V. SCHUH*
V. HRÍBALOVÁ
ELISHA ATKINS**
(WITH THE TECHNICAL
ASSISTANCE OF
L. FRANCIS AND
M. SIMUNKOVA)

Department of Biochemistry, Institute of Epidemiology and Microbiology, Prague, and Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn. 06510

THE PYROGENIC EFFECT OF SCARLET FEVER TOXIN
IV. PYROGENICITY OF STRAIN C 203 U FILTRATE: COMPARISON WITH SOME BASIC CHARACTERISTICS OF THE KNOWN TYPES OF SCARLET FEVER TOXIN†

The pyrogenic activity of scarlet fever toxin has been described a number of times. The "classic" toxin, prepared from the Dochez NY 5 strain, was found to have a double specificity, designed by Hooker and Follensby as "A" and "B".[1] More recently, Watson has analyzed the antigenic components of this toxin and found a third type, which he has denoted "C".[2]

In working with a mutant strain of streptococcus, C 203 U, which produces streptolysin O but not S, the present authors made the observation that broth culture filtrates of this strain were pyrogenic. An attempt has been made to determine the relationship of this pyrogen to other streptococcal pyrogens described previously.

MATERIAL AND METHODS

Streptococcus strains: Strains Dochez NY 5 and C 203 U were obtained from the collections of the Institute of Epidemiology and Microbiology in Prague. Strains T-28, T-18, and T-19 were kindly supplied by Dr. Watson, University of Minnesota, and strain 32369 by Dr. Julia Coffey, New York State Department of Health. All these strains were obtained in the freeze-dried state and were passaged two or three times in broth before being used.

Medium: Heart Infusion Broth (Difco) was used in part of the work; however, the medium employed predominantly was one of identical composition but prepared from beef heart infusion. This contained infusion from 500 g. beef heart (1 litre), 10 g. Tryptose (Difco), and 5 g. sodium chloride. To eliminate extraneous pyrogens it was autoclaved (121°C.) for two hours and determined to be nonpyrogenic before use.

The medium was inoculated with the individual strain and incubated for 24 hours. Supernatant fluid from culture was obtained by ultracentrifugation (Sharples) and

---

* Chief, Biochemical Dept., Inst. of Epidemiology and Microbiology, Prague.

** Professor of Medicine, Yale University.

† The work was carried out in part at Dr. Atkins' laboratory, Department of Internal Medicine, Yale University School of Medicine, during the tenure of a WHO Exchange of Research Workers Grant (three months, 1967) by one of the authors (V.S.).

Received for publication 22 May 1970.

filtration. It was not further concentrated or purified in order to prevent any component of pyrogenicity from disappearing in the purification process.

As a control in some of the experiments, erythrogenic (scarlet fever) toxin batch ET 75 or ET 76 prepared from the Dochez NY 5 strain was used; the preparation and purification of this toxin has been described earlier.[8]

Enzymes: Three enzymes were used: trypsin 1:250 (Difco), pepsin (Merck), and papain (Merck).

Rabbits: For the most part, chinchilla rabbits (weighing about 2 kg. each) from commercial breeds were employed, but some of the experiments were performed with albino rabbits (weighing about 4 kg.). Injection volumes are expressed in ml/kg. (chinchilla rabbits) or in ml/animal (albino rabbits). Temperature measurements were taken by thermoelectric probes* or with an ordinary mercury thermometer, usually in the course of five hours.

Experimental procedure: Antigenic differences in the pyrogenic substance of the individual scarlet fever toxins were determined by the method of Watson. Eight or nine daily intravenous injections of pyrogen elicit a state of so-called pyrogenic tolerance: the animal has little or no febrile reaction to a further dose of the same pyrogen, by which is meant that there is either no rise of temperature after the second hour, or the elevation is within the limit of physiological variation. Watson has shown that tolerance to the pyrogenic activity of scarlet fever toxin is immunologically specific.[2] Since tolerance of this type is dependent on the amount of

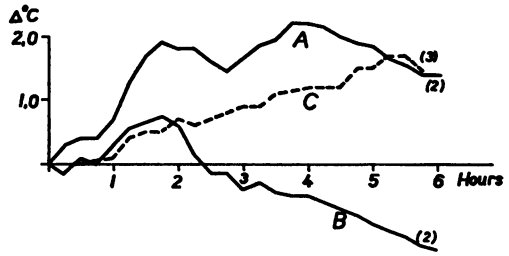

FIG. 1. Pyrogenicity of C 203 U filtrate. Abscissa: hours; ordinate: fever in °C. A: C 203 U filtrate, 1 ml/rabbit; B: C 203 U filtrate, 1 ml/rabbit—these rabbits died on the day following injection; C: C 203 U filtrate, 0.3 ml/rabbit.

