hexapeptide Val-Thr-Pro-Asn-Gly-Ala by addition of cosolvents occurs because of the decrease in the dielectric constant of the solution.¹⁹⁹ This hexapeptide deamidates by forming the cyclic imide intermediate that is presumably formed by nucleophilic attack by the main-chain peptide nitrogen after deprotonation of the peptide bond. It was suggested that the decrease in the rate of deamidation is due to the destabilization of the deprotonated peptide bond nitrogen anion in solvents with low dielectric constants. In contrast to these results, the rate of isomerization of the Asp residue in Val-Thr-Pro-Asp-Gly-Ala actually increased slightly with decrease in dielectric constant. In this case, it was postulated that in the low dielectric solvent the population of protonated aspartyl side chains increases. This results in a superior leaving group, and may balance the effect of the destabilization of the attacking nucleophile nitrogen anion.

All these experiments were performed with peptides or thermally denatured protein. Alteration of protein conformation by organic solvents may negate any of the effects observed regarding reduction of deamidation rate in native proteins. Nonetheless, these studies may prove valuable in avoiding certain salts and buffer ions, particularly phosphate, if deamidation is identified as the major route of degradation in the formulated protein.

4. Effect of Ionic Strength

The effect of ionic strength on the rate of deamidation was studied using the glutaminyl pentapeptides Gly-Leu-Gln-Ala-Gly and Gly-Arg-Gln-Ala-Gly,¹²⁸ and the asparaginyl pentapeptide Gly-Arg-Asn-Arg-Gly.¹⁹³ These studies showed that deamidation proceeds at a faster rate with higher ionic strength and supports previous work with Gln.¹⁹⁶ These peptides exhibited similar ionic strength dependence on the rates; every one unit increase in ionic strength accelerated the reaction rate by a factor of about two. Recent studies with the pentapeptides Val-Ser-Asn-X-Val and Val-X-Asn-Ser-Val (where X is one of ten different amino acids) investigated the influence of sequence, pH, buffer catalysis, and ionic strength on deamidation rate. For these peptides there was no observed buffer catalysis or dependence on ionic strength at neutral and basic pH.¹⁴² An increase in deamidation

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rate with an increase in ionic strength occurred in cytochrome C.²⁰⁰ Most parenteral formulations of proteins are isotonic and if NaCl is used as the tonicifier, then the ionic strength is sufficiently low so that the increased deamidation rates observed at high ionic strength will not be a major problem.

5. Lyophilization

Since deamidation is a hydrolysis reaction that requires water, an obvious strategy to decrease deamidation is to eliminate water. One of the most useful techniques for water removal from protein formulations is freeze-drying. The removal of all residual water in a freeze-dried product, however, often proves to be deleterious to the protein conformation and stability.¹¹⁰ Recently it has been suggested that various carbohydrates function as lyoprotectants, which essentially replace the hydrogen bonding that is lost upon removal of water.^{201,202} The residual water in typical freeze-dried formulations actually allows degradation via deamidation to occur in the solid state. This was demonstrated for the ACTH model Asn-hexapeptide. When this hexapeptide was freeze-dried from a 10 mM phosphate buffer at pH 7.4 and stored for 105 days at 37°C and 90% relative humidity, approximately 33% of the hexapeptide deamidated to Asp and iso-Asp hexapeptides.¹⁴⁹ It was suggested that there was sufficient moisture to initiate deamidation. Interestingly, when lyophilized from water there was no appreciable deamidation of the hexapeptide when stored under the same conditions as the sample that was freeze-dried from a phosphate buffer. Because residual moisture was not measured in these experiments, it is difficult to ascertain whether a difference in residual water content coupled with favorable pH for deamidation was responsible. One difficulty in this interpretation is that decreased solubility of certain buffer salts upon freezing causes large changes in pH.²⁰³ In particular, the pH of sodium phosphate solutions decreased to as low as pH 4 upon freezing, which should be within the pH range of maximum stability for the Asn-hexapeptide.¹⁹⁴ Other studies of glucagon in the dried state showed that deamidation could proceed but depended upon the pH and moisture content.²⁰⁴ Formation of the cyclic imide was also reported for Gly-Asp-Gly and Gly-Asn-Gly tripeptides in freeze-dried formulations at higher temperatures.²⁰⁵

Taken altogether, these studies suggest that removing water by freeze-drying may not be sufficient to eliminate the deamidation reaction in peptides. However, the rate of deamidation in the freeze-dried product can be controlled by proper choice of excipients^{112,116} and will usually be slower than the rates in a liquid formulation. The "trade off" in choosing a freeze-dried formulation over a liquid formulation is the extra development time for the former. In addition, other degradation routes such as aggregation may occur due to the lyophilization process. These problems need to be weighed against the benefits of reducing the deamidation on a case-by-case basis.

6. Genetic Engineering to Remove the Deamidating Amide Residue

There will undoubtedly be cases where alteration of pH, addition of salts or cosolvents, control of ionic strength and temperature, and freeze-drying will not lead to a sufficiently stabilized product. As mentioned earlier, when a number of degradation routes occur, it may be impossible to reduce all rates with appropriate formulation conditions. Such a situation can be corrected by use of recombinant DNA technology to selectively eliminate Asn residues. This is usually a last-resort strategy and can be undertaken when all other formulation attempts have failed. Such a strategy requires that an appropriate residue be used to replace the Asn without disrupting protein conformation and activity. An obvious choice of residue is Ser because of its similar size and potential hydrogen donor functional group. Recombinant derived human interleukin-1 (hIL-1 α) with a pI of 5.95 deamidated at Asn³⁶ resulting in a product with a lower pI.¹⁵⁴ Replacement of Asn³⁶ with Ser³⁶ yielded a protein that was homogeneous on isoelectric focusing (IEF) with a pI equivalent to that of undeamidated hIL-1 α .¹⁵⁵ Proton NMR and circular dichroism experiments established that this mutant protein was identical in conformation to undeamidated hIL-1 α . The activity of hIL-1 α and its Ser³⁶ mutant were identical as assessed by measurement of receptor binding affinities. The Asp³⁶ mutant had identical activity to hIL-1 α suggesting that the deamidation to α -Asp does not inactivate the protein. However, this mutant does not provide any information regarding activity of iso-Asp at this deamidation site. The Ser mutant was completely stabilized against base-catalyzed and temperature-induced deamidation. Similarly, replacement in growth hormone releasing factor of Asn⁸ with Ser⁸ resulted in a ~7-fold increase in protein stability.²⁰⁶ In this particular case, deamidation resulted in decreased biological activity.

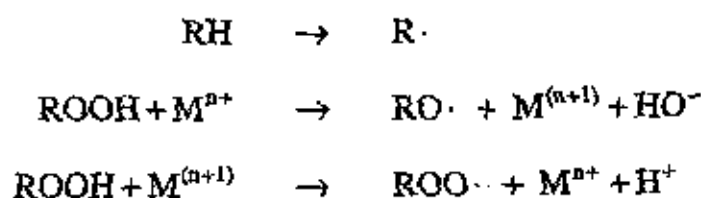
Another possible replacement residue for engineering mutant proteins that are stabilized against deamidation is a Gln residue. As previously discussed, Gln is less labile to deamidation than Asn and replaces that residue with one that has the same functional group. The main problem in this strategy is that the protein conformation would have to accommodate an extra methylene group in the side chain. Since most Asn and Gln residues are on the surface of proteins,²⁰⁷ this may not be a major problem. However, the ability of Gln residues to bind water molecules^{139,208} may have additional consequences for the packing of protein chains in the native structure.

