this treatment. The unmedicated feed samples produced fluorescence representing 1–2 ppm of thiopeptin, furnishing sufficient selectivity to determine this drug at the 5-ppm level with an intensity 33% greater than the reagent blank. The mean of the eight analyses covering the 5–20-ppm range in the feed was 95% of the known thiopeptin concentration that was added to the feed. These results demonstrate the use of this treatment for the determination of one thiazole-containing analyte and suggest that this method also should be applicable to other analogous thiazole-containing compounds of pharmaceutical and biological interest.

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Decreased Activity of Proteins Adsorbed onto Glass Surfaces with Porous Glass as a Reference

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Abstract □ The biological activity of proteins bound to controlled-pore glass surfaces was studied as a model of denaturation of biologicals upon storage in glass containers. After adsorption onto the glass for 1 week, the activities of alkaline phosphatase, catalase, and horse-radish peroxidase recovered from the glass column were 88, 63, and 97%, respectively. However, the phosphatase activity recovered after adsorption for 3 months was 14% of the total activity loaded onto the column, and the activities recovered of peroxidase and catalase were 48 and 2%, respectively. Insulin had almost full activity after adsorption for 3 months, but calcitonin activity was absent. The scission of peptide bonds of proteins eluted after adsorption for 3 months was not observed, but dissociation to the subunits was found. The proteins were active in the state adsorbed onto glass surfaces.

Keyphrases □ Adsorption—proteins, glass surfaces, activity decrease □ Proteins—adsorption onto glass surfaces, activity decrease □ Activity—proteins, decrease after adsorption onto glass surfaces

The reaction of biological materials on glass is well known (1–4). By use of controlled-pore glass (5) with a large surface area (97 m^2/g), it was shown that 5 μmoles of basic drugs and materials was adsorbed onto 97 m^2 of glass surface and that adsorption of proteins onto the surfaces was caused by amine–silanol bonding and a cooperative aggregative factor between silica and proteins (6–10). Proteins also were adsorbed in detergent solutions such as urea and guanidine hydrochloride (11). From the value of the maximum amount of protein adsorbed (233 mg/97 m^2), the amount of protein adsorbed onto the surfaces of a glass container (50 cm^2, 20 ml) and a glass injector was estimated to be >12 μg. The 12-μg/20 ml concentration must be a marginal point not affected by adsorption, and the biologicals used at lower concentrations would be affected by adsorption to glass surfaces (10).

Some biologicals composed of proteins are stored or maintained in pharmaceutical glass containers in a water medium (12). However, denaturation of proteins by their adsorptive aggregation on surfaces of glass containers upon storage for long periods is not clear. The purpose of this investigation was to show changes in the biological activity and the structure of proteins adsorbed onto glass surfaces upon storage as a model of denaturation of protein biologicals in glass containers. Enzymes and hormones were studied, and controlled-pore glass was used as a reference standard.

EXPERIMENTAL

Materials and Column Operation—The controlled-pore glass[1] was 96% silica and had a surface area of 97 m^2/g and a particle size of 100 μm. After being washed with water, 0.1% sodium dodecylsulfate, water, chromic acid mixture, and water, the glass was packed in columns (0.6

[1] CPG-10, Electro-Nucleonics, Fairfield, N.J.

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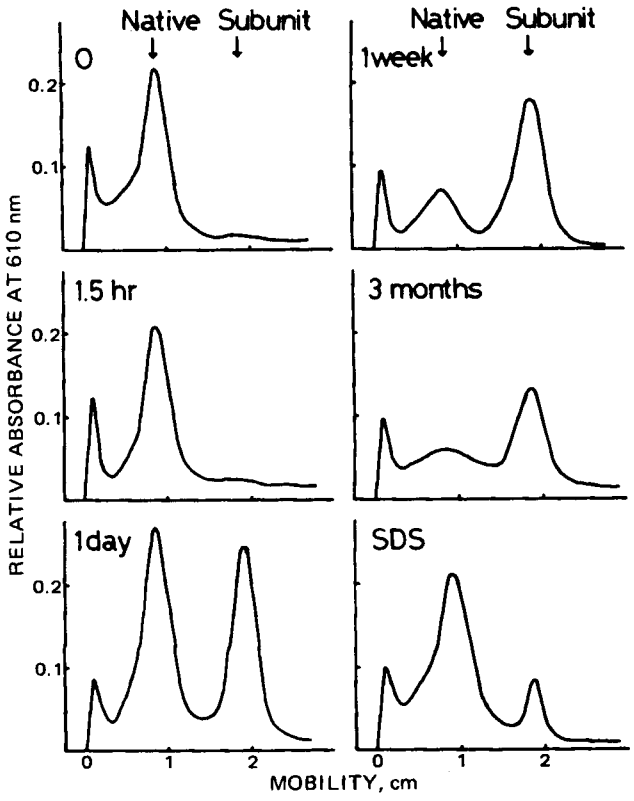