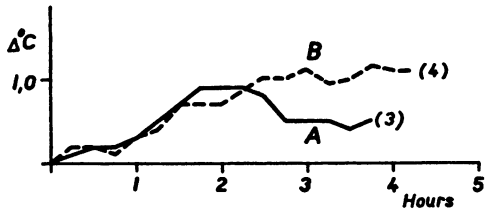

FIG. 2. Thermostability of pyrogenic effect of C 203 U filtrate. Abscissa: hours; ordinate: fever in °C. A: filtrate heated 30 min. at 65°C.; B: filtrate heated 30 min. at 96°C.; all injections i.v., 1 ml/rabbit.

* Foxboro rabbit scanning fever switch and fever recorder. Manufactured by Foxboro Co., Foxboro, Mass.

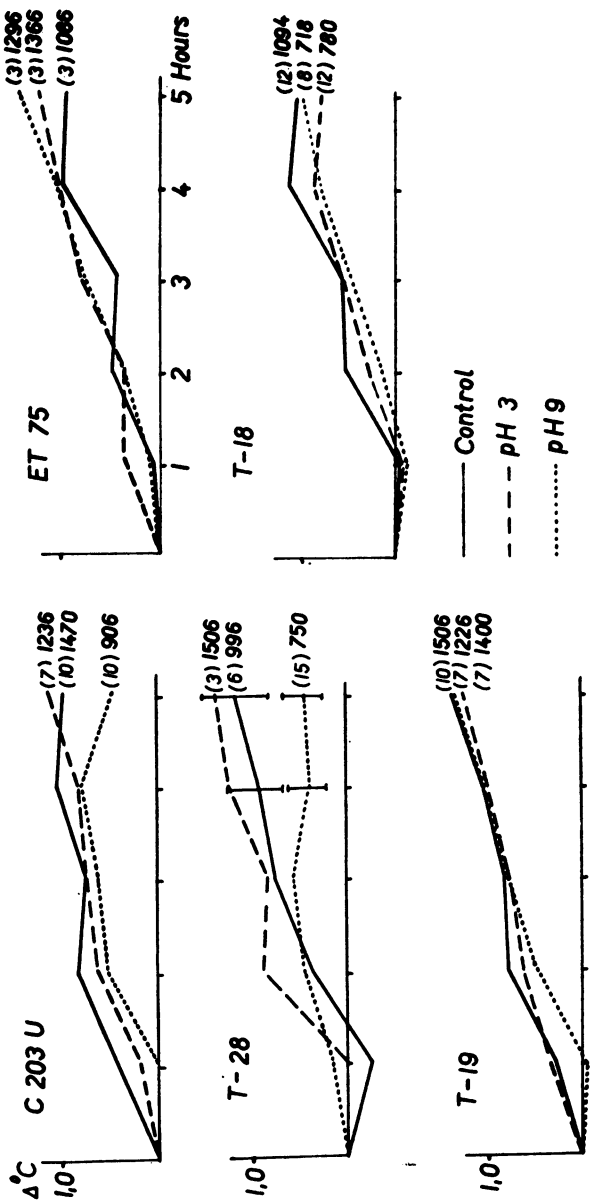

FIG. 3. The pH resistance of pyrogenic factors in filtrates of C 203 U, T-18, T-19, T-28 and ET 75 streptococcal cultures. A: ——— = control; B: - - - - - = pH 3.0; C: ..... = pH 9.0. Dosage: C 203 U—0.05 ml/kg.; T-18—0.05 ml/kg.; T-19—0.2 ml/kg.; T-28—0.1 ml/kg.; ET 75—15000 STD/kg. Figures in brackets denote numbers of rabbits in experiment.