IV. PROTEIN OXIDATION AND FORMULATION STABILITY

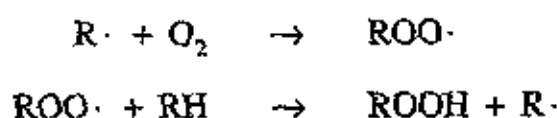
In addition to deamidation, proteins can undergo oxidation, which is one of the major degradation pathways, both in solution and lyophilized formulations. Autooxidation is generally thought of as the "uncatalyzed" thermal oxidation of substrate, and is studied infrequently because the reaction rate is slow,²⁰⁹ and often

variable. Uncatalyzed or thermal protein oxidation is actually a misnomer because the degradation rate is often governed by trace amounts of peroxide, metal ions, light, base, and free radical initiators.²¹⁰ On the other hand, protein oxidation (or catalyzed autooxidation) has been studied extensively. Even though a great deal is known about reactive oxygen species, the unknown and variable presence of the initiators makes the prediction of chemical oxidation an imprecise science. For example, free radical oxidation involves the separate effects of initiation, propagation, and termination, as shown in the following reaction scheme with metal ion M of valance n:

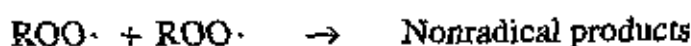
Initiation



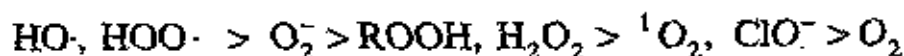
Propagation



Termination



This reaction scheme is only one of many representative oxidative pathways available. Even a cursory inspection of this scheme shows that this mechanism may afford complex kinetics in which any of the steps may be rate limiting, and where the rate-limiting step may change during the course of the reaction. Further, there are several reactive oxygen species such as singlet oxygen $^1\text{O}_2$, superoxide radical O_2^- , alkyl or hydrogen peroxide ROOH or H_2O_2 , hydroxyl radicals ($\text{HO}\cdot$ or $\text{HOO}\cdot$), and halide oxygen complexes (ClO^-).²¹¹ Singlet oxygen, which is probably the most important oxidative species in biological processes, may be formed by heat, light, or surface catalysis, and reverts to O_2 with photoemission. The generally accepted order of reactivity of oxidants commonly found in protein formulations is:



The following three sections focus on important issues pertaining to protein formulation and oxidation, such as oxidation mechanisms and reactive amino acids, effect of protein oxidation on the immune response, and formulation design considerations (liquid vs. solid).

A. Mechanisms of Oxidation

There is limited published data on the oxidation of peptides and proteins in pharmaceutical formulations because only a few proteins have been developed thus far — and not all of these are susceptible to oxidative degradation. Much of our understanding of protein oxidation comes from the chemical and biochemical literature, where the major mechanisms of protein oxidation have been described in detail. There are several reactive amino acids that may undergo oxidation: methionine, cysteine, cystine, histidine, tryptophan, and tyrosine, each with its own chemistry. Because the focus of this review is protein formulation stability, only the pertinent amino acids are discussed below. Those interested in alternate protein oxidation mechanisms, including oxidation induced by high energy radiation, photooxidation reactions, metal ion catalysis, and enzymatic oxidation by oxidases, may read general reviews in these areas.²¹²⁻²¹⁴ Shown below are the major oxidative mechanisms as they apply to protein formulation development.

1. Methionine

Methionine can oxidize to give methionine sulfoxide and, under extremely oxidative conditions, methionine sulfone. Methionine is very susceptible to autooxidation, chemical oxidation, and photooxidation. It reacts with a variety of oxidants including hydrogen peroxide, periodate, iodine, dimethylsulfoxide, light, chloramine-T, and N-chlorosuccinimide.²¹⁵

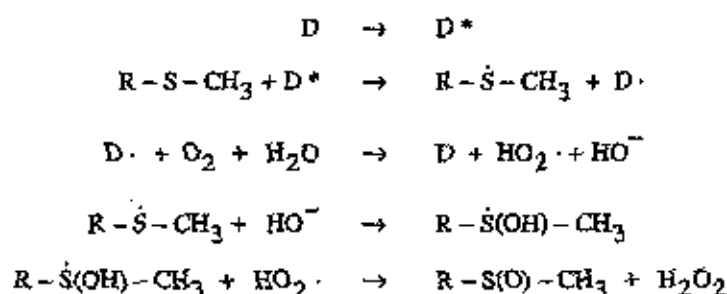
Oxidation of Methionine



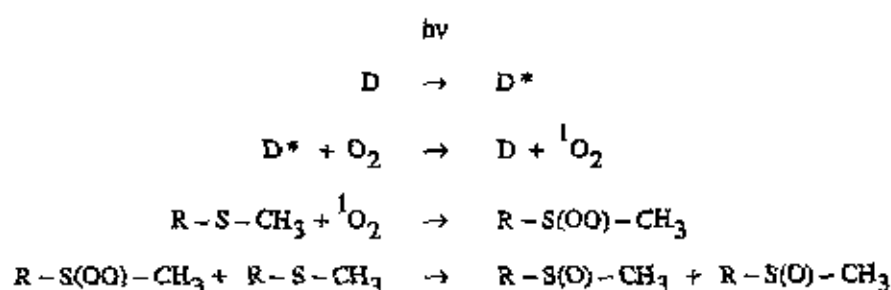
Methionine residues in native proteins show widely varying reactivity, largely because some are buried within the hydrophobic regions of the molecule and so are fairly inert, whereas some are exposed to the solvent and are thus more reactive. For example, the methionine residues in myoglobin²¹⁶ and trypsin²¹⁷ are completely unreactive under mild oxidation conditions. In many proteins, including ribonuclease A,²¹⁸ chymotrypsin,²¹⁹ pepsin,²²⁰ transferrin,²²¹ hGH,²²² and lysozyme,²²³ the methionine residues are partially exposed to solvent and react at very different oxidation rates. It is difficult to determine the effect of the primary amino acid sequence near to methionine on oxidation because the comparison of one study with another is complicated by the varied reaction conditions. Even for hydrogen peroxide, most of the studies reported to date have been carried out at different pHs and buffers, using different substrate and catalyst concentrations. These factors are important because the H₂O₂ catalyzed degradation of methionine is pH dependent,

and may follow complex kinetics.²²⁴ Methionine can also be photooxidized by a free radical pathway,²²⁵ via a singlet oxygen intermediate,²²⁶ or by nucleophilic substitution.²²⁷

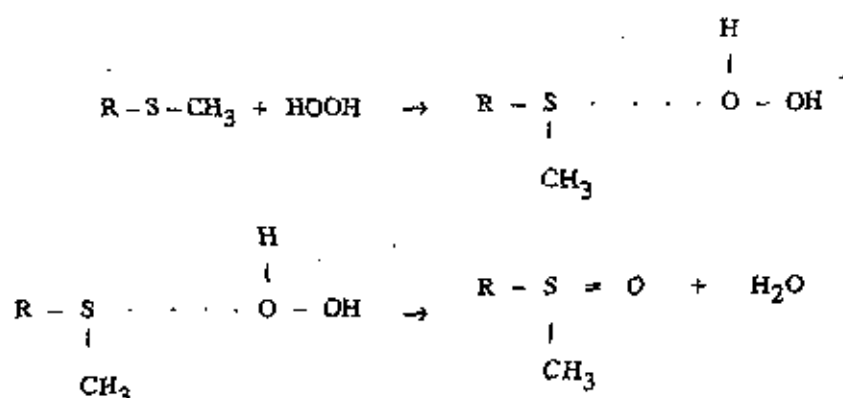
Free radical pathway



Singlet oxygen pathway



Nucleophilic substitution

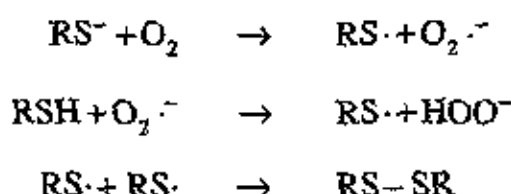


This mechanism of nucleophilic displacement is preferred over one in which there is an acid-base equilibrium with the solvent generating the species $HOOH_2^+$. This is based on the lack of salt effects,²²⁸ negative entropy of activation,²²⁷ and leaving group substituent effects.²²⁹