Figure 1—*Densitometric tracing of stained gel patterns of alkaline phosphatase on 10% polyacrylamide gel in the presence of 0.1% sodium dodecylsulfate. The times on the shoulder of each figure are those of adsorption. SDS is the sample incubated in 4* M *urea–0.1% sodium dodecylsulfate solution for 15 hr.*

× 10 cm, 1.3 g), which were used to preserve the proteins in the state in which they were bound to glass. After the column was equilibrated with buffer, the protein solution was applied on the column.

For experiments involving the retention of the activity of proteins bound to porous glass, the protein-bound columns were stored for 1 day, 1 week, 1 month, or 3 months in a cold room at 4°. After storage, the columns were eluted at a flow rate of 0.5 ml/cm^2/min, and 1-ml fractions were collected. Three columns were used for the measurement of each point, and the mean ● *SE* of the three measurements was calculated.

The enzymes and hormones used were alkaline phosphatase from *Escherichia coli*[2], horse-radish peroxidase[3], catalase[3], malate dehydrogenase[3], insulin[4], and porcine calcitonin[5]. For storage for 1 and 3 months, sodium azide was added at 0.1% to the enzyme solution as a preservative.

Activity Measurement—The activity of alkaline phosphatase was measured by the increase of the optical density at 405 nm in an assay mixture containing 0.95 *M* tromethamine [tris(hydroxymethyl)aminomethane] hydrochloride (pH 8.0), 4.8 m*M* *p*-nitrophenyl phosphate, and enzyme. Alkaline phosphatase was used after dialysis of ammonium sulfate contained in the commercial enzyme solution, and the specific activity was 10 units/mg. The activity of malate dehydrogenase was measured by the decrease of the optical density at 340 nm in the mixture of 94 m*M* phosphate buffer (pH 7.5), 0.5 m*M* oxaloacetate, 0.2 m*M* β-nicotinamide adenine dinucleotide in the reduced form, and enzyme. Commercial malate dehydrogenase was used without further purification.

The activity of peroxidase was determined according to a literature method (13) involving measurement of the time required to increase the optical density by 0.05 at 470 nm when 10 μl of 10 m*M* hydrogen peroxide was added to 3 ml of 13 m*M* guaiacol–enzyme. The activity of catalase was determined by direct measurements of the decreased light absorption at 230 nm caused by the decomposition of hydrogen peroxide by catalase.

The insulin activity was determined by measurement of the decreased glucose in rabbit serum. The glucose concentration in the serum derived from a group of six male rabbits was analyzed by a literature method using a mixture of 960 ml of 8% *o*-toluidine in acetic acid and 40 ml of 4% boric acid in water (14). Blood was drawn from the aural vein of six rabbits before and 1 hr after the injection of insulin. The rate of the fall of serum glucose after the injection compared with that before the injection was calculated, and the mean rate was taken as the glucose lowering rate.

The hypocalcemic activity of calcitonin was determined using six male rabbits (15). The sample was injected into the rabbit aural vein, and blood was drawn from another aural vein before and 1 hr after the injection. The calcium concentration in the serum was determined by atomic absorption[6] at 422.7 nm (16). The rate of fall of serum calcium after the injection compared with that before the injection was calculated. Bioassay data are expressed as the mean ± *SE*.

Other Methods—The molecular weights of the proteins were determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis (17). The acrylamide gel concentration was 10% for alkaline phosphatase, peroxidase, and catalase and 20% for insulin and calcitonin. Densitometric tracing of the stained bands on the gel was carried out using an autodensitometer[7], and the ratio of proteins on the gel was calculated from the patterns.