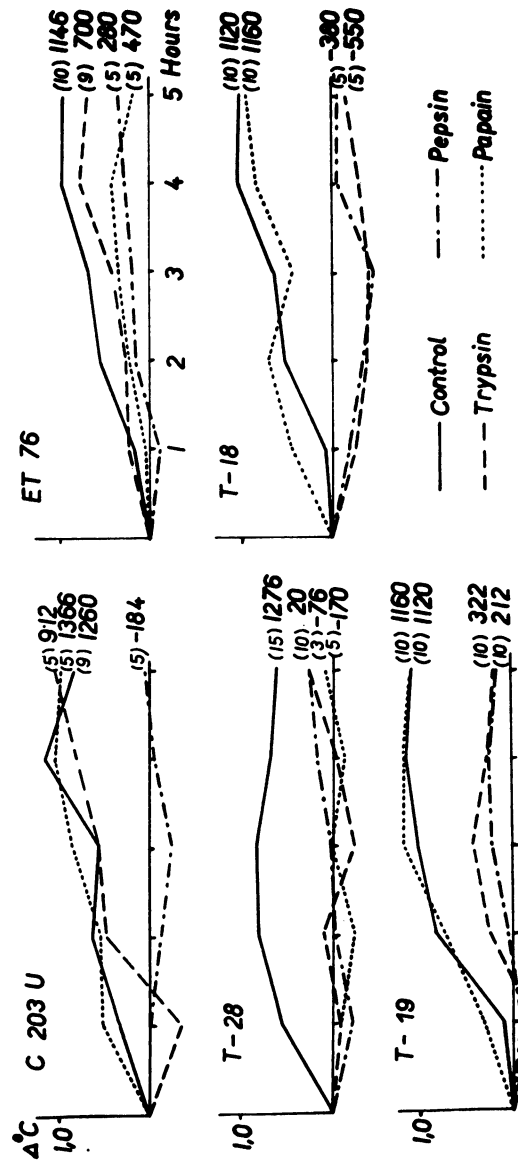

FIG. 4. Digestion of different streptococcal pyrogens by trypsin, pepsin or papain. (The filtrates had approximately 40 mg. dry matter per ml.; enzymes were added in a concentration of 1:100, in relation to weight. Their apyrogenicity, within obvious limits, was verified. Injection doses: C 203—0.05 ml/kg, T-18—0.1 ml/kg, T-28—0.05 ml/kg, T-19—0.2 ml/kg, ET 76—15000 STD/kg. Figures in brackets denote numbers of rabbits in experiment.)

pyrogen given, it is essential that all pyrogens to be tested be given in approximately the same fever-inducing dosages.

The curves in the Figures have been drawn on a scale of 1° C. and 1 hour equaling 20 mm. The area under the curve expressed in mm^2 is the "fever index" (Figs. 1-5).

Resistance to pH change: This was tested at two levels, pH 3 and pH 9. Five milliliters of filtrate were placed in cellophane tubing and dialyzed overnight against a corresponding buffer (about 130 ml.) at 4°C. The pH was then checked and adjusted back to pH 7.0.

Digestion by proteolytic enzymes: Enzyme was added in a ratio of 1:100 (ca. 40 mg/ml. crude filtrate, 4.5 mg/ml. ET 76, in relation to dry substance). Digestion was carried out as follows: by trypsin at pH 8.5, pepsin at pH 3.0, and papain (activated by cysteine) at pH 6.5 at 37°C. overnight. The pH was then readjusted to approximately 7.0. If a precipitate had formed and did not dissolve in adjusting the pH, it was removed by centrifugation and only the clear supernatant fluid was used for the injections. The precipitate was checked for its inability to adsorb pyrogenic activity by resuspending it in a small amount of saline, boiling for a few seconds, centrifuging it and testing the supernate.

RESULTS

Pyrogenicity of C 203 U filtrate. Doses of 1 ml/rabbit or 0.3 ml/rabbit were injected intravenously. Temperature measurements were taken by thermoelectric probes. The results, plotted in Fig. 1, show that the filtrate was a strong pyrogen. Fever set in after a latent period of almost 1 hour and had a protracted biphasic course. A dose of 1 ml. was close to the lethal limit, for two of the five animals receiving it died on the second day.

Nondialyzability of C 203 U pyrogenic factor. Fifteen ml. of filtrate were dialyzed (Visking, cellophane) against 15 ml. of saline in a refrigerator overnight. The dialyzate (saline) was injected into four rabbits. With one exception, the rabbits were completely afebrile. Hence the pyrogenic factor did not dialyze.