2. Cysteine

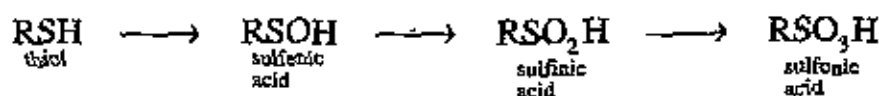
Cysteine is also easily oxidized. For example, cysteine can react with oxygen to yield cystine disulfide. The rate of cysteine oxidation is usually accelerated at higher pH where the thiol is deprotonated,²³⁰ although exceptions to this rule are known and are usually attributed to protein unfolding at lower pH with a concomitant increase in the rate of thiol oxidation.^{231,232}

Oxidation of Cysteine by Oxygen



The pH dependence on protein oxidation may be used to distinguish between cysteine and methionine oxidation. The hydrogen peroxide-catalyzed oxidation of cysteine decreases as the pH is lowered, whereas the rate of methionine oxidation increases slightly from pH 5 to 1.¹⁸⁶ Cysteine oxidation is also strongly catalyzed by transition metal ions, especially copper. The rate of cysteine oxidation is usually inversely dependent on the distance between thiol groups and neighboring electronegative groups.²⁰⁹ For example, the periodate oxidation of vicinal thiols in proximal cysteine groups to form disulfides has been observed for aldolase²³³ and other proteins.²³⁴ Once the cystine disulfide is formed, it is much less susceptible to reaction with mild oxidants (such as H_2O_2) than either methionine or cysteine, and so is often found as the stable end product in an oxidative reaction. Exceptions to this have been reported. Certain organic acids react with H_2O_2 to form the more reactive acylperoxides and peracids, which are known to oxidize cystine.²³⁵ At either low cysteine concentration, in the absence of a nearby thiol for disulfide formation, or in dye-sensitized photooxidation reactions, cysteine may also oxidize directly to give sulfenic, sulfinic, or sulfonic (cystic) acids, as observed in the oxidation of glyceraldehyde-3-phosphate dehydrogenase.²³⁶

Oxidation of Cysteine to Give Cystic Acids



In some proteins cysteine can be oxidized to give products from both pathways. For example, oxidation of α -amylase affords 30% sulfenic acid and 70% new disulfides.²³⁷

3. Histidine

Oxidation of histidine is also one of the major degradation pathways for many enzymes. The oxidation of histidine in the presence of metals is determined by the amino acids near the reactive histidine, as well as protein conformation, making the prediction of metal-catalyzed oxidation uncertain. For example, His²⁶⁹ in glutamine synthetase is far more susceptible to oxidation than other histidine residues — a surprising finding in that His²⁶⁹ is buried within the sequence Met-His²⁶⁹-Cys-His-Met where all of the residues may undergo oxidation.²³⁸ Histidine is also susceptible to photooxidation, where the proposed primary mechanism is a reaction of singlet oxygen²³⁹ with unprotonated histidine.^{240,241} The reaction rates on singlet oxygen with various amino acids has been determined, where histidine reacts 3-fold faster than tryptophan, and 6-fold faster than methionine.²⁴⁰ It has been demonstrated, using α -crystallin, that histidines react at different rates with singlet oxygen.²⁴² The different rates of oxidation occur because some of the histidines are buried and therefore inaccessible, or are protonated and therefore unreactive. An excellent review on photochemical processes involving histidine has been published.²⁴³

4. Tryptophan

Although it is generally recognized that tryptophan is easily photooxidized, there are few mechanistic studies detailing tryptophan as the predominant oxidation pathway. Photooxidation of tryptophan and tryptophan-containing polypeptides affords the kynurenine, H-formylkynurenine, glycine, alanine, serine, and aspartic acid analogs, but with poor recovery and mass balance of degradation products.²⁴⁴ At neutral pH, tryptophan and histidine typically photooxidize faster than most other amino acids, including tyrosine.²⁴³ At lower pHs, tryptophan and methionine are the predominant photoreactive amino acids.²⁴¹ Interestingly, there are only a few examples of thermal protein autooxidation where tryptophan is the predominant reactive site. The thermal side reactions of tryptophan have been reviewed, where oxidation is not as favored as iodination, ozonization, or reaction with aromatic sulfonyl halides, formic acid, or N-bromosuccinimide.²⁴⁵

B. Pharmaceutical Consequences of Protein Oxidation

1. Biological Activity

A comprehensive summary of proteins that have been oxidized is shown in Table 1. (For those interested in metal ion catalyzed protein oxidation, an excellent compilation has been published by Statman.²¹⁴) Included in Table 1 is the oxidation catalyst(s), the primary amino acid that undergoes oxidation, and whether the protein retained its biological activity after oxidation. Many of these studies were

TABLE 1
Comprehensive Summary of Protein Oxidation

Protein or Peptide	Catalyst	Reactive amino acid	Bioactivity after oxidation	Ref.
Albumin — bovine	A,F	n.d.	n.d.	316
Aldolase	B	S	no	233
Amidotransferase	G	S	no	317
Apolipoproteins A1 and AII	B	M	n.d.	318
Arginine kinase	J	M,S,W	no	246
Asparaginase	A,F	M,H,Y	partial	319
Bombesin	J	M	no	320
Brain S100b protein	G	S	n.d.	321
Calcitonin	J	M	no	322, 323
Calmodulin	C	M	no	324
Chemotactic peptide (f-MLF)	A	M	no	215
Cholecystokinin	A	M	n.d.	325
Chorionic somatomammotropin	A	M	yes	222
Chymotrypsin	A,B,F,P	M	partial	326–328
Corticotropin	A	M	no	329, 330
Corticotropin releasing factor	A	M	yes	331
Creatine kinase	J	M,S,W	no	246
α -Crystallin	G,F	M,H	n.d.	242, 332
Cytochrome C	J	n.d.	n.d.	333
Epidermal growth factor	A	M	yes	334
Erythropoietin	B	n.d.	partial	335
Fibrin	A	M	n.d.	231
Flagellin	C	M	n.d.	252
Gastrin	J	M	no	336
Glucagon	C,J	M,W	yes	262
Glucose oxidase	A	M	no	337
Glutamine synthetase	E	H	no	338–340
Gly-3-phosphate dehydrogenase	A	S	no	236
Growth hormone — bovine	C	M	yes	341, 342
Growth hormone — human	F,G	M	no, yes	112, 116, 256, 309, 343
Hemoglobin	G	n.d.	n.d.	344
Interleukin-1 β	G	S	no	166
Interleukin-2	G	M	n.d.	99
Lactalbumin	C	M	n.d.	345
Lactoperoxidase	A	n.d.	no	346
Lipoxygenase	G	M	no	224
Lysozyme	F	M	no	347
α -2-Macroglobulin	A	M	no	348
Myoglobin	J	M	n.d.	258, 349
Myosin	A	H,M,S	n.d.	350
Neuropeptide Y	A	M	partial	351
Ovoinhibitor	J	M	partial	352
Papain	A	S	no	353–355
Parathyroid hormone	A	M	no	253, 356
Pepsin	A	M	partial	220

