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Isolation of Skin Permeability Factors from Culture Filtrates of *Salmonella typhimurium*

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Enterotoxins isolated from *Vibrio cholerae* and toxigenic *Escherichia coli* cause permeability alterations in rabbit skin. Firm induration and erythema are observed within 18 to 24 h, and visualization of the reaction may be enhanced by intravenous injection of Pontamine sky blue dye. Two skin permeability factors (PF) have been found in culture filtrates of *Salmonella typhimurium*. A rapid-acting factor, produced optimally in brain heart infusion broth at 37°C by numerous *Salmonella* species, has a critical bluing time of 1 h after completion of skin testing. This rapid PF is heat stable at 100°C for at least 4 h and has no associated induration. The delayed factor is heat labile, being completely destroyed within 30 min at 75°C and causes marked induration of the rabbit skin within 18 h that is indistinguishable from the permeability reactions of *V. cholerae* and *E. coli* enterotoxins. Induration produced by the delayed PF is observed only after chromatography of the culture filtrate on a Sephadex G-100 column. Thus, the effects of the delayed PF appear to be masked or blocked by an inhibitor-like substance present in crude culture filtrates. Both early and delayed factors are estimated to have a molecular weight of at least 90,000. It is postulated that one or both of these factors may participate in the pathogenesis of *Salmonella* infections.

In recent years there has been increasing interest in the pathogenic mechanism of the enteric bacteria. Several genera of enteric pathogens have been shown to release "enterotoxins" which are responsible for the loss of fluid and electrolytes from the intestinal mucosa. The best characterized of these toxigenic enteric bacteria is *Vibrio cholerae*. The vast literature on the pathogenesis of cholera has stimulated inquiry into the possibility that other intestinal pathogens might cause diarrhea by means of an enterotoxin. Certain strains of *Escherichia coli* (6), noncholera vibrios (23), *Vibrio parahaemolyticus* (19), *Clostridium perfringens* (5), *Shigella dysenteriae* (15), and *Pseudomonas aeruginosa* (17) have been found to elaborate enterotoxins; however, the mechanism of action of the latter toxins is not as well understood (2). Of the organisms listed, *E. coli* produces an enterotoxin that is most similar in its biological characteristics to cholera toxin, and the two have been shown to cross-react immunologically (21).

Live *Salmonella* cultures have been shown to cause fluid accumulation in rabbit intestinal loop segments (14, 22). The existence of a *Salmonella* toxin responsible for the diarrhea of salmonellosis has been postulated for many years (1, 7). Crude culture filtrates of *Salmonella* species were tested by Giannella et al. (14) in the rabbit ileal loop model, but no positive responses were observed even with concentrated culture filtrates. Sakazaki et al. (20) reported that filtrates from 11 of 13 *Salmonella* strains tested yielded positive loop responses in the rabbit but were unable to observe similar results with the living cultures. More recently Koupal and Deibel described an enterotoxic factor isolated from *Salmonella enteritidis* and *Salmonella typhimurium* which caused fluid accumulation in the suckling mouse model (16). Their observations suggested that the factor was a protein derived from the cell wall or outer-membrane fraction of the cells.

The cholera enterotoxin (choleragen) has served as a model for the study of numerous other enteric toxins. It has been demonstrated that this enterotoxin elicits both fluid loss from the intestinal mucosa and permeability alterations in rabbit skin (3, 9). In the present study, we are investigating the hypothesis that *Salmonella* species may secrete a similar toxin (or toxins) responsible for all or part of the diarrhea of salmonelloses. Because higher concentrations of *V. cholerae* and *E. coli* enterotoxins are generally required to elicit a positive intestinal loop response than a positive skin test (9), it was reasoned that the skin test model might

provide a more sensitive approach to the study of *Salmonella* culture filtrates than an intestinal loop model. The results indicate that two permeability factors are elaborated by *Salmonella* species and at least one is strikingly similar to that of *V. cholerae* and *E. coli*.

MATERIALS AND METHODS

Organisms. *Salmonella typhimurium* 986 was isolated during an outbreak of diarrhea in the pediatric ward of John Sealy Hospital, Galveston, Tex. Five additional strains of *S. typhimurium*, Thax-1, LT-7, SL 1027, W 118-2, and TML, were supplied by Samuel B. Formal, Walter Reed Army Medical Center, Washington, D.C. *S. typhimurium* strains RIA and SR-11 were supplied by L. Joe Berry, University of Texas at Austin. Ten of the most frequently isolated *Salmonella* strains (excluding *S. typhimurium*) were provided by Don J. Brenner, Center for Disease Control, Atlanta, Ga. The latter isolates included *S. typhi* 3530-74, *S. javiana* 364-75, *S. panama* 219-75, *S. newport* 343-75, *S. heidelberg* 374-75, *S. infantis* 282-75, *S. enteritidis* 153-75, *S. saint-paul* 217-75, *S. agona* 184-75, and *S. derby* 113-75.

