Inflammatory Mediators and Modulators Released in Organ Culture From Rabbit Skin Lesions Produced in Vivo *by Sulfur Mustard*

II. Evans Blue Dye Experiments That Determined the Rates of Entry and Turnover of Serum Protein in Developing and Healing Lesions

SUSUMU HARADA MD,
ARTHUR M. DANNENBERG, Jr., MD,
AKIRA KAJIKI, MD, KAZUYUKI HIGUCHI, MD,
FUJIHO TANAKA, MD, and PEGGY J. PULA, AB

From the Departments of Environmental Health Sciences, Immunology and Infectious Diseases, and Epidemiology, School of Hygiene and Public Health, and the Department of Pathology, School of Medicine, The Johns Hopkins University, Baltimore, Maryland

Extravasated serum seems to be the major modulator of the local inflammatory response, because it provides both proinflammatory and antiinflammatory components. This report describes the rates of entry and turnover of extravasated serum protein in dermal inflammatory lesions produced by the military vesicant sulfur mustard (SM). Rabbits, bearing SM skin lesions, were given an intravenous injection of Evans blue dye, so that at the time of sacrifice, 2 hours later, their skin lesions were 2 hours and 1, 2, 3, 6, and 10 days of age. Evans blue labels serum albumin, a representative serum protein. By multiplying the amount of Evans blue contained in the lesions by a factor that converted micrograms of Evans blue into milligrams of serum protein, the authors could estimate the 2-hour *rate of entry* of serum protein into these lesions. Serum protein in the lesions was both bound and unbound. The *unbound* protein was extractable from the lesions into the culture fluids, and, electrophoretically, was similar in composition to serum protein. Grossly edematous peak lesions (1 day of age) contained 7.8 mg of unbound serum protein per square centimeter of skin. Healing lesions (6 and 10 days of age) contained about 4.5 mg/sq cm, and normal skin about 1.7 mg/sq cm. Lesions 1 day of age had the highest rate of serum albumin entry, and about 36% of this Evans-blue–labeled protein was unbound, ie, extractable into the culture fluids. Lesions 3 and 6 days of age had a rate of serum albumin entry that was roughly half that of 1-day lesions, and only about 13% of this entering protein was unbound. Normal skin had a very low rate of serum albumin entry, and only 8% of this entering protein was unbound. The *turnover rate* of the unbound (extractable) serum protein could be estimated from the 2-hour entry rate of the Evans-blue–labeled albumin and the total protein in the culture fluids. In 1-day lesions, about 25% of the serum protein in the culture fluids was protein which had entered during the last 2 hours, so that 100% of this unbound protein should have been replaced once in 8 hours. In contrast, in 3- and 6-day lesions, this unbound serum protein should have been replaced once in about 35 hours, and in normal skin once in 80 hours. Evans-blue–labeled serum albumin continuously entered both the bound and unbound compartments of the SM lesions, even during the healing stages. The bound serum albumin was not extractable into the culture fluids because most of it was probably encapsulated by the now nonfunctional lymphatics within the explant and loculated within connective tissue compartments. It is concluded that the amount of serum protein in acute inflammatory lesions is rather high and that it has an unexpectedly rapid turnover rate.
(Am J Pathol 1985, 121:28–38)

TO OUR KNOWLEDGE, there have been few, if any, studies on the local *turnover* of serum protein in developing and healing inflammatory lesions. Most of the studies in the literature have concerned the leakage of serum protein from the circulation into areas of inflammation[see 1–5] or the removal of serum protein from these areas by the lymphatics,[see 6,7] but not the turnover of serum proteins within the lesion itself. Such

Supported by Contract DAMD17-80-C-0102 from the U.S. Army Medical Research and Development Command, Ft. Detrick, Frederick, Maryland.

measurements were made possible by organ-culturing lesion biopsies and assaying the unbound (serum) protein that was extracted into the culture fluids.

In the present report, Evans-blue-labeled serum albumin was used in estimating the rates of entry and turnover of serum protein in inflammatory lesions produced in the skin of rabbits by the vesicant sulfur mustard (SM) (a radiomimetic alkylating agent). When SM was applied topically, epidermal cells slowly died during the first day (because of damage to their DNA), and extravasation of serum and immigration of leukocytes occurred.[8,9] A crust-covered ulcer developed in 2–3 days, and healing was nearly complete in 10 days. No abscesses developed, and no overt infection occurred. Therefore, this seemed to be an ideal model for studying an uncomplicated, slowly developing, acute inflammatory response in the skin.

The results presented herein show that the unbound extravasated serum in SM lesions had a rather rapid turnover rate. Such serum probably limits the damage caused by the proteases and oxygen radicals released by the infiltrating leukocytes.

Terminology

"Serum protein" refers to the protein that entered the inflammatory lesions from the circulation (see Discussion). It was both labeled (by Evans blue) and unlabeled and both bound and unbound (see below). In this report, labeled (and unlabeled) serum albumin is considered representative of all serum proteins.

"Labeled protein" refers to the Evans-blue-labeled protein, which was almost entirely serum albumin. This labeled serum albumin was used as a marker for serum protein in general (see Discussion). It was both bound and unbound (extractable).

"Unlabeled protein" refers to the serum protein in the lesions that was not labeled by Evans blue. Most of it was in the established lesions before Evans blue was injected intravenously. We could not determine the *bound* unlabeled serum protein in the lesions; we could only determine the *unbound* (extractable) unlabled serum protein.

"Bound protein" refers to the protein that was bound within the organ-cultured lesion and, therefore, not extractable into the culture fluids. Most of it was probably encapsulated within lymphatics or loculated within connective tissue "capsules," but some of it was probably bound by the ground substance, and interstitial proteins, such as collagen fibers (see Discussion).

"Unbound" and "extractable" are used interchangeably. Protein in the culture fluids was, by definition, unbound and extractable. Normal skin also contained an appreciable amount of unbound (serum) protein, which turned over slowly.

Materials and Methods

Production of SM Lesions

Dermal SM lesions of various ages were produced in New Zealand white rabbits,[8] as described in the preceding report.[9] After the animal was sacrificed, the pelt was removed, the lesions were measured, and 1.0-sq cm central biopsy specimens were taken and organ-cultured.[9] The cultured (and uncultured) biopsy specimens were embedded in glycol methacrylate, sectioned, stained with Giemsa, and histologically evaluated.[9]

Evans Blue Dye Injections

Scientists have used Evans blue dye (C.I. 23860) for years to estimate vascular leakage.[see 1-4] When injected intravenously, it binds firmly to serum proteins, mainly albumin.