RESULTS AND DISCUSSION

Activity of Proteins Eluted from Porous Glass Columns—Alkaline phosphatase was adsorbed onto a glass column in 0.2 *M* sodium chloride–0.01 *M* phosphate buffer (pH 7.0) and eluted with 0.2 *M* glycine at pH 9.0. After 1 mg of the phosphatase was adsorbed onto the column which can adsorb about 100 mg of protein, the column was washed with five column volumes of buffer, followed by glycine buffer (pH 9.0) for enzyme elution. After adsorption for 1.5 hr, the activity of phosphatase was completely recovered, and the specific activity was identical with that of the untreated enzyme.

The sodium dodecylsulfate–polyacrylamide gel pattern of the eluted enzyme was similar to that of the native enzyme (Fig. 1). After adsorption for 1 day in the sodium chloride–phosphate buffer, 95% of the activity of phosphatase applied on the column was eluted with the glycine solution, but 45% of the enzyme was separated into subunits with a molecular weight of 40,000 (Fig. 1). This separation did not originate from the treatment used in sodium dodecylsulfate electrophoresis, because the native phosphatase did not show the subunit pattern after incubation and treatment for 1 day in the sodium dodecylsulfate solution (Fig. 1).

In the case of adsorption for 1 week, 88% of the activity was recovered, and 61% of the total protein eluted from the column dissociated into subunits (Fig. 1). The phosphatase activity recovered after adsorption for 1 month was 62%. After phosphatase was adsorbed onto the porous glass column for 3 months, elution with glycine yielded the enzyme activity as only 14%; the yield of protein by elution with glycine was 24%. Subunits comprised 70% of the total protein eluted from the column with glycine buffer (Fig. 1).

The relationship between the activity and the binding time on glass surfaces is summarized in Table I. The half-life of the activity of phosphatase adsorbed onto glass surfaces was ~30 days. Phosphatase was eluted easily with glycine after adsorption for 1 week, and the activity was almost completely recovered. The enzyme was adsorbed strongly after standing for 3 months and was not completely eluted with glycine but was eluted with the 0.1% sodium dodecylsulfate solution, which has the strongest eluting force of proteins from the porous glass column among several buffers. The band showing a smaller molecular weight than the subunit was not found on the gel patterns (Fig. 1). Therefore, scission of the peptide bond did not occur on glass surfaces for the long preservation period. The decreased activity recovered from the glass column was believed to depend on a conformational change of proteins or desolubilization by aggregation of proteins on glass surfaces.

After adsorption of catalase for 1 week, 63% of the catalase activity was eluted from the column with the glycine solution, and residual protein was eluted with the 0.1% sodium dodecylsulfate solution. The sodium dodecylsulfate gel pattern of catalase after the 1-week adsorption showed that the subunit with a molecular weight of 6 × 10^4 was 80% of the total protein, even though the native enzyme preparation was comprised of 73% subunits by the gel pattern. The specific activity of catalase adsorbed for 1 week decreased to 75%.

After adsorption for 3 months, the catalase activity recovered from the

[2] P-L Biochemical Inc., Milwaukee, Wis.
[3] Boehringer Mannheim GmbH, Mannheim, West Germany.
[4] Fluka AG, Buchs, Switzerland.
[5] Armour Pharmaceutical Co., Chicago, Ill.
[6] AA 610 spectrophotometer, Shimadzu, Kyoto, Japan.
[7] FD-A IV, Fujiriken, Tokyo, Japan.

Table I—Protein Activity in Eluates after Adsorption onto Porous Glass Columns

Protein	Total Activity Recovered after Adsorption[a], % (Specific Activity, %)		
	1 Week	1 Month	3 Months
Horse-radish peroxidase	97 ± 1.5 (100)	77 ± 2.1 (98)	48 ± 3.1 (90)
Alkaline phosphatase	88 ± 1.1 (98)	62 ± 1.7 (92)	14 ± 1.0 (58)
Catalase	63 ± 3.2 (75)	26 ± 3.0 (73)	2 ± 0.9 (6)
Insulin	—	—	89 ± 2.3 (86)

[a] The values are given as the means of three experiments (±*SE*).

column with glycine buffer (pH 9.0) was only 2% of the total protein applied on the column. Residual catalase adsorbed onto porous glass surfaces was not eluted with the 0.1% sodium dodecylsulfate solution, and the colored band of catalase or heme was found on the top of the column. Catalase was adsorbed onto glass surfaces more strongly than was alkaline phosphatase.