TABLE 1 (continued)
Comprehensive Summary of Protein Oxidation

Protein or Peptide	Catalyst	Reactive amino acid	Bioactivity after oxidation	Ref.
Phosphoglucosylase	F	M	no	219
Plasminogen activator inhibitor	C	M	no	357, 358
α -1-Proteinase inhibitor	C	M	no	281, 359, 360
PRPP-Synthetase	D	S	partial	361
Relaxin	F	M	yes	303
Ribonuclease	D	M	partial	232, 362
Ribonuclease S	F	H	no	284
Ribosomal L12	A	M	no	363
Substance P	G	M	partial	251
Subtilisin	A,F	M	partial	364-367
Superoxide dismutase	A	n.d.	no	368
Transferrin	A,B	M,Y	partial	221
Trypsin	A	n.d.	partial	235
Trypsin inhibitor	A	M	yes	369
Trypsin/trypsinogen	A,D	M	partial	217
Venom, <i>B. asper</i> and <i>C. durissus</i>	F	n.d.	no, partial	370

Note: Oxidation catalysts are given in the second column as follows: A = hydrogen peroxide, B = periodate, C = Chloramine-T, D = iodoacetic acid, E = MAO, F = light (and sensitized photooxidation), G = autooxidation, P = peroxidase, J = miscellaneous oxidizers (see individual reference), n.d. = not determined. The primary amino acid oxidized is given in the third column using the notations: M = methionine, H = histidine, S = sulphhydryl, W = tryptophan and Y = tyrosine. Biological activity after oxidation is given in the fourth column as follows: if 10 to 90% of biological is retained, then "partial" is given in column 3, otherwise the protein is classified as active or inactive after oxidation.

carried out to determine the active site of the protein. Selective oxidation of certain amino acids, usually methionine, was used to alter biological activity as a probe for structural inference. In many cases, biological activity was only partially lost following oxidation, with 10-90% of the original activity remaining. When biological activity after oxidation was less than 10% of the parent protein (often within the limits of the assays used), the oxidized protein was considered inactive. Inspection of Table 1 shows that, in most cases, biological activity was either lost or diminished following oxidation. Loss of biological activity occurred even when the oxidation was rather mild. For example, conversion of a single methionine to methionine sulfoxide by H_2O_2 can result in a significant loss of bioactivity (see examples in Table 1 for references).

2. Conformation

There are several methods to measure changes in protein conformation, including physical, chemical, and immunological methods. One of the more

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sensitive indicators of protein conformation is the protein's ability to react with a component of the immune system, such as antibody binding. A molecule that reacts with a component of the immune system is said to be antigenic. Although this method is very dependent on the specific antibody used, review of the literature shows that oxidation usually does not alter protein antigenicity, unless the protein underwent conformational changes. For example, oxidation of arginine kinase did not affect antigenicity, but reaction of creatine kinase under similar conditions decreased the antigenicity by approximately 70%.²⁴⁶ (Arginine kinase retains much of its secondary structure upon oxidation, whereas creatine kinase does not). In other proteins that have a significant amount of tertiary structure (such as human serum albumin), oxidation or reduction affected antigenicity significantly.²⁴⁷ Similarly, the antigenic properties of hepatitis B antigen were found to depend on the conformational integrity of the protein, as identified by oxidation and reduction studies.²⁴⁸ Oxidation of *Porosipis juliflora* pollen antigen did not affect antigenicity, whereas protease digestion had a large effect.²⁴⁹ Both unoxidized and oxidized insulin B chains stimulated insulin-specific T cell hybridomas (but xenogenic insulins often did not), arguing the importance of conformation on antigenicity.²⁵⁰ Autooxidation of Substance P was found to show only a modest decrease in antigenicity as tested against two different antisera in an ELISA format.²⁵¹ Chloramine-T oxidation of flagellin, where Met¹¹¹ and Met³³¹ were completely oxidized, resulted in no change in antigenicity as measured by immunoprecipitation.²⁵² Oxidation of β -parathyroid hormone did not affect its antigenicity as measured by complement fixation, even though all biological activity was lost.²⁵³ Similarly, photooxidation of asparaginase did not result in a significant change in antigenicity.²⁵⁴ (Reduction of asparaginase also did not affect its antigenicity.²⁵⁵) Oxidation of Met¹⁴ in hGH did not affect antigenicity, as measured using a dual monoclonal antibody system.²⁵⁶ Periodate oxidation of a 400 kD mucin with the CAR-5 epitope did not alter the antigenicity as identified by using the monoclonal antibody BD-5.²⁵⁷ The complete antigenic mapping of myoglobin was performed using a number of chemical and biochemical techniques to identify antigenic determinants.²⁵⁸ Oxidation of the two methionines to methionine sulfoxide afforded a product that was antigenically and conformationally indistinguishable from native myoglobin. In another study, the product of performic acid oxidation of staphylococcal nuclease was found to be fully cross-reactive in antibody binding assays with unoxidized nuclease.²⁵⁹ Oxidation of a 20 kD allergen from *Dirofilaria immitis* did not affect antigenicity as measured by a passive anaphylaxis assay.²⁶⁰ In general, oxidation of methionine to give methionine sulfoxide does not affect protein antigenicity, probably because the gross conformational structure of the oxidized protein is close to the native structure. This is the case for many of the proteins mentioned above including myoglobin, growth hormone, and flagellin. On the other hand, oxidation or reduction of disulfide often does cause a large change in antigenicity, presumably because modification of the disulfide causes significant conformational changes. Glucagon shows decreased antigenicity after oxidation;²⁶¹ this is

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due to major conformational changes upon oxidation as verified by a change in the alpha helical content by circular dichroism.²⁶² Most proteins lose biological activity when oxidized, yet antigenicity is not affected unless the protein undergoes gross conformational changes. This suggests that antigenicity is not a sensitive indicator of protein "quality", at least when compared with biological activity. In summary, protein antigenicity is usually not affected by methionine oxidation. Oxidation of single amino acids often causes a change in biological activity, but there are only a few proteins where such a subtle change affects antigenicity. The literature on protein oxidation emphasizes that minimizing protein oxidation is crucial for maintaining biological activity of most proteins, but most oxidation reactions do not cause gross conformational changes, even when assayed using sensitive immunological methods.

