SOME QUANTITATIVE STUDIES ON A BACTERIAL PYROGEN

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The quantitative estimation of bacterial pyrogens has not, until recent years, been seriously attempted. This is mainly because interest in bacterial pyrogens has hitherto centred in ensuring their absence from solutions for injection, rather than in seeking to estimate the amount present. All the qualitative tests of any value are biological—inadequate knowledge of the chemistry of these substances having precluded the development of chemical tests. The work about to be described represents an attempt to establish a quantitative approach to the study of bacterial pyrogens, and is based upon the methods used in the older qualitative tests.

Biological tests for the presence or absence of pyrogens depend on whether the test material, when administered intravenously to rabbits or dogs, produces a rise in temperature[1,2,3,4,5,6], or a change in the number of circulating leucocytes[7,8,9,10]. But only those tests dependent on the temperature response of the rabbit have come into general use.

An essential prerequisite for quantitative studies on the temperature response of the rabbit to pyrogens is an active and stable pyrogen preparation. A number of impure solutions, and dry extracts, containing bacterial pyrogens have been described by previous workers[5,8,11,12,13,14,15,16], some of whom have attempted, with varying success, to make quantitative observations upon them. Some have claimed that impure solutions are stable[5,21,22], but others have shown that this is not always so[15]. Others again have stated—but without submitting their evidence—that even highly purified preparations are unstable[17]. In the absence, therefore, of any generally accepted stable pyrogen preparation, it was decided to prepare and study, in the first instance, a crude, dry, pyrogen-containing extract.

Part I of this paper describes the preparation of this extract, and the different methods used to measure the temperature response of rabbits to it. Part II gives an account of the various experimental studies subsequently undertaken. These were carried out over a period of 5 years and are described in the order in which they were undertaken. As the successive studies constitute a logical series the results of each are fully discussed before proceeding to the next.

Part I

Materials and Methods

Preparation of the Pyrogen Test Material

The pyrogen test preparation is a dry powdered material obtained from *Proteus vulgaris*. The method of preparation was essentially that of Robinson and Flusser[12] modified so as to give a good yield of active

material without the use of elaborate purification procedures. It makes use of the fact that bacterial pyrogen is soluble in water but insoluble in acetone[12], and that greater amounts of pyrogen appear to be produced when the bacteria are grown on natural rather than on purely synthetic media[18,19].

1 ml. of a 24-hour subculture of a strain of *P. vulgaris* (N.C.T.C. No. 6821) grown in standard Lab-Lemco medium, was diluted with 7 ml. of normal saline solution, and the whole used to inoculate a culture bottle containing solid agar medium. This medium was prepared by adding 3 per cent. of agar to the standard Lab-Lemco medium. The large volume of the inoculum suffices to moisten the surface of the medium and subsequent emulsification of the growth is facilitated. 24 bottles were used for preparing a batch of material, the medium in each bottle having an effective surface area of about 200 sq. cm. The inoculated culture bottles were incubated for 48 hours at 37° C. At the end of this period the luxuriant bacterial growth was collected as an emulsion in the smallest possible volume of normal saline solution. To keep the emulsion volume to a minimum 3 successive quantities of 20 ml. of normal saline solution were used to emulsify the growth in the first bottle; these 3 emulsions were then used, in the same order, to emulsify the growth in the second bottle; the process being continued from bottle to bottle. When the first emulsion contained approximately 9×10^9 organisms/ml. its further use was found unprofitable, so it was reserved, and the second and third much weaker ones were used as the first and second, respectively, and a fresh 20 ml. of saline solution was introduced as the third. This procedure was continued until the whole of the growth from the 24 bottles had been emulsified. The volume of the pooled emulsions from a batch was approximately 175 ml. and contained about 6×10^9 organisms/ml. The pooled emulsion was gently agitated with glass beads for 1 hour, incubated for 24 hours at 37° C., again agitated for 1 hour, and reincubated for a further 24 hours. The bacteria were then removed by centrifuging at 6000 r.p.m. for 2 hours, and filtering the supernatant liquor through a seitz filter. The filtrate, which was pale buff in colour and slightly opalescent, was immediately poured into 10 volumes of acetone containing 0·4 per cent. of acetic acid[12]. A colloidal precipitate formed immediately. This soon coagulated and was removed after 24 hours on a sintered glass filter. Since the colour of the precipitate changes from cream to brown on exposure to the atmosphere, the filtration was carried out rapidly and the precipitate immediately transferred to a vacuum desiccator. The significance of the colour change is not known but it seemed advisable to reduce atmospheric exposure to a minimum. After drying for 7 days the precipitate became hard and brittle. The exterior was brown but the interior, on fracture, was a pale buff. Several batches have been prepared by this method and the mean yield was 1·78 g. The dried material from several batches was reduced to No. 90 powder, mixed, and packed in dry glass ampoules which were sealed by fusion of the glass. This material constitutes Pyrogen Test Preparation No. 1. It has been stored between

0° and 2° C. Preliminary tests established that this preparation contained a potent pyrogen.

Methods of Measuring Pyrogenic Response

Two different methods were used, in each of which the effect of Pyrogen Test Preparation 1 on the temperature of rabbits weighing more than 1·75 kg. was measured.

Method 1. The rabbits, deprived of food for 12 to 18 hours, were weighed and placed in holders at 09.30 hours. The holder—a modified form of a type previously described[20]—consisted of a base board to which was attached an adjustable neck stock and body straps. The rabbit was placed prone on the base board, and secured by the neck-stock and the body straps, so that the hind legs overhung the end of the base board. The rectal temperature of rabbits restrained in this way can be taken by clinical thermometers with minimum disturbance, and it is only on exceptional occasions that rabbits show resentment to the restraint. In each experiment the temperature of each rabbit was taken at 11.15 hours and 11.45 hours, by inserting a thermometer in the rectum to a standard depth of 6·0 cm., and the mean of the two readings was taken as the normal for that rabbit on that occasion. The appropriate dose of Pyrogen Test Preparation 1, dissolved in 5 ml. of normal saline solution, was given through the ear-vein at 12.00 hours. At 12.15 hours a clinical thermometer was inserted in the rectum of each rabbit and left in position for 4 hours. The thermometers were retained in position by means of light elastic slings. At the end of this period they were removed and the temperature recorded on each was noted. The thermometers were re-inserted and the temperature of each animal again taken to ascertain whether it had passed the maximum. Temperature readings were continued at intervals until the remaining increase in temperature was less than half the difference between the normal and the maximum recorded. The difference between the pre-injection normal and the post-injection maximum temperature in each rabbit was used as the measure of the response.