Thermoresistance of C 203 U pyrogenic factor. Aliquots of filtrate were heated at either 65° or 96°C. in a water bath for 30 minutes. The precipitate was removed by centrifugation and the clear supernatant fluid injected in amounts of 1 ml/rabbit. Figure 2 shows that the material retained most of its pyrogenicity even after 30 minutes' exposure to temperatures close to the boiling point. Although the rise in temperature was less than in Figure 1, it still represents a considerable fever. The paradox of a lower thermoresistance at 65°C. will be discussed below.

This result strongly suggests that the pyrogenic factor belongs to the "family" of scarlet fever toxins, whose thermoresistance is well known (at least in the case of the toxin-producing strain Dochez NY 5).[4,5]

Resistance of pyrogenic factors to pH change. Culture filtrates of strains T-18, T-19, T-28, and C 203 U were tested. Partly purified scarlet fever toxin ET 75 (7.5×10^6 STD/ml.) prepared from strain Dochez NY 5

served as a control. (See METHODS.) The various doses used are given in the legend to Figure 3, which presents the results.

With all the filtrates, the rise in temperature characteristically began after a latency of about one hour, and followed a biphasic course with the second peak occurring relatively late (4-5th hour). Resistance to pH change was complete with all the filtrates as well as with the control ET 75 toxin, except for filtrate T-28 which showed some loss of activity at pH 9; difference in temperature from the controls at the fourth and fifth hour was significant (F-test, 5% level).

Digestion by proteolytic enzymes. Clear filtrate was injected in doses indicated in the legend to Figure 4. The temperature rises amounted to approximately 1°C., the peak again occurring in the fourth hour. An exception was T-28, which was inadvertently given in a slightly smaller dose. The results are presented in Figure 4. The C 203 filtrate was inactivated by pepsin only; it was markedly trypsin- and papain-stable. The T-18 and T-19 filtrates were very similar: they were both papain-resistant but were digested by pepsin. After treatment with trypsin, T-19 retained a remnant of its activity, whereas T-18 was digested completely. T-28 was the most labile toxin of the group. It was inactivated completely by all the enzymes. The control ET 76 (Dochez NY 5)toxin was only equivocally affected by trypsin; pepsin and papain had a considerably greater effect.

These results indicate, first, that all these pyrogens are probably proteins. Their varying susceptibility to the proteolytic enzymes used is evidence of different structure, probably accounted for by differences in the primary structure of the proteins. It was surprising that trypsin only slightly affected the ET 76 toxin, whereas T-19 and T-28 were almost wholly inactivated by this enzyme. By its antigenic structure, the Dochez NY 5 strain is bivalent AB with a high predominance of A^e; strains T-19 and T-28 are monovalent B and A, respectively. This difference in susceptibility to trypsin suggests that the two A pyrogens (T-28, Dochez NY 5) are not completely identical, despite other evidence for their similarity.

Cross-tolerance tests. The possibility that the C 203 U pyrogen was related to scarlet fever toxin was investigated in experiments with rabbits that were made tolerant to the respective pyrogenic substances of T-28, T-18, T-19, or C-203 U filtrates by daily injections. After developing tolerance, the rabbits were injected with a filtrate from a different strain. Since pyrogenic cross-tolerance does not occur when filtrates of unrelated microorganisms are compared,[7] this form of cross-tolerance was considered to be an argument for the identity of the agents tested in this way. The cross-tolerance experiments presented here involved all combinations of

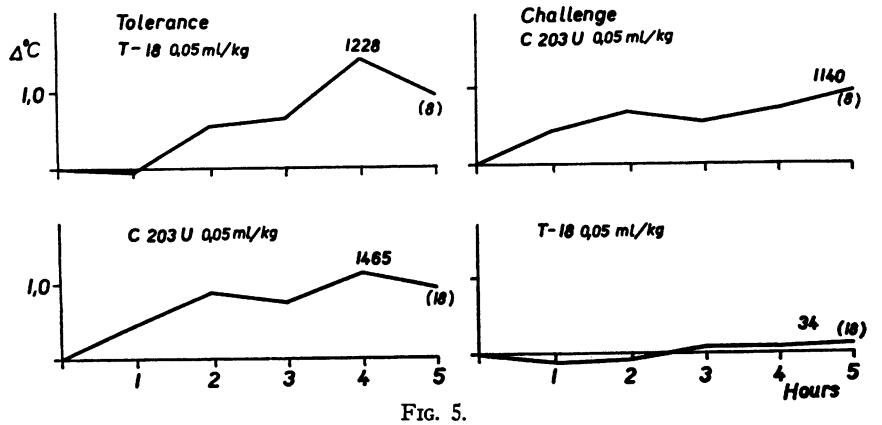

FIG. 5.

