# AN INVESTIGATION OF THE NATURE, SOURCES AND PROPERTIES OF PYROGENIC SUBSTANCES.

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# AN INVESTIGATION OF THE NATURE, SOURCES AND PROPERTIES OF PYROGENIC SUBSTANCES.

The term pyrogen is used to define a substance or mixture of substances which cause a rise in body temperature when injected into the animal body. They appear to be by-products of bacterial metabolism, and while other chemical substances of both organic and inorganic nature such as 1 per cent suspension of sulphur in liquid paraffin, tetrahydro-B-naphthylamine and dinitrophenol can also stimulate rise in body temperature and name 'Pyrogen' is not usually applied to such specific chemicals but is reserved for those fever producing substances of biological origin.

In 1868 Bergmann found that water, when injected into the bloodstream of dogs, sometimes caused pyrexia. Between that time and the close of the nineteenth century many substances were found to show this property and from such work arose the terms 'water fever' (Bergmann 1868) 'Salt fever' (Kottman 1906) 'sugar fever' (Bingel 1911), 'Salvarson fever' (Wechsellman 1911), 'tissue fever' (Hammerschlag 1890), 'protein fever' (Collmar 1887) and 'ferment fever' (Hammerschlag 1890) suggesting that water, salt, sugar, salvarson, tissue extracts, proteins and enzymes were capable of causing pyrexia when injected,

While investigating Salvarson fevers, Wechsellman (1911) discovered that the pyretic property of the salvarsan solutions was associated with the degree of bacterial contamination of the saline in which the salvarsan was dissolved and that by using freshly distilled water in the preparation of the saline no fever followed the injection. From this he inferred that the fever was not produced by the salvarsan itself but by some substance present in the water used as solvent. Later Hort and penfold (1912b) found that salt fever, salvarsan fever, sugar fever, tissue fever and ferment fever were caused by the presence of pyrogens in the water used as diluent or vehicle for the injection of the substances. They demonstrated that the active principle was not necessarily the bacterial cell itself but/

but was some substance produced as a by product of its metabolism or by its autolysis as the water retained its pyrogenic properties after the removal of the bacteria by centrifugation or filtration through Doulton filter candles (1912 a). These workers also found that this pyretic substance was heat stable and could be removed from the water by careful distillation in all-glass apparatus.

Seibert (1923 a) re-investigating the problem of pyrogenic water showed beyond doubt that bacterial contamination was the cause of the pyrogenic activity, that the pyrogen was relatively heat stable and that a baffling system must be used during distillation to prevent the carrying over of pyrogen by entrainment. In 1923 (b) Seibert claimed that protein fever was caused by bacterial pyrogen adsorbed by the protein and that fever caused by the injection of casein was due to adsorbed pyrogen derived from the bacteria in milk and that casein care-:fully isolated from freshly drawn milk was often pyrogen-free.

The field was thus narrowed down from the earlier ideas to a point at which it became clear that the pyretic property associated with many substances was probably due to the presence of an impurity, pyrogen, produced by bacterial contaminants.

It is true that other views are sometimes expressed to explain the fever and reactions often following intravenous therapy. By some it is held that reactions may be caused either by the physical properties of the solution such as Hydrogen Ion concentration or concentration of salt, by the presence of dust particles in the solution or by the presence of impurities in the solution such as Sulphur from the rubber tubing of the administration apparatus. The rate of administration and the temperature of the solution being injected are also stated to cause elevation of the temperature. Although these factors may stimulate transient changes in the body temperature and undesirable reactions during intravenous therapy it is highly probable that most febrile reactions following intravenous injection of large volumes of fluids are caused by pyrogen of bacterial origin. Both Seibert (1923 a) and Banks (1934) showed that the various factors mentioned above, such as hydrogen ion concentration and salt concentration, had little effect on the normal temperature of the rabbit.

The increased use of saline and glucose solutions and more recently the use of whole blood and plasma during the war for the treatment of haemorrhage and shock, focused attention on the febrile reactions which sometimes/

times followed the infusion of these substances. The reactions occurred most frequently during the administration of whole blood and plasma and great care was necessary in the preparation of these substances if undesirable reactions were to be avoided.

Many workers in this country and in America have investigated methods of preparing pyrogen-fre salines (Todd etal, 1941,1946, Co Tui et al, 1937, and Lees & Levy 1940) and most of these workers used water freshly distilled from a suitably baffled still and dissolved the medicament, which may itself be pyrogenic, in this water. Pyrogen introduced with the medicament was adsorbed from solution by the addition of activated carbon and this was removed by filtration through Seitz filter pads, the final solution being sterilised within a few hours of bottling. By such methods the incidence of reactions following the administration of salines practically disappeared but, unfortunately, these methods are not applicable to blood products and the incidence of reactions following their administration remains high.

The tests for the detection of pyrogen and some of the physical and chemical methods for its elimination from solution have been investigated.

The tests used so far for the detection of pyrogen are/

are based on its ability to stimulate fever within three hours of being injected intravenously into rabbits, and on its ability to cause variation in the total white blood count when injected into the rabbit.

Seibert (1923 c) investigating the source of pyrogen devised the test for its detection depending on the production of fever when injected intravenously into rabbits. She concluded that the daily variation in the rectal temperature of the normal rabbit rarely exceeded o.6°C. and that therefore any rise in temperature greater than 0.6°C. following the injection of a solution was abnormal. The temperature was determined by means of a clinical thermometer immediately before injection and each hour following it. In 1943 the United States Pharmacopoeia commission recognised in the twelfth revision of the Pharmaeopoeia the importance of a test for the detection of pyrogen in water adopting the technique for Welch et al (1943) in which also the pyrogen is detected by its fever stimulating property in rabbits. To carry out this test the solution is injected into the ear-vein of each of five rabbits and is considered pyrogenic if three or more of the rabbits show fever; a fever being indicated by a rise of 0.6°C. or more in rectal temperature.

Kuna et al (1946) modified this test to suit industrial purposes, recording the temperature by means of a thermocouple inserted in the rectum, the rabbit being immobilised throughout the test. The British Pharmacopoeia, 1948 includes a test similar in principle to the U.S.P. test but here the pyrogenic reaction is indicated by an average rise of 0.6°C. in three rabbits, the temperature being determined by either the clinical thermometer method or by the thermocouple method.