Preparation of cultures. A variety of culture media was examined for permeability factor production. Twenty-five-milliliter volumes of the following broth media were inoculated with a light suspension of *S. typhimurium* 986 harvested from heart infusion agar plates (Difco): brain heart infusion (BHI; Difco), heart infusion agar (Difco), Tryptic soy (Difco), syncase without sucrose or dextrose (11), syncase plus 1% dextrose (11), synthetic medium (11) with 2% tryptone (Difco) added (syntryp), syntryp plus 1% dextrose, and thioglycolate broth (Difco). Each medium, with the exception of thioglycolate broth, was incubated with mild shaking at 30 and 37°C for 24 h. The latter medium was dispensed into tall, slender bottles and incubated as above, but without shaking. After preliminary investigations, BHI broth was selected for use in all the remaining experiments, and all cultures were grown at 37°C with shaking for 24 h.

Preparation of filtrates. The 24-h broth cultures were harvested by centrifugation at 12,100 × *g*, and the supernatants were filtered through 0.20-μm sterile Nalgene filter units. Filtrates were either decanted into serum vials for storage or were concentrated by one of two methods. Tenfold concentration was obtained by dialysis in 20 M Carbowax (Union Carbide) followed by dialysis of the concentrate in 0.01 M Tris-hydrochloride buffer with ethylenediaminetetraacetate at pH 7.5 (13). Alternatively, filtrates were concentrated 100-fold in an Amicon ultrafiltration unit fitted with a PM30 membrane. All filtrates, both concentrated and unconcentrated, were stored at −20°C.

Column chromatography. Partial purification of selected filtrates was achieved by gel filtration through a calibrated column (1.5 by 90 cm) of Sephadex G-100 (Pharmacia) equilibrated with 0.01 M Tris-hydrochloride buffer with ethylenediaminetetraacetate at pH 7.5 (13). Two-milliliter fractions were collected at 4°C. The optical density of fractions was monitored at 280 nm. The column was calibrated using 5 mg each of protein standards (Pharmacia) with known molecular weights. Additional markers included crude *E. coli* H-10407 enterotoxin prepared in this laboratory and highly purified cholera toxin prepared by the technique of Lewis and Richardson (18) and purified by the technique of Finkelstein et al. (12). To estimate the molecular weight of the permeability factors (PFs), the elution volumes of the *Salmonella* fractions having peak biological activity, as judged by rabbit skin tests, were compared to those of the protein standards of known molecular weight.

Skin testing. Adult New Zealand albino rabbits were shaved, and the remaining hair was removed with a depilatory cream prior to skin testing. One-tenth-milliliter injections were given intradermally using 26-gauge intradermal bevel needles. After a designated time interval, a 5% solution of Pontamine sky blue dye in saline was injected intravenously to accentuate the zone of the erythematous response. Dye was given at a dose of 0.8 ml/kg. The critical time period for bluing was assessed by injecting identical samples in a timed sequence on the same animal and giving the dye immediately after the last injection (zero time). The diameter of the blue zones, which corresponds to the areas of erythema, were measured in millimeters, and the results were expressed as the area of bluing in square millimeters. Intensity of induration was graded on a scale of 1+ to 4+, and the zones of delayed bluing were approximately the same as the area of induration.

Heat stability study. A sample known to exhibit both early bluing and delayed induration with bluing was used to test the heat stability of both responses. Sealed 1-ml portions were heated at temperatures of 56, 75, and 100°C for 0, 0.5, 1, 1.5, 2, 4, and 8 h. Additional samples were autoclaved at 121°C for 15, 30, and 60 min. As each tube was withdrawn at its designated time, the sample was chilled in an ice bath and later refrigerated at 4°C. Each sample was assayed for activity by skin testing the following day.