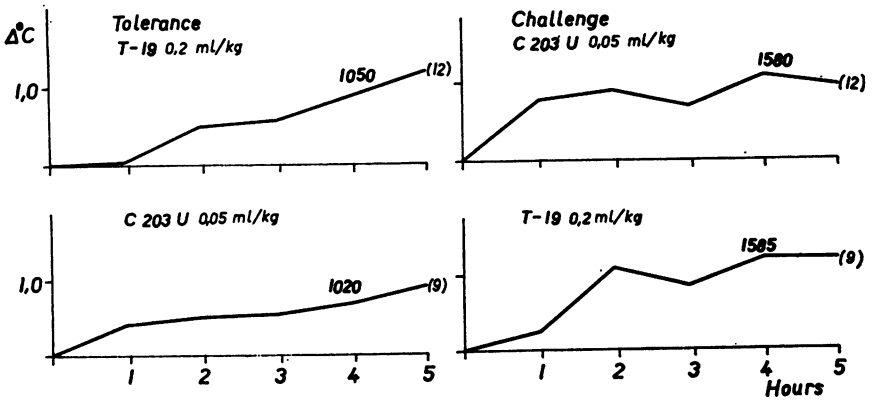

FIG. 6.

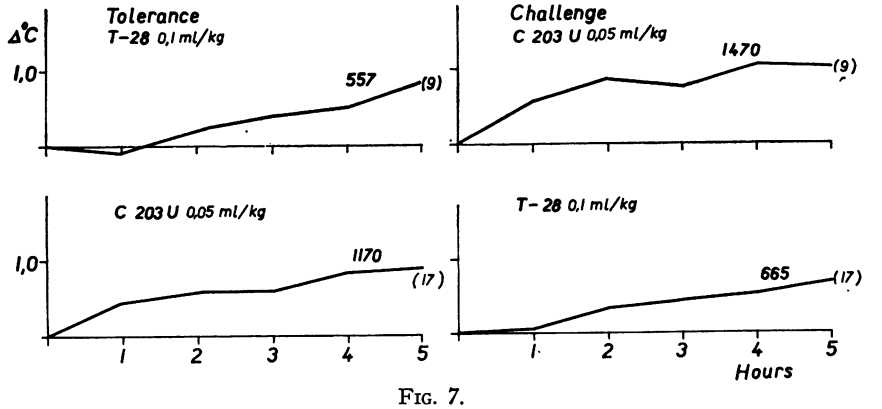

FIG. 7.

FIGS. 5-7. Cross-tolerance experiments. (Each figure gives one pair of pyrogens. Left half: temperature values after first dose of tolerance-inducing pyrogen. Temperature curves of tolerant animals are not given, but the rise never exceeded 0.4°C. Right half: temperature curves after challenge administered on the day after tolerance had been attained. Figures in brackets denote numbers of animals in experiment.)

C 203 U with monovalent filtrates T-18, T-19, and T-28. In addition, it was considered expedient to verify the identity of strains T-19 and 32369 by cross-tolerance tests. The T-19 strain produces a pyrogen that has been designated by Watson as B on the assumption that it was scarlet fever toxin.[2] The 32369 strain also is said to produce scarlet fever toxin B.[8] However, a direct comparison of both strains had not previously been performed. The results are plotted in Figures 5, 6, 7 and 8, where the technical details are also given. From the evidence in these cross-tolerance tests, the two strains, T-19 and 32369, appear to produce an identical pyrogen (see Fig. 8).

The only other instance of cross-tolerance was observed with C 203 U (induction of tolerance) and T-18 (challenge); the same system in the reverse order, however, did not produce cross-tolerance (Fig. 5).

Watson's strains have their respective pyrogenic (erythrogenic) activities denoted as follows: T-28 as A, T-19 as B, and T-18 as C. Our findings indicate that the C 203 U strain produces the pyrogenic component C and another pyrogen that appears to be distinct from the previously known types and has, therefore, been denoted D. A corresponding monovalent strain producing pyrogen D only has not been described. On the basis of these data, the pyrogenic structure of strain C 203 U has been designated as CD.

