SOME OBSERVATIONS ON THE B.P. AND U.S.P. TESTS FOR PYROGENS

By J. G. Dare

From the Department of Pharmacology, University of Leeds

Received July 14, 1953

The B.P. and U.S.P. tests for pyrogens depend on the temperature response of rabbits to intravenous administration of the test material. The methods of interpreting the data differ in the two tests but, in each, the material fails to pass the test if it produces a temperature rise greater than that prescribed. The maximum permissible response, in both the B.P. and the U.S.P., is a specified temperature rise, which is not related to any comparison with a standard preparation. Therefore, any factor which affects the magnitude of the temperature response will affect the sensitivity of the tests.

The writer has already shown that the degree of restraint imposed on the rabbits, the type of thermometer used, and the frequency of administration of pyrogen, all affect the magnitude of the rabbit's temperature response to bacterial pyrogen[1]. Thus when the degree of restraint imposed on the rabbits is severe and clinical thermometers are used, the magnitude of the response to pyrogens is much less than when the degree of restraint is slight and electrical thermometers are used. Furthermore, there is a progressive reduction in response when pyrogen is given repeatedly. The B.P. and U.S.P. specify neither the degree of restraint nor the type of thermometer to be used. Both pharmacopœias permit the repeated use of the same animals.

Two questions of practical importance arise from these considerations. The first and more obvious is; how far may the reliability of the tests be affected by failure to take these sources of variation into account? The second, which derives from the different methods of interpreting the data in the B.P. and U.S.P. tests, is; how do the relative efficiencies of the two tests compare when conducted in similar conditions? This paper seeks to provide an answer to these two questions.

Methods and Results

The data used are drawn from a large series of quantitative studies on a dry powdered bacterial pyrogen preparation from *Proteus vulgaris*. Some of these studies have been described in a previous paper[1], where details of the preparation of Pyrogen Test Preparation No. 1, and the two experimental methods used in investigating it, are given. Method 1 consists of determining the difference between the pre-injection normal and the post-injection maximum temperatures of rigidly restrained fasting rabbits weighing more than 1·5 kg., the temperatures being taken with clinical thermometers. A known dose of Pyrogen Test Preparation No. 1 is given intravenously in 5 ml. of pyrogen-free saline solution. Method 2 differs from Method 1 only in that the degree of restraint is very much

less and that electrical thermometers are used. In many experiments the rabbits were used repeatedly, an interval of at least 3 days being allowed to elapse between experiments. Thus both experimental methods meet the requirements of the B.P. and U.S.P. tests, except for the injection volume. Control experiments, however, established that this difference in injection volume did not affect the response to a standard dose of Pyrogen Test Preparation No. 1.

The mass of data available is so extensive that it is not feasible to present an analysis of all of it in this paper. Bias in selecting the data presented has been avoided, by specifying a series of typical sets of conditions under which data had been obtained, and then collecting, into groups, all data obtained under those conditions. All the groups obtained in this way are presented and analysed here, except where the total number of observations in a group happened to be less than ten. The conditions specified for each group were:—

(i) dose of Pyrogen Test Preparation No. 1;

(ii) the number of occasions on which pyrogen had been given previously, at the same dose level, at 3 or 4 day intervals;

(iii) the experimental method.

18 groups of data from experiments in which pyrogen was given and 2 groups in which pyrogen-free saline solution was given have been collected in this manner. Details for each group are given in the tables.

The variance of all observations from experiments in which pyrogen was given was analysed, and the "within groups" variance used as the variance for each group. In experiments where no pyrogen was given the data were collected in sets by experimental days, and the "within days" variance was used as the variance for each group. Given the mean and variance of each group of data, and assuming that the individual observations within a group are a random sample from a normally distributed population, the frequency with which the presence of bacterial pyrogen may be expected to be correctly detected by the two tests can be calculated as described below.

(a) *Evaluation of the data on the basis of the B.P. test*

3 rabbits are used in a test, and pyrogens are judged to be absent if the mean temperature rise above normal does not exceed 0·6° C. Now 0·6° C. in standard measure (x) for the distribution of means of groups of 3 observations is:—

$$x = \frac{0{\cdot}6 - \mu}{\sqrt{\frac{\sigma^2}{3}}}$$

when μ = mean of the distribution, and

σ^2 = variance of a single observation.