In our experiments, a 1.0% solution of Evans blue (Lot No. 213505, J.T. Baker Chemical Co., Phillipsburg, NJ) in 0.9% NaCl was injected, at a dose of 20 mg per kg of body weight, into the ear vein of rabbits, each bearing dermal SM lesions, 10 minutes and 1, 2, 3, 6, and 10 days of age. Two hours later, the animals were sacrificed in the following manner: Sodium pentobarbital (65 mg/ml) in a dose of 1.5 to 2.3 ml (depending on the size of the rabbit) was injected intravenously. After the rabbits lost consciousness, we exsanguinated them by cutting their femoral blood vessels, in order to reduce the amount of intravascular blood in the lesions.

Different lots of Evans blue varied considerably in dye content per milligram of powder. We therefore used the same lot of dye in all of our Evans blue experiments.

The findings in this report are those of the authors and should not be construed as an official Department of the Army position.

Accepted for publication May 1, 1985.

Dr. Harada and Dr. Kajiki are on leave of absence from The Second Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan.

Dr. Tanaka is on leave of absence from The First Department of Internal Medicine, Kumamoto University Medical School, Kumamoto, Japan.

Dr. Higuchi is on leave of absence from the Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University, Fukuoka, Japan.

Address reprint requests to Dr. Arthur M. Dannenberg, Jr., Johns Hopkins School of Hygiene, Baltimore, MD 21205.

Evans Blue Extractions

Before each blue-stained lesion was removed from the pelt, its average diameter was determined with calipers (see Table 1). Then the central 1.0-sq cm biopsy specimen was excised, trimmed of its underlying muscle layer, and weighed. Representative areas (1.0 sq cm) of normal skin from the same rabbit served as controls (see Figure 1 and Table 1). The Evans blue dye was extracted from each biopsy in 4.0 ml of formamide at 60 C for 72 hours, and its optical density measured at 620 nm in a spectrophotometer.[8,10] Standards of Evans blue dye in RPMI 1640 culture medium were used to convert optical density units to micrograms of Evans blue.

Evans Blue Content of Culture Fluids and Serum

To 7.5 ml of *culture fluid* in a test tube, 1.2 ml of 40% trichloroacetic acid (TCA) was added to precipitate the protein. This precipitate was centrifuged at 2500 rpm for 15 minutes and, after the supernate was decanted, dried overnight at 45 C. It was then extracted at 60 C for 3 days with 2.0 ml of formamide and read in a spectrophotometer at 620 nm. The amount of Evans blue in 1.0 ml of *serum* was determined, as described in the next paragraph for the dilute albumin standard.

Evans-blue-dyed bovine serum albumin was used as a *standard.* Small test tubes, containing 2.5 to 50 μg of Evans blue dye in 0.5 ml of culture medium RPMI 1640, were prepared. To each tube, 0.5 ml of bovine serum albumin (BSA) (4 mg per ml of RPMI 1640) was added and thoroughly mixed. (The BSA, Cat. No. A-9647, was purchased from Sigma Chemical Co., St. Louis, Mo.) The Evans-blue-dyed albumin was then precipitated with 0.14 ml of 40% TCA, dried, extracted with formamide, and read at 620 nm, as just described. In general, the ratio of Evans blue dye (extracted) to optical density (OD) units (at 620 nm) was fairly constant over a range of 0–10 μg of dye and 0.000–0.500 OD units.

Evans Blue Binding to Proteins

Part I

We investigated whether the Evans blue dye was irreversibly bound to serum proteins at the concentrations used in our experiments, namely, 20 mg/kg, or about 1 mg/ml of serum for a 3 kg rabbit (which contains about 60 ml of serum).

Two experiments were performed: a) 0.10 ml of Evans blue (10 mg/ml of 0.9% NaCl) was added to 0.90 ml of BSA (50 mg/ml of 0.9% NaCl); b) 0.10 ml of this Evans blue solution was added to 0.90 ml of normal rabbit serum, which also contains about 50 mg protein/ml.[9] The test tubes were vortexed and left 10 minutes at room temperature. Then, trichloroacetic acid (TCA) (1.0 ml of a 10% solution) was added, and the resulting precipitate was centrifuged at 2500 rpm for 15 minutes. The supernates contained no visible blue color—a result confirmed by spectroscopy at 620 nm. Thus, the binding of Evans blue by serum proteins is both rapid and complete at these concentrations.

To ascertain that Evans blue was not precipitated by TCA, we mixed 1.0 ml of Evans blue (25 μg/ml of saline) with 1.0 ml of 10% TCA. No precipitate was formed, and after the pH was readjusted to neutrality, the optical density was identical to that of the Evans blue in saline diluted with an equal quantity of water.

Table 1—Amount of Evans Blue in the Entire Sulfur Mustard Lesion at Various Times After Its Inception

Age of SM lesion	A Diameter of entire lesion (mm)	B Weight of the central 1.0-sq cm biopsy specimen (mg)	C Amount of Evans blue in the central 1.0-sq cm biopsy specimen* (μg)	D Amount of Evans blue in entire lesion calculated from surface area†‡ (Column C × πr^2)
Normal skin	–	200 ± 20	1.7 ± 0.2	–
2 hours	10.3 ± 0.2	190 ± 10	11.6 ± 2.4	9.8 ± 2.0
1 day	13.3 ± 0.7	320 ± 20	13.7 ± 1.4	18.7 ± 1.1
2 days	12.0 ± 0.4	250 ± 10	7.7 ± 0.8	8.7 ± 0.9
3 days	11.7 ± 0.3	230 ± 20	5.1 ± 0.6	5.4 ± 0.4
6 days	11.9 ± 0.9	190 ± 10	5.1 ± 0.5	5.7 ± 0.7
10 days	11.6 ± 0.7	190 ± 10	6.2 ± 0.4	6.6 ± 0.8

* These figures are plotted as the top curve of Figure 1.

† The Evans blue content of the entire lesion was calculated by multiplying the surface area of the lesion (with the formula πr^2, where r is the radius) by the amount of Evans blue in the 1.0-sq cm biopsy specimen. For these calculations, we assumed that the lesion was a flat disk instead of an ellipsoid. The means and their standard errors are listed.

‡ The figures in Column D are slightly different from those that would be obtained by using the means in columns A and C; because in Column D, the values for each of the 6 rabbits were calculated individually and then averaged.

When the experiment was repeated with a higher concentration of Evans blue (1.0 mg/ml), no visible precipitate was seen, even after centrifugation.