DISCUSSION

In this study an attempt has been made to determine the character of the C 203 U strain pyrogen. In doing so it was necessary to establish some of the basic characteristics of scarlet fever toxins other than the "classic" Dochez NY 5 toxin.

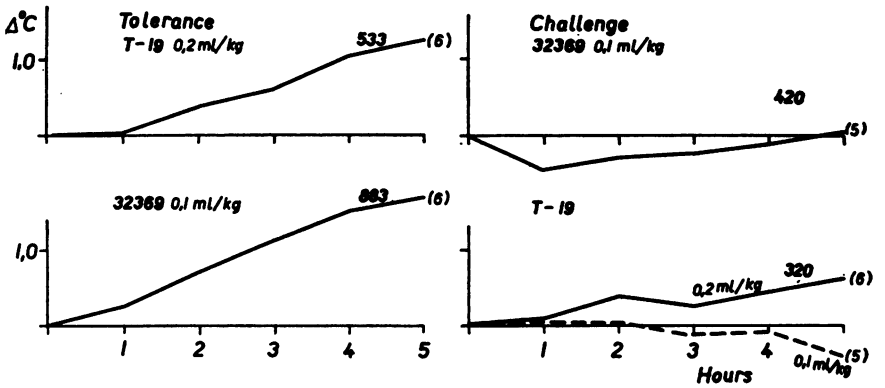

FIG. 8. Cross-tolerance between T-19 and 32369 pyrogens. (Arrangement as in Figures 5-7.)

With the use of the skin test as an indicator system for *in vitro* mixtures of toxin-antitoxin, it has been suspected for some time that scarlet fever toxins are serologically heterogeneous.[6,8,9] Coffey, whose work is the most extensive on this subject, suggests the existence of both single types and combinations.[8] The Dochez NY 5 toxin appears to have at least two specificities. This point was investigated by Hooker and Follensby, who designated the predominant component of the toxic filtrate of this strain as A and a second, minor component as B.[6] Like Coffey, they also found strains that produced toxin B only. The exact number of antigenic types of scarlet fever toxin has not yet been conclusively ascertained. Wheeler has published a table from which at least five antigenic variants (single or combinations) can be inferred, with strain Dochez NY 5 figuring as trivalent.[9] Coffey, working in the same institute, analyzed 597 strains six years later, with results also suggesting the existence of five variants and a bivalent status for the Dochez NY 5 strain.[8] In both cases, these are deductions from the data, since the authors themselves do not specify the number of antigenic variants.

These authors based their antigenic studies solely on the results of skin tests, since dermal activity was the only biological test for scarlet fever toxin known at that time. More recently, Watson has attempted to differentiate scarlet fever toxins on the basis of cross-tolerance to their pyrogenic action, which, like the results of skin tests, appear to indicate that these agents are immunologically specific.[2] He also found the Dochez NY 5 strain to be bivalent. He found a strain (T-28) that produced toxin A only, and another (T-19), producing only toxin B, and a third (T-18) producing toxin C. The respective letters need not, of course, signify single antigens but may represent groups of antigens, none of which is present in any of the other designations. Strain T-19 was identified as a toxin producer by Watson only in relation to the known bivalent Dochez NY 5 toxin.

Utilizing the technique of cross-tolerance, we have confirmed here that Watson's T-19 strain and the 32369 strain, a producer of toxin B,[8,10] produce an identical pyrogen.

Strain C 203 U appears to be a further strain (in addition to Dochez NY 5) which is at least bivalent; it possesses the pyrogenic component C (identical with strain T-18), but also a further one, distinct from Watson's A and B. Hence, it has here been designated as D, the symbol being assigned the same meaning as has been reserved for the letters A to C.

Since scarlet fever toxin heterogeneity has been demonstrated by two different methods—the skin test and pyrogenicity—and a correlation between these two activities has been shown in strain Dochez NY 5 only,

one may ask what evidence permits us to group all these substances as scarlet fever toxins. Apart from clinical experience, the assumption is based on the observations that these "toxins" are produced by streptococci isolated from scarlet fever cases, that they elicit an erythema after intradermal injection, and are neutralized by their respective antisera. Actually, only the Dochez NY toxin, or more precisely, toxin A, has been proved to be a scarlet fever toxin. It has been found to fulfil the so-called Koch criteria and has been purified[11,12] and its pyrogenicity has been demonstrated.[2] With toxin B, which has not been satisfactorily purified, our certainty is somewhat weaker, while the information relating to toxin C and the pyrogenic compenent D is very scanty.