Method 2. Electrical thermometry was used in this method so it was possible to use a simpler holder and to follow the temperature changes in detail.

Rabbits, deprived of food for 12 to 18 hours were weighed and placed in holders at 09.30 hours. The holder consisted of a base board to the front of which was attached an inclined board in which was cut a vertical slot. The rabbit was placed in the holder in a normal sitting posture with its neck in the slot. Apart from the neck-slot no restraint was placed on the rabbit. The base board was covered with wire mesh to provide a secure foothold. The thermometers were inserted in the rectum to a depth of 6 cm. and each rabbit's temperature was recorded at 12-minute intervals from 11.00 hours onwards. The mean of the first 5 temperature readings for each animal was taken as the normal for that animal on that occasion. The appropriate dose of Pyrogen Test Preparation 1 dissolved in 5 ml. of normal saline solution was given through the ear-vein at 12.00 hours. Temperature readings were

continued until the temperature for each rabbit had passed its maximum and had declined to a value less than half-way between the pre-injection normal and the post-injection maximum. The response was measured in two ways: (*a*) as for method 1; (*b*) as the total temperature effect.

In the latter, the differences between the individual observations of the post-injection temperature and the normal for each animal were plotted against the time from injection until the temperature had passed its maximum and returned to a value half-way between the pre-injection normal and the post-injection maximum. A line perpendicular to the abscissa was drawn through this point. The measure of the response was obtained from the area enclosed by the graph, abscissa and perpendicular (see Fig. 1). The use of an arbitrary criterion of when the

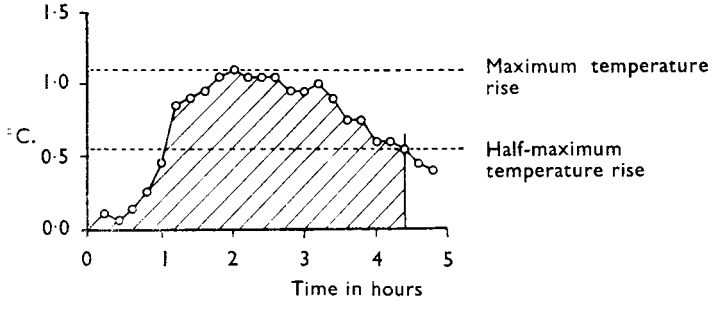

FIG. 1. Showing a typical temperature change following intravenous administration of Pyrogen Test Preparation No. 1 in a rabbit. Ordinate: temperature rise above normal. Abscissa: time in hours from injection. The shaded portion gives the measure of the total temperature effect. For further explanation see text.

response had terminated is necessary, because, no matter for how long the temperature readings continue to be taken, the temperature rarely returns to the pre-injection value. Extensive experiments established that there is a diurnal variation in the normal temperature of rabbits subjected to the experimental procedure, being minimal at 12.00 hours, and rising about 0·2° C. by 18.00 hours. This variation occurs in control experiments whether an injection of pyrogen-free saline solution is given or not. Hence, as all injections were given at 12.00 hours the "true" normal temperature for each rabbit gradually increases as the experiment continues. Whilst the mean increase with time is known, that of individual rabbits cannot be predicted with sufficient certainty, at any particular time, for it to be taken into account in determining the end of the pyrogenic response.

PART II

EXPERIMENTAL STUDIES ON PYROGEN TEST PREPARATION NO. 1

In seeking to establish quantitative methods for the estimation of bacterial pyrogen it is clear that two points have first to be determined. The first is the relationship between dose and response of the pyrogen preparation; and the second, whether the pyrogen preparation, and

solutions of it, are stable. The first two studies about to be described were undertaken to obtain information on these points in relation to Pyrogen Test Preparation No. 1. It was apparent from the very variable results obtained that unknown factors of fundamental importance were affecting the responses to pyrogen. Attempts were made to ascertain the nature of these factors and these attempts form the subject-matter of the two further studies described here.

1. *Relationship of Dose to Response of Rabbits*

Several series of experiments designed to study the dose/response curves, when the response is measured in the two ways described in Part I, have been done. For reasons which appear later in the paper the results are open to such criticism as to render them useless for the purposes originally envisaged. While there is thus no point in describing them, they nevertheless establish that the magnitude of the temperature response increases with increasing doses, and that the minimum effective dose of this preparation in the rabbit is less than 0·02 μg./kg.

2. *Stability of Solutions*

3 solutions, each containing 200 μg./ml. of Pyrogen Test Preparation 1, were prepared at widely different times and their activities compared after varying periods of storage between 0° and 2° C. The comparisons of activity were made by administering standard volumes of each solution per kg. of body weight and determining whether there were any significant differences between the mean responses to the different solutions. As all 3 Solutions were originally made up to contain the same amount of Pyrogen Test Preparation 1, equal volumes of the solutions should be equi-active if the preparation, and solutions of it, are stable. Two series of comparisons were made:—(i) Solutions A and B, 17 months and 3 months, respectively, after preparation. (ii) Solutions A, B and C, 32 months, 18 months and 1 month, respectively, after preparation. As each series of comparisons took several weeks to perform the age of each solution is given as its age halfway through the experimental series.

(i) *Comparison of solutions A and B*

12 rabbits, which had not been given pyrogen for several months, were used for the experiments. They were divided into two equal groups containing equal numbers of each sex, and the experiments were so arranged that on the first occasion solution A was given to one group and solution B to the other, the solutions being interchanged between groups on the second occasion. 3 such crossover tests were made using the same group of rabbits, the experiments being done at 3- or 4-day intervals. The volume of solution given to each rabbit was 0·005 ml./kg. In order to give such a small dose a dilution of each solution was used. These dilute solutions were prepared just before the injections were given, on each experimental day. Experimental method 1 was used.

The experimental data, and an analysis of them, are given in Tables I and II. As the analysis showed no significant differences between the

various interaction mean squares, their sums of squares have been pooled for the calculation of the error mean square. From the large probability for the test of significance between solutions it is concluded that there is no significant difference between their activities. The differences between rabbits and between tests are judged to be significant.