The ability of pyrogen to cause variation in the white blood count of the rabbit has also been used for the detection of pyrogen. Chapman (1942) maintained that a solution was pyrogenic if it stimulated a fall in white blood count of 4,000/c.mm. within 45 to 90 minutes of its intravenous injection. Young et al (1944) found that this leukopenia was followed by a definite leukocytosis within 3 to 6 hours of injection, and that a rise in white blood corpuscle count of 50 per cent, over the original count was indicative of pyrogenic activity.

Various chemical and physical methods for the removal and destruction of pyrogen in solution have been examined. Campbell and Cherkin (1945) for example destroyed the pyrogen by boiling with hydrogen peroxide, Co Tui et al (1936) and Lees & Levy (1940) introduced methods of adsorbing pyrogen from/

from solution by Seitz filtration and by use of a number of different adsorbing agents, while Collier and Paris (1947) claimed that prolonged storage was effective in some cases.

Little systematic work has been done, however, on the isolation and identification of pyrogen although Hort & Penfold (1912c) and Co Tui et al (1944) have examined a range of bacteria for their ability to produce pyrogen, and substances have been isolated from Eberthella typhosa, Ps. aeruginosa, Proteus vulgaris and B.subtilis which displayed the typical properties of pyrogen (Co Tui et al 1944, Robinson & Flusser 1944 and Rodney & Welcke 1945).

These on analysis were found to be mainly polysaccharide in nature although disagreement existed among these workers as to the presence or absence of nitrogen. Because of this disagreement doubt has been expressed whether the various substances isolated were similar to each other in chemical nature or whether each species of organism produces its own pyretic substance.

Although, as has been shown, the action of pyrogenic substances has attracted the attention of various workers since 1868, little real progress has been made in clarifying the problems related to their sources and their chemical nature. Their ability to promote powerful reaction, at what must be great dilution, in the animal body makes

it/

it clear that any attempts to isolate them from their sources will call for improvement in the measurement of the amounts present, and in this investigation an attempt has been made to improve the test so that two solutions can be compared with an accuracy sufficient to determine whether after treatment by physical or chemical means, the amount of the pyrogen present had been increased or diminished.

Experiments have also been carried out to define more closely the sources from which pyrogens are derived and the effect of treatment by physical means on their stability has also been studied.

During the work it was noticed that different types of fever curves occurred following the injection of various fractions of the one solution. These characters have been investigated and certain conclusions have been drawn from the work done which it is hoped in later chapters to show are reasonable assumptions.

## THE DETECTION OF PYROGEN.

Although Carter (1930) claimed that potassium permanganate was decolourised when boiled in pyrogenic water for a few minutes, no other chemical test for pyrogen has been demonstrated. In view of the large number of substances which decolourise potassium permanganate such a test is obviously of little value, and it is necessary, in the light of our present knowledge, to base the test for the detection of pyrogens on their action on the animal body. Two such properties have been used for this purpose, the ability to stimulate a rise in body temperature and the ability to reduce the leukocyte count when injected into the blood-stream. The test most generally used, and that used throughout this work, is the measurement of rise in body temperature of a group of animals after the intravenous administration of the solution under test.

### Choice of experimental animal.

The ideal experimental animal for pyrogen tests must possess an efficient temperature regulating mechanism and, from a practical point of view, it should be easily handled and injected intravenously.

Neither the mouse or the rat possess an efficient temperature/

temperature controlling mechanism, the normal temperature varying over a wide range (Plant and Pflugers, 1924, and Stammers 1926)

In the guinea pig, irritation of the rectum by insertion of a thermometer seems to cause rapid fluctuation of the rectal temperature and this animal is not suitable for work involving temperature measurements of this type. (Hort and Penfold 1912c)

Dogs and cats have temperature control comparable with that of the human but this advantage is offset by the difficulty in handling and in intravenous inoculation.

In many respects the rabbit is the animal of choice for, although the temperature mechanism is not as efficient as that of the dog or the cat, the handling, housing and intravenous inoculation present fewer difficulties. The rabbit was therefore used for the estimation of pyrogen throughout this work.

The rectal temperature of the rabbit may be det-:ermined by means of either a clinical thermometer or by a thermocouple inserted rectally. Both methods have been investigated and will be described separately but the thermocouple method was found to be more accurate and reliable and was used.

An examination of the Thermometer method.

The test group consisted of 5 rabbits and food was with/

with-held from one hour before the start of the experiment to assist in stabilising the body temperature. The rectal temperature was measured by means of a clinical thermometer and this temperature was regarded as the normal temperature for the rabbit from which variations throughout the experiment were measured, the solution under test being injected into the marginal ear-vein within 15 minutes of taking this rectal temperature. Variations in body temperature after the injection were detected by determining the rectal temperature each hour for three hours, or more if necessary. Although half-minute clinical thermometers were used they were found to require about one minute to reach the rectal temperature and were therefore allowed to remain inserted for two minutes. The distance of insertion was standardised at 4.3 cm. as it was found that the further the thermometer was inserted the higher, within limits, was the temperature recorded. The dose was based on the weight of the rabbit, solutions being diluted where possible so that the volume injected was 2.0 ml./kgm. of body weight, the animals were weighed to the nearest 50 gm; the diluent in all cases was sterile pyrogen-free normal saline. The needles and syringes were sterilised by boiling in pyrogen-free water/

water for thirty minutes and the syringe washed out with the solution to be tested. The solution was warmed before injection although with the low dosage of 2.0ml./kgm. of body weight the temperature of the solution was found to have little effect on the rectal temperature of the rabbit. After each experiment the syringes and needles were washed out with pyrogen-free water. Fig.1 shows two typical temperature curves obtained by the thermometer method.

Details of the preparation of the pyrogen-free saline are described in appendix 1.

