

Detection of antibodies to proteases used in laundry detergents by the radioallergosorbent test

P. J. Dor, M.D.,* M. K. Agarwal, Ph.D., M. C. Gleich, B.S.,
P. W. Welsh, B.S., S. L. Dunnette, B.S., C. R. Adolphson, M.S., and
G. J. Gleich, M.D. Rochester, Minn.

Two proteases, Esperase and Alcalase, derived from Bacillus licheniformis and B. subtilis, respectively, are used in laundry products. In testing for the prevalence of IgE antibodies to these enzymes in sera among 300 laundry product workers, we experienced two problems in the establishment of a reliable RAST for these antigens. The first problem was the propensity of the allergen, Esperase, to undergo autolysis, suggesting that solid-phase Esperase might also lose reactivity through degradation. Treatment of Esperase with phenylmethylsulfonyl fluoride stabilized the enzyme and permitted the synthesis of a stable solid-phase antigen. The second problem was the finding that sera reactive with Esperase in the RAST were also reactive with Savinase, an enzyme from B. licheniformis to which the workers were not exposed. Immunochemical analyses of the three enzymes with specific rabbit antisera by gel diffusion and by two-site immunoradiometric assay demonstrated that they were not cross contaminated to any appreciable extent. RAST inhibition demonstrated that solid-phase Esperase possessed unique allergenic determinants in that the reactivity of IgE antibodies was inhibited by low concentrations of Esperase and only by very high concentrations of Alcalase and Savinase. In contrast, the reactivity of solid-phase Alcalase was occasionally inhibited equally well by Esperase and Alcalase. Most strikingly, the reaction of IgE antibodies with solid-phase Savinase was always inhibited by comparable quantities of Esperase, Alcalase, and Savinase. Thus, the establishment of the RAST for these proteases appears to require the use of phenylmethylsulfonyl fluoride to retard autolysis, and the results must be interpreted with caution because IgE antibodies in certain sera demonstrate cross-reactivity with Alcalase and Savinase. (J ALLERGY CLIN IMMUNOL 78:877-86, 1986.)

Proteolytic enzymes used in laundry products to enhance stain removal may cause sensitization of workers with the development of allergic rhinitis and asthma.¹⁻⁶ These patients demonstrate positive skin tests, the presence of serum IgE antibodies, and positive challenge tests to the allergen. The health hazards caused by these bacterial enzymes have been reduced by encapsulation (marumerization) with an outer layer of inert ingredients that increases the size of the particle and prevents the antigen from reaching the lower respiratory tract. This process and careful attention to

Abbreviations used

PMSF:	Phenylmethylsulfonyl fluoride
KNPU:	KiloNovo protease unit
AU:	Anson unit
HSA:	Human serum albumin
SDS-Page:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PBS:	Dulbecco's phosphate-buffered saline (0.0027 mol/L KCl, 0.0015 mol/L KH ₂ PO ₄ , 0.008 mol/L Na ₂ HPO ₄ , 0.14 mol/L NaCl, pH7.5)
TSIRA:	Two-site immunoradiometric assay

From the Department of Immunology and the Division of Allergic Diseases and Internal Medicine, Mayo Clinic and Mayo Foundation, Rochester, Minn.

Supported by grants from the Clorox Co., Oakland, Calif., and from the Mayo Foundation.

Received for publication Oct. 28, 1985.

Accepted for publication April 11, 1986.

Reprint requests: Gerald J. Gleich, M.D., Department of Immunology, Mayo Clinic, Rochester, MN 55905.

*Present address: 64 Avenue Carnot, 83300 Draguignan, France.

adequate ventilation apparently have reduced sensitization of workers to enzymes that at one point in the early 1970s threatened to become widespread.⁷

A recent investigation by the National Institute for Occupational Safety and Health in a New Jersey factory demonstrated that immunologic sensitization to bacterial enzymes was occurring among some workers

exposed to the enzyme Esperase derived from *B. licheniformis*, even though the enzyme had been encapsulated.⁸ These workers developed cutaneous and respiratory symptoms, fatigue, generalized pains, a worsening of pulmonary function tests after work exposure, and the occurrence of IgE antibodies to Esperase as detected by RAST. Similar evidence for sensitization was reported in Scandinavia.⁹

Because of the need to identify sensitized workers, we established the RAST for Esperase and two other proteases, Alcalase from *B. subtilis* and Savinase from *B. licheniformis*. In the course of this work, we were confronted with two problems: (1) We found that certain sera from workers with elevated levels of IgE antibodies to Esperase also had elevated levels of IgE antibodies to Alcalase and Savinase, even though they had not been exposed to them in the workplace. (2) We found that the allergen, Esperase, could undergo autolysis, suggesting that solid-phase Esperase might also become degraded. Because of these problems, we investigated in detail the conditions for establishment of the RAST and the reactivity of sera from sensitized workers. In particular, we determined if inhibition of proteolytic activity of the enzymes by PMSF^{10,12} might yield stable reagents and thus improve the sensitivity of the RAST.

MATERIAL AND METHODS

Enzymes

Crude and purified preparations of Esperase, Alcalase, and Savinase were supplied by Novo Industri, A/S, Bagsvaerde, Denmark. Crude and purified solutions (1 ml of a 1.0 mg/ml) of Esperase contained 48.6 KNPU/gm and 180 KNPU/gm; Savinase contained 54.2 KNPU/gm and 180 KNPU/gm; Alcalase contained 11 AU/gm and 30 AU/gm. One NPU is defined as amount of enzyme that under standard conditions hydrolyses casein at a rate such that the initial rate of formation of peptides eliciting a color with trinitrobenzene sulfonic acid corresponds to the same colorimetric change that is elicited by hydrolysis of 1 μ mole of glycine per minute. One Anson unit is defined as the amount of enzyme that (at 25° C, pH 7.5, and 10 minutes reaction time) digests hemoglobin at an initial rate that results in an amount of trichloroacetic acid soluble product liberated per minute that elicits the same color with phenol reagent as 1 mEq of tyrosine.¹³

Rabbit immunization

New Zealand white rabbits were immunized with crude and purified Esperase, Alcalase, and Savinase by dissolving 400 μ g in 1 ml of sterile saline, emulsifying the saline solution with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.), and by injecting 1 ml of the mixture into the footpads initially, and subsequently at 4-week intervals intradermally. Six weeks after the last injection, an alum suspension with 400 μ g/ml of each enzyme in sterile saline with 0.1% aluminum potas-

sium sulfate was injected intradermally (0.25 ml) the first day and intravenously (0.50, 1.00, and 2.00 ml) on days 2, 3, and 4. Rabbits were exsanguinated on day 10 after the final immunization by cardiopuncture under halothane general anesthesia, and the sera were stored at -20° C.