TABLE I

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A AND B OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 17 AND 3 MONTHS RESPECTIVELY

	Response measured as maximum temperature rise in ° C. above normal					
	Test I		Test II		Test III	
Rabbit No.	Solution A	Solution B	Solution A	Solution B	Solution A	Solution B
5	0·27	0·61	0·17	0·56	0·39	0·11
6	1·44	2·70	1·12	0·78	0·61	1·00
10	0·72	0·94	0·95	0·22	0·33	0·44
41	1·22	0·83	0·78	0·67	0·44	0·67
27	0·44	0·56	0·00	0·56	1·17	0·56
20	1·12	0·83	2·00	1·17	0·50	1·22
16	0·72	0·95	0·17	0·56	0·56	0·11
29	0·33	0·44	0·06	0·06	0·33	0·06
34	0·61	1·00	0·78	0·50	0·67	0·50
11	0·27	0·33	0·33	0·95	0·89	0·78
17	1·38	1·38	0·00	0·27	0·39	0·44
43	0·89	0·61	0·38	1·12	1·78	0·72
Mean response to each solution in each test	0·78	0·93	0·56	0·62	0·67	0·55
Mean response in each test	0·86		0·59		0·61	
Mean response to each solution	Solution A = 0·67 Solution B = 0·70					

TABLE II

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE I

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	"t"	P
Main effects:						
Between solutions ..	0·01389	1	0·01389		0·2957	0·7–0·8
Between rabbits ..	6·09457	11	0·55405	3·4883		<0·001
Between tests ..	1·06470	2	0·53235	3·3516		0·05–0·01
Interactions:						
(Error)	9·05371	57	0·15883			
Totals	16·22687	71				

(ii) *Comparison of solutions A, B and C*

6 rabbits of each sex, which had not been given pyrogen during the previous 8 months, were used. Each rabbit was given each of the 3 solutions in a complete test; and the whole group was used in 3 such tests. The solutions were given randomly within the restrictions that 2 rabbits of each sex received the same treatment on each day; each rabbit received each treatment once in a complete test; the order in which the solutions were administered differed from day to day, and from animal to animal. The experimental design is given in Table III. The volume of the appropriate solution given in each experiment was

0·005 ml./kg.; fresh dilutions of each solution being prepared on each day as described above. Method 2 was used for these experiments.

The data obtained when the response is measured in terms of the maximum elevation in temperature are given in Table IV. From the mean response to each solution given in Table IV, and the analysis

TABLE III

ORDER IN WHICH SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 WERE GIVEN DURING EXPERIMENTS TO COMPARE THE ACTIVITIES OF THE 3 SOLUTIONS AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

Sex		♂						♀					
Rabbit No.		6	9	11	20	27	43	5	8	10	34	51	52
Test	Day												
I	1	C	C	A	B	A	B	C	B	A	C	A	B
	2	B	A	C	A	B	C	A	C	C	B	B	A
	3	A	B	B	C	C	A	B	A	B	A	C	C
II	1	A	B	B	A	C	C	B	C	B	A	C	A
	2	C	C	A	B	B	A	A	B	C	B	A	C
	3	B	A	C	C	A	B	C	A	A	C	B	B
III	1	C	C	A	B	B	A	A	B	B	C	C	A
	2	B	A	C	C	A	B	C	A	C	B	A	B
	3	A	B	B	A	C	C	B	C	A	A	B	C

TABLE IV

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

	Response measured as maximum temperature rise in ° C. above normal								
Solutions	A			B			C		
Tests	I	II	III	I	II	III	I	II	III
Rabbit No. 6	1·03	0·75	0·78	1·18	1·03	0·73	1·65	1·15	0·83
9	0·98	0·73	0·95	0·98	0·80	0·80	1·30	0·53	0·80
11	0·75	0·80	0·80	0·85	0·90	0·60	0·85	0·88	0·88
20	1·38	0·95	0·98	1·80	0·90	1·00	1·40	1·28	1·20
27	1·75	0·63	0·70	1·45	0·40	0·48	0·95	1·15	0·78
43	0·90	0·90	1·25	1·45	1·08	0·90	1·20	1·43	0·85
5	0·90	0·50	0·68	0·45	0·68	0·20	1·20	0·65	0·78
8	1·00	1·00	0·80	1·30	0·80	0·93	1·08	1·10	1·05
10	1·15	0·80	0·68	1·03	1·00	0·90	1·30	0·90	0·93
34	0·58	0·55	0·53	0·78	0·58	0·55	1·10	0·75	0·83
51	2·10	1·80	1·55	2·13	1·60	1·45	1·90	1·93	1·38
52	1·20	0·88	1·13	1·50	0·90	0·78	1·15	0·98	1·05
Mean for each solution in each test	1·14	0·86	0·90	1·24	0·89	0·78	1·26	1·06	0·95
Mean for each solution	0·97			0·97			1·09		

TABLE V

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE IV

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between solutions	0·3432	2	0·1716	4·4113	0·01–0·05
Between rabbits	8·0683	11	0·7335	18·856	<0·001
Between tests	2·3477	2	1·1739	30·1774	<0·001
Interactions:					
(Error)	3·5763	92	0·0389		
Totals	14·3355	107			

in Table V, it is clear that solutions A and B are equi-active, whilst solution C is significantly more active than either A or B. Again there are very significant differences between rabbits and between tests. The data obtained when the response is measured in terms of the total temperature effect are given in Table VI. The tests of significance given in Table VII indicate that there are no significant differences between solutions, or between rabbits, but that the differences between tests are significant.