3. Immunogenicity

A major concern of protein degradation is that degraded proteins, even self-proteins, may lead to an unwanted immunogenic response. Immunogenicity is the ability of a substance to stimulate or induce a detectable immune response. This response may be deleterious because antibody formation may not only neutralize the desired effect of the drug, but also may result in unwanted side effects such as anaphylactic shock after repeated administration. The immune system has evolved to recognize foreign protein, with disastrous immunological consequences if foreign proteins are not recognized.²⁶³ Both xenogenous (foreign) and autologous (self) proteins have been tested for immunogenicity. Foreign proteins, both native and modified (e.g., oxidized), should elicit an immune response. Self-proteins, however, should not give an immune response due to self-tolerance. Modified self-proteins may or may not elicit an immune response, depending on the protein and degree of modification. This last case is of particular interest to pharmaceutical scientists because it directly addresses the problem of protein stability and immunogenicity in humans. For a modified autologous protein to be recognized as foreign, it must have altered immune recognition sites, possibly due to a change in the primary sequence, or a change in protein conformation. (Note: from Section IV.B.2, oxidation does not usually affect protein conformation.) It is well established that protein conformation plays a significant role in immune response. For example, the neutralizing antibody response following immunization by gp120 is dependent on the "correct" gp120 conformation; some of the antibodies recognize conformational determinants and others recognize only primary sequence determinants.²⁶⁴ Another good example is the lack of immune response when peptide determinants are used to elicit an immune response, such as in Site A of influenza hemagglutinin.²⁶⁵ Immunization with flagellin and oxidized flagellin did not significantly alter antibody titers in rats.²⁵² Immunization of B10.BR (H2^k) mice with insulin B chain did not afford a signifi-

cant response, whereas immunization with oxidized B chain gave a large immune response.²⁶⁶ It was suggested that oxidized B chain has an epitope that forms an antigenic complex available for T cell recognition, whereas the unoxidized protein does not. There is a recent report that galactose oxidation of subunit vaccines increases immunogenicity;²⁶⁷ however this type of oxidation exerts its effects through T_H cell priming (a very specific mechanism) and does not directly address protein oxidation. Immunogenicity data have also been obtained after chronic administration of protein therapeutics in humans. A great deal of immunogenicity data on animal and human insulin have appeared in the last twenty years, but the literature data suggest that the immunogenicity differences observed are likely due to the differences between use of human and xenogenously-derived insulin.²⁶⁸⁻²⁷⁰ There is no evidence that oxidation affects the immunogenicity of insulin. It is well established that some patients form antibodies to hGH after repeated dosing of hGH. It has been reported that hGH with an additional methionine at the N-terminal end (Met-hGH) showed more antibody formation than Met-less hGH,²⁷¹⁻²⁷³ although these comparisons may not be valid because they suffer from different assay sensitivities. Usually the presence of anti-hGH antibodies does not affect growth.^{274,275} In these studies, there was no evidence of protein oxidation of the extra methionine, where increased immunogenicity may be due to the formation of hGH aggregates.¹³⁵

A search of the literature for antibody formation to autologous proteins after repeated administration shows that many proteins (for example, erythropoietin) are not mentioned at all, even though they have been administered extensively.

The effect of protein oxidation on immunogenicity is likely to be protein dependent; some proteins are better at eliciting an antibody response than others. Our immune system has evolved so that it does not recognize minor variants of autologous proteins as foreign. If it did recognize each variant, then one's own immune system would expend much energy mounting an autoimmune response against self-proteins that are partially degraded by oxidation or hydrolysis in the normal course of protein "aging" *in-vivo*. The promiscuity of immunological recognition (in this case manifested as a lack of an immunogenic response to a modified autologous protein) has been noted before²⁷⁶ — and it forms the basis of the hypothesis that minor modifications in protein structure (either primary or conformation) are allowed without an immunogenic response.

4. Biological Half-Life

One of the major hurdles in protein formulation is to minimize the amount of degraded drug product, in this case, oxidized protein. As noted above, many oxidized proteins are not biologically active, resulting in decreased formulation potency. Many oxidized proteins, even when biologically active, often show increased susceptibility to intracellular proteases^{233,277,278} and therefore may show

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altered pharmacokinetics, which is another undesirable pharmaceutical property. The proposed mechanism for the accelerated proteolysis of oxidized proteins is eventual denaturation and exposure of bulky or hydrophobic residues and sites that are susceptible to proteolytic cleavage. Incubation of oxidized proteins with cell-free extracts derived from erythrocytes, reticulocytes, skeletal muscles, cardiac muscles, hepatocytes, and bacteria strains have demonstrated accelerated degradation. Oxidized proteins have also been implicated in normal protein aging *in vivo*,^{279,280} as well as in certain disease states such as emphysema.²⁸¹

C. Formulation Parameters That Affect Oxidation

The prevention of protein oxidation in pharmaceutical formulations generally employs the following strategies: reduction in temperature, protection from light, change in formulation pH, removal of oxygen from the head space, removal of excipients that have contaminants that catalyze oxidation (such as polysorbates), and addition of antioxidants.²¹⁰ This section addresses the major methods for controlling oxidation: the effect of reduced temperatures, specifically freezing and lyophilizing, and the effect of excipients.

1. Freezing

In general, protein stability can be increased by cooling or freezing, although there have been notable exceptions.^{282,283} Usually freezing or lyophilization inhibits oxidation as it does for hydrolytic mechanisms, although the rate reductions for oxidation reactions are often not large. This is because the activation energy for many oxidation reactions is small, thus showing an insensitivity to temperature.²¹⁰ However, the opposite is sometimes true, where freezing increases the propensity for oxidation. There are several reasons why this unusual behavior may occur. As freezing proceeds, the concentration of the protein in the remaining solution increases due to exclusion of protein from the forming ice matrix. This increase in protein concentration causes a concomitant increase in the concentration of cysteine and cystine residues, with an increase in intra- or intermolecular disulfide exchange or oxidation.²⁸⁴ Sulfhydryl groups can also be readily oxidized at lower temperatures since the oxygen concentration in aqueous solution varies inversely with temperature. For example, oxygen solubility in aqueous solution at 4°C is 9.1 mL O₂/L, whereas at 25°C, oxygen solubility is 5.7 mL O₂/L.²⁸⁵ The oxygen concentration in a "partially frozen" system at -3°C was significantly higher than in solution at 0°C.²⁸⁶ This effect may be partially offset by an increase in ionic strength upon freezing; a local increase in salt concentration results in a concomitant decrease in oxygen solubility. This phenomenon has been used to stabilize oxygen-labile *C. pasteurianum* hydrogenase.²⁸⁷ The decision to freeze a pharma-

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ceutical protein product must also consider the effect of solvent upon freezing, as well as the protein-solvent interaction(s).²⁸⁸

2. Lyophilization

The effect of water on protein stabilization in the lyophilized state is often complex. Trace amounts of water in lyophilized proteins may be destabilizing, as discussed previously, largely because the water increases the rate of hydrolysis or aggregation.¹¹¹ This may also occur for oxidation reactions, e.g., with glutathione.²⁸⁹ Ribonuclease demonstrates complex protein stability in the lyophilized state; this protein showed a maximum stability (as measured by reaction of methionine) at 10% water content — it was *less* stable at lower and higher water content.^{371,372} Alternatively, carbon monoxyhemoglobin showed maximum degradation at 18% water content — it was *more* stable at lower and higher water content.²⁹⁰ Lyophilized β -galactosidase showed an increase in the water content gave higher water mobility and faster rates of degradation.²⁹¹ Oxidation may be retarded by trace amounts of water because water is a good scavenger for disrupting the propagation of free radicals.²⁹² A clear-cut example of this is the degradation of ergocaliferol, where a small amount of residual water decreased the rate of oxidation.²⁹³ The rate of oxidation may be governed by the rate of initiation, propagation, or termination, or a combination of activation energies. For example, the rate of glutathione oxidation in the solid state is independent of oxygen concentration, possibly because initiation is rate limiting.²⁸⁹ Further, the rate of oxidation in lyophilized formulations does not always correlate well with the residual head space oxygen, as shown for human growth hormone.¹¹² This has been attributed to the unknown and variable amounts of oxygen in the solid phase, but might likely be due to the oxidation rate being limited by the amount of initiator present. Photooxidation has also been observed in solid state proteins, albeit somewhat slower than in solution.²⁹⁴

3. Excipients

Protein oxidation in solution and lyophilized formulations may also be dependent on the excipients used. For example, the addition of Polysorbate 80 to lyophilized interleukin-2 formulations stabilized the product somewhat but increased the amount of protein oxidation.²⁹⁵ This oxidation was due to the high peroxide content in Polysorbate 80 remaining after purification and bleaching. Peroxide is known to increase with storage for prolonged periods, especially at higher temperatures.^{296,297} Even formulation excipients that should not catalyze oxidation (such as glycine) have been reported to show a deleterious effect on lyophilized formulation stability.¹¹²

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En vista de que el señor examinador es de la opinión de que cualquiera de los documentos D1 a D6 puede ser considerado como el arte más cercano a la presente solicitud, a continuación nuevamente nos permitimos explicar cuales son las diferencias entre dichas anterioridades y la solicitud en estudio, con el fin de corroborar que dichas diferencias no habrían sido evidentes para una persona medianamente versada en la materia técnica.