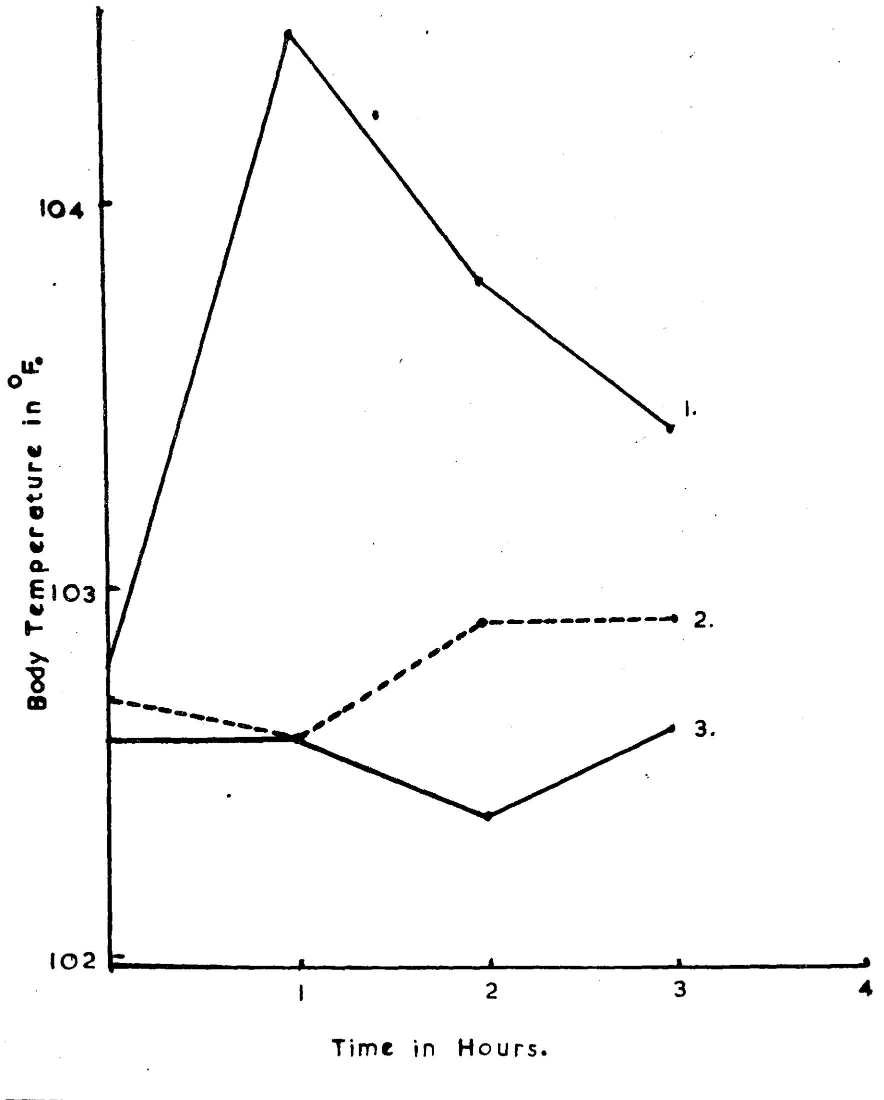


FIG.1. Showing a fever curve and normal temperature curves as obtained by the Thermometer method.

Curve 1 Fever curve.

Curves 2 and 3 Normal temperature curves.

<u>Variation of the normal body temperature of the rabbit as determined by the thermometer method</u>.

To decide whether an observed rise is a normal or an abnormal rise it is necessary to know to what extent the body temperature of the rabbit fluctuates under the conditions of the test. In this animal the temperature controlling mechanism is reported to be very sesitive to physical stimuli such as exercise or awkward handling and to emotional stimuli such as fear and excitement (Seibert 1923c). It was therefore necessary to find if the actual injection which may be accompanied by a certain amount of pain, caused any appreciable fluctuation in the rectal temperature. The rectal temperatures of the rabbits in two parallel series of tests were determined and compared in Table 1. In one set of tests the rabbits were injected with pyrogen-free saline and in the control experiments the method of handling was the same but the actual injections were omitted.

TABLE 1.

Showing the normal temperature with and without the actual injection.

| | No. of rabbits. | Aver. temp. °F. before injection. | Aver. temp. each hour after injection. | | |
|---|---|---|---|---|---|
| | | | 1. | 2. | 3. |
| Injected. | 32 | 102.7 | 102.5 | 102.5 | 102.5 |
| Not injected. | 30 | 102.8 | 102.5 | 102.5 | 102.7 |

The results show that the pain of the injection causes no detectable rise in temperature in the three hourly determinations after the injection. This has been repeatedly confirmed by the thermocouple method in which the temperature can be determined every few minutes after the injection.

In all, only seven tests on pyrogen-free saline, using five rabbits in each test, have been carried out by the thermometer method. All but one rabbit, the temperature of which rose by 1.2°F., showed a rise of less than 0.6°F. and eleven showed a fall. Considering only the rabbits which showed a rise in temperature, the average rise was 0.3°F. with a standard deviation of ±0.03°F., these results are well within the limits set by the/

by the B.P.,1948, and the U.S.P., XIII, which state that a rise must be greater than 1.0°F. before it can be considered abnormal.

Disadvantages of the thermometer method.

The thermometer method was not used as it associated with three main disadvantages:-

1. The performance of the test by this method necessitates a good deal of handling of the animal which is undesirable in some rabbits, as fluctuation of the rectal temperature is caused.
2. In agreement with the results of Lee (1939) who showed that exercise of even short duration could cause a rapid rise in rectal temperature of the rabbit, it was found that the exercise caused by chasing the rabbit about the room for a few minutes stimulated rises between 1.1°F. and 1.9°F., and, as will be shown later, immobilisation can cause an appreciable fall in rectal temperature. Thus it would seem, that in the rabbit, exercise plays an important part in the maintenance of body temperature, and variation in the amount of energy expended by the free rabbit during a test, which may last from 4 to 7 hours, could in itself cause changes in the rectal temperature. In the thermometer method, as the animal is not immobilised during the test, there is no/

no means of standardising the degree of activity either during one test or throughout a series of tests.

3. Repeated insertion of a thermometer into the rectum may set up local irritation with consequent rise in local temperature and it is therefore inadvisable to make determinations more frequently than every 45 minutes, but, as it will be shown later, the peak temperature of the fever is reached from 70 to 110 minutes after the injection, the average time being 86 minutes, and thus unless the temperature determinations are made every 10 minutes the peak temperature may easily be missed.

The details of the Thermocouple method.

In this method, which was used for later determinations, each of the five rabbits used for one test is immobilised in a wooden box and a thermo:couple fixed in the rectum by wiring it tightly to the tail, the distance of insertion in this method being standardised at 5.5 cm. After immobilisation for one hour in which time the animals usually settle down comfortably, the normal temperature of each rabbit is determined and the solution injected into the ear-vein. The rectal temperature is then measured 30,40 and 60 minutes after the injection and thereafter every 10 minutes until the peak of the fever is reached and passed/

passed. Fig.2 shows a few fever curves which are typical of those obtained by this method. The volume injected was standardised at 2.0 ml./kgm. of body weight where possible and the treatment of the needles and syringes and the preheating of the solutions were carried out as in the thermometer method.

The box used for the immobilisation of the rabbits is described in detail in appendix 2.