The percentage frequency with which mean values less than 0·6° C. will occur on such a distribution can be conveniently found from the marginal figures in a table of probits[2] corresponding to the location of the value

$(x + 5)$ in the body of the table. This figure is the percentage of occasions on which tests carried out under the same conditions will be judged to indicate that pyrogens are absent. The percentage of occasions on which pyrogens will be judged to be present is given by subtracting this figure from 100.

The results for the B.P. test given in the tables have been calculated in this way.

(b) *Evaluation of the data on the basis of the U.S.P. test*

3 rabbits are used in a test and a preliminary assessment of the data is made as follows:—

(i) If 0 rabbit exhibit a temperature rise above 0·6° C.—pyrogens judged absent.

(ii) If 1 rabbit exhibits a temperature rise above 0·6° C.—doubtful result, test must be repeated on 5 rabbits.

(iii) If 2 or 3 rabbits exhibit a temperature rise above 0·6° C.—pyrogens judged present.

In order to ascertain the proportions in which the above results occur in any particular set of conditions it is first necessary to determine the proportions of individual observations in which the temperature rise is (*a*) less than 0·6° C., and (*b*) equal to, or greater than, 0·6° C. These values, subsequently referred to as "a" and "b," respectively, are found in the same manner as for the B.P. test, except that the standard deviation of a single observation is used instead of that for the mean of three, and the values for "a" and "b" are expressed as proportions of unity instead of percentages. Thus:—

$$x = \frac{0{\cdot}6 - \mu}{\sigma}$$

then a = value from Probit table for $(x + 5)$, divided by 100, and $b = 1 - a$.

As 3 rabbits are used in a test there are 4 possible combinations of results when the individual increases in temperature are classified as greater or less than 0·6° C. The proportion of test results which will occur in each of the combinations is given by the terms of the expansion of $(a + b)^3$—"a" and "b" having the values previously determined. Hence:

a^3 = the proportion of tests which indicate that pyrogens are absent.

$3a^2b$ = the proportion of tests which have to be repeated.

$3ab^2 + b^3$ = the proportion of tests which indicate that pyrogens are present.

There is, however, a further condition attached to tests in which all three observations are less than 0·6° C., namely, that if the sum of the temperature rises exceeds 1·4° C.—that is if the mean exceeds 0·46° C.—the test must be repeated. Thus the proportion of first tests in which pyrogens will actually be found absent is less than "a^3," and the proportion

of tests which must be repeated is larger than "$3a^2b$" by the amount by which "a^3" must be diminished. To determine what proportion must be transferred it is first necessary to find what proportion of groups of three rabbits exhibit a mean temperature greater than 0·46̇° C. when no animal in the group shows an individual rise greater than 0·6° C.

Pearson[3,4] gives tables for the calculation of the mean and standard deviation of a normal distribution when only the moments of its truncated tail are known. These tables can also be used in the reverse manner. Here the standard deviation and the mean of the whole distribution are known, and those for the tail with its stump at 0·6° C. are required. The mean (m) and standard deviation (Σ) of the tail are calculated as shown below. All symbols have the same meaning as is attached to them in Pearson's Tables.

$$\Sigma^2 = \psi_1 \times d^2$$
$$m = 0{\cdot}6 - d$$
$$d = \frac{\sigma}{\psi_2}$$
$$h' = \frac{h}{\sigma} = \frac{0{\cdot}6 - \mu}{\sigma}$$

Where $\psi_1 + \psi_2$ are found from the tables by entering with h′,

μ = mean of whole distribution,

σ = standard deviation of whole distribution,

d = distance from stump to mean of tail,

m = mean of tail,

and Σ = standard deviation of tail.

The proportion of tests "a′" in which the mean is less than 0·46̇° C. and in which no individual rabbit exhibits an increase in temperature greater than 0·6° C. can now be calculated in a manner similar to that used for the B.P. test:—

$$x' = \frac{0{\cdot}46\dot{} - m}{\sqrt{\frac{\Sigma^2}{3}}}$$

then a′ = value from Probit table of (x′ + 5), divided by 100; and b′, the proportion of tests in which the mean is greater than 0·46̇° = 1 − a′. In arriving at "a′" and "b′" it has been assumed that the means of groups of three from the tail are normally distributed; this is not true, but the error introduced is small.