1.1 Altura inventiva de la solicitud en estudio sobre la anterioridad EP0841067 (D1).

Esta anterioridad reporta una preparación liofilizada de un anticuerpo monoclonal que contiene un ácido carboxílico o una sal del mismo. Además, D1 muestra que las composiciones deben tener presente ácidos carboxílicos como ácido cítrico, ácido acético, ácido oxálico, ácido succínico y mezclas de los mismos. De hecho, D1 sugiere que el ácido sea ácido cítrico y su sal sódica, como así se pudo comprobar en la página 4, entre las líneas 8 a 11.

Ahora bien, la presente invención se encuentra dirigida a una formulación acuosa estable que comprende natalizumab en una cantidad de desde 0.1 mg/ml hasta 200 mg/ml, un amortiguador de fosfato, polisorbato 80 en una cantidad de desde 0.001% hasta 2.0% (peso/volumen) y cloruro de sodio.

Si una persona versada en la materia técnica considerara las enseñanzas de D1, no podría llegar de manera evidente a la presente solicitud, ya que D1 no enseña ni sugiere que se puedan obtener formulaciones de natalizumab como las mencionadas en la solicitud en estudio, especialmente en lo que concierne a la inmunoglobulina.

1.2 Altura inventiva de la solicitud en estudio sobre la anterioridad WO98221136 (D2).

Este documento muestra sustancias farmacéuticas liofilizadas de anticuerpos monoclonales o policlonales. D2 sugiere que las composiciones contengan azúcar o una azúcar-aminoácido que puede tener la función de tensoactivo o de agente estabilizante. El ejemplo 10 ubicado en la página 33 de D2 muestra claramente que las composiciones deben tener arginina-azúcar y sustancias como el Tween 20 como tensoactivos.

En clara contraposición, la presente invención muestra una formulación acuosa estable que comprende natalizumab en una cantidad de desde 0.1 mg/ml hasta 200 mg/ml, un amortiguador de fosfato, polisorbato 80 en una cantidad de desde 0.001% hasta 2.0% (peso/volumen) y cloruro de sodio. Una persona medianamente versada en la materia técnica no habría encontrado ninguna sugerencia en D2 para dejar de utilizar azúcar o mezclas de estos con aminoácidos para realizar composiciones estables de natalizumab.

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1.3 Altura inventiva de la solicitud en estudio sobre la anterioridad US5,908,826 (D3).

Como ya se había manifestado en nuestra respuesta del 29 de abril de 2009, D3 al igual que el documento D1, reporta preparaciones de anticuerpo monoclonal liofilizadas, las cuales son el resultado de la preparación de una solución gelatinosa del anticuerpo monoclonal o en un ácido carboxílico o sal del mismo. Los ejemplos mostrados por esta anterioridad se encuentran encaminados a preparaciones de inmunoglobulinas del tipo A, G, E y M con detergentes como el Tween 20.

Teniendo en cuenta lo anterior, una persona medianamente versada en la manera técnica no habría encontrado evidente composiciones de anticuerpos, especialmente de natalizumab sin la presencia de ácidos carboxílicos, ya que D3 emplea ácidos carboxílicos como parte fundamental en sus composiciones. En consecuencia, D3 no resulta relevante para desvirtuar la altura inventiva de la presente invención.

1.4 Altura inventiva de la solicitud en estudio sobre la anterioridad US5,945,098 (D4).

D4 reporta preparaciones de inmunoglobulinas para administración intravenosa, donde dichas preparaciones son estabilizadas con aminoácidos y detergentes no iónicos junto con polisorbato y polietilenglicol. Adicionalmente, esta anterioridad enseña que las preparaciones contienen polivinilpirrolidona (PVP).

Si una persona medianamente versada en la materia tuviera en cuenta las enseñanzas de este documento, encontraría que es relevante la presencia de aminoácidos y PVP en las composiciones ya que D4 reporta específicamente en sus ejemplos I, II, III y IV ubicados en la columna 6, que el aminoácido glicina participa en todas las preparaciones junto con cantidades de polietilenglicol o con polisorbato. Por lo tanto, D4 no proporciona ningún indicio de composiciones de natalizumab como las reclamadas en la solicitud en estudio dado que D4 reporta que las preparaciones son para inmunoglobulinas, lo cual es totalmente diferente a lo reportado en la presente solicitud.

1.5 Altura inventiva de la solicitud en estudio sobre la anterioridad WO0212501 (D5).

Por su parte, la anterioridad D5 muestra anticuerpos anti-dual integrina que incluyen ácidos nucleicos que codifican dichos anticuerpos. Adicionalmente, esta referencia establece que dichos anticuerpos comprenden al menos la región variable SEQ ID 7 y 8 como se describen allí. Sin embargo, D5 no revela ni enseña formulaciones de natalizumab junto con polisorbato 80, cloruro de sodio y un amortiguador de fosfato. Por lo tanto, una persona versada en la materia no encontraría ninguna motivación en lo reportado en D5 para llegar de manera evidente a una

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composición como la reclamada en la presente solicitud.

En resumen, ninguna de las referencias citadas por el señor examinador muestra o reporta una formulación comercial o una formulación liofilizada de natalizumab como la reivindicada en la presente solicitud. De hecho, al tener en cuenta la estructura química del natalizumab, se puede concluir que la persona versada en la materia no encontraría ninguna sugerencia a partir de lo mostrado en D1 a D6 para llegar de alguna manera evidente a la solicitud en estudio, dado que el natalizumab no es estructuralmente semejante a las inmunoglobulinas e incluso a las estructuras de los anticuerpos monoclonales empleados en las formulaciones de las referencias citadas por ese despacho. De hecho, el natalizumab y las moléculas actuales tienen diferencias importantes que incluyen diferentes regiones constantes, diferentes regiones variables y diferentes blancos moleculares. Por consiguiente, no existe alguna sugerencia en el arte previo o en el conocimiento generalmente disponible a la persona medianamente versada en la materia, para sustituir natalizumab por los anticuerpos citados en las referencias.

En efecto, ninguna de las referencias proporciona alguna clase de formulación estable que contenga natalizumab y que sea apropiada para la administración terapéutica, ni existe alguna expectativa razonable de éxito en la combinación de dichos documentos. Los anticuerpos no son simplemente intercambiables de manera fácil. Las diferencias estructurales entre los anticuerpos de las referencias y el natalizumab incluyen diferencias que requieren distintas formulaciones y concentraciones a fin de llegar a formulaciones estables y adecuadas para el uso médico.