The pyrogenic activity of streptococcus C 203 U filtrate has a potent pyrogenic activity. Intravenous doses of 1 ml/rabbit were lethal to two of the five rabbits: a dose of 0.3 ml. elicited a rise in temperature of more than 1.5°C. Despite this, the latency period was relatively long, longer than with endotoxin (see Fig. 1).

The relatively long latency before onset of fever seen with the pyrogens studied here deserves some attention. A similar phenomenon has been previously noted after administration of T-18 strain toxin by Watson and Kim.[13] The present authors encountered this finding with all exogenous streptococcal pyrogens, including partly purified scarlet fever toxins and crude filtrates. Although this finding might also suggest that the substances are related and differ from the endotoxins of gram-negative bacteria,[14] culture filtrates of other microorganisms (staphylococci and tubercle bacilli as well as certain pathogenic fungi) produce biphasic fevers with a similarly delayed onset in either naturally or specifically sensitized rabbits.[15-17] Further experiments, utilizing the pyrogenic cross-tolerance techniques employed here should give useful information as to possible antigens shared in common between these diverse groups of microorganisms. Earlier work has indicated that the pyrogens in culture filtrates of tubercle bacilli (old tuberculin) and certain strains of coagulase positive staphylococci are distinct.[7]

The pyrogenicity of C 203 U filtrate is highly thermoresistant. It is still well retained after heating at nearly the boiling point (96°C.) for 30 minutes. Such thermoresistance has been observed in a partially purified scarlet fever toxin with proved pyrogenicity.[4,5] The paradoxical result obtained in the filtrate heated to only 65°C. is explicable by some previous findings. In earlier work with crude scarlet fever toxin (Dochez NY 5), the thermoresistance of the toxin was tested by skin tests. On heating the toxic filtrate to 65°C., a flaky precipitate appeared and it was found that the dermal activity of the supernatant fluid decreased. The flakes

resuspended in saline and boiled for a short interval liberated a toxin that had skin activity and that apparently had only been absorbed on the flakes (Schuh and Splitkova, unpublished results). A similar phenomenon has been described in older reports concerning the thermoresistance of staphylococcal alphatoxin.[18]

Resistance to extreme pH values is a known characteristic of scarlet fever toxin. This applies to the Dochez NY 5 toxin, for which pH resistance in the range of pH 1.08 to > 11.0 has been reported.[19] It follows from the present results that the other streptococcal pyrogens (toxins) behave similarly. Only in the case of the T-28 strain was the pyrogen partly inactivated in the alkaline pH range.

The susceptibility of scarlet fever toxin to proteolytic enzymes has been studied in toxin Dochez NY 5 (components AB) and toxin B.[6,10,19–21] The method employed was not always the same and in some cases it has not been adequately described. In most cases the criterion was skin activity; Hottle and Pappenheimer determined toxin activity by flocculation.[21] They found erythrogenic toxin (Dochez NY 5) to be resistant to trypsin, pepsin, and papain. In the present work, where the criterion of toxin activity was pyrogenicity, digestion with trypsin left pyrogen Dochez NY 5 activity almost completely unimpaired. In view of the results of other authors, the question arises whether skin activity of this toxin would likewise be preserved under the present experimental conditions. Pepsin and papain inhibited pyrogenic activity to a far greater degree. The C 203 U pyrogen also proved to be trypsin-resistant. Both these strains, i.e., Dochez NY 5 and C 203 U, possess antigenically mixed pyrogens, viz., AB and CD, respectively. It is of interest that "univalent" pyrogens A, B, and C were easily digested by trypsin. Whether trypsin-resistance is significantly related to bivalence or is merely coincidental cannot be stated at present. Toxin B has been reported by others to be easily digestible by trypsin.[6,10]

The pyrogens presented here have been classed together on the basis of certain properties (thermoresistance, pH resistance, character of pyrogenic response) that no other streptococcal product possesses. Further evidence in addition to their pyrogenicity will be necessary, however, before these substances can be unequivocally classified as scarlet fever toxins.