RESULTS

Description of rapid PF activity. It was observed that crude filtrates and partially purified preparations of *S. typhimurium* exhibit a rapid vascular permeability response. This early factor can be demonstrated only by administering the bluing dose within a critical time period after intradermal injections of culture filtrates (Fig. 1). If dye is administered within minutes after skin testing, a "false" positive reaction is seen in which both the filtrates and the media controls cause a bluing response. This is apparently due to an inflammatory response of the host (i.e., needle trauma). When the rabbits were injected with dye 1 h after skin testing, a solid blue spot appeared within 5 min as illustrated in Fig. 2. When dye was administered at this time, sterile, uninoculated BHI

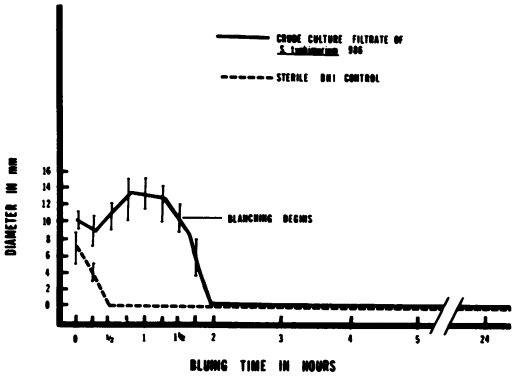

FIG. 1. *Effect of bluing time on the appearance of the rapid PF. Crude culture filtrates of S. typhimurium were injected in a timed sequence, and Pontamine sky blue dye was then given intravenously immediately after the last injection. Sterile, uninoculated BHI was also tested as a control.*

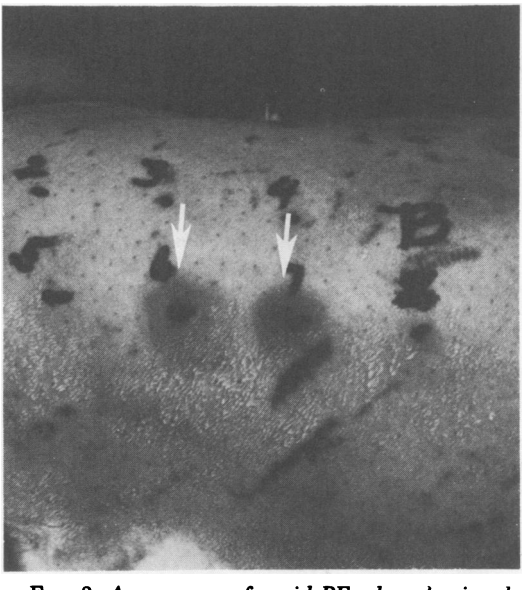

FIG. 2. *Appearance of rapid PF when dye is administered 1 h after skin testing. Arrows indicate areas of rapid bluing.*

broth controls showed no activity. This specific, rapid bluing reaction remained visible for several hours, but appeared to fade in 18 to 24 h. A typical reaction seen using a 1.5-h interval has a blanched center and bluish ringed appearance (Fig. 3). If greater than 2-h intervals are employed, the entire reaction is blanched (Fig. 1). After these observations were made, all further skin tests were performed using 1-h intervals between skin testing and dye injections.

Culture conditions for rapid PF production. Figure 4 depicts the results of a survey of culture media and incubation temperatures conducted to select the optimum growth conditions for promoting rapid PF production. Using skin PF activity of the crude filtrates as the criterion, rapid factor activity was produced in all culture media tested. The amount of rapid PF produced tended to vary with the type of culture media as well as the incubation temperature. Rapid PF was also formed under anaerobic conditions in thioglycolate broth. Rapid PF yield was the highest when *S. typhimurium* 986 was grown in BHI broth at 37°C as judged by the large area of bluing appearing around the injection site.

Survey of salmonellae for rapid PF production. BHI medium was used in a survey of 18 different *Salmonella* strains which were selected to compare production of the rapid bluing factor. Table 1 summarizes this survey. The organisms are arranged in descending order of bluing diameter. The strain of *S. typhimurium* 986 with which we have been doing extensive work elaborates the highest amount of the rapid bluing factor. The results indicate a poor correlation between strain virulence and rapid factor production, but further studies using intestinal models are required before its potential role in salmonellosis is delineated.

