ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
13 (1995) 1249–1261

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Degradation of lyophilized and reconstituted MACROSCINT® (DTPA-IgG): precipitation vs. glucosylation☆

Carla Hekman[a,*], SungAe Park[b], Wen-Yu Teng[a], Norberto A. Guzman[b], Thomas Rossi[a]

[a] *Analytical Research and Development, The R.W. Johnson Pharmaceutical Research Institute, 1000 Route 202, Raritan, NJ 08869, USA*

[b] *Protein Formulations, The R.W. Johnson Pharmaceutical Research Institute, 1000 Route 202, Raritan, NJ 08869, USA*

Received for review 21 June 1994; revised manuscript received 28 December 1994

Abstract

Diethylenetriaminepentaacetic anhydride (DTPA) conjugated to IgG (DTPA-IgG) and labeled with ^{111}In is useful for detecting focal sites of infection and inflammation (R.H. Rubin, A.J. Fischman, R.J. Callahan, B. Khaw, F. Keech, M. Ahmad, R. Wilkinson and H.W. Strauss, ^{111}In-labeled nonspecific immunoglobulin scanning in the detection of focal infection, N. Engl. J. Med., 321 (1989) 935–940). MACROSCINT® contains DTPA-IgG formulated as a lyophile from a citrate buffer containing maltose. Exposure of both reconstituted and lyophilized MACROSCINT® to intense light resulted in degradation primarily via formation of precipitating aggregates. However, lyophilized and reconstituted MACROSCINT® responded differently to thermal stress. Reconstituted MACROSCINT® subjected to thermal stress (65 °C) also degraded through formation of precipitating aggregates. In contrast, exposure of lyophilized MACROSCINT® to thermal stress (65 °C) resulted primarily in an increase in the molecular size of the MACROSCINT® DTPA-IgG monomer. This increase in molecular size was a function of both the moisture content in the vial and the amount of time for which the sample was stressed, but was not a function of the conjugation with DTPA. Monosaccharide analysis of the samples demonstrated that this increase in molecular size corresponded to an increase in the amount of glucose covalently attached to the IgG. These data suggest that the increase in molecular size as a function of thermal stress is due to the covalent attachment of maltose, which is a glucose disaccharide present in the lyophile as an excipient, to the IgG. This degradation pathway was only observed in the lyophile.

Keywords: Aggregation; Degradation pathways; DTPA-IgG; Glucosylation; MACROSCINT®; Maillard reaction; Precipitation

Abbreviations and definitions: DTPA, diethylenetriaminepentaacetic anhydride; DTPA-IgG, diethylenetriaminepentaacetic anhydride conjugated to human IgG; IgG, immunoglobulin G; SEC, size exclusion chromatography; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; reconstituted or lyophilized MACROSCINT®, DTPA-IgG formulated such that after reconstitution with saline the solution contains 1.0 mg ml^{-1} DTPA-IgG, 3.2 mg ml^{-1} sodium citrate, 1.8 mg ml^{-1} citric acid, 13.2 mg ml^{-1} maltose, 0.1 mg ml^{-1} polysorbate 80 and 2.0 ml saline.

☆ Presented at the Ninth Annual Meeting and Exposition of the American Association of Pharmaceutical Scientists (Analysis and Pharmaceutical Quality Section), San Diego, CA, 6–10 November 1994.

* Corresponding author. Tel: (908)-704-5395.

SSDI 0731-7085(95)01515-9

1. Introduction

Aggregates of IgG have been reported to fix complement and bind macrophages in a similar way to antigen–antibody complexes [1,2]. For this reason, a great number of studies on IgG aggregation and degradation have been reported in the literature [1–10, and references cited therein]. Thermal stress of IgG in solution is the most commonly reported means of inducing aggregation. This thermally induced IgG aggregation has been reported to be irreversible [1,10], and a function of both time and temperature [6], with a sharp increase in the rate of aggregation occurring at 63 °C [9]. Both soluble and insoluble aggregates have been reported depending on the sample treatment conditions and the preparation [5,6,8]. In fact, a great deal of variability has been reported in the ability of different IgG populations to aggregate [1,5] and in the stability of these aggregates once they are formed [8–10].

Aggregation phenomena are of potential concern in the preparation of antibody-based pharmaceuticals [11]. One such preparation, the MACROSCINT® imaging agent, consists of human polyclonal IgG conjugated to diethylenetriaminepentaacetic anhydride (DTPA). Subsequent to conjugation, the product is formulated in a citrate buffer containing maltose and lyophilized. DTPA-IgG, when labeled with ^{111}In and administered to patients, localizes at sites of infection and/or inflammation. These sites can be detected six to 24 h post-injection by subjecting the patient to gamma-scintigraphy [12,13].

As will be reported in this paper, the major pathway of degradation for reconstituted MACROSCINT® exposed to both light and heat, and for lyophilized MACROSCINT® exposed to light was aggregation and precipitation. Although similar findings have been reported for other proteins [14,15] and for intact human IgG [1,5–10] treated with heat or extremes of pH, the data in this report demonstrate that exposure of either reconstituted or lyophilized IgG proteins to intense light also results in degradation through precipitation.

In contrast, MACROSCINT® lyophilized in the presence of maltose and subjected (as a lyophile) to thermal stress showed no tendency to precipitate, and only slight evidence of aggregation. The principal change observed under this condition was a gradual increase in size of the DTPA-IgG monomer. This increase in size, too small to be attributed to the formation of dimers or trimers, was a function of the time over which the sample was subjected to stress and to the moisture content of the sample. The molecular size change was not a function of the conjugation with DTPA, as judged by experiments demonstrating that a similar increase in molecular size could be observed with both polyclonal and monoclonal IgG. However, a correlation was observed between the molecular size and the amount of glucose bound to the IgG products. The data indicate that this increase in size was due to the covalent attachment of the excipient maltose to the IgG by a non-enzymatic glucosylation reaction.

2. Materials and methods

2.1. Materials

Potassium chloride, sodium chloride, potassium phosphate monobasic, sodium phosphate dibasic, phosphoric acid, bromphenol blue, Brilliant Blue R, and glycerol were from Sigma (St. Louis, MO). Tris-(hydroxymethyl)aminomethane, glycine, sodium dodecyl sulfate, and SDS-PAGE molecular weight standards were from Bio-Rad (Melville, NY). Polyacrylamide gels (4–15% linear gradients) were from Bio-Rad or Jule (New Haven, CT). MACROSCINT® was supplied by the R.W. Johnson Pharmaceutical Research Institute. Human polyclonal IgG was Gamimune® N from Miles (Elkhart, IN). Murine monoclonal IgG was supplied by Ortho Biotech Inc. SEC columns were TSK 3000 SW (column dimensions 7.5 mm × 30 cm, 10 μm particle size) from Phenomenex (Torrance, CA). Prior to SEC analysis, samples were filtered through Gelman acrodisc filter units (0.45 μm). The column employed for monosaccharide analysis was a Dionex CarboPac PA1 analytical column with a CarboPac PA1 guard column (Sunnyvale, CA).