Biuret assay

The protein content of enzyme solutions was determined by biuret assay¹⁴ standardized with human serum albumin (Sigma Chemical Co., St. Louis, Mo.). The concentration of human serum albumin was determined by absorbance at 277 nm, $E_{1\%}^{1\text{cm}} = 5.3$.

Measurement and inhibition of protease activity

The protease activities of Esperase, Alcalase, and Savinase were assessed by casein proteolysis as described by Arnon.¹⁵ Briefly, 1 ml samples of enzyme varying from 0.5 mg/ml to 0.005 mg/ml in Tris buffer (hydroxymethyl aminomethane, HCl, 0.05 mol/L, pH 8.0) were added to 1 ml of casein solutions of 1 mg/ml in the same buffer at timed intervals in Pyrex (Corning Glass, Corning, N.Y.) 1380 tubes. After 10 minutes at 37° C, 3 ml of 5% trichloroacetic acid was added. After a brief vortex mixing, the tubes were incubated for 40 minutes at room temperature. The assay tubes were centrifuged in a Beckman J21 centrifuge (Beckman Industries, Irvine, Calif.) at 40,000 $\times$ g for 20 minutes, and nonprecipitable absorbance at 280 nm was measured. For analysis of the effect of PMSF (Sigma Chemical Co.), 50 μ l of PMSF solution in isopropanol was added to the enzyme solution for 10 minutes before adding casein. The PMSF inhibition experiments were performed at concentrations ranging from 20 mmol/L to 0.004 mmol/L.¹²

Gel filtration on Sephadex G-75 column chromatography analysis

Esperase was dissolved in 0.01 mol/L PO_4 , 0.3 mol/L NaCl, pH 7.7, at 25 mg/ml concentration and centrifuged at 40,000 $\times$ g and 4° C. The supernatant was analyzed on a Sephadex G-75 column calibrated with marker proteins including ferritin, chymotrypsin, cytochrome C, and ¹²⁵I. Two milliliters of Esperase was applied, and 1.4 ml fractions were collected. Fractions from the first absorbance peak were pooled and reanalyzed on the same column.

Spectrophotometric analysis

Fresh solutions of pure Esperase and Alcalase (1 mg/ml) and crude Savinase and Alcalase (1 mg/ml) were examined in a Cary 219 (Varian Associates, Inc., Palo Alto, Calif.) spectrophotometer at 20° C; scan range was 330 to 240 nm, 10 nm/sec/cm. Elastase, 1 mg/ml, was used as a standard.

SDS-PAGE

Enzymes, 10 to 200 μ g and with or without PMSF treatment, final concentration 0.2 mmol/L to 20 mmol/L, were analyzed by electrophoresis in 8% polyacrylamide gels, pH 7.4, containing 1% SDS as described by Fairbanks et al.¹⁶ Samples were reduced by use of 40 mmol/L of dithio-

erythritol, final concentration, and gels were stained with Coomassie brilliant blue. In one experiment, Esperase, Savinase, and Alcalase in 0.2 mmol/L of PMSF were mixed directly in 1% SDS at 56° C.¹⁷

Immunodiffusion

Ouchterlony double diffusion was performed in 4% agarose (Indubiose A45, Ind. bio. Fse 92, Clichy, France) in PBS with 100 ppm merthiolate (Thimerosal, crystalline, Sigma Chemical Co.) by use of varying concentrations of each enzyme with or without PMSF inactivation (20 mmol/L, 2 mmol/L, or 0.2 mmol/L final concentration).

Performance of RAST

Esperase, Alcalase, and Savinase were coupled to CNBr-activated Avicel microcrystalline cellulose (Brinkman Instruments, Westbury, N.J.) to provide solid-phase antigens as described previously.¹⁸ The maxi-RAST procedure was used unless otherwise specified. To test the effect of inactivation with PMSF, active and inactivated enzymes were coupled to microcrystalline cellulose. The enzyme samples inactivated by PMSF were incubated with a final concentration of 0.2 mmol/L of PMSF in borate buffer (0.05 mol/L, pH 8.0) (sodium borate, Fischer Scientific Co., Springfield, N.J., diluted in H₂O) or Tris buffer (0.05 mol/L, pH 8.0) for 1 hour at 37° C. To prepare solid-phase antigens at varying ratios of antigen to cellulose, PMSF, 2 mmol/L, final concentration, was used, and various quantities of enzymes, from 1.0 mg to 100 mg were coupled to 100 mg microcrystalline cellulose.

The maxi-RAST procedure used 500 µl of enzyme-cellulose (1 mg/ml), 50 µl of serum, and 450 µl of RAST diluent (0.1 mol/L KH₂PO₄-K₂HPO₄, pH 7.4, containing 0.2% bovine serum albumin [fraction V], 1% Tween 20, and 0.1% sodium azide); it was compared with the mini-RAST procedure¹⁹ that used 100 µl of enzyme-cellulose, 50 µl of serum, and 100 µl of RAST diluent. In each test, at least three negative control sera were used, and results are expressed in percent of total counts added as well as percent of negative control (see below). After performance of these preliminary tests, sera of workers were analyzed for presence of specific IgE to the three enzymes by RAST with the optimal conditions established above.

RAST-inhibition procedure

The specificity of IgE antibodies in sera from patients with high levels of IgE antibodies to the enzymes was tested by RAST inhibition²⁰ with the mini-RAST method. Purified Esperase, 180 KPNU/gm, Alcalase, 30 AU/gm, and Savinase, 180 KPNU/gm solutions containing 2 mmol/L of PMSF in borate buffer were tested for their inhibitory activities in concentrations from 0.1 µg per tube to 1000 µg per tube (1 µg/ml to 10⁶ µg/ml).