Now the proportion of all tests in which no rabbit exhibits a temperature increase of more than 0·6° is a^3. Hence the proportion of all tests in which no rabbit shows a temperature rise of more than 0·6° C. whilst at the same time the mean rise is greater than 0·46̇° C., is

$$a^3 \times b'.$$

This is the proportion of test results which must be transferred, from those

previously interpreted as establishing that pyrogens are absent, to those which have to be repeated. These proportions now become:—

$$\text{pyrogens absent} = a^3 - (a^3 \times b')$$
$$\text{repeat test required} = 3a^2b + (a^3 \times b').$$

The U.S.P. requires that repeat tests shall be carried out on 5 rabbits, and the repeat test is considered as establishing the absence of pyrogens if not more than 1 rabbit exhibits a temperature rise of 0·6° C. or more. The proportion of tests in which 0, 1, 2, etc., rabbits show a rise of 0·6° C. or more, when 5 rabbits are used, is given by the terms of the expansion of $(a + b)^5$—where "a" and "b" have the values previously determined. Reasoning as before, there are 6 possible combinations of results from 5 rabbits, of which the sum of the terms $a^5 + 5a^4b$ gives the proportion of tests in which pyrogens will be found absent. This is the proportion of tests using 5 rabbits, in which 0 or 1 rabbit will exhibit a temperature rise of 0·6° C. or greater. Conversely, $1 - (a^5 + 5a^4b)$ gives the proportion of tests in which 2 or more rabbits will exhibit a temperature rise of 0·6° C. or greater. Now the probability of two independent events occurring simultaneously is the product of their individual probabilities. Hence the proportion of all tests in which pyrogens will be found absent, as the result of a repeat test, will be the product of (i) the proportion of tests which have to be repeated, and (ii) the proportion of tests, using 5 rabbits, in which not more than 1 rabbit exhibits a temperature rise of 0·6° C. or greater, i.e.,

$$[3a^2b + (a^3 \times b')] \times (a^5 + 5a^4b).$$

The probability of either of two events occuring on a particular occasion is the sum of their probabilities. Hence the proportion of all tests under a particular set of conditions in which pyrogens will be found absent, is the sum of the proportions of tests in which pyrogens will be found absent in either (i) the first test, or (ii) the repeat test, i.e.,

$$a^3 - (a^3 \times b') + (3a^2b + (a^3 \times b')) \times (a^5 + 5a^4b)$$

By a similar argument it can be shown that the proportion of all tests in which pyrogens will be found present is:—

$$3ab^2 + b^3 + [3a^2b + (a^3 \times b')] \times [1 - (a^5 + 5a^4b)].$$

Multiplication of the two probabilities by a 100 gives the results in expected percentage frequencies.

The results for the U.S.P. test given in the tables have been calculated in this way.

Discussion

The data given in Table I are from groups of rabbits experimented upon for the first time, and where method 1 was used. The data given in Table II, columns 2, 4, 6, 9 and 12, are also from groups of new rabbits, but where method 2 was used. It is evident from the tables, and from Figure 1, that the frequency with which pyrogen will escape detection by either the B.P. or the U.S.P. test is much greater with method 1 than with method 2. From Figure 1 it can also be seen that when method 2 is

used, pyrogen can be detected with certainty (P = 0·99), when it is present in only about one fifteenth the amount that can be detected with method 1. These results also show that the magnitude of the response, previously shown[1] to depend on the experimental technique for doses of 1 and 2 μg./kg. of body weight is equally affected by differences in technique for