2.2. Size exclusion chromatography (SEC)

The SEC method employed was an isocratic HPLC procedure. The mobile phase was 2.7 mM potassium chloride, 0.14 M sodium chloride, 1.5 mM potassium phosphate, and 8.1 mM sodium phosphate (pH 7.2). The column utilized was a Phenomenex TSK 3000 SW SEC column. The injection volume, flow rate, and temperature were 10 μl, 0.7 ml min^{-1}, and 25 °C, respectively. Detection was via absorbance at 280 nm. Quantification of the

DTPA-IgG content in MACROSCINT® was determined by comparison of peak areas in the sample(s) to that of an IgG reference standard of known concentration. Quantification of percentage monomer, dimer, or oligomer DTPA-IgG, and DTPA-IgG degradation product(s) was determined as a percentage of the total peak area in the sample chromatogram. A Waters 600E Solvent Delivery System was employed together with a Waters 490E detector and Waters 717 autosampler (Waters Corp., Milford, MA).

2.3. SDS-PAGE

Samples were prepared and electrophoresed on 4–15% linear gradient polyacrylamide gels according to Laemmli [16] at 100 V until the tracking dye reached the bottom of the gel. The gels were then removed, stained in 10% methanol, 10% acetic acid and 0.05% Brilliant Blue R, and destained in 5% methanol and 10% acetic acid. The mini-protean II electrophoresis system from Bio-Rad was employed throughout these studies.

2.4. Monosaccharide analysis

Samples of MACROSCINT®, human polyclonal IgG, and murine monoclonal IgG were dialyzed into the same buffer system used to formulate MACROSCINT® (12.7 mg ml^{-1} sodium citrate dihydrate, 7.1 mg ml^{-1} citric acid, anhydrous, 0.4 mg ml^{-1} Polysorbate 80, 5.3% maltose). These samples (0.5 ml) were then lyophilized and stressed at 65 °C in the presence of 20% added moisture (see below) for 23–72 h. Subsequent to stressing, the samples were reconstituted with 2.0 ml 0.9% sodium chloride, USP, and dialyzed extensively into water to remove the free maltose. They were then dried on a Speedvac® (Savant) and hydrazinolysed using the Oxford GlycoPrep™ 1000 (Oxford Glycosystem, Rosedale, NY). The carbohydrate portion was collected from each sample and dried by Speedvac®. Each sample was dissolved in 100 µl of deionized water to which 287 µl of 2 M trifluoroacetic acid was added. The samples were hydrolyzed at 100 °C for 15 h after which they were cooled to room temperature and dried by Speedvac®. The samples were then reconstituted with 300 µl of water and injected into a Dionex Glycosystem HPLC containing Dionex CarboPac PA1 analytical and guard columns. Elution was performed isocratically over 25 min using 15 mM sodium hydroxide as eluant. The flow rate was 0.8 ml min^{-1}. Elution was monitored using a pulsed amperometric detector.

2.5. Forced degradation of MACROSCINT®

Heat and light treatment of reconstituted MACROSCINT®

Vials of MACROSCINT® were reconstituted with 2.0 ml 0.9% sodium chloride, USP, and the contents combined to provide a homogeneous sample. Subsequent to mixing, the combined sample was divided into aliquots in polystyrene round bottom tubes with caps. Each aliquot was tightly capped. One aliquot, which served as a control, was stored at 4 °C throughout the course of the study. Thermal stress was accomplished by maintaining the samples at 37 °C, 50 °C, or 65 °C for time periods ranging from 5 min to 11 days, after which the samples were cooled to 4 °C prior to analysis. Alternatively, photo stressing was accomplished by exposure of samples in capped, borosilicate vials in a Suntest CPS lightbox (Heraeus DSET Laboratories) which was water cooled at 25 °C. Exposure was at maximum intensity (725 W m^{-2}) for 4 or 6 h. After removal from the light box, samples were stored at 4 °C until analysis.

Heat and light treatment of lyophilized MACROSCINT®

In order to prepare samples free from the fill volume variability encountered for MACROSCINT®, the contents of several vials of MACROSCINT® were combined[1]. These contents were then distributed into borosilicate vials with screw caps. One aliquot, which served as a control, was stored at 4 °C throughout the course of the study. Thermal stress was accomplished by maintaining the vials at 65 °C for time periods ranging from 2 to 72 h, after which the samples were cooled to 4 °C prior to analysis. Additional studies, in which various amounts of moisture (5–50%) were introduced into the vials prior to heating, were also conducted. In these cases, moisture was introduced by pipetting the desired amount of water (<20 µl) onto the cap prior to

[1] It should be noted that comparable results were obtained when these experiments were conducted using intact lyophilized MACROSCINT® cakes rather than the dispersed cakes utilized here. Dispersed cakes were employed to provide a homogeneous sample free from fill volume variability in MACROSCINT® vials.

sealing the vial. Photo stressing was accomplished by exposure of vials in a Suntest CPS lightbox (water cooled at 25 °C) at maximum intensity (725 W m^{-2}) for 2 or 4 days. Subsequent to removal from the light box, samples were stored at 4 °C until analysis. The vial contents were reconstituted with 2.0 ml 0.9% sodium chloride, USP, prior to analysis by SEC or SDS-PAGE.

3. Results

3.1. Degradation by precipitation

MACROSCINT® was exposed to intense light (725 W m^{-2}) for 4–6 h (reconstituted) or for up to 4 days (lyophilized). In addition, reconstituted MACROSCINT® was subjected to thermal stress (65 °C) for up to 2 h. Subsequent to treatment, the lyophilized samples were reconstituted with saline. All samples were then analyzed by SEC and SDS-PAGE. The results are presented in Figs. 1–4 and summarized in Tables 1 and 2.

The SEC chromatograms (Figs. 1 and 2) of both reconstituted and lyophilized MACROSCINT® samples exposed to intense light revealed a loss of between 70 and 90% of the DTPA-IgG protein over the course of the study (Tables 1 and 2). Although a change in monomer/dimer/aggregate distribution is also evident in these SEC chromatograms, the extent of protein loss is far greater than the change in monomer content. As indicated by the SDS-PAGE profiles (Figs. 4(A) and 4(B)), which revealed the presence of precipitated and aggregated protein that was unable to penetrate the polyacrylamide gel in both the reconstituted and lyophilized samples, this protein loss is a result of precipitation in these samples. In the SDS-PAGE profile of the lyophilized MACROSCINT® considerable "smearing" was also evident from the top of the gel through the DTPA-IgG monomer band. This "smearing" effect is indicative of the partial dissociation of the precipitated DTPA-IgG in the SDS.