Part II

In a similar experiment, we incubated the same concentrations of Evans blue, BSA, and normal rabbit serum as in Parts Ia and Ib for 10 minutes at 37 C (instead of at room temperature) to determine which serum proteins bound the Evans blue. The Evans blue solutions were then electrophoresed on two identical slabs of agarose gel.[11] One of the slabs was stained with Coomassie blue to identify the various serum proteins; the other slab was left unstained so that the location of the Evans blue dye could be matched with the bands on the Coomassie-blue-stained preparation. Over 95% of the Evans blue was bound to the albumin fraction of serum. A faint but distinct Evans-blue-dyed band was discernible in the β-globulin fraction (see Part IV, below). The other serum fractions were essentially unstained by Evans blue.

Part III

A third experiment was performed to determine stability of the Evans blue–protein binding. Once bound to one protein, could Evans blue leave and bind to another protein? Evans blue–serum and Evans blue–BSA complexes were prepared at 37 C, as described in Part II above. Then an equal quantity of undyed BSA and undyed rabbit serum was added, respectively, to each, and they were further incubated at 37 C. Aliquots were removed at 1, 8, and 24 hours and electrophoresed on agarose slabs, as described in Part II. The unlabeled BSA did not remove Evans blue from the β-globulin fraction of labeled serum; and the β-globulin fraction of unlabeled serum did not remove Evans blue dye from the labeled BSA. In other words, the binding of Evans blue to these serum proteins seemed irreversible, at least under these conditions.

Part IV

A fourth experiment was performed to determine which protein fractions of the organ-culture fluids were labeled with Evans blue. Nine 1-day SM lesions were produced in the skin of a rabbit. Evans blue was injected intravenously 2 hours before the rabbit was killed. The lesions were removed and organ-cultured for 24 hours. Then the culture fluids were pooled, concentrated by Amicon filtration,[11] and electrophoresed on two identical slabs of agarose gel, as just described in Part II (above). Two approximately equal protein fractions had been stained by the intravenous Evans blue: a) the expected albumin fraction and b) an unexpected, diffuse

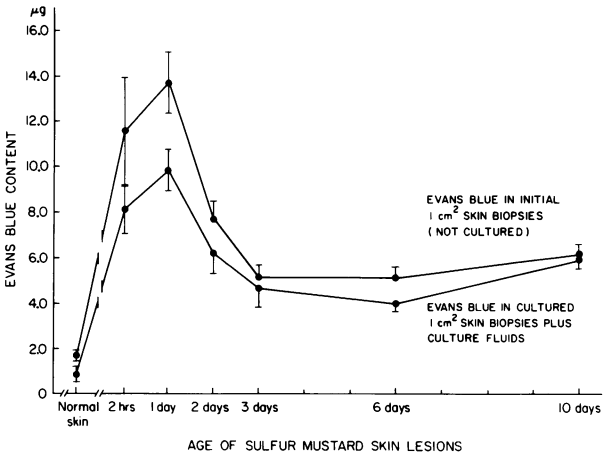

Figure 1—Evans blue content in 1.0-sq cm central biopsy specimens of rabbit dermal sulfur mustard lesions of various ages. The Evans blue is bound to (and is a measure of) the serum protein (albumin) that entered the lesions during the 2 hours before the animal was sacrificed. The *top graph* depicts the micrograms of Evans blue per 1.0-sq cm explant, extracted by formamide from 12 lesions at each time period (two lesions from each of 6 rabbits). The *bottom graph* depicts *a*) the micrograms of Evans blue per 1.0-sq cm explant, extracted by formamide from 18 organ-cultured lesions at each time period (three lesions from each of 6 rabbits after 3 days in culture), *plus b*) the Evans blue extracted by formamide from protein (in the first-, second-, and third-day culture fluids) precipitated by 5% trichloroacetic acid (TCA). The slight discrepancy was probably due to some hydrolysis of Evans-blue–bound serum protein by tissue proteases during the 3 days of culture. Such hydrolysis could have produced TCA-soluble Evans-blue–bound peptides.

This figure clearly shows that the early SM lesions (2 hours, 1 day, and 2 days of age) had more extravasation of (Evans-blue–labeled) serum protein (albumin) than healing lesions (3 and 6 days of age). (For the "not-cultured" biopsies, using the one-tailed, paired-sample Student *t* test, *P* was <0.001 for 1-day lesions versus 3- or 6-day lesions, and *P* was ≤0.025 for 2-hour or 2-day lesions versus 3- or 6-day lesions. The means and their standard errors are depicted. The slight rise at 10 days occurred only with some rabbits and was not statistically significant.

β-globulin fraction. These findings suggest that part of the Evans blue-labeled serum albumin within 1-day SM lesions was altered in such a manner that it electrophoresed with the β-globulin fraction. (In rats, specific antibody identified albumin in this β-fraction,[12] and albumin dimer formation was suggested as the cause of the altered electrophoretic mobility.[12])

Estimate of the Amount of Evans Blue in the Entire SM Lesion

We used 1.0-sq cm explants of the SM lesions as the basis of quantitation throughout this series of reports.[9,11,13] The amount of Evans blue in the *entire* lesion can be estimated by multiplying the amount of Evans blue in the central 1.0-sq cm biopsy by the surface area of the lesion (Table 1). The 2-hour Evans blue lesions were slightly smaller than the 1.0-sq cm biopsies, and the 1- and 2-day lesions were somewhat larger, especially the 1-day lesions (which were markedly

edematous). The others were approximately the same size. If the reader is interested in values for the entire SM lesion, he or she can use Table 1 to obtain these values from the tables and figures that we are presenting.

Protein Determination

The total protein in the culture fluids was determined by Bradford's Coomassie blue procedure (Bio-Rad Laboratories, Richmond, Calif), as described in our accompanying article.[9] The Evans blue in the culture fluids was too dilute to interfere with this procedure.

Statistics

The one-tailed, paired-sample Student *t* test was used. The data were first examined to determine whether they were normally distributed. In almost all instances, they were, and outliers were rare. In the figures and tables, the means and their standard errors are shown.

Results

Entry of Serum Protein Into SM Lesions

Evans blue dye was injected intravenously into rabbits bearing dermal SM lesions of various ages, and the animals were killed 2 hours later. Almost immediately, the Evans blue tagged the circulating serum albumin (see Materials and Methods). Thus, during this 2-hour period, the amount of serum protein that entered the lesions (represented by the tagged albumin) was proportional to the amount of dye that could be extracted from the lesions.

The greatest accumulation of Evans blue dye occurred in 2-hour, 1-day, and 2-day lesions (Figure 1). A reduced accumulation (about 40% of peak values) occurred in 3- and 6-day lesions. The slight rise in 10-day lesions was not statistically significant nor reproducible in a similar but smaller experiment.