TABLE I

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHOD 1 WITH NEW RABBITS

		Dose of Pyrogen Test Preparation No. 1 in μg./kg. of rabbit's body weight				
		0·3	0·6	1·0	2·0	6·0
Number of observations in each group .. Mean response in °C.		10 0·81	11 1·06	12 1·02	16 1·10	11 1·26
Within Group Variance = 0·1273						
Percentage of tests in which pyrogen will escape detection	B.P.	15·98	1·28	2·07	0·77	0·07
	U.S.P.	2·25	0·06	0·12	0·04	<0·01
Percentage of tests in which pyrogen will be detected	B.P.	84·02	98·72	97·93	99·23	99·93
	U.S.P.	97·75	99·94	99·88	99·96	>99·99

lower doses. Hence, to achieve uniform sensitivity in the pharmacopœial tests, the degree of restraint which it is permissible to impose upon the animals, and the type of thermometer used to measure the temperature, must be specified. When the degree of restraint is minimal, and electrical thermometers are used, the efficiency of both tests is maximal.

The upper part of Table II gives data from experiments in which groups of rabbits were given pyrogen repeatedly at three or four day intervals: all the data for each dose level were collected from the same rabbits. The

TABLE II

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHOD 2 AND SHOWING THE EFFECT OF REPEATED ADMINISTRATION OF PYROGEN

		Dose of Pyrogen Test Preparation No. 1 in μg./kg. of rabbit's body weight											
		0·02		0·08		0·40			1·00			2·00		
Number of previous administrations of pyrogen		0	2	0	2	0	2	4	0	2	4	0	2	4
Number of observations in each group Mean response in °C.		18 0·74	18 0·61	18 1·15	18 0·83	12 1·30	12 1·07	12 0·61	24 1·46	24 1·15	24 0·89	16 1·83	16 1·35	16 1·22
Within Group Variance = 0·1273														
Percentage of tests in which pyrogen will escape detection	B.P.	19·91	47·29	0·36	12·21	0·03	1·11	47·29	<0·01	0·38	7·67	<0·01	0·01	0·14
	U.S.P.	4·50	14·15	0·02	1·80	<0·01	0·05	14·15	<0·01	0·01	0·53	<0·01	<0·01	<0·01
Percentage of tests in which pyrogen will be detected	B.P.	80·09	52·71	99·64	87·79	99·97	98·89	52·71	>99·99	99·62	92·33	>99·99	99·99	99·86
	U.S.P.	95·40	85·85	99·98	98·20	>99·99	99·95	85·85	>99·99	99·99	99·47	>99·99	>99·99	>99·99

results are given in the lower part of Table II, and are illustrated graphically in Figure 2. They reveal, very clearly, the effect of repeated administration on the sensitivity of the tests. They show, indeed, that the minimum dose, detectable with certainty (P = 0·99), by either pharmacopœial test, suffers a 27-fold increase by the fifth successive administration of pyrogen. This is the situation which obtains when the same dose of pyrogen is given on successive occasions.

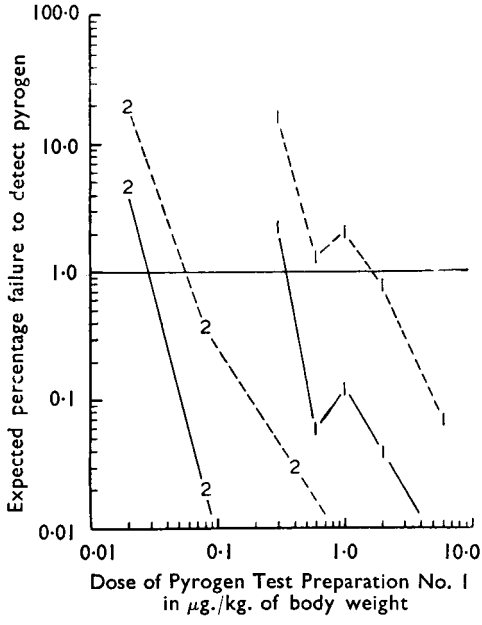

FIG. 1. Showing the percentage frequency of failure to detect pyrogen in the B.P. and the U.S.P. tests, with experimental methods 1 and 2, and with rabbits given pyrogen for the first time. Broken lines, B.P. tests: solid lines, U.S.P. tests. Points indicated by numerals 1 and 2 are from experiments with methods 1 and 2, respectively. The dose corresponding to the point at which each line intersects the ordinate at the 1 per cent. level is the smallest dose that can be detected with certainty (P = 0·99).