TSIRA for Esperase, Alcalase, and Savinase

Purified rabbit antibodies to Esperase, Alcalase, and Savinase were prepared by affinity chromatography on columns of Esperase-Sepharose 4B-CL, Alcalase-Sepharose 4B-CL, or Savinase-Sepharose 4B-CL, respectively. To pre-

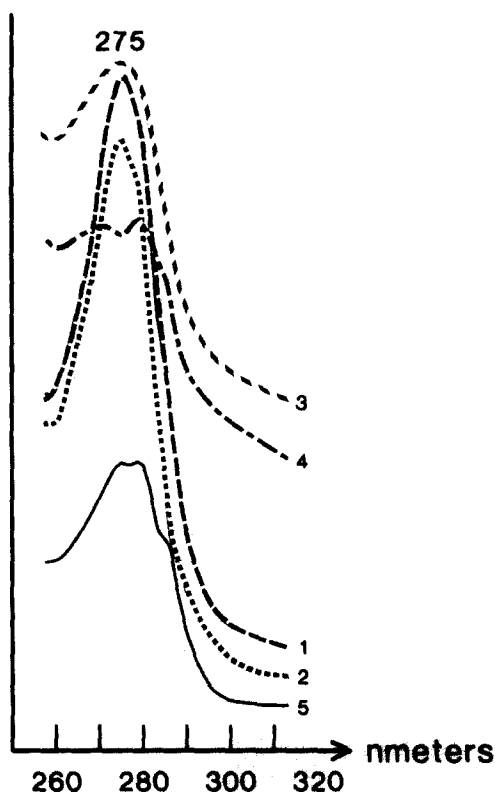


FIG. 1. Spectra of purified and crude enzymes. Purified Esperase, 1 mg/ml in saline buffer (line 1); purified Alcalase, 1 mg/ml in saline buffer (line 2); crude Alcalase, 1 mg/ml in saline buffer (line 3); crude Savinase, 1 mg/ml in saline buffer (line 4); and crude elastase, 1 mg/ml (20°) (line 5).

pare these immunosorbent materials, we reacted CNBr-activated Sepharose with Esperase, 10 mg/ml, PMSF, 2 mmol/L, final concentration, in 20 ml total volume. The same quantities of Alcalase and Savinase were used in separate experiments. Rabbit antisera were applied to the column, and the column was washed with borate, pH 8.0, 0.1 mol/L overnight. Bound antibody was desorbed with 0.05 mol/L of glycine HCl buffer, pH 3.0; peak absorbance (280 nm) fractions were pooled, dialyzed against PBS, and centrifuged. The supernatants were concentrated with an Amicon PM30 (Amicon Corp., Danvers, Mass.) membrane. Protein concentration was determined by absorbance with $E_{1\text{cm}}^{1\%} = 14.0$ for IgG, and samples were stored at -26° C. Affinity-purified antibody was radiolabeled with the iodine monochloride method.²¹

TSIRA has been described in general terms elsewhere.²² We used Immunlon 2 (Dynatech Corp., Alexandria, Va.) microtitration U-bottom plates that were coated with antibody in 0.015 mol/L of phosphate, pH 8.1, overnight at 4° C and washed three times with RAST wash solution (0.1 mol/L KH₂PO₄ - K₂HPO₄, pH 7.4, containing 1% Tween 20). Samples diluted in RAST diluent were incubated overnight at 4° C. The plates were washed five times in RAST wash, and radiolabeled ¹²⁵I affinity-purified antibody in RAST diluent solution was added to the well. After 18 hours

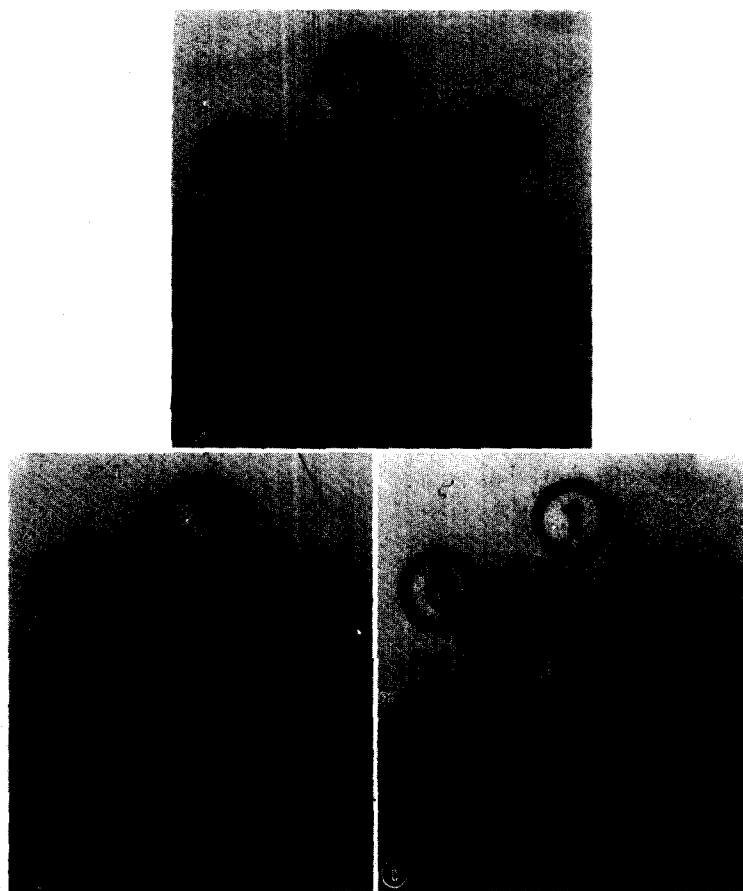


FIG. 2. Ouchterlony immunodiffusion analysis followed by Coomassie blue staining. **A,** Rabbit anti-Espersase (X), Espersase 1 mg/ml (1) and 0.5 mg/ml (2), Alcalase 1 mg/ml (3) and 0.5 mg/ml (4), Savinase 1 mg/ml (5) and 0.5 mg/ml (6). **B,** Rabbit anti-Savinase (X), Espersase 1 mg/ml (1) and 0.5 mg/ml (2), Savinase 1 mg/ml (3) and 0.5 mg/ml (4), Alcalase 1 mg/ml (5) and 0.5 mg/ml (6). **C,** Rabbit anti-Alcalase (X), Espersase 1 mg/ml (1) and 0.5 mg/ml (2), Savinase 1 mg/ml (3) and 0.5 mg/ml (4), Alcalase 1 mg/ml (5) and 0.5 mg/ml (6).

the plates were washed 10 times in RAST wash and counted in a Packard gamma scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Standard curves were established with purified enzymes, and crude and purified enzyme preparations were tested for contamination and/or cross-reactivity.