Unbound Serum Protein in SM Lesions of Various Ages

The unbound (serum) protein in the organ-cultured dermal SM lesions was extracted by the culture fluids. This unbound protein falls into two categories: a) Evans blue-*labeled* protein (albumin) (which entered the lesions during the 2-hour period between the injection of Evans blue and the sacrifice of the animal) and b) *unlabeled* protein (which was in the lesions prior to the injection of Evans blue).

Evans-Blue-Labeled Albumin

In SM lesions, Evans blue is always attached to unbound and bound serum albumin and is never free. By measuring the Evans blue extracted by the culture fluids and the Evans blue remaining in the lesion explants after 3 days of culture, we could determine the *relative amounts of unbound and bound serum albumin* in these lesions.

Our results are presented in Table 2. The culture fluids extracted 23 to 36% of the total (bound plus unbound) Evans blue from 2-hour, 1-day, and 2-day lesions, but only extracted 11–16% from 3-, 6-, and 10-day lesions. In other words, a greater percentage of Evans-blue–labeled serum albumin was *unbound* when the lesions were largest and most edematous, and their ground substance was in a sol state (see Discussion). Conversely, a greater percentage was *bound* in healing lesions, where the gel state of ground substance had been reestablished.

Table 2 also compares the amount of Evans-blue–labeled albumin extracted from the lesions by first-, second-, and third-day culture fluids, respectively. A mean of 85% was extracted by first-day culture fluids, and most of the remainder was extracted by second-day culture fluids.

Unlabeled Unbound Protein

By measuring the total protein in the culture fluids and subtracting the Evans-blue–labeled protein, we could calculate the amount of unlabeled unbound protein. Evans-blue–labeled protein (represented by Evans-blue–labeled serum albumin) contained 0.54 mg of protein for every 1.00 μg of Evans blue (see Table 3).

The concentrations of both total protein and Evans-blue–labeled protein were highest in the culture fluids of 1-day lesions (Table 3 and Figure 2). Subsequently, the total unbound protein dropped slowly to 55% of peak values, and the Evans-blue–labeled unbound protein dropped rapidly to 15% of peak values.

Thus, after the first-day peak, "fresh" serum protein apparently entered SM lesions at a decreased rate; and, therefore, "older" serum protein constituted a larger percentage of the serum protein within such lesions (Table 3, Column D).

The slight rise in the leakage of serum protein in 10-day lesions was not always present, nor was it statistically significant. The increased leakage in some of the healing lesions was probably due to the scratching and irritation of such lesions by the rabbit.

Turnover of Unbound Serum Protein in SM Lesions

The data just described suggest that early lesions have a rather high serum protein turnover. Evans-blue–labeled serum albumin comprised nearly all of the serum albumin entering during the life of a 2-hour lesion. Such lesions were started 10 minutes before the

Table 2—Amount of Bound and Unbound Evans-Blue–Labeled Serum Albumin in Sulfur Mustard Lesions During Their Development and Healing*

	A	B		C		D		E	F	G	
Age of SM lesion	Total amount of Evans blue in the 1-sq cm lesion biopsy specimen† (μg)	Amount of Evans blue in first-day culture fluids		Amount of Evans blue in second-day culture fluids		Amount of Evans blue in third-day culture fluids		Amount of Evans blue in all culture fluids (unbound) (μg)	Evans blue bound within the lesions‡ A–E (μg)	Percent of unbound and bound Evans-blue–labeled serum albumin in the SM lesions*§	
		μg	B/E	μg	C/E	μg	D/E			Unbound E/A	Bound F/A
Normal skin‖	0.87 ± 0.36	0.05 ± 0.04	71%	0.02 ± 0.02	29%	0	0	0.07 ± 0.06	0.81 ± 0.31	8%	93%[c]
2 hours	8.13 ± 1.04	2.02 ± 0.44	81%	0.37 ± 0.09	15%	0.11 ± 0.06	4%	2.49 ± 0.51	5.63 ± 0.68	31%	69%
1 day	9.85 ± 0.94	2.77 ± 0.25	79%	0.61 ± 0.11	18%	0.12 ± 0.07	3%	3.50 ± 0.33	6.34 ± 0.66	36%	64%
2 days	6.20 ± 0.86	1.19 ± 0.20	83%	0.20 ± 0.07	14%	0.04 ± 0.03	3%	1.43 ± 0.19	4.77 ± 0.71	23%	77%
3 days	4.67 ± 0.83	0.46 ± 0.07	88%	0.04 ± 0.03	8%	0.02 ± 0.02	4%	0.53 ± 0.09	4.14 ± 0.77	11%	89%
6 days	4.00 ± 0.36	0.51 ± 0.11	88%	0.05 ± 0.03	9%	0.02 ± 0.01	3%	0.57 ± 0.13	3.44 ± 0.38	14%	86%
10 days	5.91 ± 0.37	0.81 ± 0.22	88%	0.10 ± 0.03	11%	0.01 ± 0.01	1%	0.92 ± 0.22	4.99 ± 0.29	16%	84%
Average for lesions			85%		12%		3%			22%	78%

* After intravenous injection, Evans blue attaches to and therefore labels serum albumin (see Materials and Methods). In these experiments, each microgram of Evans blue represented 0.36 mg of Evans-blue–labeled serum albumin, ie, the 0.54 mg of serum protein in Column C of Table 3, multiplied by 66.2% for the serum albumin component.[see 11]

† The amount of Evans blue remaining in the 1.0-sq cm lesion explant after 3 days in culture *plus* the amount of Evans blue in the first-, second-, and third-day culture fluids. These figures are plotted as the bottom curve in Figure 1.

‡ The figures in Column F are slightly different from those that would be obtained by using the means in Columns A and E, because in Column F, the values for each of the 6 rabbits were listed individually and then averaged. In column G, 93%, rather than 92%, appears for the same reason.

§ In these experiments, the protein extractable into the culture fluids was represented by *unbound* serum albumin. Most of the *bound* serum protein (albumin), ie, bound within the organ-cultured lesion, was probably encapsulated within the lymphatics (see Discussion). The *P* values for the percentages in *unbound* Evans blue-labeled serum albumin (listed in Column G) are as follows: normal skin versus 1- or 2-day lesions, $P < 0.001$; normal skin versus 3-, 6-, or 10-day lesions, $P < 0.03$; 1-day lesions versus 2-, 3-, 6-, or 10-day lesions, $P < 0.001$; 2-day lesions versus 3-, 6- or 10-day lesions, $P < 0.01$.

‖ Normal skin from the six rabbits with SM lesions used in this experiment.