Partial purification of the PFs. To achieve partial purification of this rapid bluing factor from culture media and other bacterial products, a sample of concentrated, cell-free filtrate from *S. typhimurium* strain 986 was chromatographed on a Sephadex G-100 column. Figure 5 illustrates the elution pattern of the concentrate. A control concentrate prepared from uninoculated BHI yielded a similar optical density pattern, but the first peak was not present. We found that when alternate fractions were skin tested, the rapid bluing factor activity was associated with the first peak. As previously mentioned, with rapid bluing activity of unchromatographed crude *Salmonella* filtrates no additional reaction (i.e., induration) is observed after the initial response, and the bluing is often not detectable by 18 to 24 h. However, in the case of the G-100 chromatographed fractions surrounding the first peak, a totally different permeability response appeared after 18 h. The response was characterized by a raised area of firm induration accompanied by bluing. The area of bluing coincided with an area of marked induration which was indistinguishable from that of a cholera toxin reaction. This peak of induration activity corresponds to the first peak seen in Fig. 5. A comparison of skin test reactions of crude filtrates before and after chromatography is seen in Fig. 6. These observations have been confirmed repeatedly with

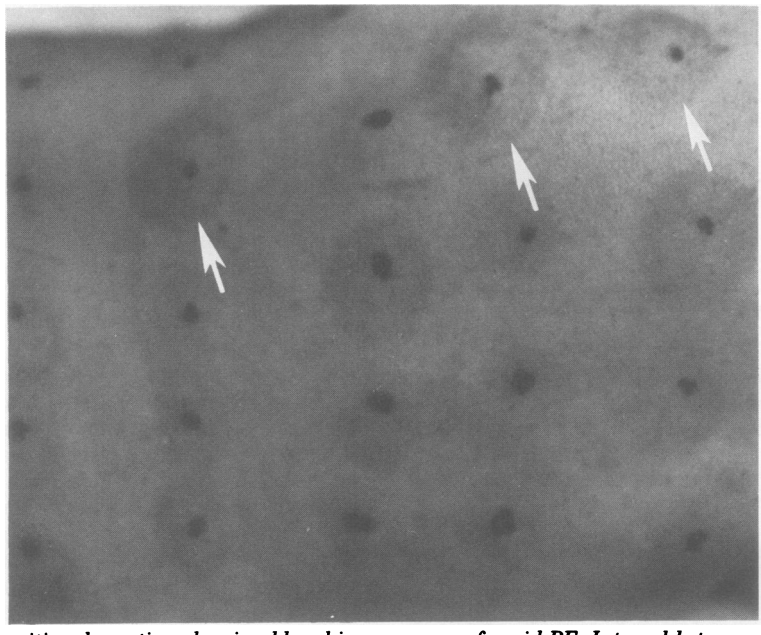

FIG. 3. *Transitional reaction showing blanching response of rapid PF. Interval between skin testing and injection of dye was 1.5 h. Arrows indicate examples of a transitional bluing response.*

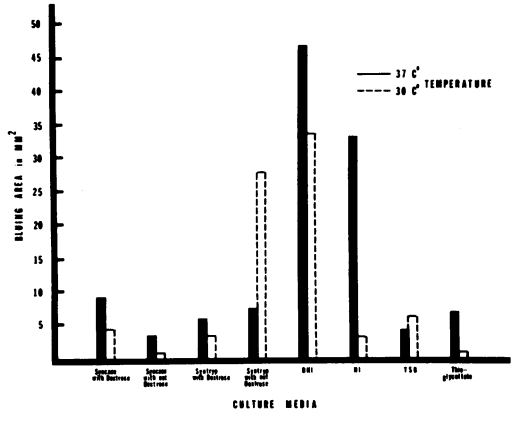

FIG. 4. *Survey for effect of growth media and incubation temperature on production of rapid PF. S. typhimurium 986 was grown at two different temperatures in a variety of media. Cultures were incubated for 24 h, and all were shaken for aeration except for the thioglycolate broth.*

both concentrated *S. typhimurium* filtrates as well as unconcentrated filtrates which had been chromatographed. Thus, it is clear that chromatography on Sephadex G-100 separates out an inhibitory substance (not yet characterized) and reveals the presence of two PFs, one rapid in onset and the other delayed. The delayed bluing factor is accompanied by marked induration, whereas the rapid bluing factor is not.

Heat stability determinations. The heat stability characteristics of the two factors are quite different. The rapid factor is heat stable at 56°C for 8 h (the longest interval tested) and at 75 to 100°C for up to 4 h. It also survives autoclaving for 15 min. In contrast to the rapid bluing factor which is heat stable, the delayed bluing factor is heat labile, as shown in Fig. 7. The delayed bluing and induration factor is destroyed within 30 min at 75 and 100°C. It is also destroyed in 15 min by autoclaving. At 56°C, it gradually loses both bluing and induration activity such that within 4 h, the majority of bluing and induration activity has been destroyed. Figure 8 typifies the heat-stable, rapid bluing factor. This photograph was taken within 2 h after skin testing. After 24 h (Fig. 9), early bluing has faded, and the delayed bluing and induration response has appeared but only in those preparations heated at 56°C for less than 4 h.