RESULTS

Physicochemical properties of Espersase, Alcalase, and Savinase

Initial analyses of Espersase, Alcalase, and Savinase by SDS-PAGE to determine the purity of the preparations did not demonstrate bands, even though large amounts of enzyme, up to 375 μ g, were applied. With most proteins, an application of 1 to 5 μ g of protein is sufficient for visualizing the band after staining with Coomassie brilliant blue. In these experiments enzymes were dissolved in borate or phosphate buffer, and the content of the enzyme was expressed as mass

per volume. Biuret analysis of these enzyme solutions demonstrated protein concentrations varying from 5.6 mg/ml for crude Savinase to 15 mg/ml for purified Espersase. In addition, no bands were observed by SDS-PAGE, even though Espersase was dissolved directly in 1% SDS at room temperature, 37° or 56° C. Because the enzyme might degrade during the experiment, we treated enzyme solutions with PMSF, 2 mmol/L, but we did not obtain a band, even when the gels were stained with silver nitrate.²³

Because of these findings, we suspected that the enzymes were either undergoing proteolysis and/or were present in lower concentrations than we anticipated, on the basis of mass of powder dissolved. Fig. 1 illustrates spectral analysis of Espersase solutions with the expected absorbance peak at 277 nm. Analysis of enzymes by immunodiffusion (Fig. 2) illustrates the presence of precipitin bands with the homologous antisera and no evidence of cross-reactivity.

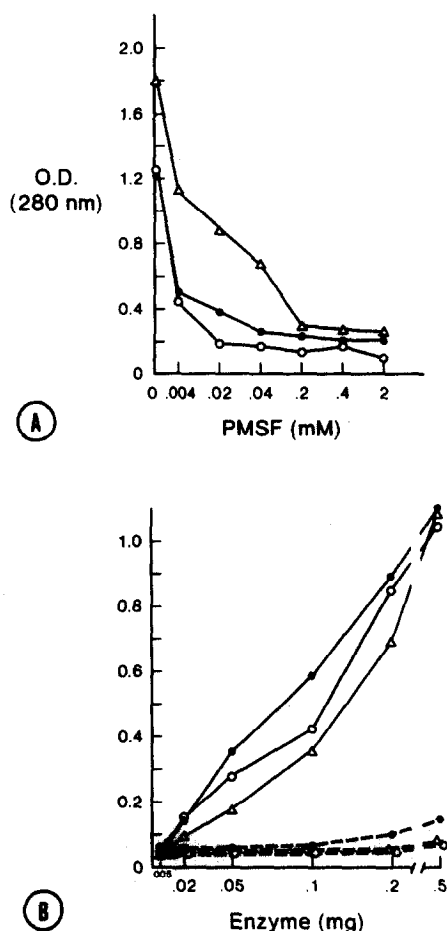


FIG. 3. Enzymatic activity of Esperase, Alcalase, and Savinase as measured by absorption at 280 nm of nonprecipitable (= denatured) casein after application of trichloroacetic acid to mixture of enzyme, casein, and PMSF. **A**, The variable is the concentration of PMSF. Enzyme concentration is 0.5 mg/ml (total volume 1 ml). For a concentration of 0.2 mmol/L of PMSF and the above enzymes, activity is inhibited >80%. The remaining activity with 0.2 mmol/L of PMSF is 17.6% for Esperase, 20% for Alcalase, and 12% for Savinase. **B**, The variable is the concentration of each of the three enzymes in solutions containing 0.2 mmol/L of PMSF (---) or containing no (-) PMSF. There is a dose-related response curve of proteolytic activity as a function of enzymatic content of the solution. For the highest enzymatic concentration tested (0.5 mg/ml), PMSF inhibition is again >80% (remaining activity 14% for Esperase and 8% for Alcalase and Savinase). On both **A** and **B**, open triangles represent Esperase, dots for Alcalase, and open circles for Savinase.

These results demonstrate that antigenically reactive material was contained in the enzyme preparations.

Gel filtration of Esperase on G-75 Sephadex column demonstrated two peaks, the first peak at the expected elution volume for Esperase and a second peak at a higher elution volume and hence lower molecular weight. When the first peak was pooled, concentrated,

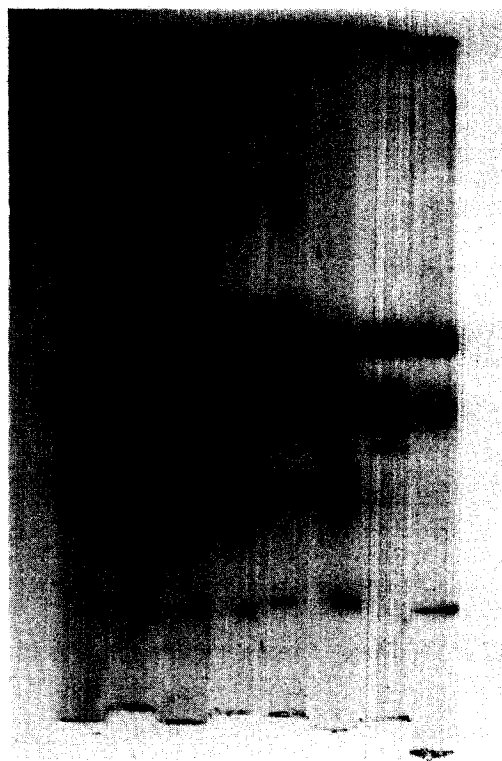


FIG. 4. Molecular weight determination by SDS-PAGE analysis of enzyme preparations. The left gel (gel 1) illustrates the marker proteins that are, from the top: bovine serum albumin (69,000 daltons), ovalbumin (49,000 daltons), trypsinogen (24,000 daltons), β -lactoglobulin (18,000 daltons), and cytochrome C (12,400 daltons). From left to right: Alcalase preparations, Sigma Chemical Co., St. Louis, Mo., 50 μ g (gel 2), pure 50 μ g (gel 3), crude 50 μ g (gel 4); Esperase preparations: pure 50 μ g (gel 5), crude 50 μ g (gel 6); Savinase preparations: pure 25 μ g (gel 7), crude 50 μ g (gel 8). All enzymes were treated with 20 mmol/L of PMSF, reduced with dithiothreitol, and electrophoresed in 8% polyacrylamide gel. Relative migration values were the ratio of the distance migrated for each protein relative to the distance migrated by the pyronin Y tracking dye (bottom). Molecular weights were calculated by the least squares regression line ($R^2 = 0.996$).

allowed to stand for 3 days, and rechromatographed on G-75, two peaks similar to the first gel filtration were observed (results not presented). These results suggested that the preparations of Esperase spontaneously degraded when it was concentrated and rechromatographed at pH 7.7.