Table 3—Unbound (Serum) Protein Turnover in Sulfur Mustard Lesions*†

Age of SM lesions (1.0-sq cm explants)	A Evans blue extracted into 1-, 2- and 3-day culture fluids (μg)	B Total protein in 1-, 2- and 3-day culture fluids (mg)	C Evans blue-labeled protein in these culture fluids (A × 0.54) (mg)	D 2-hour (serum) protein (C) entry as percent of total protein (B) in these culture fluids (C/B × 100%)	E Total extractable (serum) protein entering the lesions in 24 hour at 2-hour rate (C × 12) (mg)	F No. of turnovers in 24 hours: total extractable protein entering the lesions (E) divided by total extractable protein present (B)‡ (E/B)	G Time required to turn over once the extractable (serum) protein in the lesions $\left(\frac{24\text{ hr}}{F}\right)$
Normal skin§	0.07 ± 0.06	1.7 ± 0.3‖	0.04 ± 0.03	2.4%	0.5 ± 0.4	0.3 ×	80 hours
2 hours	2.49 ± 0.51	3.0 ± 0.5‖	1.34 ± 0.28	44.7%	16.1 ± 3.4	5.4 ×	4 hours
1 day	3.50 ± 0.33	7.8 ± 1.0	1.89 ± 0.18	24.2%	22.7 ± 2.2	2.9 ×	8 hours
2 days	1.43 ± 0.19	6.0 ± 0.7	0.77 ± 0.10	12.8%	9.2 ± 1.2	1.5 ×	16 hours
3 days	0.53 ± 0.09	5.4 ± 0.6	0.29 ± 0.05	5.4%	3.5 ± 0.6	0.6 ×	40 hours
6 days	0.57 ± 0.13	4.7 ± 0.4	0.31 ± 0.07	6.6%	3.7 ± 0.8	0.8 ×	30 hours
10 days	0.92 ± 0.22	4.3 ± 0.4	0.50 ± 0.12	11.6%	6.0 ± 1.4	1.4 ×	17 hours

* The 6 rabbits used in this experiment were not the same 6 used in the preceding paper.[9]

† For this table, we have assumed 1) that the *unbound* Evans-blue–labeled *serum albumin* was representative of *all unbound serum protein* in the lesions, and 2) that the unbound proteins in the lesions were almost all *serum* protein, rather than tissue protein (see Discussion). To convert "serum protein" in this table to "serum albumin" (which is what we labeled with Evans blue), multiply the figures in Columns B, C, and E by 66.2%.[see 11] The figures in the other columns would be unchanged. A complete analysis of this table appears as the Appendix of this report.

‡ This is the 24-hour turnover rate if the 2-hour rate were maintained (see footnote in Appendix). This rate is plotted in Figure 3. The *P* values for the number of turnovers in 24 hours (listed in Column F) are as follows: normal skin versus 1-day lesions, $P < 0.001$; normal skin versus 2-, 3-, 6-, or 10-day lesions, $P < 0.02$; 1-day lesions versus 3- or 6-day lesions, $P < 0.001$; 1-day lesions versus 2- or 10-day lesions, $P < 0.01$; 2-day lesions versus 3-day lesions, $P < 0.001$; 2-day lesions versus 6-day lesions, $P < 0.01$.

§ Normal skin from the 6 rabbits with SM lesions used in this experiment. The average total protein of 1.7 mg extracted from 200 mg normal explants[9] by culture fluids compares favorably with the 8.0 mg of serum albumin extracted from 1.0 g of rat skin by homogenization.[12]

‖ The 1.7 and 3.0 figures were really 1.73 and 3.03 (see Appendix).

intravenous Evans blue injection.* In contrast, a 24-hour lesion had a 22-hour influx of unlabeled serum albumin and only a 2-hour influx of Evans-blue–labeled albumin entering during its lifetime. One can use these findings to estimate the turnover of unbound serum protein (albumin) in SM lesions of various ages (Table 3). If we assume that 2-hour entry of Evans blue into 1-day SM lesions was continued at this rate for 24 hours, then these 1-day lesions turned over their unbound serum protein about 2.9 times in 24 hours. Similarly, 2-day lesions turned over their unbound serum protein about 1.5 times in 24 hours; 3- and 6-day lesions, about 0.7 times; and 10-day lesions, about 1.4 times (Table 3, Column F, and Figure 3). As mentioned above, the rise at the 10-day figure was not always present. The differences in these turnover rates between 1-day lesions and 3- or 6-day lesions were highly significant ($P < 0.001$).

These results can also be expressed as the time required for one complete turnover of the serum protein in the lesions (Table 3, Column G). One-day lesions turned over their protein once in about 8 hours; 2-day lesions, once in about 16 hours; 3- and 6-day lesions, once in about 35 hours. Although the turnover rate of serum protein in the *healing* lesions decreased markedly, the *total* unbound serum protein within these lesions decreased relatively little (Figure 2).

Turnover of Serum Protein in Normal Skin

Normal skin also contained unbound serum protein.[9] Specifically, in these experiments 1.7 ± 0.3 mg of such protein was extracted from 1.0-sq cm biopsy specimens of the normal skin between the SM lesions (Table 3). The turnover of this unbound serum protein (measured as serum albumin) was slow, compared with that in acute inflammatory lesions, ie, once every 80 hours in contrast to once every 8 hours in the 1-day inflammatory lesions (Table 3; also see Figure 3).

These experiments were repeated with normal skin from 4 rabbits without SM lesions. Their skin contained about 3 times the total amount of Evans blue (per 1.0 sq cm) as the normal skin of the SM rabbits represented in Table 2, Column A; about 10 times the amount of *unbound* Evans blue (extracted by the culture fluids)

* In 2-hour lesions, a negligible amount of serum protein should have extravasated during the 10 minutes before the Evans blue injection. SM injury tends to be delayed, so such leakage should not have started immediately. At most, the first 10 minutes would represent 8% of the total leakage into the 2-hour lesions. (Such lesions were really 130 minutes old).