Por lo tanto, nuevamente nos permitimos decir que no existía una expectativa razonable de éxito en las anterioridades para producir la invención en estudio. Aún más, una persona medianamente versada habría tenido que hacer más que una experimentación rutinaria para llegar a la formulación de natalizumab reclamada en esta solicitud, ya que el natalizumab era conocido por ser un agente bastante difícil para formularlo en un producto estable y seguro para la administración en pacientes. La combinación específica y la concentración de los ingredientes de la formulación reclamada fueron encontradas únicamente después de muchos años de investigación y de experimentación y de ensayos clínicos en seres humanos, lo cual va más allá del mero nivel de ensayo y error.

Adicionalmente, la formulación reivindicada resuelve las carencias de estabilidad notadas en la técnica anterior y cumple con un requerimiento médico que hasta la fecha no se había podido satisfacer, y ha logrado además un éxito comercial. La presente formulación cubre una formulación comercial en los Estados Unidos de América comercializada bajo la marca Tysabri® y en Europa para el tratamiento de las formas de recaída de esclerosis múltiple.

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En la Esclerosis Múltiple EM, este medicamento es recomendado para pacientes que han tenido respuesta inadecuada en terapias previa, así como también para el tratamiento de la enfermedad activa de Crohn desde moderada hasta severa.

En este sentido, una persona versada en la materia al momento de la presentación de la prioridad de la solicitud en estudio reconocería que existía una necesidad de proporcionar una formulación estable de natalizumab. Es decir, que el problema de la presente invención habría podido ser identificado por dicha persona, sin embargo, dicha persona no habría sido capaz de crear dicha formulación con base en lo mostrado en el arte previo.

En efecto, como ya se demostró anteriormente, la técnica anterior muestra que los anticuerpos no son fácilmente intercambiables entre sí dentro de formulaciones, como así se expone y se evidencia en el anexo 1 titulado "Critical Reviews in Therapeutic Drug Carrier Systems" de Cleland y colaboradores 10(4):307-377 (1993), en donde se muestra que cada anticuerpo posee una serie de características físicas únicas que difieren en gran manera e incluyen el punto isoeléctrico, la solubilidad y las condiciones bajo las cuales el anticuerpo se aglomera. Por lo tanto, el arte previo falla en enseñar una formulación estable que contenga natalizumab o en cómo llegar a dicha formulación.

Sobre este particular, respetuosamente se hace énfasis sobre el hecho que una formulación que comprende natalizumab, solución amortiguadora de fosfato, polisorbato y cloruro de sodio no había sido nunca reportada ni sugerida en el estado del arte. Por lo tanto, la evaluación de la altura inventiva realizada por el señor examinador corresponde más a un enfoque en retrospectiva o hindsight como se le conoce ampliamente en el campo de la Propiedad Intelectual, dado que el señor examinador está buscando de manera retrospectiva la composición reclamada en los documentos del arte previo.

No es obvio simplemente sustituir natalizumab dentro de las formulaciones de la técnica anterior. El natalizumab es un anticuerpo IgG4 monoclonal humanizado el cual hace blanco en las sub-unidades alfa-4 de los receptores de superficie celular $\alpha 4\beta 1$ y $\alpha 4\beta 7$. Sin embargo, los anticuerpos de todos los documentos de la técnica anterior son diferentes. El natalizumab tiene blancos moleculares distintos, regiones constantes diferentes y regiones variables distintas en relación con los anticuerpos citados en las referencias. De hecho, los anticuerpos no son fácilmente intercambiables en las formulaciones como se demuestra en el artículo del anexo 1.

Las diferencias únicas de los diferentes anticuerpos requieren de formulaciones distintas para lograr el máximo de eficacia terapéutica como también de estabilidad en el producto. Por consiguiente, una persona medianamente versada en la materia no habría podido predecir "a priori" si una formulación sería efectiva para natalizumab o algún otro anticuerpo.

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Las referencias citadas por esa oficina, enumeran ingredientes de formulación pero no enseñan por qué esos ingredientes podrían ser especialmente beneficiosos con anticuerpos particulares o con características químicas y físicas determinadas de dichos anticuerpos. En este sentido, es necesario señalar que existe un número ilimitado de formulaciones que podrían ser desarrolladas mediante la selección de ingredientes en varias concentraciones. Sin embargo, no existe ninguna sugerencia en la técnica anterior en cuanto a qué combinación específica de ingredientes podría hacer que una formulación de natalizumab fuera estable.

En consecuencia, como se mencionó anteriormente, no existe sugerencia en ninguno de los documentos citados por el señor examinador que combinado con el conocimiento general de una persona medianamente versada en la materia logran conducir a sustituir los anticuerpos de las referencias por natalizumab. Además, tampoco existe alguna expectativa de éxito razonable en hacer dicho intercambio.

Como ya se indicó, los anticuerpos no son fácilmente intercambiables, dado que las diferencias estructurales entre el anticuerpo de la técnica anterior (D1 a D6) y el natalizumab incluyen diferencias en las regiones constante y variable como también en el blanco molecular. Tales diferencias requieren de formulaciones distintas y de concentraciones diferentes para lograr formulaciones estables y adecuadas para uso médico.

La presente invención no es el resultado de una experimentación rutinaria, toda vez que una persona medianamente versada en la materia habría tenido que hacer más que ensayos rutinarios para lograr la formulación reclamada, dado que el natalizumab resulta ser una sustancia bastante difícil para su formulación en productos estables y seguros para la administración en pacientes. La combinación específica y los ingredientes fueron encontrados luego de años de experimentación y ensayos clínicos en seres humanos.

En este sentido, es deseo del solicitante poner a consideración del señor examinador el anexo 2 titulado "Preformulation Study" hecho por Celltech Biologics para Athena Neurosciences, una compañía adquirida por el solicitante en el año de 1996.

La formulación reclamada, cuando se emplea en el producto comercial, contiene natalizumab, polisorbato, solución amortiguadora de fosfato y cloruro de sodio. Como se discute más abajo, la experimentación que se realizó con la formulación de la presente solicitud, no es el producto de la simple experimentación rutinaria o del ensayo/error. Si se hubiera empleado la simple experimentación rutinaria, la presente formulación no habría sido desarrollada o probada como se discute más abajo. De hecho, la frase "ensayo y error" implica que la persona versada intenta y experimenta y con base en los resultados vistos en dicha experimentación intenta un nuevo experimento, continuando dicho proceso hasta que se alcance el objetivo. Sin embargo, este concepto únicamente funciona si los resultados de los experimentos son capaces de proporcionar una guía correcta. En el caso de la

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presente formulación, los resultados de estudios para determinar excipientes capaces de proporcionar una formulación estable y susceptible de uso para el natalizumab, a menudo condujeron a los investigadores en la dirección equivocada.

En 1994, durante el proceso de desarrollo de la formulación de natalizumab, el estudio de preformulación "Preformulation Study" fue realizado para monitorear la estabilidad de quince formulaciones que comprendían acetato, histidina y soluciones amortiguadoras de fosfato. Además, se estudiaban los efectos del cloruro de sodio y del Tween 80 (polisorbato 80) en la estabilidad de la formulación. Los resultados mostraron que el anticuerpo natalizumab fue, al menos inicialmente, estable de manera relativa en las formulaciones evaluadas (durante estudios posteriores se encontró que este no era el caso, también dando sustento al argumento que el simple ensayo y error no era suficiente para el desarrollo de esta formulación). Adicionalmente, los estudios mostraron que el uso de cloruro de sodio y de Tween 80 afectaba de **manera desfavorable** la estabilidad.

