Studies on Bacterial Pyrogenicity. I. Quantitative Basis.*† (24350)

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The bacterial origin of heat-stable pyrogenic substances that may contaminate biological solutions was firmly established by Seibert(1). It has since been demonstrated that many species of both Gram negative and Gram positive bacteria elaborate pyrexial substances(2) and that the most potent of these pyrogens are associated with the Gram negative species(3,4). Much effort designed to isolate and chemically characterize the pyrexic principal(s) has been described(2,5). However, little quantitative information exists describing the pyrogenic capacity of intact organisms added to solutions(3,6). This report is concerned with the effect of injection of autoclave-sterilized suspensions of varying numbers of certain species of bacteria on body temperature of rabbits.

Materials and methods. Adult albino male and female rabbits obtained from a local source were used for pyrogen tests. Rabbits weighed between 3 and 4 kg; the animals were caged and tested in a room by themselves. All organisms were tested in a previously unused group of rabbits. This was done to avoid the problem of acquired tolerance to intravenously introduced bacteria. Such tolerance may be manifested by decreased response to the pyrogenic substances(5). Rabbits were accommodated to the laboratory situation by initial control tests. Animals which could not be easily accommodated were discarded. In each experiment was included one set of rabbits injected with known nonpyrogenic saline. This group served as a control for technic and other external factors, including noise, temperature and humidity(6). *Pyrogen tests* were carried out according to technics described in Pharmacopeia of U. S., 15th Revision, (U.S.P. XV) with the following exceptions: animals were caged either individually or in pairs; syringes, needles and glassware were boiled in detergent solution, then rinsed with freshly distilled water and sterilized in oven at 170°C; solutions to be tested were at room temperature rather than 37°C; temperature readings were taken prior to injection and at 15 minutes, 1 and 2 hours after injection. Results were interpreted as per U.S.P. XV, *i.e.*, the test was called positive if each animal showed a rise of 0.6°C or more above its control temperature or if the sum of maximum increase for the 3 animals equalled or exceeded 2.1°C. A portable, battery powered direct reading thermistor probe was employed for determining rectal temperatures. The accuracy of the instrument was routinely checked before use. Organisms employed were obtained from freshly isolated cultures or from available stock cultures. The bacteria were grown on agar slants, and incubated at 37°C for up to 18 hours. Growth was harvested from a number of slants by gently emulsifying with saline. The saline suspensions were filtered through sterile glass wool and pooled. Such procedure was employed in an effort to minimize addition of autolyzed material that might be present in broth. Furthermore, organisms prepared in this fashion formed a homogeneous suspension which could be readily counted. Counts were made on quantitatively diluted aliquots in a Petroff-Hauser chamber; at least 625 organisms were counted. Employing the calculated population estimate, suspensions of organisms designed to contain definite numbers per ml were prepared in 0.9% NaCl, 5% dextrose and U.S.P. Ringer's solution. The solutions containing the living suspended bacteria were again divided. Portions were filtered through membrane filters (Millipore, type HA, grid) and these filters were then cultured on human blood agar plates incubated at 37°C to yield estimates of the relation between the microscopic and viable organism counts. Other portions of the suspensions were sterilized by

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autoclaving at 120°C for 15 minutes. The sterilized solutions were then tested for pyrogenic response in rabbits, *i.e.*, 3 rabbits were injected with 10 ml/kg of the material under test. Organisms tested to date are: *Escherichia coli* (4 strains), *Paracolobactrum* species, *Aerobacter aerogenes, Klebsiella pneumoniae, Achromobacter* species, *Proteus vulgaris, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus globigii, Staphylococcus epidermidis, Alcaligenes fecalis, Brucella abortus, Flavobacterium arborescens, F. suaveolens, F. aquatile, Hemophilus influenzae, Serratia marcescens, Streptococcus viridans, Pasteurella multocida, Corynebacterium pseudodiphtheriticum, Vibrio metschnikovii, V. comma, Salmonella typhimurium.*

Results. Table I is from a typical experiment with a freshly isolated strain of *E. coli* suspended in saline and tested for pyrogenicity by the methods described. None of these solutions can be adjudged pyrogenic according to the U.S.P. interpretation, although the solution containing 1000 organisms per ml (or 10^6 per liter) tends in this direction. By the more sensitive method of interpretation of U.S.P. XIV, however, this solution would be labeled presumptively "pyrogenic."

Table II shows pyrogen test results obtained with another strain of *E. coli* suspended in 5% dextrose. In this case, it is seen that

TABLE I. Effect of Injecting Rabbits Intravenously, 10 ml/kg, with *E. coli** in 0.9% NaCl. Solution autoclaved prior to injection.