Horse-radish peroxidase was not adsorbed onto the glass column in 0.2 *M* sodium chloride–0.01 *M* phosphate buffer (pH 7.0) but was adsorbed in a distilled water medium. Therefore, peroxidase was eluted with the sodium chloride–phosphate buffer from the column on which the enzyme was loaded in a water medium. After adsorption for 1 week, the recovery of the peroxidase activity was 97% of the initial activity. The recovery of the activity after adsorption for 3 months was 48%, and the residual protein adsorbed onto glass surfaces was eluted with the 0.1% sodium dodecylsulfate solution. The mobility of the eluted peroxidase on a gel did not change from that of the native protein.

The eluting order of proteins by adsorption chromatography was peroxidase, phosphatase, and catalase (7), and the adsorption of peroxidase was weak. The recoveries of the activity after adsorption for 3 months were 48% for peroxidase, 14% for phosphatase, and 2% for catalase (Table I). The stronger the proteins adsorbed onto glass surfaces, the lower was the activity recovery. The retention of the activity of proteins did not relate to the molecular weight and isoelectric point but instead to the inherent properties of proteins such as solubility and aggregative force. Since γ-globulin in serum was adsorbed strongly onto glass and the elution of the γ-globulin fraction from a porous glass column by adsorption chromatography was difficult, as reported previously (7), adsorption of antiserum samples onto the surface of glass containers must be considered. The inactivation of biologicals composed of proteins upon storage must be caused partially by adsorption onto the surface of glass containers.

Insulin (0.5 mg, 10 units) was dissolved in 0.5 ml of 0.01 *N* hydrochloric acid–1.5% glycerin isotonic solution and applied on the porous glass column. Insulin was adsorbed well onto porous glass in this condition (10). After adsorption for 3 months, adsorbed insulin was eluted with 0.2 *M* glycine (pH 9.0), and the pH of the eluate was adjusted with hydrochloric acid to pH 2. For injection, the protein concentration of insulin in the solution was estimated from the absorbance at 280 nm. The solution containing insulin was diluted to a concentration of 0.05 mg/ml with a 1.5% glycerin isotonic solution (pH 2.6), and 0.5 ml was injected subcutaneously into each rabbit. The molecular weight of insulin in the eluate from the column was studied by sodium dodecylsulfate gel electrophoresis using 20% gel, but the mobility on the gel was identical to that of native protein, and the scission to the A-chain and B-chain was not found. In a dose of 0.025 mg/rabbit, native insulin lowered the rabbit serum glucose concentration to 41.7 ± 10.3%, and insulin in the column eluate after adsorption for 3 months lowered the concentration to 36.3 ± 4.1%. The value of 36.3% was less than that of native insulin, but no significant difference between the two values was found. Insulin was not inactivated by adsorption for 3 months.

The calcium lowering rate of native porcine calcitonin was 12.7 ± 0.7% 1 hr after the injection of 4 MRC units/kg of rabbit. Calcitonin (100 MRC units) was dissolved in 1 ml of water and loaded onto a porous glass column, and the column was stored for 3 months at 4°. The column was eluted with 0.2 *M* glycine (pH 9.0), and the eluate of five column volumes was collected. The eluate was used for the hypocalcemic activity assay. The dose of 4 MRC units/kg of rabbit to be injected was calculated on the assumption that the eluate contained the total amount of calcitonin (100 MRC units) applied on the column. However, the hypocalcemic rate at 1 hr after the injection, 2.6 ± 1.4%, was insignificant, even though the band of calcitonin was found on the sodium dodecylsulfate–polyacrylamide gel of the eluate. After elution with glycine, the column was eluted with the 0.1% sodium dodecylsulfate solution, but the gel pattern of the eluate did not contain the band of calcitonin. Finally, active calcitonin was not recovered from the porous glass column after adsorption for 3 months.

These adsorption experiments for long periods showed that enzymes and hormones had a specific strength of activity on glass surfaces. Moreover, drugs might be inactivated on surfaces of glass containers upon storage. In this experiment, 96% silica glass was used, but proteins show different properties on other glass, such as soda-lime glass, from those on 96% silica glass. The stability of enzymes in the buffer at 4° was examined as a control experiment, and the enzymes stored for 3 months in the solution containing 0.1% sodium azide were fully active and the dissociation to the subunit was not found because the commercial enzymes used were stable and pure. Twenty-milliliter glass containers are able to adsorb about 10 μg of proteins. Therefore, for many biologicals composed of proteins at high concentration, the protein drugs in the containers might be inactivated more slowly than the time showed in these experiments.