Sobre este particular, se dirige respetuosamente la atención del señor examinador a la Sección 5.0 que concluye que la adición de 150 mM de cloruro de sodio o de 0.02% de Tween 80 tenía el efecto de acelerar el proceso de degradación. El estudio recomendó que se considerara la formulación que contenía acetato, histidina y fosfato. El estudio además puso de manifiesto que eran recomendables el fosfato y la histidina sobre la sal debido a que la adición de la sal a la formulación de acetato tenía un efecto perjudicial sobre la estabilidad de la formulación.

También se notó que en estos estudios, el cloruro de sodio fue empleado en ciertas formulaciones para evaluar el efecto de la fuerza iónica, pero no fue probado junto con polisorbato 80. Los investigadores concluyeron que uno o el otro de estos dos excipientes, era influyente de la estabilidad de las formulaciones en estudio, pero no fueron intentadas las formulaciones que contenían ambos excipientes, como el caso de la presente formulación reclamada.

Por consiguiente, este estudio brinda sustento al argumento de que la formulación reclamada en la solicitud en estudio no es un simple resultado de ensayo y error. Aquí el solicitante realizó ensayos y los resultados, de hecho, se apartaron y condujeron a los investigadores lejos de la eventual formulación disponible comercialmente de hoy en día.

En consecuencia, por lo menos se requirió de un paso inventivo con el fin de ir desde estos estudios hacia la presente formulación reclamada.

Luego de estos estudios y basados en sus hallazgos, el solicitante intentó una formulación que contenía natalizumab e histidina. La histidina se agregó a la formulación, como sugerencia de los resultados del estudio de preformulación. No obstante, esto fue también una guía equivocada. En la prueba de formulaciones en 1994 y 1995, el solicitante obtuvo resultados bastante nublados ya que las

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formulaciones no fueron ni estables ni susceptibles de uso médico.

Sin embargo, a pesar de lo que enseñaba el estudio de preformulación, el solicitante intentó emplear cloruro de sodio para dirigirse al problema de la isotonicidad. No obstante, se observó que sucedía la precipitación de proteína y la conglomeración en las muestra de formulación. Se intentó entonces una *segunda formulación* que contenía natalizumab, histidina, cloruro de sodio y polisorbato 80. A pesar de lo que se había visto previamente, el solicitante intentó la adición de polisorbato 80 ya que se había observado que el polisorbato 80 actuaba como un inhibidor del proceso de degradación. No obstante, se descubrió que la histidina puede reaccionar con el polisorbato 80 en presencia de trazas de metal, lo que resultaba en la degradación del polisorbato 80 e impurezas en la solución.

La próxima formulación contenía natalizumab, fosfato de sodio 8monobásico y dibásico), cloruro de sodio y polisorbato 80. Esta sustitución resolvió el problema de la reacción de la histidina con el polisorbato 80 en presencia de trazas de metal. Esta última formulación fue empleada en ensayos clínicos que fueron realizados durante un número de años. Por lo tanto, a pesar de los resultados de los estudios previos que mostraban los perjuicios del polisorbato 80 y del cloruro de sodio y la recomendación de la histidina, el solicitante produjo una formulación que contiene polisorbato 80, fosfato y cloruro de sodio. Esta combinación poco probable de excipientes proporciona una formulación que produce una base estable para la formulación de natalizumab en un escenario médico. Por lo tanto, no era ni obvio ni evidente a partir de ensayos rutinarios obtener una formulación de natalizumab como la reclamada en la solicitud en estudio.

Finalmente, respetuosamente nos permitimos decir que la citación de 6 documentos por parte del señor examinador pone de manifiesto que la presente solicitud no es evidente para una persona medianamente versada en la materia pues no habría sido evidente para dicha persona combinar tantos documentos para llegar a las formulaciones reclamadas en la solicitud en estudio.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: "*habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares*" y "(...) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso", respectivamente (El subrayado es nuestro).

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En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



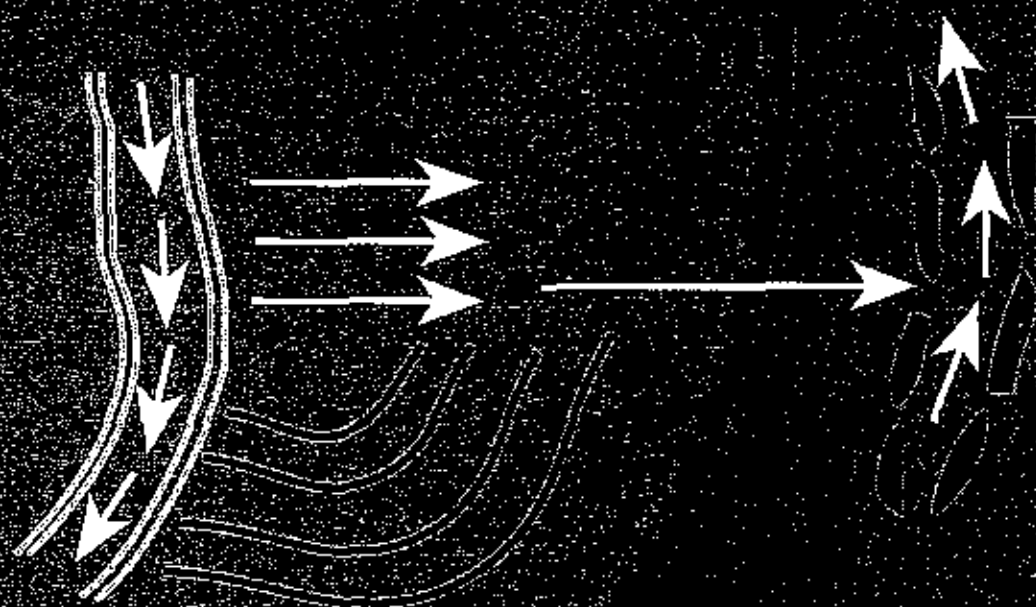
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Editor

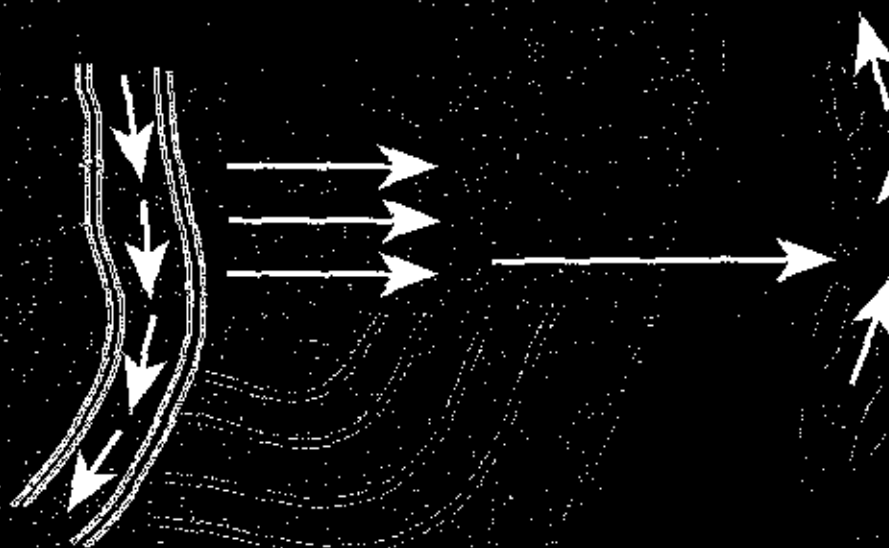
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