The question immediately arises as to what happens when the successive doses are varied. To determine this, a first large dose of 10 μg./kg. of Pyrogen Test Preparation No. 1 was given to each of several groups of 12 rabbits. A second smaller, but effective dose of 0·2 μg./kg. was given to each of the groups of animals, after a different interval of time for each group. Even after the longest interval of 17 days, on giving the smaller dose the temperature increase was barely significant, and both the B.P. and U.S.P. tests consequently failed to detect the pyrogen.

Thus the repeated administration of small doses of pyrogen greatly reduces the sensitivity of the tests, whilst a single administration of a large dose will render invalid a subsequent test in which the same rabbits are used. It was established, in another series of experiments, that repeated administration of pyrogen-free saline had no effect on the esponse to a dose of pyrogen given subsequently. Thus to maintain the sensitivity of tests it is necessary to exclude only rabbits which have previously been given pyrogen. It is, therefore, suggested that the repeated use of the same rabbits be permitted, provided that no rabbit used in a test where pyrogen was judged to be present be used again.

From the results illustrated in Figures 1 and 2 it is clear that the frequency with which pyrogen will be detected by the U.S.P. test is always greater than by the B.P. test. This is owing to the different methods of interpreting the data. The frequency with which pyrogen, at any stated

dose level, will escape detection, can be reduced to any desired value by suitably reducing the permissible temperature rise. But if the permissible temperature rise is reduced too far, the normal fluctuations in temperature may be large enough to be mistaken for pyrogen responses. The advantage gained by the increase in frequency with which pyrogen is detected, when present in small amounts, will then be more than offset by the frequency with which it will be judged to be present when, in fact, absent. It is therefore pertinent to determine whether the greater efficiency of the U.S.P. test—in which lower total temperature increases than those required by the B.P. test often result in pyrogen being judged present—is achieved only by a corresponding increase in the number of pyrogen-free samples which are declared by the test to contain pyrogen. To determine this point data from experiments in which pyrogen-free saline was given were analysed. It was not known whether both methods would lead to the same results, so two groups of data were gathered together, in one of which method 1 was used and in the other method 2. These results are given in Table III.

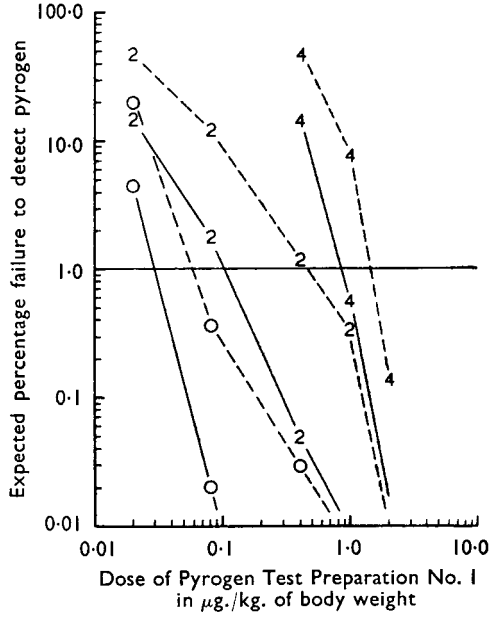

FIG. 2. Showing, with method 2, the reduction in efficiency in the B.P. and the U.S.P. pyrogen tests, when pyrogen is given repeatedly to the same rabbits at 3 or 4 day intervals. Broken lines, B.P. tests; solid lines, U.S.P. tests. Numerals indicating points on the graphs give the number of previous occasions on which the same dose of pyrogen had been given. The progressively higher dose levels at which the succeeding lines cut the ordinate at the 1 per cent. level shows the progressive reduction in efficiency of the tests as the number of previous administrations of pyrogen increases.

There is a slight diurnal variation in the normal temperature of the rabbit under the experimental conditions, the temperature being minimal at noon and rising by an average of 0·15° C. by 3 p.m. The temperature of each rabbit also exhibits random fluctuations. Hence, as in all cases the difference between the pre-injection normal and the post-injection maximum temperatures is the measure of the response, and as all injections were given at noon, the mean temperature change in each group is positive. The means for each group are not very different, but the "within days" variance for experiments in which method 1 was used is greater than that for method 2. Despite this, the proportion of test results indicating the presence of pyrogen when it is in fact absent, do not

differ materially for the two methods. The results thus show that neither the B.P. nor the U.S.P. tests are likely, with either experimental method, to result in the condemnation of pyrogen-free preparations.