Therefore, we used a standard casein assay to determine the concentration of PMSF needed to inactivate the enzymes. Fig. 3, A illustrates that >80% enzyme activity was destroyed by 0.2 mmol/L of PMSF, and at this concentration a plateau of inactivation was reached. Increasing the PMSF concentration to 2 mmol/L elicited only a slight additional reduction of enzyme activity. Fig. 3, B illustrates the

TABLE I. RAST results as function of enzyme solid-phase support ratio*

	Esperase (mg)/cellulose (mg)				Alcalase (mg)/cellulose (mg)				Savinase (mg)/cellulose (mg)			
	1/100	10/100	50/100	100/100	1/100	10/100	50/100	100/100	1/100	10/100	50/100	100/100
Negative controls												
1	0.7	1.3	2.0	2.5	1.2	1.2	0.7	0.8	1.0	1.8	2.5	2.6
2	0.4	0.7	1.4	2.5	0.6	0.5	0.6	0.7	0.6	1.0	1.5	2.4
Mean	0.55	1.0	1.7	2.5	0.9	0.85	0.65	0.75	0.8	1.4	2.0	2.5
Positive controls												
1	2015	700	482	307	240	415	455	430	250	235	210	161
2	1300	640	500	315	245	255	240	250	200	227	188	165
3	400	250	190	145	150	145	120	125	180	140	115	115
Mean	1238	530	391	256	212	272	272	268	210	201	171	147

*In these experiments crude enzymes in the amounts presented were coupled to 100 mg of activated microcrystalline cellulose and tested in the maxi-RAST procedure. Results are expressed for the negative controls as percent of total counts added, and for positive controls as the ratio of counts bound by positive serum to counts bound by negative control sera $\times 100$. Analyses were performed in duplicate.

TABLE II. Analyses of sera for IgE antibodies to enzymes*

Test Sera	Esperase results	Alcalase results	Savinase results
1	30.28†(4912)‡	11.51(4612)	1.36(567)
2	20.60(3341)	14.43(5783)	4.38(1825)
3	17.41(2925)	15.97(6397)	0.57(238)
4	19.68(3192)	9.63(3859)	4.95(2060)
5	4.78(775)	12.00(4809)	9.20(3833)
6	18.72(3037)	5.89(1424)	0.40(170)
7	30.43(4936)	15.09(6045)	4.07(1696)

*These sera were chosen because they display marked reactivity with enzymes and demonstrate the patterns of this reactivity.

†Results expressed as percent of counts bound.

‡Results expressed as ratio of percent of counts bound by test serum to percent of counts bound by negative control serum times 100. Values <200 are regarded as negative.

control, demonstrating dose-dependent digestion of casein by enzymes and the effect of 0.2 mmol/L PMSF on proteolytic activity of different concentrations of the three enzymes. These results demonstrated that a small amount of enzyme activity survived treatment with 2 mmol/L of PMSF. Therefore, we tested whether a concentration of 20 mmol/L of PMSF could inactivate enzymes in order that bands could be visualized by SDS-PAGE. Fig. 4 indicates that after treatment in 20 mmol/L of PMSF, protein bands could be visualized by SDS-PAGE. Each enzyme demonstrated a single major band with molecular weights of 26,000 for Esperase and 28,000 for both Alcalase and Savinase. A series of less strongly staining bands was noted for each enzyme at low molecular weights. Therefore, the enzyme preparations indeed contained the protein, and the initial failure to observe bands in SDS-PAGE apparently was due to proteolytic digestion of enzyme denatured in SDS by small amounts of remaining active enzyme.

Detection of IgE antibody by RAST

Our initial RAST studies had demonstrated a loss of antigenic reactivity of Esperase solid-phase antigen during a 3- to 4-week period, and after we were aware of the capacities of the enzymes for autolysis in solution, we examined several different solid-phase Esperase preparations by the mini-RAST. Comparison of three freshly prepared solid-phase Esperase suggested that Esperase treated with 2 mmol/L of PMSF was a better reagent than untreated solid-phase Esperase. We did not use 20 mmol/L of PMSF inactivation because previous Ouchterlony studies demonstrated enzyme immunoreactivity was reduced with 20 mmol/L of PMSF inactivation (data not presented).

Next, we compared the maxi- and mini-RAST procedure and found no difference between them; we used the maxi-RAST subsequently, except for the RAST-inhibition experiments. We also determined the optimal enzyme-cellulose ratio by coupling varying amounts of enzyme to the same quantity of cellulose.

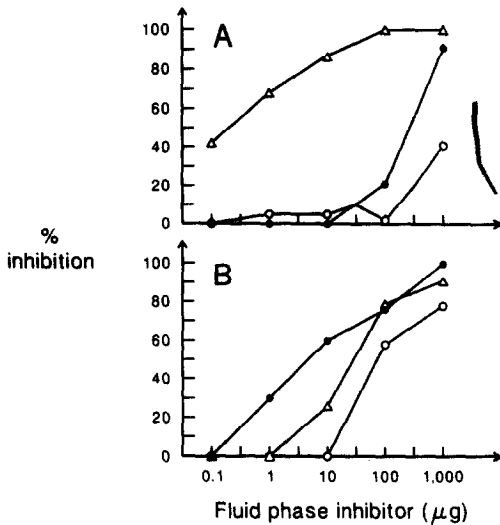


FIG. 5. RAST inhibition with serum from patient 1. **A**, Inhibition of solid-phase Esperase by fluid-phase enzymes in micrograms per tube. Symbols for fluid-phase enzymes are Esperase, open triangle; Alcalase, closed circle; Savinase, open circle. **B**, Analogous inhibition experiment for solid-phase Alcalase.

Table I presents the results. Note that the larger amounts of Esperase and Savinase increased binding of IgE by normal serum, whereas the opposite was observed for Alcalase. The experiment suggested that 1 mg of Esperase or Savinase per 100 mg of cellulose and 10 mg of Alcalase per 100 mg of cellulose were optimal, and these ratios were used to make the solid-phase reagents for subsequent experiments.

Table II demonstrates the results of analysis of sera from workers exposed to enzymes. More than 800 sera from exposed individuals were tested for IgE antibodies to the enzymes, and Table II presents those sera with the highest levels. All seven sera had elevated IgE antibody levels for both Esperase and Alcalase, and six of the seven sera had elevated levels for Savinase as well. These workers were exposed to Esperase and Alcalase, but not Savinase.