Reconstituted MACROSCINT® samples stressed at 65 °C and analyzed by SEC revealed some minor differences when compared to the MACROSCINT® light treated samples (compare the SEC profiles in Figs. 1–3). However, a very similar overall pattern of degradation was observed, i.e. loss of total protein by SEC, due to precipitation, with a slight (relative to the protein loss) change in monomer/dimer/aggregate distribution (Fig. 3). It is interesting to note that for these samples a pattern was evident in the monomer/dimer/aggregate distribution as a function of stress. The aggregate content in these samples increased initially, but was followed by loss of aggregates as they precipitated (Table 1). These data suggest that when MACROSCINT® is subjected to conditions which encourage the formation of aggregated material, a critical concentration is reached at which continued accumulation of aggregates ceases and precipitation begins. The SDS-PAGE profile (Fig. 4(C)) of these heat-treated MACROSCINT® samples revealed the presence of a small amount of aggre-

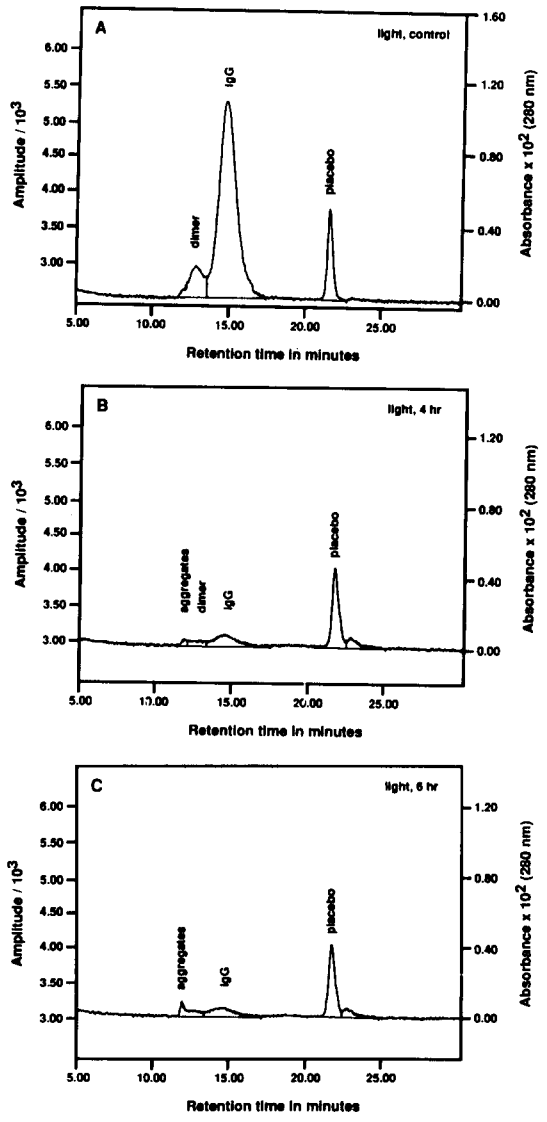


Fig. 1. SEC chromatogram of reconstituted MACROSCINT® control (A), reconstituted MACROSCINT® treated with light (725 W m^{-2}) for 4 h (B), and reconstituted MACROSCINT® treated with light for 6 h (C).

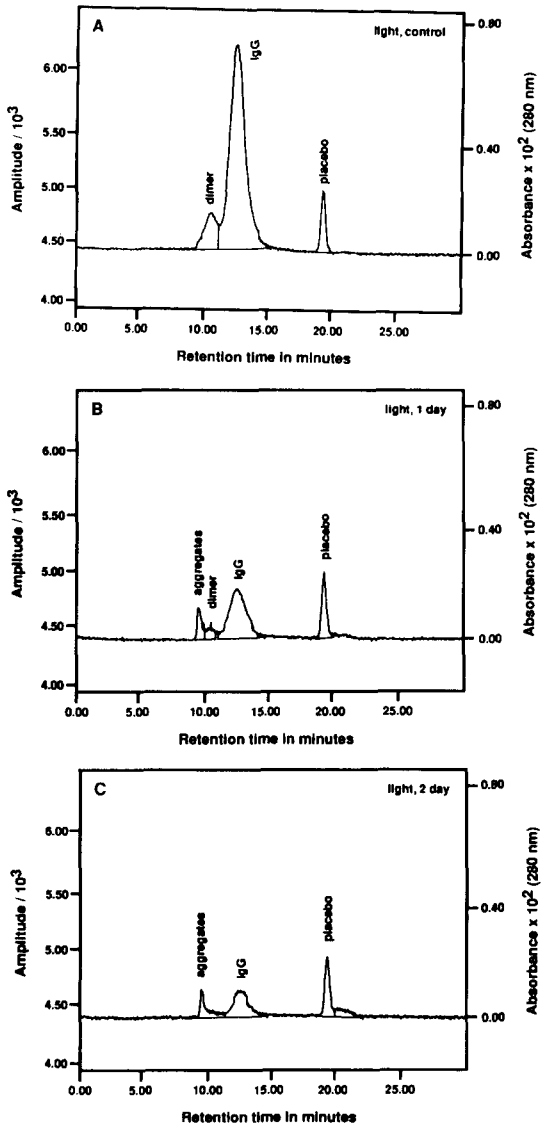


Fig. 2. SEC chromatogram of lyophilized MACROSCINT® control (A), lyophilized MACROSCINT® subjected to light (725 W m^{-2}) for 1 day (B), and for 2 days (C).

gated and precipitated material which was unable to penetrate the polyacrylamide gel in the samples heated for both 30 and 45 min. However, when the band intensities of the heat-treated samples on the SDS-PAGE gels are compared with the control, it is clear that the majority of the protein can be accounted for as DTPA-IgG monomer and dimer rather than as insoluble aggregate/precipitate. These data indicate that the precipitates present in the heat-treated samples are, to some extent, soluble in SDS. Together with the data from the light-treated samples (Figs. 1, 2, 4(A) and 4(B)), these results suggest that the ability of the SDS to solubilize and dissociate the aggregated/precipitated DTPA-IgG is a function of the conditions under which the sample is stressed.