Estimations of molecular size. A preliminary estimation of the molecular size of the two factors has been made (Fig. 10). Each elutes close to the void volume on a Sephadex G-100 column. The molecular weight of both factors is at least 90,000 compared to cholera toxin, which behaves as if it were 61,000 on this column, and *E. coli* H-10407 PF which behaves like a molecule of 140,000 molecular weight. Further analysis, using a similar column of Sephadex G-150 calibrated with the same protein standards

TABLE 1. *Survey of different Salmonella species for production of rapid PF*[a]

Species		Rabbits	Bluing area		
			Range (mm^2)	Mean area (mm^2)	Intensity
Salmonella typhimurium	986	6	55–78	73.6	+++
Salmonella typhimurium	986	6	18–113	72.4	+++
Salmonella typhimurium	986	6	33–113	72.0	+++
Salmonella agona	184-75	6	49–133	71.3	+±
Salmonella infantis	282-75	5	33–104	69.4	++±
Salmonella javiana	364-75	6	33–103	66.3	++±
Salmonella heidelberg	374-75	6	33–86	64.3	++
Salmonella saint-paul	217-75	5	27–78	62.2	++
Salmonella typhimurium	TML	6	28–78	58.1	+++
Salmonella typhimurium	W 118-2	6	24–165	54.8	+
Salmonella enteritidis	153-75	5	33–78	54.5	+++
Salmonella newport	343-75	6	28–94	53.6	+±
Salmonella typhi	3530-74	6	33–86	50.8	++
Salmonella typhimurium	LT-7	6	20–113	40.1	+
Salmonella typhimurium	Thax	6	28–79	33.0	++
Salmonella panama	219-75	5	0–63	30.8	+
Salmonella derby	113-75	6	20–78	24.9	++
Salmonella typhimurium	SR 11	6	0–55	26.9	+
Salmonella typhimurium	SL 1027	6	0–49	23.2	++±
Salmonella typhimurium	RIA	6	0–57	14.3	+

[a] All organisms were grown in BHI at 37°C for 24 h in a shaker incubator.

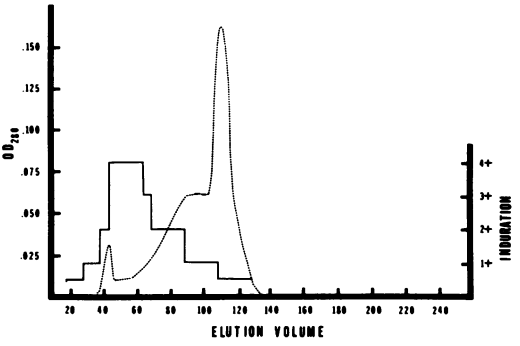

FIG. 5. *Elution pattern of S. typhimurium 986 crude culture filtrates from a Sephadex G-100 column. The dotted curve traces the optical density at 280 nm. The bar graph is representative of the intensity of induration after 24 h when these chromatographed fractions were skin tested.*

(Pharmacia), indicated the molecular size of both factors to be approximately 84,000.

Dose response. A dose response curve for the rapid-acting and the delayed-acting factors is shown in Fig. 11. The preparation tested was a filter-sterilized, BHI fermentor-grown culture filtrate of *S. typhimurium* which had been concentrated by ultrafiltration on a PM30 membrane. This membrane filtration step apparently removed a portion of the inhibitory properties usually encountered in the crude culture filtrates. In this instance, delayed induration and bluing could be detected before chromatography of the concentrated filtrates. Fivefold serial dilutions of the filtrate were made using Tris buffer as diluent. All dilutions were tested