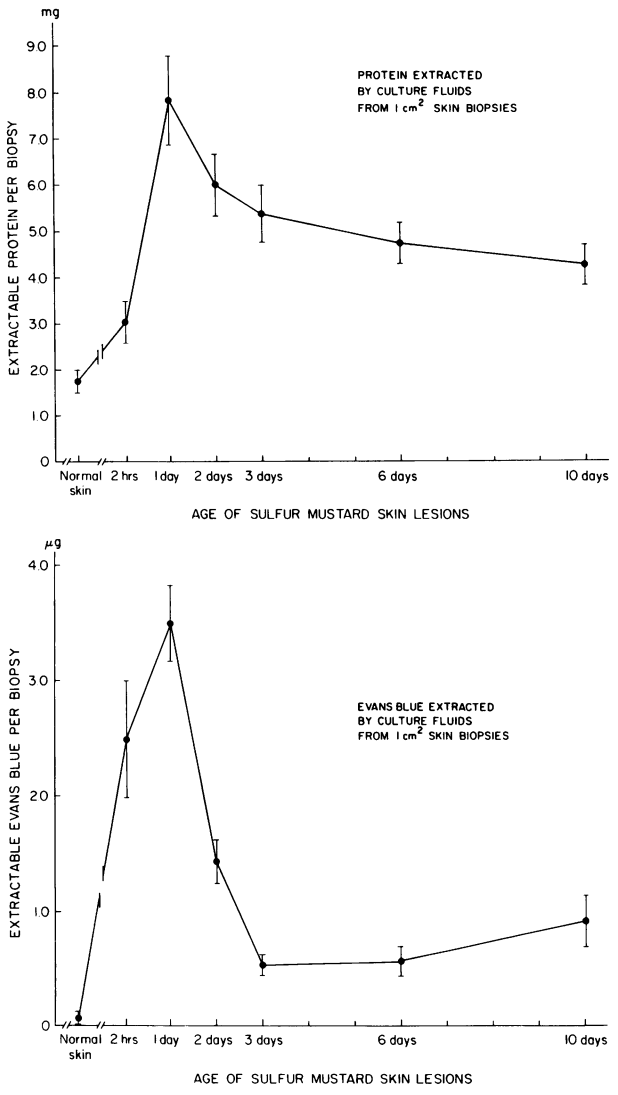

Figure 2—Amount of protein (top graph) and Evans blue (bottom graph) extracted by culture fluids from 1.0-sq cm central biopsy specimens of rabbit dermal sulfur mustard lesions of various ages. As stated in Figure 1, the Evans blue is bound to (and is a measure of) the serum protein (albumin) that entered the lesions during the 2 hours before the animal was sacrificed. Each 1.0-sq cm biopsy specimen was cultured in 2.5 ml; the first-, second-, and third-day culture fluids were assayed for protein and Evans blue, and the totals from all 3 days of culture are depicted in the graphs. Note that the *protein* extracted from the lesions by the culture fluids *decreased only slightly* as the lesions healed. (For 1-day SM lesions versus 3- and 6-day SM lesions, P was <0.02.) Note also that the Evans blue extracted from the lesions by the culture fluids *decreased markedly* as the lesions healed. (For 1-day SM lesions versus 3- or 6-day SM lesions, P was <0.001.) During the healing process, not only did *less* Evans-blue-labeled (serum) protein enter (see Figure 1), but that which did enter was evidently more firmly bound (Table 2). The 6 rabbits used in this experiment were not the same 6 used in our accompanying paper.[9]

(see Table 3, Column A); and about the same amount of *total unbound* protein (extracted by the culture fluids).

Thus, the normal skin of rabbits from different experiments showed considerable variation in Evans blue

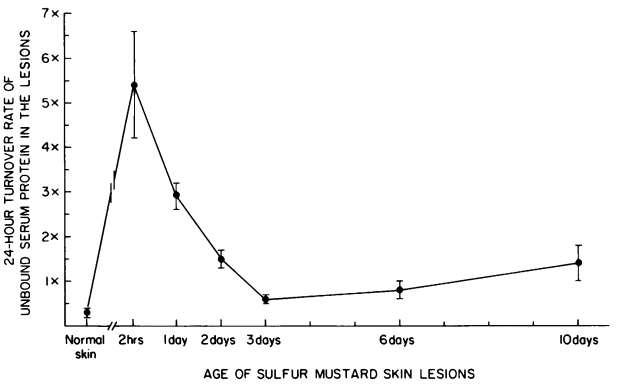

Figure 3—Daily turnover rates of unbound serum protein in normal skin and in developing and healing sulfur mustard skin lesions. The 24-hour turnover rates were extrapolated from 2-hour turnover rates (see Table 3 and Appendix). The P values for this figure are presented in footnote‡ of Table 3.

content and in the amount of Evans blue extracted by the culture fluids. One would also expect variations in the turnover rates of serum protein in normal skin. For this reason, we believe that each rabbit should provide its own normal skin controls.

Discussion

Serum, extravasated into local inflammatory sites, contains potentially *proinflammatory* factors,[14,15] eg, complement components, kininogen and plasminogen, and *antiinflammatory* factors,[16] eg, inhibitors of chemotaxis,[17,18] antiproteases (α_1-antiproteinase, α_2-macroglobulin, and α_2-antiplasmin),[19] ceruloplasmin (an antioxidant),[20] and kininase.[14] Since extravasated serum is such an important modulator of the inflammatory response, we measured the rates of entry and turnover of serum protein in developing and healing inflammatory skin lesions (produced by SM) before we measured some of the specific mediators that were present.

Two hours before rabbits (bearing dermal SM lesions of various ages) were killed, Evans blue dye was injected intravenously. This dye immediately labeled the circulating serum albumin (see Materials and Methods). This labeled albumin was used as a measure of the 2-hour rate of serum protein entry into the SM lesions. Within the lesions, 64–89% of the entering labeled albumin was bound (Table 2), but the remainder was unbound and could be extracted by the culture fluids along with unbound, unlabeled (serum) protein, much of which had previously entered the lesions.

This model allows some definite conclusions to be made on the entry, distribution, and turnover of serum albumin in developing and healing SM lesions and allows some tentative conclusions for the other serum proteins that accompanied this serum albumin.

Definite Conclusions

a) The leakage of serum albumin into peak lesions (2 hours to 1 day) was about twice that into healing lesions (3–10 days) (Figure 1 and Table 1). *b*) The leakage into healing lesions was about three times that into normal skin. *c*) Within the lesions, an average of 20% of the extravasated serum albumin was unbound, ie, extractable into the culture fluids (Table 2). *d*) This unbound serum albumin had an unexpectedly high turnover rate: once every 4–8 hours in peak lesions, and once every 30–40 hours in healing lesions (Table 3). Even during the healing stages of these acute inflammatory lesions, appreciable amounts of serum protein still entered and left the site (Figure 3 and Table 3). Therefore, serum protein could play a major modulating role in *all* stages of the inflammatory process.

Tentative Conclusions

a) The serum albumin/globulin ratio in the culture fluids was about the same as in serum itself.[11] Therefore, the turnover of the unbound serum albumin might reflect the turnover of the unbound serum protein in general. *b*) The *bound* Evans-blue–labeled serum albumin (and probably other serum proteins) also had an appreciable turnover rate. These two tentative conclusions warrant further discussion.