In an attempt to determine whether these heat-induced changes were unique to temperatures of 63 °C or higher [5,8,9], additional experiments were conducted in which reconstituted MACROSCINT® was treated at 37 °C and 50 °C. The results (Fig. 5) illustrated that loss of total protein by SEC was observed not only at 65 °C, but was also detected at other, lower temperatures. However, at the lower temperatures the effects were less extreme. Thus, for example, while treatment at 65 °C resulted in greater than 85% loss of protein by SEC after 45 min, at 50 °C a time

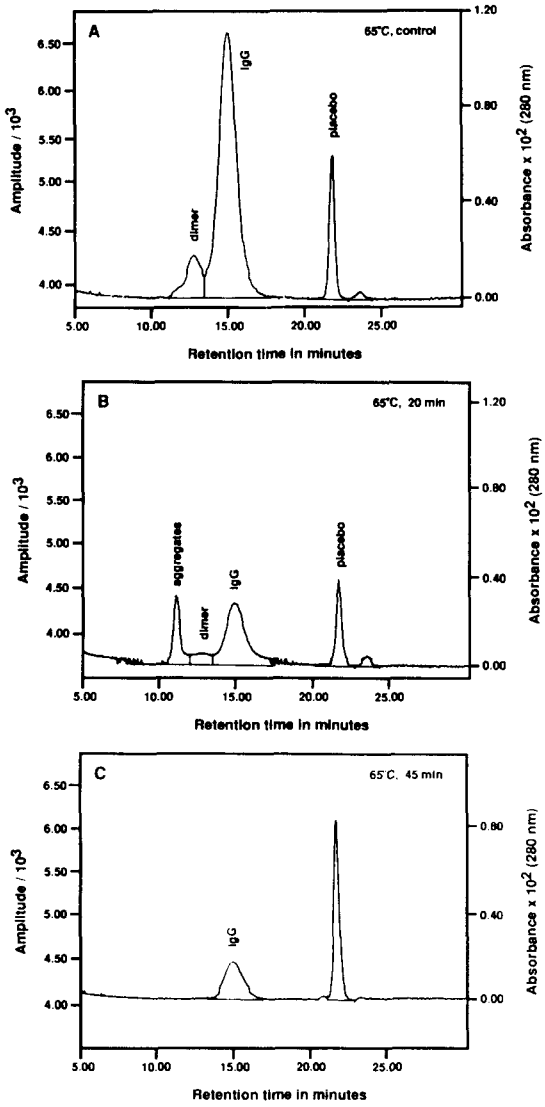
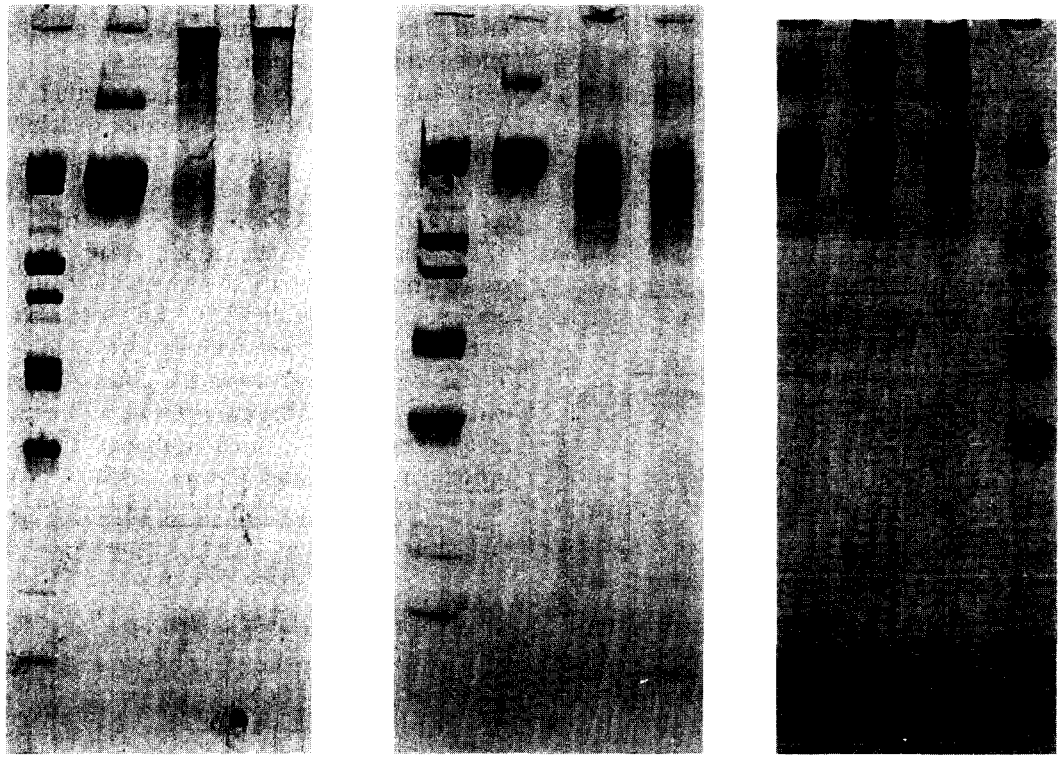


Fig. 3. SEC chromatogram of reconstituted MACROSCINT® control (A), reconstituted MACROSCINT® heated at 65 °C for 20 min (B), and reconstituted MACROSCINT® heated at 65 °C for 45 min (C).



(A) (B) (C)

Fig. 4. SDS-PAGE gels of light (725 W m^{-2}) and heat (65 °C) treated MACROSCINT®. Lanes from left to right are as follows: (A) molecular weight standards, MACROSCINT® control, reconstituted MACROSCINT® treated with light for 4 and 6 h; (B) molecular weight standards, MACROSCINT® control, lyophilized MACROSCINT® treated with light for 1 and 2 days; (C) MACROSCINT® control, reconstituted MACROSCINT® treated with heat for 30 and 45 min, molecular weight standards. Molecular weight standards include Myosin (MW 200 000), β-galactosidase (MW 116 250), phosphorylase B (MW 97 400), bovine serum albumin (MW 66 200), ovalbumin (MW 45 000), carbonic anhydrase (MW 31 000), and soybean trypsin inhibitor (MW 21 500).

period of 16 h was required before the total DTPA-IgG concentration dropped below 60%. 11 days of treatment was necessary at 37 °C to yield a 14% loss in total DTPA-IgG.

These data together illustrate that light treatment of reconstituted or lyophilized MACROSCINT® and heat treatment of reconstituted MACROSCINT® induce the formation of precipitating DTPA-IgG aggregates which, because they either precipitate out of solution or are removed by filtration of the sample prior to SEC, are evident by SEC as a loss of total protein. Depending on the time and severity of the stress, SDS demonstrates some limited ability to solubilize and dissociate these precipitating aggregates.

3.2. Degradation with increased DTPA-IgG monomer size

Thermal degradation of lyophilized MACROSCINT® was accomplished by stressing samples at 65 °C for 23 and 72 h. Subsequent to treatment, the samples were analyzed by SEC and SDS-PAGE. The resulting chromatograms and SDS-PAGE gels are illustrated in Figs. 6 and 7, respectively. The data are summarized in Table 3.