Rabbit No.	No. *E. coli*/ml	Temp., °C: Before inj.	After inj.: 15 min.	1 hr	2 hr	Δ†
33A	10	39.2	39.2	39.0	39.3	+ .1
33B		39.6	39.8	39.4	39.4	+ .2
34A		39.4	39.4	39.4	39.5	+ .1
34B	10^2	39.6	39.7	39.7	39.5	+ .1
35A		39.4	39.4	39.6	39.7	+ .3
35B		39.3	39.3	39.4	39.5	+ .2
36A	10^3	39.4	39.4	40.4	40.4	+1.0
36B		39.4	39.4	39.8	39.6	+ .4
37A		39.4	39.3	39.3	39.3	− .1
38A	0	39.7	39.6	39.7	39.6	− .1
38B		39.4	39.4	39.5	39.4	+ .1
39A		39.4	39.6	39.7	39.4	+ .3

Room temp., 24°C.

* Wild strain, recent isolate.

† Largest difference (increase) between pre- and post-inj. temperature readings.

TABLE II. Effect of Injecting Rabbits Intravenously, 10 ml/kg, with *E. coli** in 5% Dextrose. Solution autoclaved prior to injection.

Rabbit No.	No. *E. coli*/ml	Temp., °C: Before inj.	After inj.: 15 min.	1 hr	2 hr	Δ
14A	10^2	39.2	39.4	39.4	39.5	+ .3
14B		39.8	39.8	40.0	39.7	+ .2
15A		39.4	39.8	39.7	39.8	+ .4
12B	10^3	39.2	39.2	39.7	39.7	+ .5
13A		39.8	40.3	41.1	40.7	+1.3
13B		39.8	40.0	40.0	40.1	+ .3
16A	10^4	39.7	40.6	41.1	40.5	+1.4
16B		39.2	39.8	39.7	39.6	+ .6
17A		39.7	39.8	40.7	40.6	+1.0
1A	0	39.5	39.4	39.5	39.5	− .1
1B		39.7	39.7	39.8	39.7	+ .1
2A		39.3	39.5	39.5	39.6	+ .3

Room temp., 24°C.

* Stock strain, Dept. Bacteriol.

10^3 organisms per ml yielded a presumptively positive result while 10^4 organisms per ml yielded an unequivocally positive result. In addition, control tests on these animals injected with uncontaminated nonpyrogenic saline 72 hours following the initial tests yielded completely negative results.

The rabbits with which the results shown in Table II were obtained were used for the following experiment. A fresh suspension of the *E. coli* was prepared in saline, 10^4/ml and 10^5/ml. The suspensions were promptly filtered through membrane filters and the filtrates tested for pyrogenicity, with negative results.

Table III summarizes results obtained with organisms tested in more than 160 experiments involving over 600 rabbits. In each experiment a control group was included. Inevitably, results were not obtained in the smooth fashion indicated by the Table, but had to be filled in as the opportunity was available. In some cases data have been excluded because control animals yielded positive results or because one set of data excluded significance of other results. For example, in initial tests with *H. influenzae* suspended in dextrose a positive reaction was obtained with 10^2 organisms/ml in a series in which 10^3 organisms/ml yielded a negative result. On repetition of these tests, both suspensions were negative.

TABLE III. Pyrogen Test Results in Rabbits Injected Intravenously, 10 ml/kg, with Autoclave Sterilized Suspensions of Bacteria.

Test organism	Solution*	Pyrogen test results with: 10/ml†	10^2/ml	10^3/ml	10^4/ml	10^5/ml
E. coli (4 strains)	S	—	—	2 + 2 —	3 + 1 —	
" (2 ")	D		—	1 + 1 —		
" (2 ")	R		—	+		
Paracolobactrum sp.	S, D	—	—	—		
A. aerogenes	"	—	—	—	+	
K. pneumoniae	S, D, R		—	—	—	
Achromobacter sp.	"		—	—	—	
Pr. vulgaris	"	—	—	—		
Ps. aeruginosa	S, D	—	—	—		
	R		—	+		
B. subtilis	S	—	—	—	+	
	D, R		—	—	—	
B. globigii‡	S, D		—	—	—	—
	R			—	—	—
S. aureus	S, D, R		—	—	—	
S. epidermidis	"	—	—	—		
Al. fecalis	"		—	—	+	+
Br. abortus	S, D		—	—		
	R		—	+		
F. arborescens	S, D, R		—	—	—	
F. suaveolens	"		—	—	—	
F. aquatile‖	"		—	—	—	
H. influenzae	S, R		—	+		
	D			—		
S. marcescens	S, D, R		—	—		
P. multocida§	"		—	—	—	—
C. pseudodiphtheriticum§	"		—	—	—	—
V. metschnikovii§	"		—	—	—	—
V. comma§	"		—	—	—	+
S. typhimurium	S			—	+	+

* S = saline; D = 5% dextrose; R = U.S.P. Ringer's. † No. of organisms/ml.
‡ Spore suspension. § Grown on blood agar. ‖ Growth from plate incubated at room temperature for 72 hr.