TABLE VI

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

	Response measured as the total temperature effect (see Fig. 1)								
Solutions	A			B			C		
Tests	I	II	III	I	II	III	I	II	III
Rabbit No. 6	2·07	1·17	2·50	1·99	2·56	1·66	5·69	2·58	1·95
9	1·83	2·35	2·25	2·23	1·88	2·19	5·19	1·19	2·76
11	2·21	2·35*	3·63*	1·79	2·95	2·11*	1·68	3·09*	2·99*
20	2·89	1·80	3·31*	5·36	1·96	2·78	2·62	2·52	2·96
27	4·93	2·63*	2·24*	2·89	1·17	2·33*	2·15	2·94	2·19*
43	1·83	1·93	2·81	5·55	2·78	2·44	2·14	3·48	1·84
5	1·65	1·55*	2·76	0·85	2·18	0·59	4·65	1·12	2·73*
8	2·20	2·70	2·04	3·81	1·95	2·16	2·31	2·83	3·30
10	3·03	1·73	1·61	2·08	2·37	2·71	3·29	2·01	1·99
34	1·08	1·20	1·47	1·56	1·25	1·49	4·00	1·88	2·75*
51	3·74	2·17	1·88	3·03	2·01	1·66	2·60	6·40	2·07
52	2·47	1·44	2·15	3·70	1·38	1·61	1·96	1·66	1·92
Mean for each solution in each test	2·49	1·92	2·39	2·89	2·04	1·98	3·02	2·64	2·46
Mean for each solution	2·27			2·30			2·71		

* These observations are of doubtful accuracy because the temperature was returning to normal so slowly that the duration of action could not be precisely determined.

TABLE VII

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE VI

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between solutions	5·4612	2	2·7306	2·8770	0·1–0·05
Between rabbits	11·2710	11	1·0246	1·0795	>0·2
Between tests	9·5116	2	4·7556	5·0106	0·01–0·001
Interactions:					
(Error)	87·3253	92	0·9491		
Totals	113·5691	107			

Discussion. When the response was measured in terms of the maximum elevation in temperature, no differences in the activity of solutions A and B were found in either of the two series of tests; but solution C was found to be significantly more active than either of the others. An examination of the ages of each of the solutions in the two tests reveals that solution B was already 3 months old when it was first compared with solution A, whereas solution C was only 1 month old when it was compared with solutions A and B. This suggests that loss in activity occurs in solutions immediately after preparation, but that the rate of inactivation rapidly diminishes, so that after 3 months it has become negligible. Whether the loss in activity is due to chemical decomposition of the pyrogen,

or to physical factors, is not known; but as the solutions were stored in 2-ml. glass ampoules some adsorption on the glass may have occurred. Calculation shows that for the adsorption of half the Pyrogen Test Preparation 1 contained in an ampoule, the concentration at the surface of the glass would only be $1{\cdot}5 \times 10^{-5}$ g./sq. cm., so that adsorption may account for the whole of the observed loss in activity.

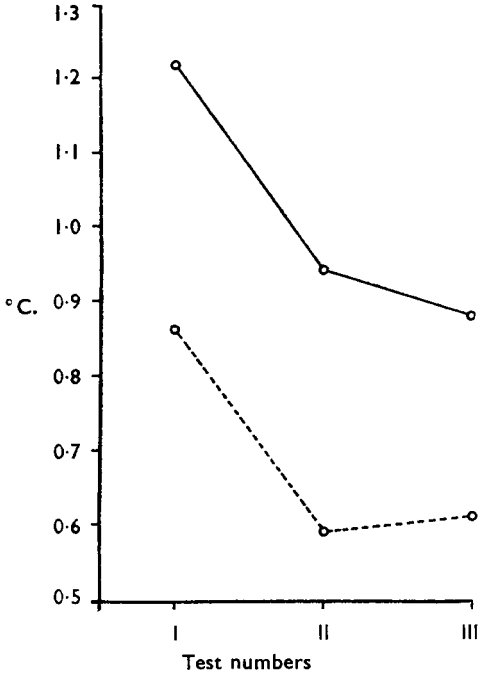

FIG. 2. Showing the mean responses in each test of two series of experiments comparing the activities of Pyrogen Test Preparation No. 1 solutions after storage. Ordinate: mean temperature rise above normal in °C. Abscissa: test number. The broken line shows the mean responses when solutions A and B were compared using method 1 (see Table I): solid line those when solutions A, B and C were compared using method 2 (see Table IV).

In the second series of experiments the response was also measured in terms of the total temperature response. No significant differences between the activities of the 3 solutions were found by this method. But these results are less valuable than those obtained when the response was measured in terms of the maximum elevation of the temperature above normal, because the standard deviation obtained when the total temperature response was measured was ±44 per cent. of the mean as against only ± 20 per cent. when the maximum temperature elevation was used. Furthermore, temperature readings must be taken for more than 6 hours after the injection in order to measure the total response, and quite often the temperature returns to normal so slowly that it is not possible to determine the end of the response with any precision (see Table VI). There is no evidence in these experiments of any loss in the activity of the Pyrogen Test Preparation No. 1 stored in the dry form.

3. *Effect of Repeated Administration on the Response of the Rabbit*

The finding that there were consistent and significant differences in the response from test to test in both series of experiments (see Tables II and V) was unexpected, so the data were critically examined to see whether the cause of this could be determined.

The mean response for each test has been plotted in Figure 2 and it is obvious that in each series of experiments there is a progressive reduction

in the response as test succeeds test. The regularity of this reduction in both series of tests seems to preclude any possibility of it being attributable to random variation. To determine whether the reduction was also consistent from day to day, the daily means from the second series of experiments were plotted (Fig. 3) —a procedure which is justified in virtue of the experimental design. The regular reduction during the first few days, after which the response is fairly constant, is very striking. An examination of the data shows that the effect is common to all the rabbits.

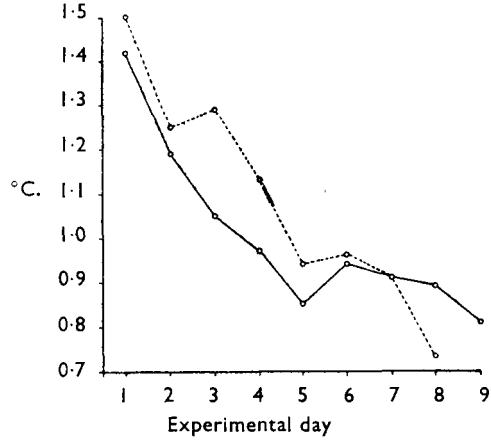

FIG. 3. Showing the mean responses to a standard quantity of Pyrogen Test Preparation No. 1 given repeatedly to each of two groups of rabbits at 3- or 4-day intervals. Ordinate: mean temperature rise above normal. Abscissa: experimental day. The solid line shows the mean response, on each experimental day, in the series of experiments comparing by method 2, the activities of solutions A, B and C: it may be derived, by the use of Table III, from the data in Table IV. The broken line shows the mean responses on each experimental day for the data given in Table IX.