The SEC chromatograms of these heat-treated lyophilized MACROSCINT® samples revealed a very different pattern of degradation than was observed for any of the other stressed samples discussed above. No loss of total protein by SEC was observed even after the lyophilized MACROSCINT® had been exposed to 65 °C for 72 h. Degradation was, instead, revealed (by SEC) as both an increase in soluble aggregate content and a decrease in retention time of the DTPA-IgG monomer peak (relative to the IgG standard). This decrease in retention time was unique to the lyophilized MACROSCINT® exposed to thermal stress (compare Tables 1–3), not being observed for reconstituted MACROSCINT exposed to thermal stress or lyophilized MACROSCINT® exposed to intense light,

Table 1
Heat and light induced degradation of reconstituted MACROSCINT®

Treatment	Pro. conc. (SEC)[a]	% Protein recovered	% Monomer[b]	% Dimer[b]	% Aggregate[b]	% Degraded[b]	Rel. Ret. time[c]
Light control	0.94	100	89	11	0	0	0.988
Light 4 h	0.09	9.1	71	20	9	0	na[d]
Light 6 h	0.09	9.1	65	0	35	0	na
65 °C control	0.99	100	88	12	0	0	0.987
65 °C 10 min	0.34	34	89	8	3	0	0.987
65 °C 15 min	0.28	28	86	7	7	0	0.986
65 °C 20 min	0.30	30	70	6	24	0	0.985
65 °C 30 min	0.13	13	100	0	0	0	0.987
65 °C 45 min	0.14	14	100	0	0	0	0.990

[a] Protein concentration by SEC = DTPA-IgG monomer + dimer + aggregation/degradation products.
[b] Determined as percentage of total sample peak area. % DTPA-IgG aggregate is soluble aggregate only (evident in the SEC profile).
[c] Relative retention time (relative to polyclonal IgG standard).
[d] Owing to sample precipitation, a reliable retention time could not be obtained for the DTPA-IgG monomer in these samples.

both of which were shown to degrade via formation of insoluble aggregates and precipitates. Unfortunately, because of the extent of protein precipitation in the reconstituted MACROSCINT® exposed to intense light, it was not possible to obtain an accurate retention time for the residual DTPA-IgG monomer in these samples. Therefore, it is still possible that the degradation of these reconstituted MACROSCINT® samples via aggregation/precipitation is accompanied by a shift in SEC retention time. However, the similarity in pathway of degradation between the light- and heat-treated reconstituted samples contradicts this.

The decrease in retention time observed for the thermally stressed lyophilized MACROSCINT® is indicative of an increase in the size of the DTPA-IgG monomer. However, the extent of this size increase for the monomer is not sufficient to indicate the conversion of the DTPA-IgG monomer to dimers or soluble aggregates. SDS-PAGE (Fig. 7) analysis of these same samples confirmed an increase in molecular size of the DTPA-IgG monomer, which was thought to reflect either a conformational change in the DTPA-IgG under these conditions or a reaction with the maltose present as an excipient in the product. Because a conformational change should not be evident under the denaturing conditions employed for SDS-PAGE, the latter suggestion seemed more likely.

Both of these changes, increased size of the DTPA-IgG monomer and formation of soluble aggregates, were found to be a function of moisture content in the samples, as illustrated in Table 4. Thus, as more moisture was added to the vial, the retention time of the monomer peak decreased steadily (up to 20% moisture), indicating increased monomer size. In addition, the percentage of soluble DTPA-IgG aggregate (by SEC) increased with increasing moisture levels. At 50% moisture, the retention time of the DTPA-IgG monomer peak once again increased. This reversal in the direction of the reaction time corresponds to a sudden drop in protein recovery by SEC, suggesting that at some point between 20% and 50% moisture the pathway of degradation changes from one of increased monomer size to one which also involves precipitation, as was observed and reported above for reconstituted and lyophilized MACRO-SCINT® exposed to light or for reconstituted MACROSCINT® treated at 65 °C.

In addition to being moisture dependent, these changes (increase in DTPA-IgG monomer size with formation of soluble aggregates) also varied as a function of time at 65 °C. This is illustrated in Figs. 8 and 9 for lyophilized MACROSCINT® containing 20% added moisture. While the total protein concentration by SEC (data not shown) varied by no more than 3.5% throughout the course of this study (72 h), the percentage of soluble DTPA-IgG aggregate increased by 17% (Fig. 9) and the retention time (increase in IgG monomer size) decreased from 0.986 (relative to the IgG standard) to 0.920. The correlation between time and effect was relatively linear for

Table 2
Light induced degradation of lyophilized MACROSCINT®

Treatment	Pro. conc. (SEC)[a]	% protein recovered	% Monomer	% Dimer	% Aggregate	% Degraded	Rel. Ret. time
Light control	0.69	100	88	12	0	0	0.984
Light 1 day	0.21	30.4	76	9	14	1	0.983
Light 2 days	0.13	18.8	58	0	26	16	0.981
Light 4 days	0.13	18.8	59	6	22	13	0.983

[a] Protein concentration by SEC = DTPA-IgG monomer + dimer + aggregation/degradation products.

soluble aggregate content (correlation coefficient = 0.967). However, the change in retention time vs. time at 65 °C does not seem to be a linear function (correlation coefficient = 0.917). Instead, this curve appears to approach a plateau after 39 h of incubation. This lack of linear fit is consistent with the conjugation of the DTPA-IgG with the maltose in the MACROSCINT® lyophile, as is demonstrated in greater detail below, because any reaction between a ligand (maltose) and a finite number of binding sites (DTPA-IgG) is expected to reach equilibrium with time. SDS-PAGE gels (data not shown) confirmed the apparent increase in molecular weight of these samples, with a 10% difference in calculated molecular weight between the control and the 72 h sample.

These data together indicate that the unique changes seen in MACROSCINT® as a result of stressing the lyophile at 65 °C, increased size of the DTPA-IgG monomer and formation of soluble aggregates, are both moisture and time dependent.

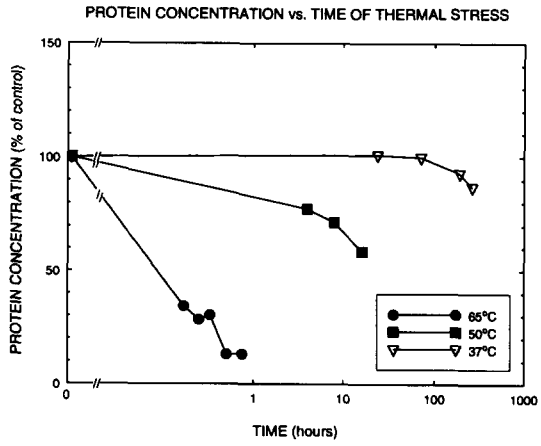


Fig. 5. Analysis of reconstituted MACROSCINT® treated at 37 (triangles), 50 (squares), and 65 °C (circles) by SEC.