The thermocouple circuit consisted of ten thermocouples, each of which could be switched into the circuit by means of a rotary switch, a reference junction immersed in a thermostatically controlled water-bath at about 35°C. with a temperature fluctuation of ±0.01°C. about the mean, and a calibrated Cambridge 'Spot' galvanometer of 50 ohms resistance to measure the difference in temperature between the thermocouple inserted in the rabbit and the reference junction. Details of the thermocouple circuit used and of the calibration and accuracy of the circuit are given in appendix 3.

The thermocouple method has a number of advantages over the thermometer method:

1. Once the animal is immobilised in the test-box no further handling is required.

2. Immobilisation assures the necessary standardisation of the amount of energy expended by the animal in movement/

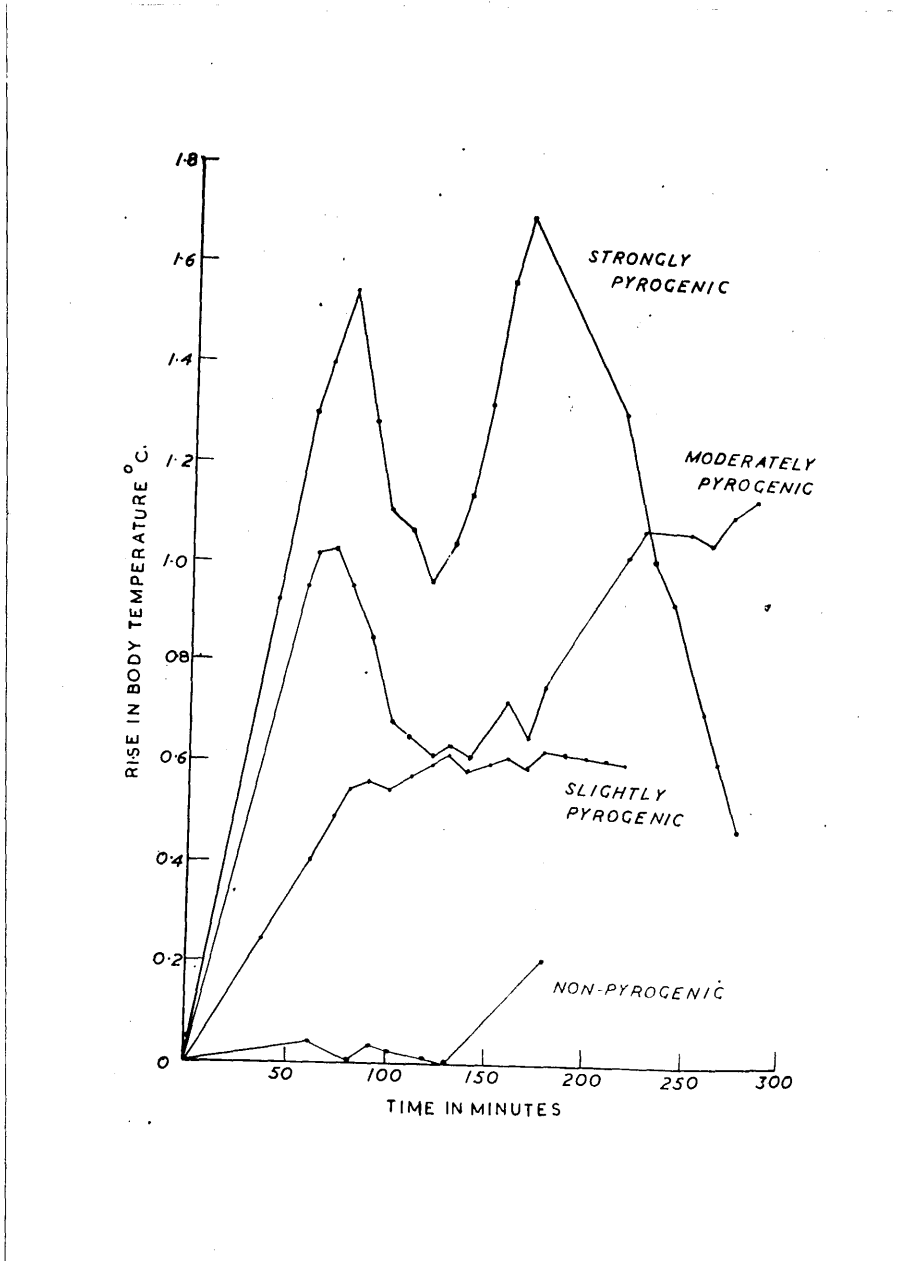


FIG. 2. Typical fever curves as obtained by the Thermocouple method.

movement. This is important as the body temperature fluctuates rapidly in response to exercise.

3. To detect the peak temperature it is necessary to determine the temperature every ten minutes, such frequent insertion of a thermometer causing irritation might lead to false results and in the U.S.P.,XIII method, in which the thermometer method is used, it is stated that the temperature should only be determined once each hour after injection. Fig.3 shows fever curves obtained by determining the temperature every ten minutes by the thermocouple method, the errors in these particular curves which would be caused by taking the temperature each hour only have been shown by joining the hourly points. The B.P.,1948 advocates determinations at least every 45 minutes after the injection, and Fig. 3 also shows how this has some effect on the readings but does not in all cases eliminate the error.

Of the two methods described it is clear that the thermocouple method is more accurate and reliable although it calls for more equipment and greater skill in maintaining its accuracy.

Assessment of results using the thermocouple method.

After the injection of pyrogen into a group of five rabbits the average rise in body temperature for the group can be calculated in two different ways:

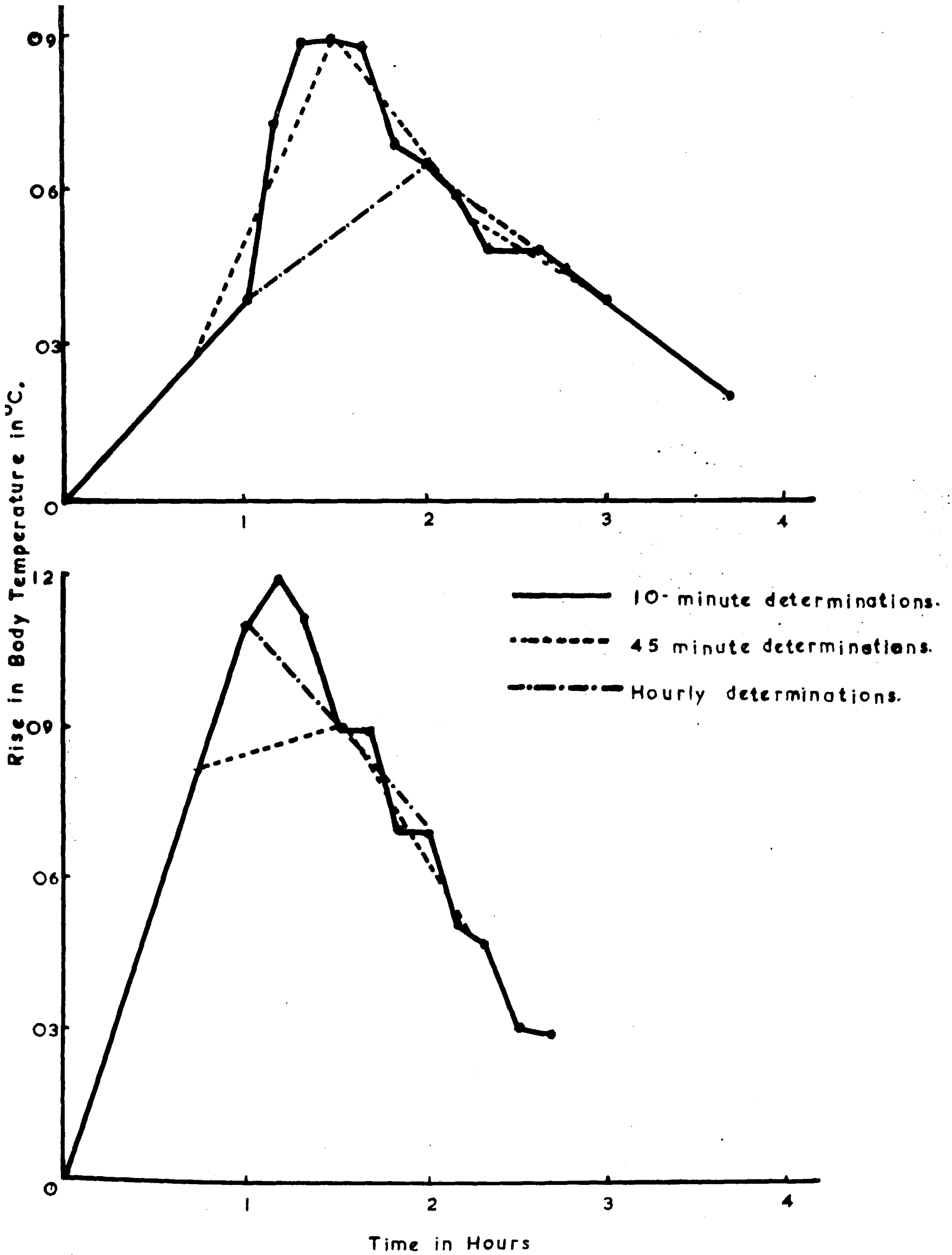


FIG.3. Showing error introduced by B.P.1948,and U.S.P.XIII, methods.

a. The average maximum rise, which is the average of the maximum rises attained by each of the five rabbits within 3½ hours of injecting the solution.

b. The maximum average rise, here the average rise in temperature for all of the five rabbits at each ten-minute determination is calculated and the maximum of these average rises is the maximum average rise. Due to the fact that the time to reach the peak temperature varies from rabbit to rabbit the maximum average rise is often lower and never greater than the average maximum rise as is shown in the following results from 15 experiments:-

| | |
|---|---|
| Average maximum rise | 1.06°C. |
| Maximum average rise | 1.00°C. |

The difference of the two results from each of these experiments ranges from 0.00 to 0.12°C. and although this difference is not very great in the test for the detection of pyrogen it is definitely significant in the quantitative test. As it was found that the average maximum rise was more consistent than the maximum average rise it was decided to use the former both in testing for the presence of pyrogen and in estimating the relative quantities present in a solution.

As the principle of the test lies in the detection of an abnormal rise in body temperature it is important to/

to know to what extent the body temperature of the normal rabbit can rise when subjected to the conditions of the test without the injection of pyrogen. Of 177 results obtained by testing pyrogen-free saline solutions, 89 per cent. of the animals showed a rise of less than 0.4°C., the average rise for all of the rabbits being 0.23°C. with a standard deviation of 0.13°C. There is therefore a 1-in-3 chance that any one normal rabbit will show a rise of 0.36°C. (mean + standard deviation) or more and only a 1-in-250 chance that five normal rabbits will show an average rise as great as this and thus, under the conditions of the test used in this work, an average rise of 0.36°C. or more in five rabbits is considered to be an abnormal rise.

Summary of the test for the detection of pyrogen.

There are two separate procedures which can be used in the test for the detection of pyrogen:

1. The thermometer method, in which the rectal temperature is determined by a clinical thermometer, and,
2. The thermocouple method, in which the rectal temperature is determined by a thermocouple.

After experimental investigation it was found that the thermocouple method showed many advantages over the thermometer method and this method was therefore used throughout the work.

Using five rabbits in a test group pyrogenic activity is indicated by an average maximum rise of 0.36°C. or more the chance of a false positive result being about 1-in-250.

The normal temperature of the rabbit.

Lee (1939) has shown that, provided the thermocouple is inserted more than 5 cm., there is little or no diurnal variation in temperature and that feeding and environmental temperature have very little effect on the body temperature of the rabbit although rapid fluctuation in environmental temperature did cause fluctuation in the rectal temperature of the rabbit. Our findings agree with those of Lee, it was found that when the thermocouple was inserted 5.5 cm. into the rectum of the immobilised rabbit, environmental temperature and feeding had little effect on the rectal temperature of the rabbit, diurnal variation in temperature could be ignored in this work as all of the actual pyrogen tests were carried out at the same time of day, starting at 9.30 a.m. each day. How much the disappearance of these accepted variations in animal temperature was due to the distance of insertion and how much to the immobilisation was not investigated. It was found necessary, after investigation, to standardise the distance of insertion of the thermocouple to 5.5 cm. as variations in the depth of insertion caused distinct variation in the temperature recorded, the deeper the insertion the higher within limits was the temperature recorded. Exercise and repeated insertion of the thermocouple were the only two factors observed/

observed to consistently cause rapid fluctuation in the temperature.

Comparison of the normal temperature as determined by the thermometer method and by the thermocouple method gave a series of results which are summarised in Table II. The results in this table were obtained by using the same twenty rabbits for a series of tests over a six-month period, the temperature being determined in the tests of the first two months at hourly intervals by the thermometer method, the rabbits being free in their cages throughout the experiments, in the remaining four months the temperature was determined by the thermocouple method in which the animals were immobilised.