The most obvious explanation of the regular reduction in response is either (*a*) that solutions rapidly lose their activity, or (*b*) that rabbits exhibit tachyphylaxis to pyrogen. The data were examined to determine whether either of these views was tenable.

The mean responses to each solution in each test in the second series of experiments are given in Table IV and have been plotted in Figure 4. It is obvious from Figure 4 that a similar reduction in response occurs for each solution. Hence, if this is due to progressive loss in activity,

TABLE VIII

TESTS OF SIGNIFICANCE FOR DIFFERENCES BETWEEN MEAN RESPONSES TO SOLUTIONS A AND B IN THE FIRST TEST AND SOLUTION C IN THIRD TEST IN EXPERIMENTS COMPARING THE ACTIVITIES OF THE 3 SOLUTIONS AFTER DIFFERENT PERIODS OF STORAGE. DATA TAKEN FROM TABLES IV AND V

Solution A, first test Solution C, third test	$t_{(92)} = \frac{1{\cdot}14 - 0{\cdot}95}{\sqrt{0{\cdot}0389(1/12 + 1/12)}}$	$= 2{\cdot}3567 \quad P = 0{\cdot}02$
Solution B, first test Solution C, third test	$t_{(92)} = \frac{1{\cdot}24 - 0{\cdot}95}{\sqrt{0{\cdot}0389(1/12 + 1/12)}}$	$= 3{\cdot}5971 \quad P = <0{\cdot}001$

then each solution must be losing activity at approximately the same rate. The mean ages of solutions A, B and C, at the time of the tests, were 31 months, 18 months and 1 month, respectively. Now if these 3

solutions, of widely differing ages but stored under identical conditions, were all losing activity at the same rate at the time their activities were compared, it is reasonable to infer that they have all been losing activity continuously at the same rate throughout their lives. But, if this is so, the response elicited by solutions A and B during the first test would be expected to be less, not greater, than the response to solution C in the third test. That the responses to solutions A and B in the first test were significantly greater than the response to solution C in the third test is shown in Table VIII. Thus the data do not support the hypothesis that the reduction in response in successive experiments is due to loss in activity.

The data are, however, compatible with the hypothesis that the effect is due to a progressive reduction in sensitivity when pyrogen is given repeatedly to rabbits.

To confirm this conclusion another group of 12 rabbits, which had never before been given pyrogen, was submitted to a series of experiments in which the same dose of solution B as had been used in the earlier experiments was given. The experiments were performed at 3- or 4-day intervals, and method 2 was used to determine the temperature responses. The data and analysis of variance are given in Tables IX and X. There are again significant differences between the responses on different experimental days. That these differences are due to a progressive

TABLE IX

DATA FROM EXPERIMENTS TO DETERMINE THE EFFECT OF REPEATED ADMINISTRATION OF A STANDARD DOSE OF 0·005 ML./KG. OF SOLUTION B OF PYROGEN TEST PREPARATION NO. 1 AT INTERVALS OF 3 OR 4 DAYS ON THE TEMPERATURE RESPONSE OF RABBITS

	Response measured as maximum temperature rise in ° C. above normal							
Experimental days ..	1	2	3	4	5	6	7	8
Rabbit No. 010 ..	1·23	1·15	0·95	1·03	0·73	0·88	0·70	0·70
259 ..	1·85	1·60	1·40	1·35	0·95	1·13	0·83	0·80
277 ..	1·53	1·40	1·75	1·78	1·60	1·58	1·38	1·18
453 ..	1·33	0·95	1·18	0·95	0·90	0·60	0·88	0·63
468 ..	1·45	0·90	1·10	0·88	1·05	0·75	0·88	0·60
662 ..	1·78	1·50	1·38	1·48	1·20	1·33	1·15	0·85
274 ..	1·90	1·55	1·43	0·93	0·73	0·93	0·80	1·05
457 ..	1·23	1·00	1·35	1·08	0·95	0·83	0·73	0·63
458 ..	0·95	0·95	1·15	0·78	0·80	0·63	0·73	0·58
460 ..	1·90	1·63	1·18	1·10	0·88	1·10	0·93	0·78
467 ..	1·60	1·70	1·75	1·23	1·08	1·15	1·18	0·78
651 ..	1·25	0·70	0·80	1·00	0·38	0·60	0·70	0·13
Means	1·50	1·25	1·29	1·13	0·94	0·96	0·91	0·73

TABLE X

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE IX

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between experimental days	5·2870	7	0·7553	25·6034	<0·001
Between rabbits	5·1943	11	0·4722	16·0068	<0·001
Interaction:					
(Error)	2·2727	77	0·0295		
Totals	12·7540	95			

reduction in the mean response is evident from Figure 3. Examination of the data from individual rabbits again shows that the effect was common to all the rabbits (see Table IX).

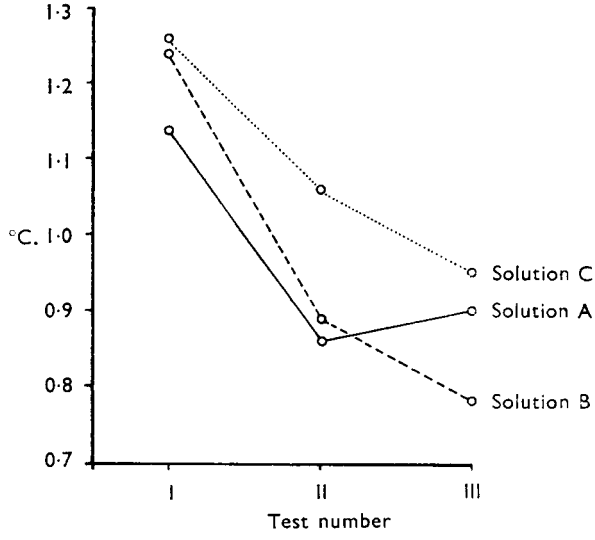

FIG. 4. Showing the mean response in each of the 3 tests of the series comparing solutions A, B and C by method 2 (see Table IV). Ordinate: mean temperature rise above normal. Abscissa: test numbers.