3.3. Comparison of degradation for lyophilized polyclonal IgG, monoclonal IgG, and MACROSCINT®

Samples of polyclonal IgG, monoclonal IgG, and MACROSCINT® were dialyzed into citrate

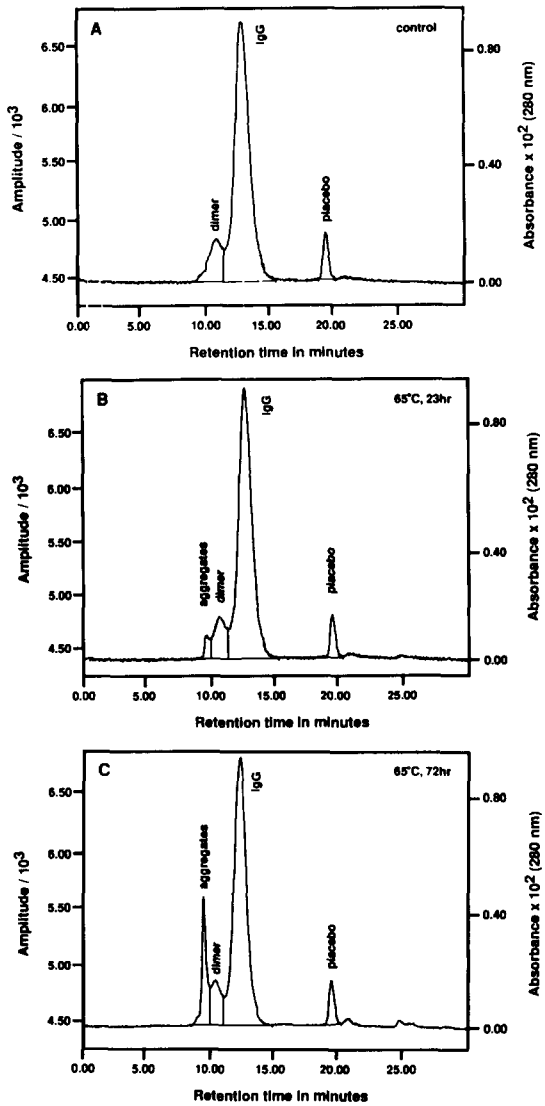


Fig. 6. SEC chromatogram of lyophilized MACROSCINT® control (A), and lyophilized MACROSCINT® subjected to heat (65 °C) for 23 h (B), and for 72 h (C).

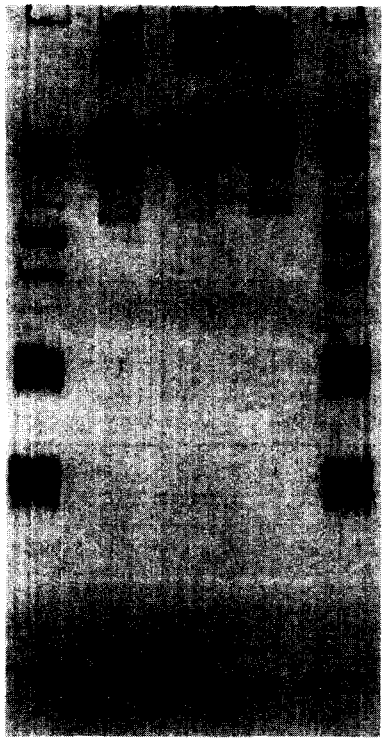


Fig. 7. SDS-PAGE gel of lyophilized MACROSCINT® subjected to treatment with heat (65 °C). Lanes from left to right are as follows: molecular weight standards, MACROSCINT® control, MACROSCINT® treated at 65 °C for 23 h, MACROSCINT® treated at 65 °C for 72 h, molecular weight standards.

buffer without maltose or into citrate buffer with maltose. Both the maltose-free and maltose-containing samples were lyophilized and stressed at 65 °C in the presence of 20% (added exogenously) moisture. The samples were then reconstituted and subjected to analysis by SEC. The results are presented in Table 5 and illustrate that in the absence of maltose extensive degradation of the polyclonal IgG, monoclonal IgG, and MACRO-SCINT® was observed as a result of the formation of precipitating aggregates (protein recoveries by SEC <2%). However, the presence of maltose in the lyophile prevented this precipitation phenomenon (protein recoveries by SEC were between 76 and 116%). Changes in these IgG products were, nevertheless, still apparent in that the retention times of the IgG monomers by SEC were down to approximately 0.91–0.92 (relative to the IgG standard). An increase in the percentage of dimer and soluble aggregate was also observed.

These data indicate that the mode of degradation observed for lyophilized MACRO-SCINT® exposed to thermal stress is not unique to DTPA-IgG, but is also seen for polyclonal and monoclonal IgG lyophilized under similar conditions. In addition, since in the absence of maltose degradation by precipitation is observed, these data add support to the hypothesis that the maltose present as an excipient in the preparation may be responsible for the apparent shift in molecular size of the IgG monomer peaks.

3.4. Correlation between increase in IgG monomer size and glucose conjugation to the IgG

In order to pursue the hypothesis that conjugation with the excipient maltose (a glucose disaccharide) may be responsible for the increase in size of thermally stressed, lyophilized IgG, samples of polyclonal IgG, monoclonal IgG, and MACROSCINT®, lyophilized in the presence of maltose, were subjected to thermal stress at 65 °C and 20% moisture for 23–72 h or at 40 °C for 18 months (MACROSCINT® only). These samples, together with control samples, maintained at 4 °C, were subjected to monosaccharide analysis. The results demonstrated that when the monosaccharide analyses of the control samples (lyophilized in the presence of maltose, then maintained at 4 °C) were compared to the monosaccharide analyses of the thermally stressed samples, a distinct peak, with a retention time corresponding to glucose, was present in the thermally stressed samples but not in the control samples (Fig. 10). These data (summarized in Table 6) also demonstrate that the amount of glucose bound to the DTPA-IgG was a function of the time over which the samples were stressed, as was also observed above for the SEC retention times of these same samples. These results together indicate that during the thermal stress of polyclonal IgG, monoclonal IgG or MACROSCINT® which have been lyophilized in the presence of maltose (a glucose disaccharide), the maltose in the cake conjugates to the IgG. This is a likely explanation for the decreased retention time of the IgG monomers in the thermally stressed, lyophilized IgG and MACROSCINT® samples. The data presented in Fig. 11, illustrating a linear relationship (correlation coefficient 0.969) between the SEC relative retention time for both human polyclonal IgG and the MACROSCINT® DTPA-IgG monomer and the glucose conjugated to the protein, add further support to this hypothesis.

Table 3
Heat induced degradation of lyophilized MACROSCINT®

Treatment	Pro. conc. (SEC)[a]	% Protein recovered	% Monomer	% Dimer	% Aggregate	Rel. ret. time[b]
23 h control	0.81	100	88	12	0	0.987
65 °C, 23 h	0.84	103.6	86	11	3	0.961
72 h control	0.79	100	88	12	0	0.986
65 °C, 72 h	0.81	102.5	76	10	14	0.935

[a] Protein concentration (mg ml^{-1}) by SEC = DTPA-IgG monomer + dimer + aggregation/degradation products.
[b] Relative retention time (relative to polyclonal IgG standard).

Table 4
Effect of moisture on degradation of lyophilized MACROSCINT® (65 °C, 23 h)

Moisture added	Pro. conc. (SEC)[a]	% Protein recovered	% Monomer	% Dimer	% Aggregate	Rel. ret. time[b]
Control[c]	0.81	100.0	88	12	0	0.987
0%	0.84	103.6	86	11	3	0.961
5%	0.84	104.2	87	11	2	0.949
10%	0.83	102.3	78	11	11	0.948
20%	0.84	103.3	83	11	6	0.947
50%	0.65	80.2	68	8	24	0.962

[a] Protein concentration (mg ml^{-1}) by SEC = DTPA-IgG monomer + dimer + aggregation/degradation products.
[b] Relative retention time (relative to polyclonal IgG standard).
[c] Control sample was stored without added moisture at 4 °C throughout the course of the study.