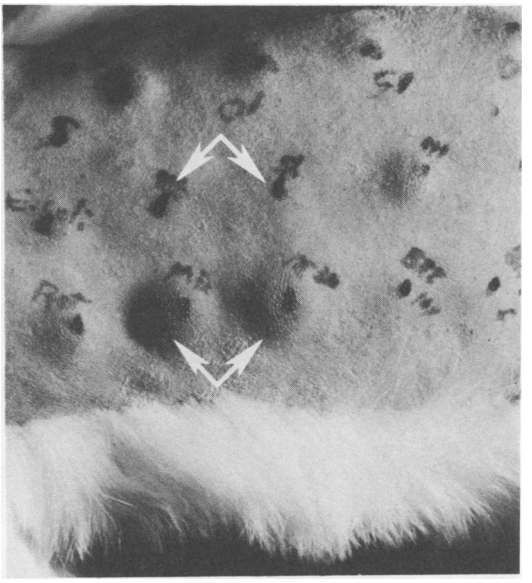

FIG. 6. *Comparison of crude filtrate before and after chromatography. The lower arrows point to the blue areas of induration caused by crude unconcentrated filtrate after chromatography on Sephadex G-100. The upper arrows point to the sites of injection of crude unconcentrated filtrates that were not chromatographed.*

in triplicate on each of four rabbits, and the areas of bluing of the rapid-acting factor were measured 2 h after skin testing (1 h after the dye was administered). This reaction gradually faded during the next several hours. After 24 h, the induration response was optimal and accompanied by a bluing reaction. The zones of the latter bluing response, which generally corresponded to the zones of induration, were then measured. The data presented in Fig. 11 show that two different response curves were derived. The correlation coefficients for the rapid bluing factor and the delayed bluing factor were 0.63 and 0.88, respectively. An analysis of variance for the two lines was done to test the degree of parallelism. The slopes of the rapid and the delayed dose response curves were found to be significantly nonparallel ($P = 0.02$) using the test for equality of the two variances (F). The different slopes of the response curves are highly suggestive of two separate mechanisms for the two bluing reactions.

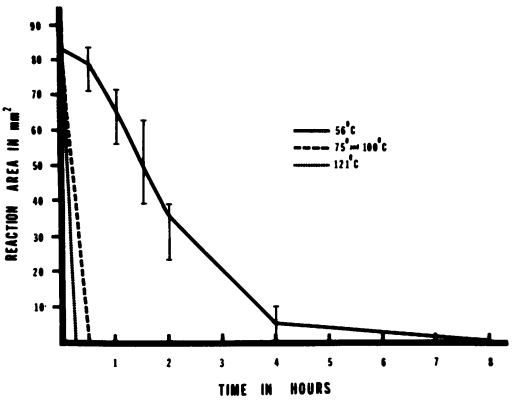

FIG. 7. *Heat inactivation curve for the delayed bluing and induration factor from crude culture filtrates of S. typhimurium 986. Portions of a concentrated pool of active G-100 fractions were subjected to four temperatures for a variety of time periods. Skin tests were used to assay for loss of biological activity, and the area of induration was recorded in square millimeters.*

DISCUSSION

In the present study, two skin PFs were found in culture filtrates of *S. typhimurium*. One factor, designated the "rapid-acting PF," was found to be widely distributed among the *Salmonella* species. The rapid PF was observed optimally only when Pontamine sky blue dye was injected within 1 h after skin testing with crude or chromatographed culture filtrates. Administration of the dye later than 2 h after skin testing resulted in a negative reaction or a