These confirmatory experiments were begun 2 months after finishing the second series of comparisons of activity. Hence, if the reduction in response found in the second series was due to loss of activity in the solutions, the mean response on the first day of the confirmatory series should not be greater than the mean response to solution B on the last day of the second series, and should be significantly less than the response to solution B on the first day in the second series. On the other hand, if the reduction in response is due to reduction in sensitivity resulting from the repeated administration of pyrogen, then the mean responses on the first day in both series should not differ significantly; but the mean response on the first day of the confirmatory series should be significantly greater than the mean response on the last day of the previous series. The appropriate data have been taken from Tables IV and IX and are given in Table XI. The analysis of variance reveals that there are significant differences between days, but it is evident that the mean responses on the first day in each series do not differ significantly, whereas the mean response on the last day of the second series is significantly less than either of these obtained on the first day of either series. It is thus impossible to escape the conclusion that there is a significant reduction in the response to bacterial pyrogen when it is given repeatedly at short intervals to the same rabbits.

When the experimental methods used in this work were first defined it was assumed, from the reports of previous workers, that a constant response could be repeatedly elicited by a standard dose of bacterial pyrogen. From the large body of data collected in the U.S.P. Commission's *Collaborative Studies on Pyrogens*[5,11,21], the writers noted that

"there was a slight trend to a lower response" when a large dose of pyrogen was given repeatedly, but that no such effect was observed when a sequence of different doses was given. That the "trend" was regarded as unimportant is shown by the fact that the test designed during that work and subsequently included in the U.S.P., permits the repeated use of the same rabbits. Evidence has been given[17] from which it was

TABLE XI

Observation	Response in ° C. to 0·005 ml./kg. of solution B		
	Data reconstructed from Tables III and IV (Rabbits previously used but rested for 8 months)		Data from Table IX (New rabbits)
	Test I First day	Test III Last day	First day
1	1·80	0·80	1·23
2	1·45	0·60	1·85
3	1·30	1·45	1·53
4	1·50	0·20	1·33
5			1·45
6			1·78
7			1·90
8			1·23
9			0·95
10			1·90
11			1·60
12			1·25
Means	1·51	0·76	1·50

Analysis of variance

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Between days	1·6977	2	0·8489	6·9355	0·01–0·001
Within days	2·0808	17	0·1224		
Total	3·7785	19			

suggested that the reduction in response observed in the U.S.P. studies was due, at least in part, to the rabbits becoming accustomed to the procedure, the implication being that rabbits thoroughly experienced in the procedure would not exhibit this trend. Wylie and Todd[15], on the other hand, found no change in the magnitude of the response when rabbits were used repeatedly over long periods.

Such conclusions are not in agreement with ours*, but insufficient data are presented by some of these workers[15,17] to enable one to judge whether their results are capable of a different interpretation. Welch *et al.*[21], however, in the U.S.P. studies, present a comprehensive summary of their data, analysis of which reveals that what they describe as a "trend" is in fact a very highly significant shift in response level. Furthermore, their statement that after an interval of 3 to 4 weeks the "response was approximately equal to the initial" is an over-statement, for it was intermediate between the first and the later responses, and still significantly less than the first one. Full recovery of sensitivity clearly does not occur

* *Note added in press:* The writer's attention has been drawn to a paper by Beeson (*J. exp. Med.*, 1947, **86**, 29) who reports that the response, when measured as the total temperature effect, suffers a progressive reduction when the same rabbits are repeatedly used. This seems to be the first reference to this phenomenon.

in 4 weeks. That the effect is only temporary, however, is established by our work, for rabbits which had been given much pyrogen were found after a rest of 8 months to give the same response as rabbits which had never had any (see Table XI).

The fact that each succeeding response is affected by the previous experimental history of each rabbit does not invalidate any of the conclusions drawn in this paper, because in the experiments described here the doses used did not vary widely, and because the design of the experiments was such that the magnitude of the variation from this source was known. In experiments where a series of widely differing doses is used a very different situation exists, and the drawing of any but the most general conclusions from such work is open to criticism. For this reason our own early results on the dose response curve of rabbits to pyrogen have been discarded as virtually useless, and new studies on this subject, which make use of the facts just described, and of others yet to be discussed, are in progress at the time of writing.

4. *Effect of Varying the Experimental Method on the Magnitude of the Rabbit's Response to Pyrogen*

In method 1 the holders used are such that the rabbits are subjected to severe restraint. These holders were adopted because it has been stated that in completely immobilised animals the normal temperature settles to a lower steady value than that otherwise obtained and that the magnitude of the response to pyrogens is correspondingly increased[17]. Clinical thermometers were used in method 1 as a temporary measure, pending the availability of electrical thermometers. In the meantime, however, doubts had arisen as to the advisability of subjecting the rabbits to such severe restraint. Hence, when the electrical thermometers were ready, the opportunity of using holders in which the rabbits were subjected to less restraint was utilised. These are the conditions described in method 2. Thus the methods differ in two major respects—in the degree of restraint imposed on the rabbits, and in the type of thermometer used.

From different experiments with each method it appears that the magnitude of the response is considerably greater with method 2. This is illustrated in Figure 2, which shows the results for two series of experiments in each of which a different method was used, although the doses given were the same. It was not, of course, known how much each of the two factors which had been deliberately changed was contributing to the apparent difference in the magnitude of the response. Furthermore, it was not known if variations in other unknown factors might also have contributed to the difference, since the various experiments using the two methods were done at widely separated times. It was therefore desirable to determine, by experiments conducted simultaneously, whether the apparent difference was real, and, if so, what was the contribution of each of the two variable factors to it.

There are four possible ways in which the two factors can be combined: (*a*) rigid restraint + clinical thermometers; (*b*) slight restraint + electrical

TABLE XII

DATA FROM EXPERIMENTS TO DETERMINE THE MAGNITUDE OF THE RABBIT'S TEMPERATURE RESPONSE TO PYROGEN TEST PREPARATION NO. 1 WHEN DIFFERENT EXPERIMENTAL METHODS ARE USED