TABLE II.

Comparison of the normal temperatures as determined by the clinical thermometer method (free rabbit) and the thermocouple method (immobilised rabbit.)

| | Immob-ilised rabbit | Free rabbit |
|---|---|---|
| No. of temperature determinations . . . . . . . . . . . | 119 | 125 |
| Average normal temperature . . . . . . . . . . . . . . . | 38.5°C | 39.2°C |
| Standard deviation of average normal temperature . . . . . . . . . . . | 0.5°C | 0.28°C |
| Percentage of results below 38.2°C.. . . . . . . . . . | 21 | 0 |
| " " " between 38.2 and 38.8°C.. . . . . | 51 | 12 |
| " " " of 38.9°C. and over . . . . . . | 29 | 88 |

The results show that immobilisation causes a fall in the rectal temperature of the twenty rabbits, in the individual rabbits this fall ranged between 0.2 and 1.4°C., the average fall being 0.7°C. Immobilisation, however, had very little effect on the temperatures of the seven albino rabbits of the group, this, by increasing the range of normal temperatures of the immobilised rabbits, accounted for the greater standard deviation from the average normal of the immobilised rabbits. In the United States Pharmacopoeia XIII it is stated that a rabbit with a normal temperature outside the range of 38.9 to 39.8°C. should not be used in pyrogen testing, but Table II shows that although only 12 per cent. of the temperature determinations by the thermometer method are outside this range, by the thermocouple method 72 per cent. are outside this range.

## The normal temperature of the rabbit as determined by the thermocouple method.

When using a rabbit in a pyrogen test the rectal temperature was ordinarily determined before the injection of the solution. By compiling these results over a period of two years the results in Table III were obtained. This table shows the average temperature and the standard deviations of the averages for 55 rabbits. From the 1561 temperature determinations on the 55 rabbits the weighted average/

## TABLE III

Average temperatures with Standard Deviations for 55 rabbits.

| No.of rabbit | Average normal temp. °C | Stand. Deviat. | No.of results | No.of rabbit | Average normal temp. °C | Stand. Deviat. | No.of results |
|---|---|---|---|---|---|---|---|
| 1 | 38.79 | 0.28 | 35 | 31 | 38.71 | 0.32 | 41 |
| 2 | 38.54 | 0.22 | 9 | 33 | 38.28 | 0.44 | 32 |
| 3 | 38.53 | 0.20 | 15 | 34 | 38.22 | 0.32 | 24 |
| 4 | 38.64 | 0.22 | 45 | 35 | 38.12 | 0.28 | 38 |
| 6 | 38.32 | 0.33 | 32 | 36 | 38.49 | 0.40 | 23 |
| 7 | 38.26 | 0.46 | 61 | 37 | 38.45 | 0.36 | 29 |
| 8 | 38.54 | 0.17 | 7 | 38 | 38.36 | 0.32 | 24 |
| 9 | 38.27 | 0.39 | 61 | 39 | 38.36 | 0.37 | 24 |
| 10 | 38.27 | 0.44 | 44 | 40 | 38.42 | 0.22 | 29 |
| 11 | 38.66 | 0.22 | 61 | 41 | 38.91 | 0.32 | 28 |
| 12 | 38.87 | 0.25 | 54 | 42 | 38.20 | 0.32 | 24 |
| 13 | 38.30 | 0.32 | 59 | 43 | 38.60 | 0.37 | 22 |
| 14 | 38.71 | 0.36 | 36 | 44 | 38.30 | 0.35 | 21 |
| 16 | 38.66 | 0.32 | 40 | 45 | 38.42 | 0.26 | 23 |
| 17 | 38.71 | 0.35 | 32 | 46 | 38.69 | 0.17 | 20 |
| 18 | 38.56 | 0.41 | 37 | 47 | 38.90 | 0.55 | 6 |
| 19 | 38.65 | 0.20 | 41 | 48 | 38.75 | 0.26 | 23 |
| 20 | 38.62 | 0.22 | 34 | 49 | 38.53 | 0.20 | 11 |
| 21 | 38.80 | 0.28 | 40 | 52 | 38.23 | 0.39 | 12 |
| 23 | 38.28 | 0.32 | 43 | 53 | 38.04 | 0.30 | 9 |
| 25 | 38.92 | 0.33 | 24 | 55 | 38.25 | 0.30 | 10 |
| 26 | 38.10 | 0.35 | 50 | 56 | 38.57 | 0.20 | 2 |
| 27 | 38.78 | 0.32 | 65 | 58 | 38.40 | 0.22 | 8 |
| 28 | 38.39 | 0.32 | 33 | 60 | 38.43 | 0.26 | 7 |
| 29 | 38.90 | 0.22 | 27 | 61 | 38.19 | 0.35 | 9 |
| 30 | 38.76 | 0.26 | 45 | 63 | 38.91 | 0.14 | 9 |
| 66 | 38.23 | 0.37 | 9 | 64 | 38.21 | 0.26 | 5 |
| | | | | 65 | 38.59 | 0.36 | 9 |

average temperature was calculated to be 38.5°C., the individual determinations ranging from 37.3°C. to 39.62°C. The range of average temperatures was 38.04°C. to 38.92°C. This weighted average temperature is lower than that determined by other workers Seibert (1923c) 39.05°C., Lee (1939) 39.6°C. and Frothingham & Minot (1912) 39.9°C., but these workers used the thermometer technique in which the animal was not immobilised. The average of 39.2°C. obtained by the thermometer technique in this work (Table II) is comparable with their results. The standard deviation of the average temperature for each rabbit (Table III) gives an indication of the day-to-day variation in the normal temperature of each of the rabbits, and it can be seen that a deviation of 0.6°C. from the mean on any one day in a rabbit would be quite usual.

## QUANTITATIVE TEST.

Although the test for the detection of pyrogen, as described in the previous chapter, is sensitive, such a test is not sufficient for a careful investigation of the subject. For comparative purposes it is necessary to have some means of detecting slight differences in the pyrogenic activity of two or more solutions and the preparation of a Standard was first undertaken.

### Preparation of Standard pyrogenic solutions.

In preparing a standard two difficulties were encountered;

1. The chemical nature of the substance, or substances, involved are not known, and
2. From previous workers it would seem that different species of bacteria may produce different pyretic substances, differing not only in chemical nature but also in the type of fever which they stimulated.