Analyses of the specificity of antibodies to enzymes

The cross-reactivity of IgE antibodies to enzymes was tested by RAST inhibition for sera 1 to 7 (presented in Table II). Typical results are illustrated in Figs. 5 to 7, and Table III summarizes the results. Fig. 5 illustrates the reactivity patterns of serum No. 1 with solid-phase Esperase and Alcalase. With solid-phase Esperase (Fig. 5, A), 50% inhibition was achieved with approximately 0.2 μg of fluid-phase Esperase. In contrast, 250 μg of fluid-phase Alcalase (1250-fold excess) and >1000 μg of fluid-phase Savinase (>5000 excess) were required for 50% inhibition. The reactivity of IgE antibodies with solid-phase

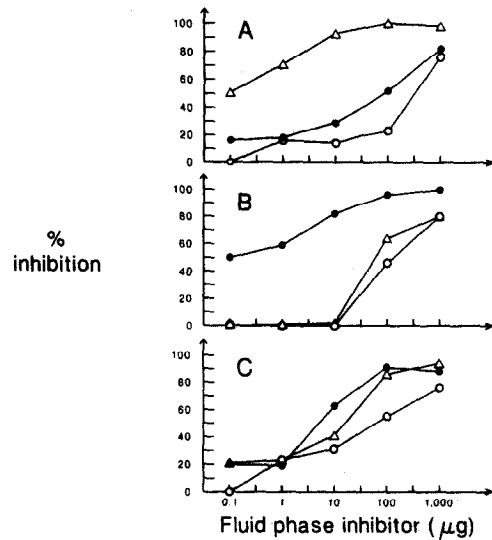


FIG. 6. RAST inhibition with serum from patient 4. **A**, Inhibition of solid-phase Esperase by fluid-phase enzymes in micrograms per tube. Symbols for fluid-phase enzymes are the same as in Fig. 5. **B**, Inhibition of solid-phase Alcalase. **C**, Inhibition of solid-phase Savinase by fluid-phase enzymes.

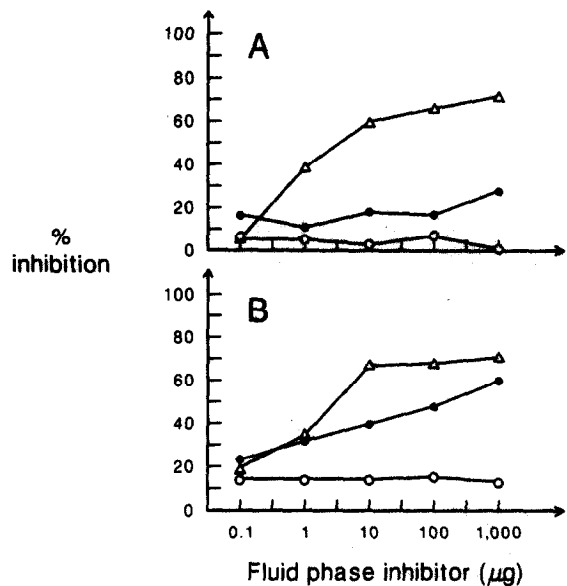


FIG. 7. RAST inhibition with serum from patient 6. **A**, Inhibition of solid-phase Esperase by fluid-phase enzymes. Symbols for fluid-phase enzymes are the same as in Fig. 5. **B**, Inhibition of solid-phase Alcalase by fluid-phase enzymes. Solid-phase Savinase inhibition was not presented because of the low amount of specific IgE in the serum from patient 6.

Alcalase (Fig. 5, B) was inhibited by all three enzymes at more comparable quantities, although fifteenfold more fluid-phase Savinase and sixfold more fluid-phase Esperase were required for inhibition. Fig. 6 illustrates the inhibition when serum No. 4 was

TABLE III. Reactivity of IgE antibodies to enzyme by RAST inhibition

Serum	Solid-phase Esperase			Solid-phase Alcalase			Solid-phase Savinase		
	Fluid-phase			Fluid-phase			Fluid-phase		
	Esperase*	Alcalase	Savinase	Esperase	Alcalase	Savinase	Esperase	Alcalase	Savinase
1	0.2	250	>1000†	28	4.7	70	—‡	—	—
2	0.7	300	>1000	40	0.3	300	16	28	4
3	0.1	150	800	56	0.1	>1000	—	—	—
4	0.1	100	300	60	0.1	100	15	5	56
5	—	—	—	10	1.0	50	18	2	18
6	0.3	>1000	>1000	0.3	20	>1000	—	—	—
7	0.8	550	350	250	0.5	300	1	1	0.3

*Quantity in micrograms per tube required for 50% inhibition interpolated from semilog plots of percent inhibition versus quantity of fluid-phase enzyme. Serum specimen Nos. 1, 4, and 6 are illustrated in Figs. 6 to 8.

†No inhibition observed at highest concentration tested.

‡Not done.

tested with solid-phase Esperase (*A*), Alcalase (*B*), and Savinase (*C*). With solid-phase Esperase, the results are comparable to those described above for patient 1. With solid-phase Alcalase, approximately 1000-fold more fluid-phase Esperase and Savinase were required for inhibition than was needed for fluid-phase Alcalase. With solid-phase Savinase, rather comparable quantities of all three enzymes were required for 50% inhibition. Fig. 7 illustrates the inhibition patterns when serum No. 6 was tested with solid-phase Esperase and Alcalase. Fig. 7, *A* illustrates results with solid-phase Esperase; only fluid-phase Esperase was an effective inhibitor. Fig. 7, *B* illustrates inhibition of solid-phase Alcalase; the heterologous fluid-phase Esperase produced more inhibition than the homologous fluid-phase Alcalase.