4. Discussion

The major pathway of degradation for lyophilized or reconstituted MACROSCINT® exposed to intense light or for reconstituted MACROSCINT® exposed to thermal stress is via the formation of precipitating aggregates. Degradation through this pathway is evident principally as a loss of total protein by SEC. The data suggest that when samples of MACROSCINT® are subjected to conditions which promote the formation of aggregated material, a critical concentration is reached at which continued accumulation of soluble aggregates ceases and precipitation begins. Degradation of IgG products as well as other protein-based pharmaceuticals by aggregation and precipitation has been well documented in the literature [1,5–11,14,15]. However, these studies have been conducted primarily on proteins in solution by subjecting them to stress with either heat or extremes of pH. In this report we demonstrate that degradation by aggregation and precipitation is also applicable to lyophilized IgG products, and can result from photo stressing as well as thermal stress.

In contrast, both lyophilized MACROSCINT® and lyophilized polyclonal and monoclonal IgG exposed to thermal stress at temperatures of 40 °C and above degrade through a very different pathway, characterized by both an increase in molecular size of the IgG (or DTPA-IgG) monomer and an increase in IgG (or DTPA-IgG) soluble aggregate con-

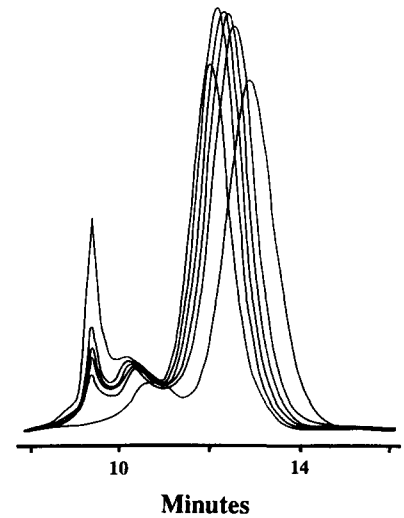


Fig. 8. SEC chromatograms (overlaid) of MACROSCINT® treated at 65 °C/20% moisture for 0, 8, 16, 24, 39, and 72 h (right to left).

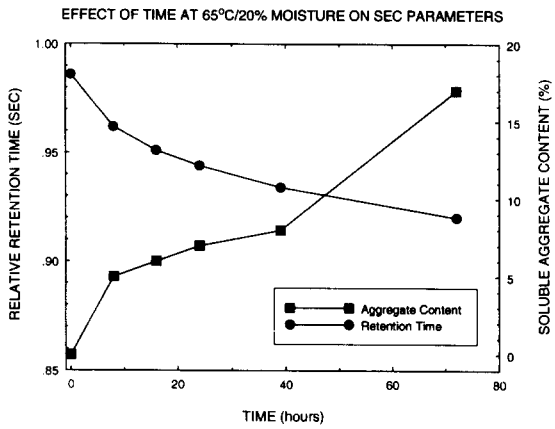


Fig. 9. Effect of time of stress at 65 °C on SEC relative retention time (circles) and soluble aggregate content (squares) for MACROSCINT® samples containing 20% added moisture.

centration. The molecular size change and soluble aggregate accumulation observed to accompany storage of lyophilized MACROSCINT® at higher temperatures is a function of both product moisture content and time of storage. The molecular size change has been demonstrated to correlate with a conjugation between the IgG products and the maltose present as an excipient in the product. Such non-enzymatic glucosylation of proteins has long been known to occur in vivo with a number of proteins due to the ubiquitous nature of the reducing sugars in the body and the nonspecificity of the reaction with amino groups. Increased levels of this posttranslational modification in diabetes have been correlated with hyperglycemia [17,18]. A possible involvement of non-enzymatic glucosylation in DNA damage, diabetes-related pathogenesis and aging has also been postulated [19,20].

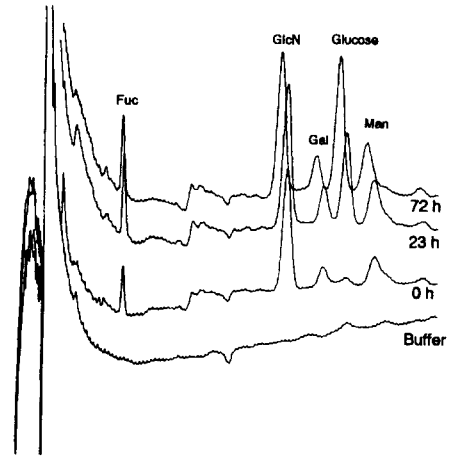


Fig. 10. Monosaccharide analysis of MACROSCINT® stressed at 65 °C, 20% added moisture for 0 h (second scan from bottom), 23 h (third scan from bottom), and 72 h (top scan). Bottom scan represents the buffer blank. Peak identification in order of elution: fucose (Fuc), glucosamine (GlcN), galactose (Gal), glucose, and mannose (Man).

Although the mechanism of maltose addition to these IgG samples is far from clear, since the

Table 6
Monosaccharide analysis of polyclonal IgG, monoclonal IgG, and MACROSCINT®

Sample name	Glucose[a]
MACROSCINT® control	0
MACROSCINT®, 65 °C/20% moisture, 23 h	4468000
MACROSCINT® 65 °C/20% moisture, 72 h	7385000
MACROSCINT® 40 °C, 18 months	12865000
Polyclonal IgG control	0
Polyclonal IgG 65 °C/20% moisture, 72 h	5526000
Monoclonal IgG control	1223500
Monoclonal IgG 65 °C/20% moisture, 72 h	4547500

[a] Peak area of glucose peak from monosaccharide analysis.

Table 5
Degradation of polyclonal IgG, monoclonal IgG, and MACROSCINT®, lyophilized with and without maltose

	Without maltose		With maltose			
	mg ml^{-1} (SEC)	Rel. ret. time[a]	mg ml^{-1} (SEC)	Aggregate/dimer (%)	Monomer (%)	Ref. ret. time[a]
Polyclonal IgG control	1.044	1.003	1.028	0	100	1.003
Polyclonal IgG, 20%, 65 °C, 72 h	<0.01	na[b]	1.191	18.6	72.0	0.920
Monoclonal IgG control	0.607	1.020	0.858	0	100	1.018
Monoclonal IgG, 20%, 65 °C, 72 h	<0.01	na	0.991	3.4	93.9	0.907
MACROSCINT® control	0.802	0.988	0.945	9.5	90.5	0.988
MACROSCINT®, 20%, 65 °C, 72 h	<0.01	na	0.719	40.5	52.7	0.915

[a] Retention time of monomer peak relative to polyclonal IgG standard.
[b] Owing to sample precipitation, a reliable retention time could not be obtained for the DTPA-IgG monomer in these samples.