Groups of rabbits	Rabbit No.	Replicate sets of experiments									Totals of residual sums of squares
		I			II			III			
		Experimental days			Experimental days			Experimental days			
		1	2	3	4	5	6	7	8	9	
1 ♀	71	(*a*) 0·72	(*c*) 1·63	(*b*) 1·43	(*b*) 1·22	(*c*) 1·47	(*a*) 0·56	(*a*) 1·00	(*c*) 0·71	(*b*) 1·03	
	72	(*c*) 0·91	(*b*) 1·59	(*a*) 0·56	(*a*) 0·89	(*b*) 1·35	(*c*) 1·04	(*b*) 1·32	(*a*) 0·78	(*c*) 0·97	
	73	(*b*) 1·39	(*a*) 1·28	(*c*) 0·73	(*c*) 0·73	(*a*) 0·78	(*b*) 0·70	(*c*) 0·87	(*b*) 1·27	(*a*) 0·39	
Residual sums of squares ..				0·0699			0·0374			0·0170	0·1243
2 ♀	74	(*b*) 1·26	(*a*) 1·11	(*c*) 1·15	(*a*) 0·39	(*b*) 1·55	(*c*) 1·09	(*c*) 1·34	(*b*) 1·37	(*a*) 1·11	
	75	(*c*) 0·83	(*b*) 1·75	(*a*) 1·22	(*b*) 1·16	(*c*) 1·07	(*a*) 0·78	(*a*) 1·00	(*c*) 0·89	(*b*) 1·17	
	76	(*a*) 0·89	(*c*) 1·11	(*b*) 0·98	(*c*) 0·87	(*a*) 0·89	(*b*) 0·74	(*b*) 0·73	(*a*) 0·39	(*c*) 0·70	
Residual sums of squares ..				0·1385			0·2097			0·0192	0·3674
3 ♂	77	(*b*) 1·17	(*a*) 1·33	(*c*) 0·19	(*c*) 0·72	(*a*) 0·67	(*b*) 1·00	(*c*) 0·77	(*b*) 0·94	(*a*) 0·94	
	78	(*a*) 1·06	(*c*) 0·90	(*b*) 1·50	(*b*) 2·35	(*c*) 1·81	(*a*) 1·89	(*b*) 2·00	(*a*) 1·50	(*c*) 1·62	
	79	(*c*) 1·51	(*b*) 1·81	(*a*) 0·81	(*a*) 0·44	(*b*) 1·31	(*c*) 0·63	(*a*) 0·89	(*c*) 0·81	(*b*) 1·07	
Residual sums of squares ..				0·4224			0·0756			0·0300	0·5280
4 ♂	80	(*c*) 1·06	(*a*) 1·28	(*b*) 1·40	(*a*) 0·94	(*b*) 1·44	(*c*) 1·26	(*b*) 1·32	(*c*) 1·45	(*a*) 1·06	
	81	(*a*) 0·67	(*b*) 1·84	(*c*) 1·31	(*c*) 1·40	(*a*) 1·50	(*b*) 1·66	(*c*) 1·67	(*a*) 1·67	(*b*) 1·72	
	82	(*b*) 1·98	(*c*) 1·29	(*a*) 0·61	(*b*) 1·37	(*c*) 1·27	(*a*) 0·78	(*a*) 1·22	(*b*) 1·49	(*c*) 1·36	
Residual sums of squares ..				0·2900			0·0434			0·0052	0·3386
Total residual sums of squares				0·9208			0·3661			0·0714	
Mean responses to each method			(*a*) 0·96 (*b*) 1·52 (*c*) 1·05			(*a*) 0·88 (*b*) 1·32 (*c*) 1·11			(*a*) 1·00 (*b*) 1·29 (*c*) 1·10		

Letters in parenthesis indicate the method used, and the following figures are the measures of the responses—for full explanation see text.

thermometers; (*c*) rigid restraint + electrical thermometers; (*d*) slight restraint + clinical thermometers. Of these combinations, (*d*) involves handling of the rabbits each time the temperature is observed, and as this would constitute a further variation in technique, the combination was regarded as impracticable. Thus it was not possible to design an experiment which would give precise information on the contribution of each factor.

The fundamental unit of the design adopted was a 3 × 3 latin square, in which the rows were rabbits, the columns days, and the letters methods.

TABLE XIII

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE XII

Replicate sets	Items	Degrees of freedom	Sums of squares		Mean squares	Variance ratios	P
I	Total between methods	8	2·5442		0·3180		
	Main effect between methods	2		2·0620	1·0310*	12·0023	<0·001
	Interactions between methods within groups	6		0·4822	0·0804		
	Total between days	8	1·4201		0·1776		
	Main effect between days	2		1·1050	0·5525		
	Interactions between days within groups	6		0·3151	0·0525		
	Total between rabbits	8	0·5518		0·0690		
	Residual	8	0·9208		0·1151		
	Residual + interactions	20		1·7181	0·0859*		
II	Total between methods	8	1·3935		0·1742		
	Main effect between methods	2		1·1900	0·5950†	16·7135	<0·001
	Interactions between methods within groups	6		0·2035	0·0339		
	Total between days	8	0·5840		0·0730		
	Main effect between days	2		0·4423	0·2212		
	Interactions between days within groups	6		0·1417	0·0236		
	Total between rabbits	8	3·5365		0·4421		
	Residual	8	0·3661		0·0458		
	Residual + interactions	20		0·7113	0·0356†		
III	Total between methods	8	0·6713		0·0839		
	Main effect between methods	2		0·5203	0·2602‡	13·2755	<0·001
	Interactions between methods within groups	6		0·1510	0·0252		
	Total between days	8	0·2172		0·0272		
	Main effect between days	2		0·0483	0·0242		
	Interactions between days within groups	6		0·1689	0·0282		
	Total between rabbits	8	2·3054		0·2882		
	Residual	8	0·0714		0·0089		
	Residual + interactions	20		0·3913	0·0196‡		

*, †, ‡, indicate the mean squares used in calculating the variance ratios given above.

4 groups of rabbits were used—2 of males, and 2 of females. Each group was used in 3 replicate sets of experiments. The complete design is given in Table XII. The experiments were done at 3- to 4-day intervals, a standard dose of 0·01 ml./kg. of solution C being given each time. The response was measured in terms of the maximum increase in temperature above normal. Experiments using combinations (*a*) and (*b*) were conducted as described under methods 1 and 2, respectively. Experiments using combination (*c*) were conducted as described for method 2, except that the holders used were those described for method 1.

The experimental data are given in Table XII and the analysis of variance in Table XIII.