In the absence of any information on these two points it was decided to use a solution of pyrogen prepared from Pseudomonas aeruginosa as the standard. In the course of this work five standards have been prepared.

Standard 1. was a suspension of Pseudomonas aeruginosa (B.pyocyanea) in water, obtained by scraping the growth from an agar slope into a few ml. of sterile water and adding, with precautions to prevent contamination, to 4 litres/

litres of sterile freshly distilled water. After incubation at 37°C. for 16 days the solution was passed through ordinary filter paper to remove any trace of the agar added with the organism. The filtrate was made isotonic with blood by the addition of 0.9 gm. per 100 ml. of pure sodium chloride and distributed in 100 ml. quantities in 6 fl.oz.screw-cap bottles. The bottles were sealed by plugging with cotton-wool and screwing on metal caps and the final product ster:ilised by autoclaving at 115°C. for thirty minutes. When stored at room temperature and in darkness this solution retained its potency for at least six months. (see Table VIII)

Standard II. Synthetic medium composed of acid-hydrolysed gelatine to which had been added the missing essential amino-acid and growth factors, was inoculated with Pseudomonas aeruginosa. After incubation at 37°C. for 48 hours, the bacterial cells were removed by filtration through a Berkefeld Filter Candle of porosity W. The filtrate was distributed in 1 ml.amber anpoules and sterilised by autoclaving at 115°C. for thirty minutes. This Standard, when freshly prepared, was about 100 times the strength of Standard I, but rapidly lost potency even when stored at just above freezing temperature.

Standards III and IV. These were made in the same way as Standard 1 but the final product was in each case too dilute/

dilute to be of any practical value as it required injection of too large a volume to stimulate a reaction in the quantit-:ative range.

Standard V Standard II had had the advantage over the other standards in that it could be prepared in a relatively short time (48 hours) and could be diluted to whatever dose was required while the volume injected was standardised at 2.0 ml./kgm. of body weight, however, due to its high concentration the rate of destruction on storage was very rapid. Standard V was therefore prepared by growth of Ps. aeruginosa in synthetic medium as for Standard II, but the filtrate was diluted 1 in 150 before bottling and sterilising and thus brought to the same strength as Standard 1 in the hope that at such concentration the rate of destruction would be slow. This Standard was prepared near the end of the work and therefore no long-term examination of the rate of loss of potency was possible but no deterioration in potency was detected during two months of its use and it was therefore much more stable than Standard II.

The quantitative test. The proceedure used was identical with that used in the determination of the presence of pyrogen in a solution, as already described. In the qualitative test a positive result was indicated by an average rise of 0.36°C. or more in five rabbits, in the quantitative test however, the average maximum rise was measured and by

use/

use of a dose/reaction curve for a standard pyrogenic solution the potency of the solution under test could be compared with the Standard.

Construction of test groups.

Animal variation in biological methods is the sum of two variables, namely.

1. the inherent difference in sensitiveness between rabbits,
2. the day-to-day variation in individual response.,

and it is therefore usual to use a large number of animals in a test-group in an attempt to diminish the variation caused by the differences in sensitiveness of the animals and to standardise the conditions of testing, housing and diet in an attempt to diminish the day-to-day variation. As the rabbit is the animal used in this work it is impracticable to use large numbers in each test-group, fortunately, as the animals could be used repeatedly, the average response for each animal and the day-to-day variation from that response could be determined and by this means an approximate value for the two variables mentioned above could be obtained (Table XV, page 119 ). As the standard deviation from the average response for each rabbit (Table XV) was quite small it was thought possible to construct test groups of appoximately equal sensitiveness with 5 rabbits in each test group. In practice this was found to be quite possible. The animals to be grouped were injected with a given dose of a/

a standard pyrogenic solution and the response of each animal calculated as a percentage of the average response of all the rabbits injected, from five or six such experiments the average percentage response for each animal was calculated, and from these results groups of approximately equal sensitiveness were constructed. On three separate occasions thirty rabbits were grouped in fives and only two groups were different in reaction from the others, in such cases the group was either re-arranged or not used for quantitative work. Each group when so constructed was then standardised with a Standard pyrogen solution as will be described later. Rabbits new to the test were liable to give rather erratic responses in the first test or two and this must be taken into account when calculating the average percentage response.

Having grouped the rabbits in sets of five, experiments were carried out to determine:-

1. To what extent any one group of rabbits varied in response to a given dose of Standard pyrogen and whether the different groups constructed responded equally to a given dose of the Standard.

2. The relationship between the dose of pyrogen and the reaction produced.

3. The maintenance of the response by groups of rabbits to a Standard over a period of time.

These points determined the accuracy of the comparison

of/

of two solutions, whether both these solutions were tested on the same group on different days or, as is often more convenient on different groups on the same day.

<u>Variation in response of groups of rabbits to the Standards.</u>

Of the five Standards prepared only I, II and V were of any practical value. Unfortunately these three Standards could not be tested on the same groups of rabbits and thus no direct comparison of their strengths could be made. The results obtained for various doses of each Standard are shown in Tables IV, V and VI. In the light of the experiences gained by the use of Standards I and II it was thought unnecessary to test any more than two dose levels of Standard V, this being sufficient to standardise the groups and to construct the log dose/reaction curve which will be shown later to be a straight line. These tables of results show that the individual group reactions do not vary greatly, that the groups within each set of results respond equally to the Standards and that the variation of the average reactions within a group is as great as the variation of average reactions between groups. It would thus appear that the reaction of one of the constructed groups could be directly compared with the reaction of another. The results also show that there is much less variation in response to doses stimulating rises/

## TABLE IV

Reactions obtained using Standard I, showing the average maximum rises for each group in °Centigrade.

| Group | Dose in ml./kgm. of body weight. | | | | | |
|---|---|---|---|---|---|---|
| | 5.0 | 2.0 | 1.0 | 0.5 | 0.33 | 0.25 |
| I | 1.31<br>1.26 | 0.98<br>0.98 | — | — | 0.72 | 0.89 |
| II | 1.20<br>1.27 | 1.05 | 0.68 | 0.83<br>0.54 | 0.54 | — |
| III | 1.32<br>1.16 | 1.02<br>0.97 | 0.80 | 0.71 | 0.61 | 0.50 |
| Average | 1.25 | 1.00 | 0.74 | 0.69 | 0.62 | 0.70 |

## TABLE V

Reactions obtained using Standard II, showing the average maximum rises for each group in °Centigrade.