Activity of Proteins in State Adsorbed onto Glass—After enzymes were adsorbed onto a glass column, the substrate solution was loaded onto the column at a constant flow rate, and the absorbance of the product in the eluate was measured. Malate dehydrogenase (23 μg) in 0.1 *M* phosphate buffer (pH 7.4) was adsorbed onto the column, and the substrate solution containing nicotinamide adenine dinucleotide in the reduced form and oxaloacetic acid was applied on the column at a flow rate of 1.2 ml/min/cm^2. The decreased absorbance in the eluate at 340 nm was 0.41, and thus confirmed that malate dehydrogenase on glass surfaces was active. The reaction rate of the enzyme immobilized on glass was about 1/600 compared with that in the free solution from the flow rate data, the thickness of the band adsorbing the enzyme, and the enzyme concentration in the band.

Alkaline phosphatase (3.4 μg) was adsorbed onto the column, and the column was washed with five column volumes of the 0.2 *M* sodium chloride–0.01 *M* phosphate buffer (pH 7.0). The substrate solution containing 5 m*M* *p*-nitrophenyl phosphate was applied on the column at a flow rate of 1.2 ml/min/cm^2, and the absorbance of the eluate at 405 nm was measured. The apparent reaction rate of the enzyme on glass surfaces was about one-fifth of that in the free solution. The absorbance of the solution was measured at 405 nm at 0 and 1 hr, but it had not changed. Therefore, the enzyme was not eluted and was not contained in the eluate. The change of the absorbance was caused by digestion with the enzyme bound to porous glass surfaces.

Peroxidase (2 mg) dissolved in a water medium was adsorbed onto the glass column, and the substrate solution containing guaiacol and hydrogen peroxide was loaded on the column. The eluate developed a brown color, confirming the reaction with peroxidase on the column. The activity of catalase bound to the porous glass column was ascertained by the evolution of gas (oxygen) with elution of the hydrogen peroxide solution. Bovine serum albumin (1 μmole) bound to glass surfaces adsorbed 0.42 μmole of aspirin and 0.59 μmole of sulfamethoxazole in 0.15 *M* sodium chloride–0.01 *M* phosphate (pH 6.0) by frontal analyses. After adsorption of albumin for 1 week, the amounts of aspirin and sulfamethoxazole bound to albumin (1 μmole) were 0.35 and 0.16 μmole, respectively. The reason for the lower values than those obtained using albumin coupled on agarose (18) must be that the site on albumin which adsorbs drugs was used for adsorption to glass silanol.

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Synthesis and Anti-Inflammatory Evaluation of 3-Methylthio-1,2,4-triazines, 3-Alkoxy-1,2,4-triazines, and 3-Aryloxy-1,2,4-triazines

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Abstract □ To develop nonacidic, nonsteroidal anti-inflammatory agents without GI complications, a series of asymmetric triazines was synthesized and evaluated for anti-inflammatory efficacy in the carrageenan-induced pedal edema assay. Toxicity was estimated by determination of approximate LD_{50} values in mice. Twenty-five compounds possessed activity comparable to the standard, indomethacin. Thirteen of the 25 compounds were selected for dose–response evaluation in the carrageenan assay based on their relative toxicity and anti-inflammatory activity. Neurotoxicity of the 13 triazines was estimated by determination of NTD_{50} values in mice. Five of the 13 compounds tested in the dose–response assay were active in terms of anti-inflammatory efficacy (ED_{50} values) and lack of overt neurotoxicity (NTD_{50} values) when compared to indomethacin. To determine the effect of these five developmental triazines on chronic inflammation, they were evaluated in the adjuvant-induced polyarthritis assay. One was comparable to indomethacin in reducing adjuvant-induced inflammation in this assay.