Unbound Serum Protein

Were we justified in using the entry rate of Evans-blue–labeled serum albumin in the lesions as a rough measure of the turnover rate of serum protein in general? The answer is probably *yes*, as far as *unbound* serum protein is concerned, because serum and culture fluids showed similar two-dimensional gel electrophoretic patterns.[11]

The constancy of the composition of the unbound proteins in the lesions does not, however, assure identical *turnover* rates for each electrophoretic fraction: globulin is larger than albumin and may enter and leave the lesions more slowly than albumin. In other words, the levels of each fraction in the extractable fluids may remain relatively constant and yet may have different turnover times. We do believe, however, that the turnover time of serum albumin measured in this report *is in the general range* of the turnover time of the serum globulins. They both enter *inflammatory sites* because of vascular leakage and both leave primarily because of drainage via the lymphatics. Such leakage and drainage should be affected only slightly by the differences in the size of their protein molecules.

Bound Serum Protein

Serum protein (albumin) continually entered and was bound in SM lesions of all ages, but the lesions did not continually grow in size. Only the 1- and 2-day lesions showed gross edema. Therefore, from the third day on, the amount of serum protein that entered and bound was probably equivalent to the amount of bound serum protein that disappeared from the lesions. In other words, the *bound* serum protein probably turned over at an appreciable rate. Quantitation of this rate was impossible: we could not measure the size of the *bound* serum protein pool in the lesions, because many non-serum proteins were present.

Most of the bound (nonextractable) serum protein in the explants was probably encapsulated within nonfunctional lymphatics (see below). Some was probably loculated within connective tissue compartments, or "capsules."[21] Some was probably bound to the ground substance; and a small amount was probably adsorbed onto collagen (and elastic) fibers.

Nevertheless, the high rate of *entry* of labeled serum protein (albumin) into the bound serum protein pool leads to several interesting speculations. Bound serum proteins (at least the bound albumin) are not in equilibrium with the unbound serum proteins extractable into the culture fluids. Only 11–36% of the Evans-blue–labeled serum albumin in the SM lesions was extracted into culture fluids over 3 successive days (Table 2). Therefore, 64–89% of the serum albumin seemed to be tightly bound.

How, then, did these "bound" serum proteins turn over? We believe that they left via the lymphatics and also were endocytosed and digested by cells in the inflammatory lesions, especially the macrophages and fibroblasts. Evidently, the protein in the lymphatics was not free to diffuse into the culture fluids, but was sealed in place (ie, bound) by the lymphatic endothelium. *In vivo*, the contents of the lymphatics are propelled along valved channels by external movement (and contractions of the larger lymphatics).[6,7] *In vitro*, no such movement occurs locally, and the larger lymphatics have been cut, so they could not create suction on the smaller lymphatics.

The *unbound serum protein* (the turnover of which is described in this report) probably plays a more functional role in inflammation than the bound serum protein. The unbound protein is freely diffusible and, therefore, available to provide local inflammatory mediators (or inhibitors) throughout the lesion. In contrast, most of the *bound serum protein* may formerly have been unbound serum protein that had already played its role and is now leaving the site via the lymphatics.

Finally, *normal* skin contained appreciable amounts of unbound and bound serum protein (Tables 2 and 3). The amount of this protein (and probably its turnover) varied from experiment to experiment, probably because of skin thickness, hair density, room temperature, humidity, time of feeding, and the animal's age and activity. Extravasated serum protein probably serves an important function in the physiology of normal skin.

The distribution and turnover of extravascular serum proteins (especially albumin) have been extensively studied in normal experimental animals[6,12,22,23] and even in man.[see 24,25] In the body as a whole, the amount of serum albumin found extravascularly is four to five times that found intravascularly.[12] Of all the tissues, skin and muscle contain the most extravascular albumin.[12,22]

Appendix

Comments on Table 3

Column A lists, in micrograms, the amount of Evans blue dye found in first-, second-, and third-day culture fluids (added together) from 1.0-sq cm SM lesion explants of various ages. The procedure for precipitating the proteins from the culture fluids and determining their Evans blue content is described in Materials and Methods.

Column B lists, in milligrams, the amount of "serum" protein found in first-, second-, and third-day culture fluids (added together) from these explants, determined by direct measurement with the Bio-Rad reagent. Some of this extractable protein could have been released from lesion components other than serum, but over 90% was probably of serum origin.[see 11]

Column C lists the protein equivalent to the Evans blue label in the culture fluids. These results were derived by multiplying the micrograms of Evans blue (Column A) by 0.54 and changing micrograms to milligrams (ie, multiplying by 540).

The factor 0.54×10^3 was derived as follows. Ten minutes after the application of SM to the skin (for 2-hour lesions), Evans blue was injected intravenously. It bound to the serum albumin immediately, so that *almost all of the serum albumin leaking into the 2-hour lesions was labeled with Evans blue.* The amount of protein extracted into the culture fluids of 1.0-sq cm normal skin and 2-hour SM skin explants was 1.7 and 3.0 mg, respectively, by direct measurement (Column B) with the Bio-Rad reagent (see Materials and Methods). Thus, 2.49 μg Evans blue found in the 2-hour lesions (Column A) was equivalent to 1.34 mg (really "3.03" minus "1.73–0.04") of unbound protein that we now call "Evans-blue-labeled protein" because of its labeled serum albumin content. (The "0.04 mg" is the unbound Evans-blue-labeled albumin in normal skin, which we must subtract from the 1.73 mg of total unbound protein in normal skin to obtain a value for the *unlabeled* unbound protein in normal skin.) The factor 0.54 (ie, 1.34 mg of unbound "Evans-blue-labeled protein" divided by 2.49 μg of Evans blue) converts micrograms of Evans blue into milligrams of Evans-blue-labeled protein.

We then used this factor in Column C to obtain the milligrams of protein in the culture fluids for the 1-, 2-, 3-, 6-, and 10-day SM lesion explants. Into *these* lesions, Evans-blue-*labeled* (serum) protein had extravasated for only 2 hours, and *unlabeled* serum protein had previously extravasated for the rest of each lesion's life, ie, for 22, 46, 70, 142, and 238 hours, respectively. (The factor was similarly used for the normal skin explants.)

Column D lists the extractable (serum) protein (unbound Evans-blue-labeled protein) entering each lesion for the 2-hour period as a percent of the total extractable protein (total unbound protein) in each lesion.