FIG. 8. *Rapid bluing demonstrated within 2 h in heat stability study.*

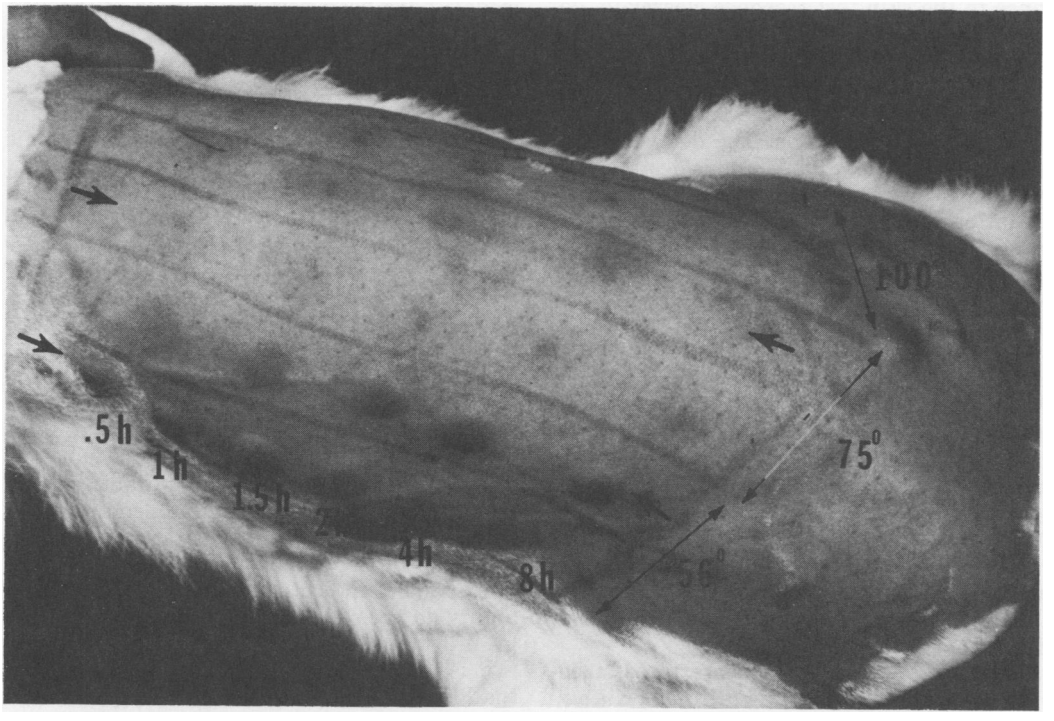

FIG. 9. *Delayed induration and bluing demonstrated within 24 h in heat stability study.*

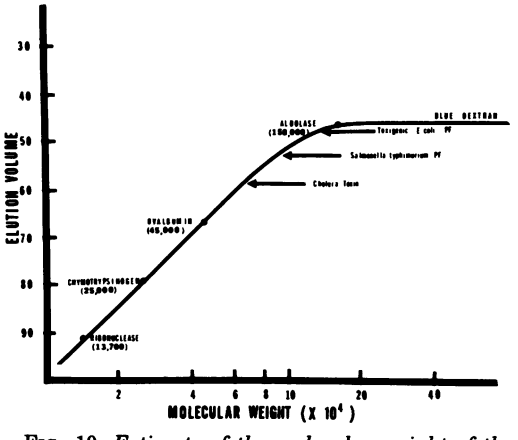

FIG. 10. *Estimate of the molecular weight of the PF from chromatographed crude culture filtrates of S. typhimurium. A Sephadex G-100 column was calibrated using proteins with known molecular weights. Crude culture filtrates of S. typhimurium 986, toxigenic E. coli H-10407, and highly purified cholera toxin were each chromatographed.*

"blanched reaction." No induration was observed with the rapid PF reaction, and the rapid bluing response tended to fade after several hours. The rapid PF activity was found to be heat stable for at least 4 h at 100°C and survived autoclaving for 15 min. The rapid PF has a molecular weight of at least 90,000 as determined by Sephadex G-100 chromatography.

In contrast, the second PF, designated the "delayed PF," is characterized by delayed onset of induration accompanied by bluing. The firm, raised induration has a remarkable resem-

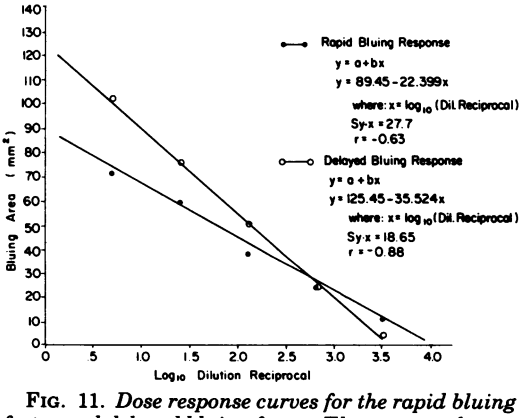

FIG. 11. *Dose response curves for the rapid bluing factor and delayed bluing factor. The test sample was diluted by serial fivefold dilutions, and the reaction zone areas were measured at 2 h (1 h after bluing) and 24 h after skin testing.*

blance to the skin test response classically described for cholera toxin (3, 4) and the heat-labile enterotoxin of *E. coli* (8). When cholera toxin is injected into an adjacent site, it is not possible to distinguish between the appearance of the two toxin reactions. The delayed PF is characterized as heat labile and is destroyed in 30 min at 75 and 100°C. The molecular weight of the latter toxin also appears to be approximately 90,000.

Interestingly, the delayed PF activity is not seen in crude unconcentrated culture filtrates of *Salmonella*. Chromatography on Sephadex G-100 revealed the presence of the induration activity. Similar results were observed using PM30 membrane filtrations. Thus, it appears that some inhibitor-like substance is separated from the induration factor. A search for the "inhibitor" among the column fractions is in progress. When crude *S. typhimurium* culture filtrate material is added back to chromatographed filtrates prior to skin testing, the induration reaction is noticeably diminished. The inhibitory substance is not found in uninoculated media or buffer diluent.