As the 3×3 latin square was the fundamental unit in the experimental design, the data from each square were analysed separately into sums of squares arising from variations between methods, between days, between rabbits, and the residual portion. It was originally intended to treat the data as from one large experiment by summing the various sums of squares. There are, however, very large and systematic changes in the residual sums of squares from unit to unit, the residual sum of squares consistently becoming less for every group of rabbits in succeeding replicate units (see Table XII). When the residuals are summed over groups for each replicate set there are significant differences between the sums, but when they are summed over replicates for each group there are no significant differences. In consequence of this the combined data from the 4 groups have been treated as a separate set of experiments for each replicate.

TABLE XIV

TESTS OF SIGNIFICANCE FOR DIFFERENCES BETWEEN MEAN RESPONSES FOR EACH METHOD IN EACH REPLICATE SET OF EXPERIMENTS

	Replicate sets		
	I	II	III
Differences due to varying both degree of restraint and type of thermometer (methods (*a*) and (*b*))	$t_{(20)} = \frac{1{\cdot}52 - 0{\cdot}96}{\sqrt{0{\cdot}0859(^1/_{12} + ^1/_{12})}}$ $= 4{\cdot}6815$ $P = <0{\cdot}001$	$t_{(20)} = \frac{1{\cdot}32 - 0{\cdot}88}{\sqrt{0{\cdot}0356(^1/_{12} + ^1/_{12})}}$ $= 5{\cdot}7135$ $P = <0{\cdot}001$	$t_{(20)} = \frac{1{\cdot}29 - 1{\cdot}00}{\sqrt{0{\cdot}0196(^1/_{12} + ^1/_{12})}}$ $= 5{\cdot}0788$ $P = <0{\cdot}001$
Differences due to varying type of thermometer, degree of restraint being constant (methods (*a*) and (*c*))	$t_{(20)} = \frac{1{\cdot}05 - 0{\cdot}96}{\sqrt{0{\cdot}0859(^1/_{12} + ^1/_{12})}}$ $= 0{\cdot}7524$ $P = 0{\cdot}4\text{–}0{\cdot}5$	$t_{(20)} = \frac{1{\cdot}11 - 0{\cdot}88}{\sqrt{0{\cdot}0356(^1/_{12} + ^1/_{12})}}$ $= 2{\cdot}9866$ $P = 0{\cdot}01$	$t_{(20)} = \frac{1{\cdot}10 - 1{\cdot}00}{\sqrt{0{\cdot}0196(^1/_{12} + ^1/_{12})}}$ $= 1{\cdot}7513$ $P = 0{\cdot}1$
Differences due to varying the degree of restraint, using the same type of thermometer (methods (*b*) and (*c*))	$t_{(20)} = \frac{1{\cdot}52 - 1{\cdot}05}{\sqrt{0{\cdot}0859(^1/_{12} + ^1/_{12})}}$ $= 3{\cdot}9291$ $P = <0{\cdot}001$	$t_{(20)} = \frac{1{\cdot}32 - 1{\cdot}11}{\sqrt{0{\cdot}0356(^1/_{12} + ^1/_{12})}}$ $= 2{\cdot}7269$ $P = 0{\cdot}02\text{–}0{\cdot}01$	$t_{(20)} = \frac{1{\cdot}29 - 1{\cdot}10}{\sqrt{0{\cdot}0196(^1/_{12} + ^1/_{12})}}$ $= 3{\cdot}3275$ $P = 0{\cdot}01\text{–}0{\cdot}001$

The various pooled sums of squares for each replicate set, which are given in Table XIII, are each based on 8 degrees of freedom. Of the 8 degrees of freedom for the sum of squares between methods within each replicate set, only 2 are directly concerned with differences between methods, the remaining 6 being interactions. For each replicate set these sums of squares have been appropriately partitioned and, as in no case is the interaction sum of squares significantly different from the residual sum of squares, these 2 have been pooled, providing a residual sum of squares based on 14, instead of 8, degrees of freedom. The pooled sums of squares for differences between days for each replicate set were analysed in the same way, and as none of these interaction sums of squares were found to differ significantly from the appropriate residual sum of squares, these interaction sums of squares were also pooled with the residual sums of squares, thus providing a residual sum of squares based on 20 degrees of freedom. No such partitioning of the sums of squares for differences between rabbits is possible. The details of the analysis are given in Table XIII. From the tests of significance it is concluded that there are significant differences between

the magnitudes of the responses obtained when the experimental method is varied.

Tests of significance for differences between the mean response to each method in each replicate set of experiments are given in Table XIV. It is evident from these tests that the differences in the magnitude of the response obtained in the different experimental circumstances are real, and that both the degree of restraint and the method of taking the temperature affect the response. Although the tests for differences in magnitude of the response caused by varying the method of taking the temperature when the degree of restraint is constant are not all significant (see Table XIV), the differences are all in the same direction; and when the probabilities from these tests are combined by a standard method[23] the differences are highly significant.

When the degree of restraint is minimal, and when electrical thermometers are used for measuring the temperature changes, the response is maximal.

Summary

1. The absence of a stable preparation suitable for use in the development of quantitative methods for the study of bacterial pyrogens is pointed out.

2. A simple method giving a good yield of a highly pyrogenic dry extract from *Proteus vulgaris* is described.

3. Two methods of measuring the pyrogenic response are described.

4. Experimental studies on (i) the temperature response of rabbits to various doses of the pyrogen preparation, (ii) the stability of solutions of the pyrogen preparation, (iii) the effect upon the response of repeated administration of the preparation, (iv) the effect upon the response of varying the experimental procedures, are described and discussed. Standard statistical methods have been used in the analysis of the results.

5. The temperature response increases with increasing doses. The minimal effective dose of the dry extract is less than 0·02 μg./kg.

6. Solutions containing 200 μg./ml. of the pyrogen preparation are still highly active after several years storage, though there is evidence that some loss of activity occurs immediately following their preparation.

7. There is no evidence of loss of activity of the dry extract over a period of 5 years.

8. When bacterial pyrogen is given repeatedly to rabbits, at intervals of a few days, the temperature response diminishes progressively during the first few successive administrations. While the effect is only temporary the rabbits must be rested for considerably more than 4 weeks before it passes off.

9. The magnitude of the pyrogenic response is affected by the degree of restraint imposed on the rabbits, and by the type of thermometer used. The maximum response is obtained when the degree of restraint is minimal and when electrical thermometers are used.

10. The maximum increase in temperature above normal is a more useful measure of pyrogenic effect than is a measure which, in addition, also takes account of the duration of the temperature rise.

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