Keyphrases □ Triazines, asymmetric—synthesis, evaluation of anti-inflammatory activity □ Anti-inflammatory agents—asymmetric triazines, synthesis, evaluation of activity □ Polyarthritis, adjuvant induced—assay, asymmetric triazines

In recent years, the literature on nonsteroidal anti-inflammatory agents has increased dramatically. From 1966 to 1976, 807 new compounds from 262 research laboratories were identified as new nonsteroidal anti-inflammatory agents. However, clinical reports were available on only 65 drugs; and of those that have been marketed, only a few have been commercially successful. GI irritation continues to be the principal complication with most developmental, clinical, and commercial nonsteroidal anti-inflammatory drugs.

The occurrence of GI effects in humans has been demonstrated by numerous investigators with administration of various salicylates (1–4), phenylbutazone (5, 6), indomethacin (7), tolmetin (8), naproxen (9), and ibuprofen (10). The ongoing search for novel classes of nonsteroidal anti-inflammatory agents in part reflects the continued inability to separate anti-inflammatory efficacy from GI toxicity. Although considerable controversy concerns the etiology of this toxicity, it generally is agreed that gastric irritation is associated, directly or indirectly, with the acidic nature of these drugs and their metabolites (11, 12).

DISCUSSION

To eliminate GI complications while maintaining anti-inflammatory activity, a series of 81 asymmetric triazines was synthesized and evaluated for potential anti-inflammatory efficacy. The carrageenan-induced pedal edema assay was utilized to detect primary level activity; acute toxicity was estimated by determination of LD_{50} values in mice. A dose–response carrageenan assay and neurotoxicity evaluation were used to determine the ED_{50} and NTD_{50} values of those compounds active at the primary level. Neurotoxicity was estimated by determination of NTD_{50} values in mice. Compounds that were comparable to indomethacin in the secondary stage of evaluation were tested in the adjuvant-induced polyarthritis assay to determine their effect on a chronic inflammatory condition.

Synthesis—3-Methoxy-5-substituted-1,2,4-triazines (III*a*–III*v*) and 3-alkoxy-5-substituted-phenyl-1,2,4-triazines (IV*a*–IV*l*) were synthesized. Melting points and recrystallization solvents are shown in Tables I–III. The 3-methylthio-1,2,4-triazines (II*a*–II*v*) served as common intermediates in the synthesis of all 3-alkoxy- and 3-aryloxy-1,2,4-triazines (III*a*–III*v*, IV*a*–IV*l*, X*a*–X*h*, and XI*a*–XI*i*). The 3-methylthio intermediates were synthesized according to the method of Paudler and Chen (13) with modifications by Heilman *et al.* (14).

Treatment of II with sodium methoxide in refluxing methanol resulted in nucleophilic displacement of the methylthio function to afford the corresponding 3-methoxy-5-substituted-1,2,4-triazines (III*a*–III*v*). Reaction of II with a variety of sodium alkoxides refluxing in dioxane afforded the corresponding 3-alkoxy-5-phenyl-1,2,4-triazines (IV*a*–IV*l*).

5,6-Disubstituted-3-alkoxy-1,2,4-triazines (VII*a*–VII*h* and VIII*a*–VIII*i*) were synthesized. Melting points and recrystallization solvents are shown in Tables IV and V. Cyclization of symmetrical 1,2-diones (V*a*–V*h*) under basic conditions with methylthiosemicarbazide hydrogen iodide afforded the 3-methylthio-5,6-disubstituted-1,2,4-triazines (VI*a*–VI*h*). Nucleophilic displacement of the methylthio group with the appropriate sodium alkoxide produced the desired 3-alkoxy-5,6-disubstituted-1,2,4-triazines (VII*a*–VII*h* and VIII*a*–VIII*i*).

Biology—The effect of the asymmetric triazines on the inflammatory response was evaluated at the primary level in the carrageenan-induced pedal edema assay (15). Carrageenan injected into the plantar tissue of the hindpaw of Sprague–Dawley rats produces an edematous condition, which simulates in part the inflammatory process found in human arthritis (16–18). Nonsteroidal anti-inflammatory drugs such as indomethacin, phenylbutazone, and aspirin inhibit the formation of this edema (19, 20).

Compounds were administered orally, using 0.25% methylcellulose as the vehicle. Five rats were used per dose, with the reported percent reduction in inflammation represented by the average of the reduction produced in the five animals. Compounds were administered at levels expected to be subtoxic by consideration of their approximate, measured LD_{50} values. The LD_{50} values in mice were determined in a standard,

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