A similar relationship appears to exist between the PF and "blanching factor" of *E. coli*, as described by Finkelstein (10). This investigator described the isolation of these two factors and pointed out that the *E. coli* blanching factor tended to diminish the activity of the *E. coli* PF. In contrast, we have shown that the blanching reaction in *Salmonella* crude culture filtrate is a phenomenon of timing of dye injection. By administering the dye later than 1 h after skin testing, it was possible to demonstrate a blanching reaction. We are not sure of the relationship of this blanching reaction to the *Salmonella* "inhibitor-like" substance responsible for inhibition of the bluing and induration factor demonstrable in G-100 chromatographed *Salmonella* culture filtrates.

The inhibitor substance is interesting in that it does not appear to be firmly bound to the delayed PF, since it can be separated by column chromatography. Further study is required to determine if it is truly an inhibitor or possibly another PF which acts as a vasoconstrictor and counteracts the effects of the delayed PF. It should be pointed out that the presence of the inhibitory substance tends to hinder the study of the delayed PF, since no rapid technique has yet been found to reliably remove it, other than column chromatography.

It is difficult at this time to compare the PFs described here to the enterotoxic factor reported by Koupal and Deibel (16), since no experimental comparisons of the factors in the two animal models have been performed. Both of the PFs eluted close to, but not in, the void volume on a Sephadex G-100 column and have an approximate molecular size of 90,000, whereas the enterotoxic factor was reported to be contained in the void volume. In addition, the enterotoxic factor was demonstrated to be particulate and a constituent of the bacterial cell membrane. In contrast, both PFs appear to have molecular weights of 90,000, suggesting they are not particulate. With regard to heat stability, one of the PFs is heat stable, and the other is heat labile. The enterotoxic factor was reported to be heat labile (80°C for 30 min). Although these comparisons suggest a difference between the reported enterotoxin factors and these PFs, it is premature to reach a definite conclusion.

Giannella et al. (14) have presented substantial data with *Salmonella* mutants which strongly support the concept that penetration of the epithelial surface by the bacteria is a necessary step in the pathogenesis of the infection. Their studies indicate that strains lacking this ability do not cause fluid accumulation in rabbit intestinal loops.

It is tempting to suggest that one or both of the PFs described in this study may play a role in the pathogenesis of *Salmonella* infections, since such a relationship exists between the enterotoxins and PFs of *V. cholerae* and *E. coli*. The presence of such factors can be related to the findings of previous investigations and the pathogenic process may involve both invasion and toxin production. Confirmation of this hypothesis will require further testing of these factors in intestinal models.

There are several possible reasons why these PFs have not been elucidated by other investigators. (i) The concentrations are quite low, and recently we have noticed some variability in production or detection of both factors that may be related to cultural conditions (i.e., media lot number) or seasonal variability in the response of rabbits. (ii) The biological activity of the delayed induration is counteracted by an inhibitor in the culture filtrates. (iii) The rapid factor acts so rapidly that it would be easily overlooked in the standard skin test technique in which blue dye is injected after 18 to 24 h.

We are seeking to understand further the function these PFs may have in the virulence of *Salmonella*. The actual pathogenic role of the substances elaborated by *Salmonella* species is unknown at present, but the biological properties studied thus far in this laboratory suggest a probable role in fluid and electrolyte loss during intestinal infections. Similarly, the factors may also be involved in systemic toxicity of

septicemic-type infections, but further studies are necessary to substantiate the latter speculations.

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Addendum

Sephadex G-100 chromatographed fractions containing both the delayed and rapid PFs have recently been added to Chinese hamster ovary cells. The cells were observed to elongate in a manner identical to those treated with cholera toxin. The inhibitor-like effect of crude culture filtrates on the delayed PF seen in rabbit skin was also a phenomenon observed with Chinese hamster ovary cells (i.e., there was no reaction in Chinese hamster ovary cells unless the crude filtrate was first chromatographed). Moreover, this morphological effect on Chinese hamster ovary cells produced by the *Salmonella* toxins was neutralized by a monospecific rabbit antitoxic serum made against purified choleragen but not by the preimmunization serum. Similarly, the delayed induration effect in rabbit skin produced by the same toxin preparations was also neutralized by the same antitoxic serum but not by the preimmunization serum. Thus, the latest observations provide evidence for an intriguing similarity in mode of action and antigenicity between the *Salmonella* PFs and the toxin of *V. cholerae*.

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