TABLE III

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHODS 1 AND 2 WHEN PYROGEN-FREE SALINE IS GIVEN

		Method 1	Method 2
Number of observations in group		55	84
Mean response in °C.		0·20	0·22
Within day variance		0·0282	0·0094
Percentage of tests in which pyrogen will be found present	B.P.	0·01	<0·01
	U.S.P.	0·02	<0·01
Percentage of tests in which pyrogen will be found absent	B.P.	99·99	>99·99
	U.S.P.	99·98	>99·99

SUMMARY

1. The effect of varying the experimental conditions upon the sensitivity of the B.P. and the U.S.P. pyrogen tests has been examined, and a comparison of the efficiency of the two tests has been made.

2. It is found that the sensitivity of both tests is maximal, (*a*) when the rabbits have not previously been given pyrogen, (*b*) when the degree of restraint is minimal, and (*c*) when electrical thermometers are used.

3. It is, therefore, recommended that in pharmacopœial descriptions of pyrogen tests, (*a*) the further use of rabbits which have once been used in a test in which pyrogen has been found present be forbidden, (*b*) the maximum degree of restraint which may be imposed on the rabbits be specified, and (*c*) the use of electrical thermometers should be required.

4. The U.S.P. test detects smaller amounts of pyrogen than does the B.P. test.

5. The frequency with which pyrogens will be judged present when they are, in fact, absent, by either the B.P. or the U.S.P. test, is so small as to be negligible.

6. It is suggested that the U.S.P. method of interpreting the test data should replace the method used in the B.P.

I wish to thank Professor W. A. Bain for advice, and the Medical Research Council for a grant towards part of the expenses incurred, during this work.

REFERENCES

1. Dare, *J. Pharm. Pharmacol.*, 1953, **5**, 528.
2. Fisher and Yates, *Statistical Tables for Biological, Agricultural and Medical Research*, 3rd Ed., Oliver and Boyd, London.
3. Pearson, *Tables for Statisticians and Biometricians*, Part I, 2nd Ed., University College, London, 1924.
4. Pearson, *ibid.*, Part II, 1st Ed., University College, London, 1931.

DISCUSSION

The paper was presented by THE AUTHOR.

MR. W. P. LEGGETT (Liverpool) asked whether the author could offer an alternative method which did not involve the use of rabbits, because if manufacturers used animals once only many thousands more rabbits would be needed than were obtainable. Was the decline in response associated with any particular pyrogen? It was well established that there were many different pyrogens derived from different bacterial species. He also asked whether the author agreed that there were occasions when rabbits did not respond to pyrogens and, if so, whether the percentage of rabbits which did not respond was significant.

MR. G. A. STEWART (Dartford) said that he had used the author's pyrogen preparation in a number of studies, and had found a variation in sensitivity between sexes and breeds. Male rabbits were more sensitive than female rabbits to pyrogen, and Flemish rabbits were found to be most sensitive. When repeated doses of pyrogens were given to female rabbits (the experiment had not been carried out with male rabbits), he had found an increase in sensitivity to the pyrogens on days 2 to 6, and than it began to fall off; this was contrary to the author's report. Eventually a slight tolerance was produced, but after a few weeks rest sensitivity returned. He agreed with the idea of using standard pyrogens on test rabbits. Some rabbits were initially very insensitive to pyrogens so if they were not tested initially, rabbits could be introduced into the test which would bias the results. He asked whether the author had noticed any difference between sex and breed, and on what diet he kept his rabbits. Also, what was the relationship between the animal tests and clinical findings? He wondered whether the author had tested his own pyrogen preparation in humans.