These results suggested that either antibodies from certain patients were cross-reactive with the enzymes and/or the enzymes were cross contaminated. In all RAST-inhibition analyses, highly purified enzymes, Esperase, 180 KNPU/gm, Alcalase, 30 AU/gm, and Savinase, 180 KNPU/gm, were tested. These preparations (and also the crude enzymes; see Fig. 1) did not cross-react when they were tested with rabbit antisera by immunodiffusion. To investigate further the question of cross contamination, the cross-reactivity of the enzymes was investigated by the TSIRA. Fig. 8, *A* illustrates that antigen cross contamination and cross-reactivity were extremely low when plates were coated with anti-Esperase. The average background count was 386 cpm. With pure Alcalase and pure Savinase, only two points exceeded this: Alcalase, 1.4 times background, and Savinase, 1.3 times background. Therefore, even at 100,000-fold excess levels of Alcalase and Savinase, very little antigenic cross-reactivity or cross contamination was detected with

anti-Esperase. Fig. 8, *B* illustrates that antigenic cross-reactivity and cross contamination were low when plates were coated with anti-Alcalase. More than 500-fold excess of crude Savinase was needed to produce binding with the anti-Alcalase, and more than a 5000-fold excess of crude Esperase was needed to produce binding with the anti-Alcalase. Fig. 8, *C* illustrates that antigenic cross-reactivity and cross contamination were low when plates were coated with anti-Savinase. Esperase and Alcalase both failed to bind to anti-Savinase, and even at the highest concentrations tested, pure Alcalase had counts of only 1.5 times the average background binding level (265 cpm).

DISCUSSION

Initial experiments suggested (1) that these enzymes spontaneously degraded and (2) that sera with IgE antibodies to Esperase also contained IgE antibody activity to Alcalase and Savinase. We suspected that the loss of potency with time of the solid-phase enzyme preparations might be due to autolysis of enzymes. The finding of serologic cross-reactivity might have been due to enzyme cross contamination or might be due to cross-reactivity among the enzymes.

We investigated the autolysis of these enzymes by SDS gel electrophoresis and by gel filtration. We found that autolysis readily occurred and that autolysis could be limited by a final solution concentration of 20 mmol/L of PMSF. Because immunodiffusion studies demonstrated that 20 mmol/L of PMSF had an adverse effect on immunoreactivity, we selected 2 mmol/L of PMSF as the final concentration in preparing enzyme solutions for RAST and TSIRA experiments.

Table III summarizes our RAST-inhibition results to investigate allergenic cross-reactivity and cross

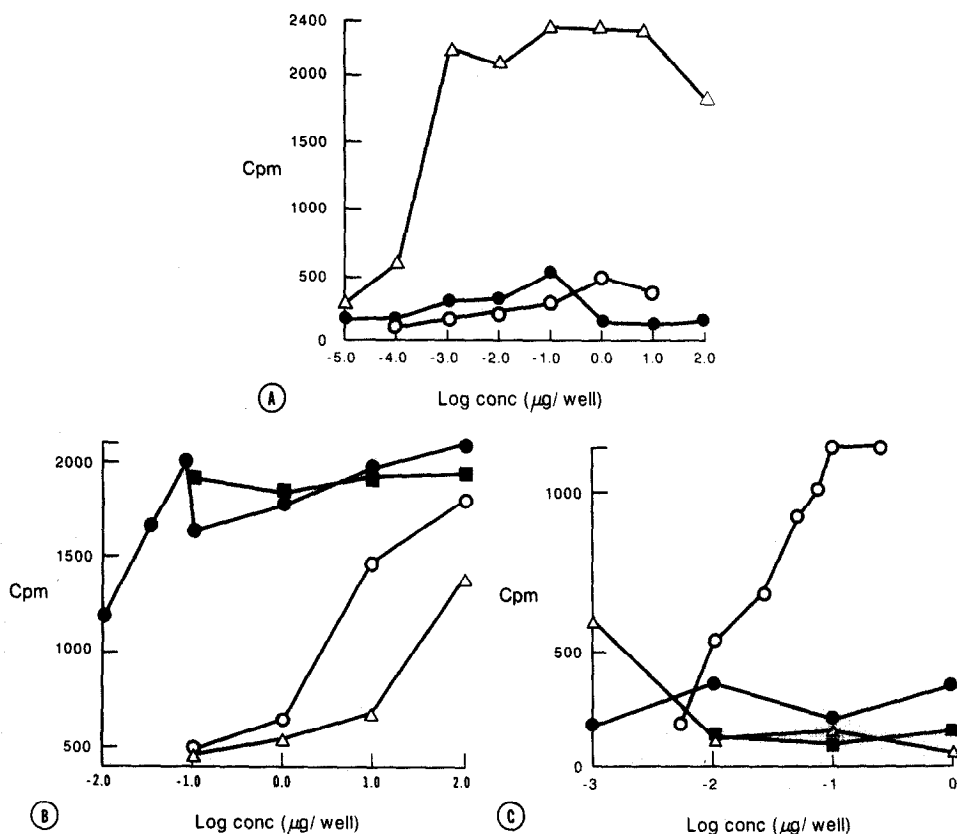


FIG. 8. TSIRA results. **A**, Plates coated with rabbit anti-Esperease. **B**, Coated with anti-Alcalase. **C**, Coated with anti-Savinase. **A**, The binding of pure Esperase (Δ), 180 KNPU/gm; pure Alcalase (●), 28.8 AU/gm; and pure Savinase (○), NOVO kg 24/3-83, 25.11.76. The background binding by RAST diluent was 386 cpm. **B**, The binding of an Alcalase sample (●); Clorox (Oakland, Calif.), 1.47 AU/gm; crude Alcalase (■), 11 AU/gm; crude Esperase (Δ), 52 KNPU/gm; and crude Savinase (○), 54.2 KNPU/gm. The background binding by RAST diluent was 470 cpm. **C**, The binding of Savinase (○), 4.02 KNPU/gm; crude Esperase (Δ), pure Alcalase (●), and crude Alcalase (■). The background binding by RAST diluent was 265 cpm.

contamination. The six sera tested with solid-phase Esperase demonstrated marked specificity for Esperase; comparison of enzyme quantities for 50% inhibition indicated that Alcalase and Savinase contained, at most, 0.3% Esperase. Thus, Esperase is quite pure and has unique allergenic determinants. The TSIRA studies confirmed the antigenic purity of Esperase. Fig. 8, *B* illustrates that Esperase contains <0.01% of Alcalase, and Fig. 8, *C* illustrates that Esperase contains <1% of Savinase.

The seven sera tested by RAST inhibition with solid-phase Alcalase demonstrated three patterns of reactivity with fluid-phase Esperase: (1) slight inhibition (sera 2, 3, 4, and 7) <0.8%, (2) moderate inhibition (sera 1 and 5) about 10% to 20%, and (3) marked inhibition (serum 6) in which fluid-phase Esperase was a more potent inhibitor than the homologous Alcalase. Fig. 8, *A* illustrates that Alcalase had <0.001% contamination with Esperase, and Fig. 8, *C* illustrates Alcalase contained <1% of Savinase.