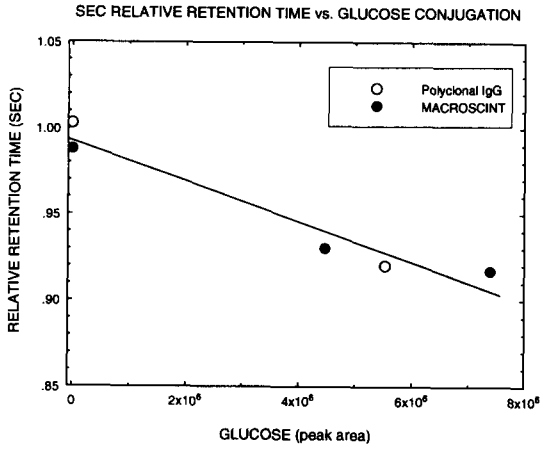


Fig. 11. Correlation of IgG relative retention time by SEC vs. glucose conjugation for IgG (○) and MACROSCINT® (●). Samples of MACROSCINT® and human polyclonal IgG lyophilized in the presence of maltose were stressed at 65 °C in the presence of 20% added moisture for various periods of time up to 72 h. The samples were then reconstituted and subjected to SEC analysis and monosaccharide analysis.

lyophilized samples which conjugate with maltose in the course of thermal stress also develop a slight brownish/yellow color, one possible mechanism by which this conjugation may occur is the Maillard reaction. This reaction, which is also known as the "browning reaction", has been reported to occur between other proteins or amino acids and reducing sugars including fructose, glucose, and lactose [21–24]. It has also been observed during heat treatment and prolonged storage of foods [25]. In this report we have demonstrated that the reaction between proteins and reducing sugars also plays a significant role in the stability and degradation of protein-based pharmaceuticals lyophilized in the presence of reducing sugars.

References

[1] T. Jøssang, J. Feder and E. Rosenqvist, Heat aggregation kinetics of human IgG, J. Chem. Phys., 82 (1985) 574–589.

[2] J. Tschopp, Kinetics of activation of the first component of complement (C1) by IgG oligomers, Mol. Immunol., 19 (1982) 651–657.

[3] H.N. Eisen, in Immunology: An Introduction to Molecular and Cellular Principles of the Immune Responses, 2nd edn., Harper & Row, Philadelphia, PA, 1980.

[4] K. James, C.S. Henney and D.R. Stanworth, Structural changes occurring in 7S γ-globulins, Nature, 202 (1964) 563–566.

[5] J.H. Morse, The aggregation of γ-myeloma proteins, J. Immun., 95 (1965) 722–729.

[6] I. Oreskes and D. Mandel, Size fractionation of thermal aggregates of Immunoglobulin G, Anal. Biochem., 134 (1983) 199–204.

[7] V.P. Zav'yalov, G.V. Troitsky, A.P. Demchenko and I.V. Generalov, Temperature and pH dependent changes of Immunoglobulin G structure, Biochim. Biophys. Acta, 386 (1975) 155–167.

[8] D. McCarthy, D.H. Goddard, P.H. Embling and E.J. Holborow, A simple procedure for assessing the stability of heat-aggregated IgG preparations, J. Immunol. Methods, 41 (1981) 75–79.

[9] D. McCarthy, D.H. Goddard, B.K. Pell and E.J. Holborow, Intrinsically stable IgG aggregates, J. Immunol. Methods, 41 (1981) 63–74.

[10] E. Rosenqvist, T. Jøssang and J. Feder, Thermal properties of human IgG, Mol. Immun., 24 (1987) 495–501.

[11] M.C. Manning, K. Patel and R.T. Borchardt, Stability of protein pharmaceuticals, Pharm. Res., 6 (1989) 903–918.

[12] R.H. Rubin, A.J. Fischman, R.J. Callahan, B. Khaw, F. Keech, M. Ahmad, R. Wilkinson and H.W. Strauss, ^{111}In-labeled nonspecific immunoglobulin scanning in the detection of focal infection, N. Engl. J. Med., 321 (1989) 935–940.

[13] W. Oyen, R. Claessens, J. Van Horn, J. van der Meer and F. Corstens, Scintigraphic detection of bone and joint infections with indium-111-labeled non-specific polyclonal human immunoglobulin G, J. Nucl. Med., 31 (1990) 403–412.

[14] S.A. Charman, K.L. Mason and W.M. Charman, Techniques for assessing the effects of pharmaceutical excipients on the aggregation of porcine growth hormone, Pharm. Res., 10 (1993) 954–962.

[15] J.A. Schrier, R.A. Kenley, R. Williams, R.J. Corcoran, Y. Kim, R.P. Northey, Jr., D. D'Augusta and M. Huberty, Degradation pathways for recombinant human macrophage colony-stimulating factor in aqueous solution, Pharm. Res., 10 (1993) 933–944.

[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227 (1970) 680–688.

[17] J.B. Henry, in Clinical Diagnosis and Management, 18th edn., W.B. Saunders Co., Philadelphia, PA, 1991.

[18] S.R. Thorpe and J.W. Baynes, Nonenzymatic glycosylation of proteins in vitro and in vivo, in M.I. Horowitz and W. Pigman (Eds.), The Glycoconjugates, Vol III, Academic Press, New York, 1982, pp. 113–131.

[19] B.S. Kristal and B.P. Yu, An emerging hypothesis: synergistic induction of aging by free radicals and Maillard reactions, J. Gerontol., 47 (1992) B107–B114.

[20] V.M. Monnier, D.R. Sell, R.H. Nagaraj, S. Miyata, S. Grandhee, P. Odetti and S. Ibrahim, Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia, Diabetes, 41 (1992) 36–41.

[21] F.B. Whitfield, Volatiles from interactions of Maillard reactions and lipids, Crit. Rev. Food Sci. Nutr., 31 (1992) 1–58.

[22] W. Dills, Protein fructosylation: fructose and the Maillard reaction, Am. J. Clin. Nutr., 58 (suppl) (1993) 779S–787S.

[23] E. Tarelli, P.H. Corran, B.R. Bingham, H. Mollison and R. Wait, Lysine vasopressin undergoes rapid glycation in the presence of reducing sugars, J. Pharm. Biomed. Anal., 12 (1994) 1355 - 1361.

[24] G. Suárez, R. Rajaram, A.L. Oronsky and M.A. Gawinowicz, Nonenzymatic glycation of bovine serum albumin by fructose (fructation), J. Biol. Chem., 264 (1989) 3674–3679.

[25] T. Henle, H. Walter and H. Klostermeyer, Evaluation of the extent of the early Maillard-reaction in milk products by direct measurement of the Amadori-product lactuloselysine, Z. Lebensm. Unters. Forsch., 193 (1991) 119–122.