DR. F. HARTLEY (London) said that the author had led himself to the conclusion that every rabbit must be a detector of pyrogens. That might be true for his particular standard pyrogen preparation, but if no rabbits could be used a second time, it had to be assumed that every one was a perfect detector of all types of pyrogen. That was not true in the experience of those who had examined a whole range of unknown types of pyrogenic materials rather than specially prepared standard preparations of particular pyrogens. Before the author could go so far as to express any comparison between sensitivities and particular testing methods, it was necessary for the study to be repeated with different pyrogens. He was not convinced that the author had a basis for any recommendation of switching from the present qualitative type of test in the B.P. In laboratories where it was common practice to establish first the sensitivity of the animals used, a resettling period of 4 to 5 weeks was adequate.

MR. E. ADAMS (Plymouth) said that the paper indicated one disadvantage of the fever test not apparent with the leucocyte method. In a paper published in the Transactions of the New York Academy of Science, it was stated that the response in the leucocyte method could be additive with further doses. It was possible to superimpose one response

upon another and obtain a rise in white blood cell count of from 16,000 to 67,000 per cu. mm.

PROFESSOR J. P. TODD (Glasgow) said that the author had carried out a series of tests which he had repeated at intervals of 3 or 4 days, and he had given one series of figures. Those figures he interpreted as indicating a diminution of sensitivity to the pyrogenic substances. He (Professor Todd) and his colleagues had also carried out over the last 6 or 7 years a series of tests including such tests as those described. It had, for example, in the last year been found necessary to repeat the doses at 3 or 4 day intervals, and that was coupled with the leucocyte response. The temperature response in no way agreed with the author's results. There were the usual fluctuations, but they did not, in his opinion, indicate any diminution of the sensitivity. In 1948 Wylie and himself published a paper in which that point was investigated. A crude complex mixture of pyrogens was used in order to get some increase in temperature by sensitisation of the rabbit, but that did not happen. The leucocyte response appeared to him to show possibilities of providing in the near future a test which was very much more accurate than the temperature test. Of course, it was necessary to be satisfied that a high leucocyte response was indicative of high pyrogenic activity. He asked what the author meant by an "electrical thermometer," pointing out that for many years he had been satisfied with the simple thermocouple. The B.P. test for pyrogens was, in his view, possibly the crudest test which ever got into the B.P.

MR. J. W. LIGHTBOWN (Mill Hill) said that an expert committee of the World Health Organisation were instigating an international investigation into the assessment of pyrogens, and they had obtained two pyrogen samples which they intended to distribute for examination. One was a preparation of *proteus* and the other a polysaccharide, more highly purified, prepared from *Chromobacterium prodigiosum*. Some years ago he was concerned in some experiments with pure polysaccharide in an investigation of the Schwartzman reaction, which depended on repeated injection of bacterial products into an animal. If pure polysaccharide were injected into the skin and 24 hours later a second dose were given there was necrosis at the site of injection. If both injections were given intravenously there was diffuse hæmorrhage throughout the organs, and on the second injection there was also a fall in temperature. If the polysaccharide were used in a dose below about 1 μg./kg. there was a strong pyrogen response. If a dose above 10 μg./kg. were used there was a decrease in temperature. It would be interesting to know whether the author had observed anything like that with *proteus*, and whether high doses gave no response at all or decreased responses. There was a possibility of antibody production due to repeated doses of the *proteus* preparation. With the polysaccharide, which was free from protein, after frequent injections precipitins were detected in the plasma which would eliminate the pyrogenic effect. It would be interesting to know whether the author's animals had produced any antibody which might account for a decrease in response.

MR. K. L. SMITH (Nottingham) said it was a pity that the author had applied so much restraint to the animals. In his laboratory when a clinical thermometer was used the operators nursed the animals in their laps. He was surprised that the variance of the rise in temperature in the method using more restraint was the same as when less restraint was applied. There was some justification for reducing the critical temperature of the author's pyrogen test. It was difficult to increase the dose of distilled water and the critical level could be reduced, but he did not suggest that the U.S.P. method should be accepted *in toto*. Before the test was made more restrictive, it was desirable to consider its object. There must be a great deal of water used with intravenous injections on a large scale which was never submitted to a pyrogen test at all, but he did not think that the incidence of pyrogenic response was great. It was impossible to decide in manufacture, where distilled water was being continuously produced, what constituted a batch. The B.P. test could not be followed exactly.