Discussion. The data show that in no instance has any solution to which has been added 10 or 100 organisms/ml yielded a pyrexic response. The data suggest that at least 1000 organisms/ml of the most pyrogenic species are necessary to induce a positive test result in rabbits. Among 3 species of *Flavobacterium* and one of *Achromobacter,* which species are the most common contaminants we have encountered in hospital distilled water, as many as 10^4 organisms/ml have yielded negative U.S.P. pyrogen tests. Even 10^5 organisms per ml were negative in the case of *P. multocida, C. pseudodiphtheriticum,* and *V. metschnikovii. B. subtilis* yielded a positive test in saline in a concentration of 10^4 organisms/ml, while 10^5 spores/ml of *B. globigii,* currently considered to be identical with *B. subtilis,* were nonpyrogenic.

In view of the fact that freshly distilled water, collected under ordinary conditions of cleanliness in a large general hospital, with no unusual precautions taken to exclude air borne contamination, contained from 0 to 30 organisms/liter, up to 24 hours after distillation, it seems likely that these observations have practical application in pyrogen testing.

Seibert(1) enunciated the concept that "a bacterial count of a water may not be a true index of its pyrogenicity." This hypothesis applies to water which has been allowed to stand for indefinite periods. Contaminating organisms may grow in the presence of organic contamination. Organisms do lyse and release the somatic pyrogenic substances. However, Seibert's concept does not apply to

freshly distilled water, kept for no longer than a working day and employed for preparation of hospital solutions, if no extensive exogenous contamination occurs during processing. If bacterial contamination does occur, the extent is mirrored by the relatively simple methods of membrane filter culture. The membrane filter procedure thus may be employed as a screen test for pyrogenicity. Details of such a procedure will be published.

Summary. Multiple tests on solutions to which have been added known numbers of various bacterial species, suggest that 1000 or more organisms/ml must be present before the U.S.P. rabbit pyrogen test will yield a positive result. The potentiality is inferred of a simple culture procedure as a screen test for pyrogenicity of solutions to be employed for parenteral infusion.

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Relation of Ethyleneimmonium Ring Stability to Adrenergic Blocking Activity in Two Haloalkylamines.* (24351)

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A previous report(1) described the adrenergic blocking and cholinergic properties of N, N-dimethyl, 2 - chloro - 2 - phenylethylamine (DMEA). Comparative study of the 1-methyl analog (N,N-dimethyl,2-chloro-2-phenyl-1-methylethylamine) (M-DMEA) has raised questions concerning the metabolic fate of these compounds.

Methods. The experimental procedures were the same as used in the original study (1).

Results. Intramolecular rearrangement. In water solution, M-DMEA and DMEA liberated a full equivalent of chloride, indicating formation of an ethyleneimmonium ring, which in turn reacted with an equivalent of thiosulfate ion. The reactivity with thiosulfate declined with time, as the ring presumably was hydrolyzed to an alcohol. Comparison was made of speed with which M-DMEA and DMEA underwent these changes, by serial titration of chloride liberation and thiosulfate uptake. Since reaction rates varied with pH and temperature, tests were made in buffered solution (pH 7.4) at 37°C. As shown in the Table, formation of the ring form of DMEA was virtually complete within minutes and hydrolysis was complete within 1½ to 2 hours. In the case of M-DMEA, formation of the ethyleneimmonium ring was again rapid, at the same rate as for DMEA. How-

TABLE I. Rates of Intramolecular Transformations *In Vitro*.

	DMEA		M-DMEA	
Time after sol.	Chloride liberation	Thiosulfate uptake	Chloride liberation	Thiosulfate uptake
	(eq.)			
2 min.	.90	.98	.94	.98
5	.96		.97	
10	.98		.96	
30		.46		
60		.19		.89
90		.03		
2 hr		.00		.82
4				.76
6				.70
18				.41

In buffered solution (pH 7.4) at 37°C.

* Supplies of DMEA and M-DMEA were furnished by Eli Lilly Research Labs. All doses of DMEA and M-DMEA are given in terms of hydrochlorides.