Therefore, when the Alcalase RAST-inhibition results are considered together with the TSIRA results, we observed appreciable allergenic cross-reactivity for three of seven sera.

The four sera tested by RAST inhibition with solid-phase Savinase demonstrated marked cross-reactivity with both Esperase and Alcalase. Indeed, in patients 4 and 5, heterologous fluid-phase Alcalase was a more potent inhibitor than homologous fluid-phase Savinase. The TSIRA results in Fig. 8, *A* demonstrate that Savinase has <0.001% contamination with Esperase, and results in Fig. 8, *B* demonstrate that Savinase contains 0.1% of Alcalase. Therefore, allergenic cross-reactivity rather than antigen cross contamination appears to explain the results.

We thank Dr. Juraj Hostynek of the Clorox Co. for suggesting the use of 20 mmol/L of PMSF in the experiments analyzing enzymes by SDS-PAGE, Dr. F. Prendergast for performing the spectrophotometric analyses of the enzymes, and Mrs. Linda H. Arneson for secretarial assistance.

REFERENCES

1. Flindt MLH: Pulmonary disease due to inhalation of derivatives to *Bacillus subtilis*-containing proteolytic enzyme. *Lancet* 1:1177, 1969
2. Newhouse ML, Tagg B, Pocock SJ, McEwan AC: An epidemiologic study of workers producing enzyme washing powders. *Lancet* 1:689, 1970
3. Greenberg M, Milne JF, Watt A: Survey of workers exposed to dusts containing derivatives of *Bacillus subtilis*. *Br Med J* 2:629, 1970
4. Pepys J, Hargreave FE, Longbottom JL, Faux J: Allergic reactions of the lung to enzymes of *Bacillus subtilis*. *Lancet* 1:1181, 1969
5. Shapiro RS, Eisenberg BC: Sensitivity to proteolytic enzymes in laundry detergents. *J ALLERGY* 47:76, 1971
6. Bernstein IL: Enzyme allergy in populations exposed to long-term, low-level concentrations of household laundry products. *J ALLERGY CLIN IMMUNOL* 49:219, 1972
7. Medical Research Council Pneumoconiosis Unit Symposium Report. Cardiff, U.K., May 4-5, 1976 Biological effects of proteolytic enzyme detergents. *Thorax* 31:621, 1976
8. Liss GM, Kominsky JR, Gallaher JS, Melius J, Brooks SM, Bernstein IL: Failure of enzyme encapsulation to prevent sensitization of workers in the dry bleach industry. *J ALLERGY CLIN IMMUNOL* 73:348, 1984
9. Zachariae H, Hoegh-Thomsen J, Witmeur O, Wide L: Detergent enzymes and occupational safety. *Allergy* 36:513, 1982
10. Inoue M, Horiuchi S, Morino Y: Inactivation of glutamyl transpeptidase by phenylmethanesulfonyl fluoride, a specific inactivator of serine enzymes. *Biochem Biophys Res Commun* 82:1183, 1978
11. Sekar V, Hageman JH: Specificity of the serine protease inhibitor phenylmethylsulfonyl fluoride. *Biochem Biophys Res Commun* 89:474, 1979
12. Polgar L, Bender ML: The reactivity of thiol subtilisin, an enzyme containing a synthetic functional group. *Biochemistry* 6:610, 1967
13. Anson ML: The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *J Gen Physiol* 22:79, 1939
14. Kabat EA, Mayer MM: Estimation of protein with the biuret and ninhydrin reactions. In Kabat EA, Mayer MM, editors: *Experimental immunochemistry*, ed 2. Springfield, Ill., 1967, Charles C Thomas, Publisher, p 559
15. Arnon R: Assay of proteolytic activity. In Perlman GE, Loraud L, editors: *Methods in enzymology*. New York, 1970, Academic Press, p 226
16. Fairbanks G, Steck TL, Wallach DFH: Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606, 1971
17. Weber K, Osborn M: The reliability of molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. *J Biol Chem* 244:4406, 1969
18. Gleich GJ, Jones RT: Measurement of IgE antibodies by the radioallergosorbent test. I. Technical considerations in the performance of the test. *J ALLERGY CLIN IMMUNOL* 55:334, 1975
19. Gleich GJ, Adolphson CR, Yunginger JW: The mini-RAST: comparison with other varieties of the radioallergosorbent test for the measurement of IgE. *J ALLERGY CLIN IMMUNOL* 65:20, 1980
20. Adolphson CR, Gleich GJ, Yunginger JW: Standardization of allergens. In Rose NR, Friedman H, Fahey JL, editors: *Manual of clinical immunology*, ed 3. Washington, D.C., 1986, American Society of Microbiology, pp 652-59
21. Bale WF, Helmkamp RW, Davis TP, Izzo MH, Goodland RL, Contreras MA, Spar IL: High specific activity labeling of protein with ^{131}I by the iodine monochloride method. *Proc Soc Exp Biol Med* 122:407, 1966
22. Hales CN, Woodhead JS: Labeled antibodies and their use in the immunoradiometric assay. In Vunakis HV, Langone JJ, editors: *Methods in enzymology*. New York, 1980, Academic Press, p 334
23. Merrill CR, Goldman D, Sedman SA, Ebert MH: Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* 211:1437, 1981

AVAILABLE NOW! The FIVE-YEAR (1981-1985) CUMULATIVE INDEX TO THE JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY can be purchased from the Publisher for \$42.00. This comprehensive, 156-page reference guide is a current presentation of all topics included in the JOURNAL from January 1981 to December 1985 (volumes 67-76)—the past 10 volumes. It incorporates complete references to over 672 original articles, abstracts, case reports, letters, editorials, and CME articles. It features 1,525 Subject Headings, under which there are 5,316 references. Each subject entry lists the complete article title, author(s), volume, page, and year of publication. It also includes 4,780 Author Entries, listing contributors, along with their respective titles, author-to-author referral, volume, page, and publication date.

To purchase, call or write: The C.V. Mosby Company, 11830 Westline Industrial Dr., St. Louis, Missouri 63146, or telephone FREE 1-800-325-4177, Customer Service (in Missouri call collect at 314-872-8370, Customer Service). Please reference book code number 2605-6 when placing your order. PREPAYMENT REQUIRED. Make checks payable to The C.V. Mosby Company. (All payments must be in U.S. funds drawn on a U.S. bank.) Price: \$42.00 in the U.S., \$44.75 international (price includes mailing charges).