DR. G. SOMERS (London) agreed that large amounts of water were injected which had never been submitted to the pyrogen test. Although water for injection should be pyrogen free when being placed into ampoules and subsequently sterilised, the material was able to derive pyrogens from the glass container itself. Therefore, some requirement should be made, as in the sterility test, that a percentage of the final product should be tested. In the design of pyrogen tests it was necessary to take a practical view; large batches of material could not be rejected because the temperature of 3 rabbits went up. It was not unknown for rabbits to show false rises due to excitement and similar causes.

MR. T. D. WHITTET (London) supported the suggestion that the B.P. should be more specific in regard to the restraining apparatus in the pyrogen test. In a recent paper it had been shown that not only did severe immobilisation cause hyperthermia, but that rabbits having unrestricted freedom could show a rise in temperature of 1 to 1½° in an hour.

MR. BROOM (Nottingham) said there was no evidence whether the permitted temperature rise in the B.P. or U.S.P. was right or not. There was real need of collaboration between pharmacologist and clinician to ascertain the correlation, if any, between the response in animals and in humans.

DR. G. E. FOSTER (Dartford) pointed out that the fundamental requirement of a biological test was that the standard preparation should be the same as the test preparation, but he was not sure that that had been fulfilled in the present case.

DR. J. G. DARE, in reply, said he knew of no more convenient animal than the rabbit and it was not suggested that, in routine testing, animals should be used only once. In the literature there were now reports of pyrogens from 4 different bacterial species having been examined for tachyphylaxis and it was observed in each case. In his experience only very rarely did rabbits fail to respond to doses of pyrogen within the normal effective range, and no rabbit has been found which failed to respond more than once, but the minimum effective dose varied widely. Part of the superiority of the U.S.P. test was due to the fact that when a

threshold dose of pyrogen is given it will be discovered even when a relatively insensitive rabbit is included, because the data are assessed by the number of individual responses.

The dose response curve for males appeared to be steeper than for females, but the minimum effective dose for each appeared to be similar, so that for qualitative tests either sex is suitable. He had made no comparison of different breeds of rabbits. An ordinary standard diet with greens and oats had been used. He had not given pyrogens day after day, but Beeson reported that when this was done there was a progressive reduction in response. Studies on human beings were in progress, and it was hoped to report on these shortly.

He was not clear what Dr. Hartley meant by a "perfect detector." But it would seem that he wished to specify that particular rabbits must only be used for pyrogen testing after they have been shown to be sensitive to pyrogens from all sources. This was clearly impossible. For practical purposes it had to be assumed that any rabbit will react to pyrogen if the dose is sufficient: nor is there any evidence to the contrary. The data in the paper showed, however, that when pyrogen had been given previously the "sufficient" dose may be very much greater than if no pyrogen had been given before. Dr. Dare said he made no recommendation to change from the qualitative type of test in the B.P.; his recommendations were still for a purely qualitative type of test and they would not make the test any more stringent than the present U.S.P. test.

He wished to draw Professor Todd's attention to the references, given in his earlier paper, to other work in which a diminution in sensitivity to pyrogens was reported. Favorite and Morgan have also reported that effect in man. As a generally applicable qualitative test, leucocyte changes could have only a limited value, because many of the preparations tested would contain substances other than pyrogens which had profound effects on leucocytes. Furthermore, Favorite and Morgan observed that to produce the same degree of leucopœnia the dose of pyrogen had to be increased on each occasion. The thermocouple was, of course, only one of many electrical devices that might be used as thermometers.

A purified polysaccharide from *Chr. prodigiosum* is one of the preparations which have been shown by others to exhibit tachyphylaxis in rabbits. With greater than maximum effective doses of his preparation serious distortion of the normal response pattern occurred.

In the U.S.P. test no sample passes without further examination if the mean is greater than 0·46° C. Thus Mr. Smith's suggestion that a lower temperature could be used is clearly included, whilst the advantage of making use of individual temperature changes is retained. He agreed that tests on the final product should be required, especially if it was to be given intravenously.

With regard to the permitted rise of temperature, it was hoped to obtain information on this point when the results from the human studies were related to those from rabbits.

The B.P. and U.S.P. tests were qualitative tests which did not involve any comparison with a standard preparation.