Column E lists the total extractable (serum) protein (total unbound Evans-blue-labeled protein) that would have entered the culture fluids, assuming that the rate of entry at 2 hours continued for 24 hours.†

Column F compares the total extractable (serum) protein (total unbound Evans-blue-labeled protein) entering in 24 hours (*at the 2-hour rate,*† shown in E) with the total extractable (serum) protein (shown in B) found in each lesion. Thus, it indicates for the SM lesions the number of turnovers of extractable (serum) protein in 24 hours. For example, there was 7.8 mg of extractable (unbound) (serum) protein in the 1-day lesions (Column B), but 22.7 mg entered in 24 hours (Column E). Therefore, the unbound protein existing in the lesions would have been replaced 2.9 times in 24 hours, if one assumes an unchanged turnover rate† (Figure 3).

Column G lists the *turnover rate,* ie, the time required (in hours) for one turnover of the unbound (serum) protein in the lesions. For example, if the unbound (serum) protein in the 1-day lesions is replaced 2.9 times in 24 hours (Column F), it would take about 8 hours (24 hours divided by 2.9) to replace it once.

The figures in *Columns B, C, and E* can be converted to milligrams of serum albumin, by multiplying each

† We realize that this 2-hour rate of (serum) protein entry was decreasing each day. Thus, the turnover rates listed apply only to the time when the lesions were removed from the animal and are *not* to be considered rates that continued for 24 hours.

of them by 66.2%.[see 11] The protein in both rabbit serum and the culture fluids averaged about 66.2% of albumin.[11]

References

1. Menkin V: Dynamics of Inflammation: An Inquiry into the Mechanism of Infectious Processes. New York, The MacMillan Company, 1940, pp 24–48
2. Spector WG, Willoughby DA: The Pharmacology of Inflammation. New York, Grune & Stratton, 1968
3. Florey HW (ed): General Pathology. Philadelphia, W.B. Saunders, 1970, pp 22–39; 40–123
4. Wilhelm DL: Chemical mediators, The Inflammatory Process. Edited by BW Zweifach, L Grant, RT McCluskey. 2nd edition. Vol II. New York, Academic Press, 1973, pp 251–301
5. Willoughby DA: Mediation of increased vascular permeability in inflammation,[4] pp 303–331
6. Zweifach BW, Silberberg A: The interstitial-lymphatic flow system, Cardiovascular Physiology III; International Review of Physiology. Vol 18. Edited by AC Guyton, DB Young. Balitmore, University Park Press, 1979, pp 215–260
7. Casley-Smith JR: The response of the microcirculation to inflammation, The Cell Biology of Inflammation. Handbook of Inflammation. Vol 2. Edited by G Weissman. Amsterdam, Elsevier/North-Holland Biomedical Press, 1980, pp 53–82
8. Vogt RF Jr, Dannenberg AM Jr, Schofield BH, Hynes NA, Papirmeister B: Pathogenesis of skin lesions caused by sulfur mustard. Fund Appl Toxicol (suppl) 1984, 4:S71–S83
9. Dannenberg AM Jr, Pula PJ, Liu L, Harada S, Tanaka F, Vogt RF Jr, Kajiki A, Higuchi K: Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced *in vivo* by sulfur mustard: I. Quantitative histopathology; PMN, basophil, and mononuclear cell survival; and unbound (serum) protein content. Am J Pathol 1985, 121:15–27
10. Yamamoto T, Kambara T: A protease-like permeability factor in guinea pig skin: I. Partial purification and characterization. Biochim Biophys Acta 1978, 540:55–64
11. Harada S, Dannenberg AM Jr, Vogt RF Jr, Myrick JE, Tanaka F, Merkhofer RM, Pula PJ: Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced *in vivo* by sulfur mustard: III. Electrophoretic protein fractions and trypsin-inhibitory capacity of the culture fluids. (Manuscript submitted)
12. Katz J, Sellers AL, Bonorris G, Golden S: Studies on the extravascular albumin of rats, Plasma Protein Metabolism: Regulation of Synthesis, Distribution and Degradation. Edited by MA Rothschild, T Waldmann. New York, Academic Press, 1970, pp 129–154
13. Tanaka F, Harada S, Dannenberg AM Jr, Pula PJ, Merkhofer RM, Hugli TE: Inflammatory mediators and modulators released in organ culture from rabbit skin lesions produced *in vivo* by sulfur mustard: IV. Chemotactic and chymotrypsin-like esterase activities of the culture fluids. (Manuscript in preparation)
14. Colman RW: Formation of human plasma kinin. N Engl J Med 1974, 291:509–515
15. Kaplan AP: Coagulation, kinins, and inflammation, Cellular Functions in Immunity and Inflammation. Edited by JJ Oppenheim, DL Rosenstreich, M Potter. New York, Elsevier/North-Holland, 1981, pp 397–410
16. Lewis DA: Endogenous antiinflammatory factors. Biochem Pharmacol 1948, 33:1705–1714
17. Maderazo EG, Ward PA, Woronick CL, Quintiliani R: Partial characterization of a cell-directed inhibitor of leukotaxis in human serum. J Lab Clin Med 1977, 89:190–199
18. Kreutzer DL, Hupp JR, McCormick JR: Elevation of serum chemotactic factor inactivator activity during acute inflammatory reactions in rabbits and humans. Ann NY Acad Sci, 1982, 389:451–453
19. Laskowski M Jr, Kato I: Protein inhibitors of proteinases. Ann Rev Biochem 1980, 49:539–626
20. Goldstein IM, Kaplan HB, Edelson HS, Weissmann G: Ceruloplasmin: An acute phase reactant that scavenges oxygen-derived free radicals. Ann NY Acad Sci 1982, 389:369–378
21. Rodbard S, Feldman P: Functional anatomy of the lymphatic fluids and pathways. Lymphology 1975, 8:49–56
22. Reeve EB, Chen AY: Regulation of interstitial albumin,[12] pp 89–109
23. Rosenoer VM, Rothschild MA: The extravascular transport of albumin,[12] pp 111–127
24. Rothschild MA, Oratz M, Schreiber SS: Extravascular albumin. N Engl J Med 1979, 301:497–498
25. Parving H-H, Hansen JM, Nielsen SL, Rossing N, Munck O, Lassen NA: Mechanisms of edema formation in myxedema: Increased protein extravasation and relatively slow lymphatic drainage. N Engl J Med 1979, 301:460–465

Acknowledgments

We are grateful to Dr. Robert F. Vogt, Jr., for performing the agarose gel electrophoresis experiments described in Materials and Methods and for his review of the manuscript; to Dr. Bruno Papirmeister, of the U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, for his discussion on sulfur mustard injury; to Dr. Helen Abbey, of our Biostatistics Department, for advice on the statistical treatment of our data; and to Ilse M. Harrop for typing and editing the manuscript.