SUMMARY

Various biological properties of the pyrogens belonging to the so-called "scarlet fever" group of streptococcal toxins were investigated. By their thermoresistance, pH resistance and the febrile response they induce, these agents appear to resemble each other and to be separable from other strep-

tococcal products. On the basis of fever studies, there appear to be at least four separate pyrogenic agents, each representing a different antigenic component. A new pyrogen, tentatively designated as "D," has been found in culture filtrates of a mutant strain of streptococcus (C203U). Its relationship to three previously described streptococcal pyrogens has been defined.

REFERENCES

1. Hooker, S. B. and Follensby, E. M.: Some toxins of scarlatinal streptococci. *J. Bact.*, 1932, *23*, 85-86. (Abstract, 33rd Annual Meeting of Society of American Bacteriologists.)
2. Watson, D. W.: Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. *J. exp. Med.*, 1960, *111*, 255-284.
3. Schuh, V. and Hríbalová, V.: The pyrogenic effect of scarlet fever toxin. II. Leukocytic pyrogen formation induced by scarlet fever toxin or salmonella paratyphi B endotoxin. *Fol. microbiol.*, 1966, *11*, 112-122.
4. Dick, G. F. and Dick, G. H.: Scarlet fever toxin in preventive immunization. *J. Amer. med. Assoc.*,, 1924, *82*, 544-545.
5. Ando, K.: On heatstability of scarlet fever and diphtheria toxins; preliminary report. *J. Immunol.*, 1930, *19*, 223-225.
6. Hooker, S. B. and Follensby, E. M.: Studies of scarlet fever; different toxins produced by hemolytic streptococci of scarlatinal origin. *J. Immunol.*, 1934, *27*, 177-193.
7. Bodel, P. T. and Atkins, E.: Studies in staphylococcal fever: IV. Hypersensitivity to culture filtrates. *Yale J. Biol. Med.*, 1964, *37*, 130-144.
8. Coffey, J. M.: Further observations on toxigenic properties of hemolytic streptococci. *J. Immunol.*, 1938, *35*, 121-130.
9. Wheeler, M. W.: Notes on antigenic activity of hemolytic streptococci from different types of infection. *J. Immunol.*, 1932, *23*, 311-314.
10. Stock, A. H. and Lynn, R. J.: Preparation and properties of partially purified erythrogenic toxin B of group A streptococci. *J. Immunol.*, 1961, *86*, 561-566.
11. Dick, G. F. and Dick, G. H.: Etiology of scarlet fever. *J. Amer. med. Assoc.*, 1924, *82*, 301-302.
12. Stock, A. H.: Studies on hemolytic streptococcus; further purification and concentration of scarlet fever toxin. *J. Biol. Chem.*, 1942, *142*, 777-783.
13. Watson, D. W. and Kim, Y. B.: Modification of host responses to bacterial endotoxins. I. Specificity of pyrogenic tolerance and the role of hypersensitivity in pyrogenicity, lethality, and skin reactivity. *J. exp. Med.*, 1963, *118*, 425-446.
14. Atkins, E.: Pathogenesis of fever. *Physiol. Rev.*, 1960, *40*, 580-646.
15. Atkins, E.: Studies in staphylococcal fever. II. Responses to culture filtrates. *Yale J. Biol. Med.*, 1963, *35*, 472-488.
16. Hall, C. H., Jr. and Atkins, E.: Studies on tuberculin fever. I. The mechanism of fever in tuberculin hypersensitivity. *J. exp. Med.*, 1959, *109*, 339-359.
17. Braude, A. I., McConnell, J., and Douglas, H.: Fever from pathogenic fungi. *J. clin. Invest.*, 1960, *39*, 1266-1276.
18. Landsteiner, K. and Rauchenbichler, R. von: Über das Verhalten des Staphylolysins beim Erwärmen. *Zschr. Immunf.*, 1909, *1*, 439.
19. Barron, E. S. G., Dick, G. F., and Lyman, C. M.: Chemical nature of scarlet fever toxin. *J. Biol. Chem.*, 1941, *137*, 267-282.
20. Kodama, T.: Studies on toxic fractions of hemolytic streptococci; preparation of hemolytic streptococcal toxin of high value and separation of different toxic fractions. *Kitasato Arch. exp. Med.*, 1936, *13*, 101-117.
21. Hottle, G. A. and Pappenheimer, A. M., Jr.: Quantitative study of scarlet fever toxin-antitoxin flocculation reaction. *J. exp. Med.*, 1941, *74*, 545-556.