| Group | Dose in ml./kgm. of body weight. | | | |
|---|---|---|---|---|
| | 0.08 | 0.04 | 0.02 | 0.01 |
| IV | 1.26 | 0.95<br>1.03 | 0.74 | 0.38 |
| V | 1.21 | 1.02<br>0.94 | 0.75 | 0.55 |
| VI | 1.15 | 0.97 | — | 0.40 |
| VII | 1.20 | 1.03 | — | 0.54 |
| Averages | 1.20 | 0.99 | 0.74 | 0.47 |

TABLE VI

Results obtained using Standard V, showing the average maximum rises for each group in °Centigrade.

| Group | Dose in ml./kgm. of body weight. | |
|---|---|---|
| | 2.0 | 4.0 |
| VIII | 0.93 | 1.11, 1.13, 1.08 |
| IX | 0.83, 0.82 | 1.09, 1.12, 1.11, 1.06 |
| X | 0.84, 0.94 | 1.17 |
| XI | 0.85 | 1.09, 1.15 |
| **Averages.** | 0.87 | 1.11 |

rises of 0.75°C. to 1.30°C. than in the responses to doses stimulating rises below 0.75°C. and thus for comparison purposes this is the useful response range. Due to the rapid deterioration in Standard II only a limited number of results, determined in rapid sequence, could be relied upon.

The relationship between the dose and the effect.

When the relationship between the dose and the average effect, as recorded in Table IV was plotted a curve was obtained Fig. 4 which is similar to that for other pharmacologically active substances which stimulate a measurable reaction. Thus it was probable that the curve relationg the logarithms of the doses to the average reactions would tend to be linear; this, however, was only found to be so for the points corresponding to the reactions between 0.75°C. and 1.30°C. as is shown in Fig. 5. The curve representing all dose levels in Table IV could be calculated but as the reactions between o.75°C. and 1.30°C. form the useful quantitative range it is probably more accurate to use only these points in calculating the regression line. This assumption is strongly supported by the fact that the regression lines for Standards II and V through the same range of points are almost parallel to that for Standard I. The equations for these lines are:

| | |
|---|---|
| Standard I | $Y = 0.76 + 0.71X$ |
| Standard II | $Y = 0.53 + 0.75X$ |
| Standard V | $Y = 0.63 + 0.80X$ |

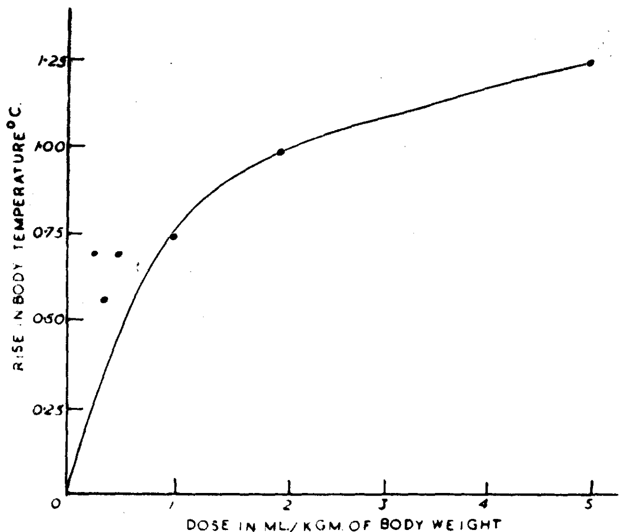


FIG.4. Relationship between the dose of Standard I and the reaction stimulated.

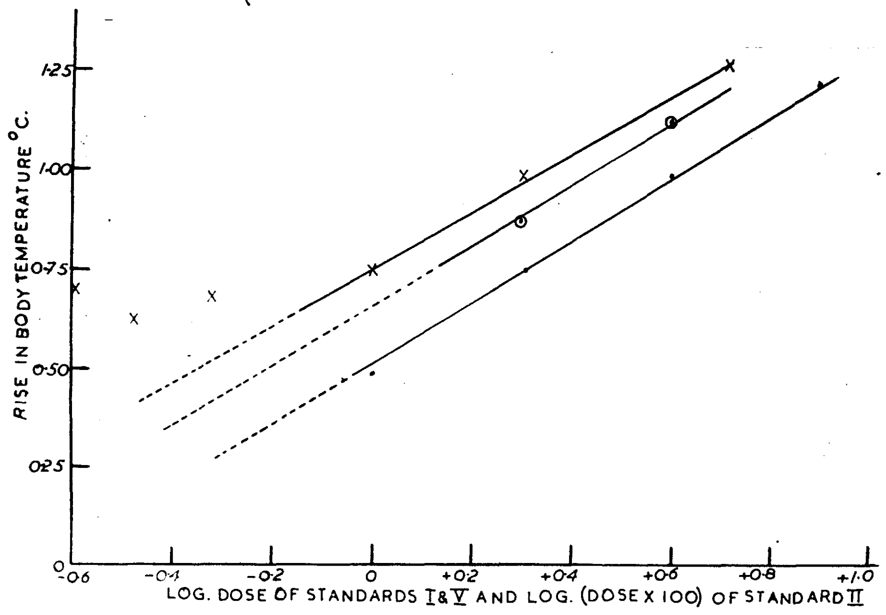


FIG.5. Relationship between the log. of the dose and the reaction stimulated.

Standard I X  Standard II •  Standard V ⊙

The fact that three different Standard solutions on three entirely different sets of animals show almost parallel regression lines would suggest that the method devised for the comparison of two solutions is of value.

Accuracy of the method.

Assuming the regression lines in Fig. 5 to be correct it is possible to assess the accuracy of the results in Tables IV, V and VI. By comparing the theoretical dose, found by use of the regression line for the appropriate Standard, with the actual dose the percentage error can be determined, as is shown in Table VII.

The average percentage error (weighted) when the results in the quantitative range of 0.75°C. to 1.30°C. are considered is 10 per cent. with a Standard deviation of 7.5 per cent. Therefore in 22 repititions of the test on five rabbits one result can be expected to deviate by more than 25 per cent. from the true result and probably only 1-in-100 will deviate by more than 30 per cent. from the true result. The greater percentage errors for the lower dose levels show the importance of not considering suitable for comparison purposes reactions below 0.75°C. when only five rabbits are used in each test.

## TABLE VII

Accuracy of Quantitative Test.

| Average rise in °C. | Dose in ml./kgm. of body weight. | | Percentage error | Aver. %age error | |
|---|---|---|---|---|---|
| | Actual | Theoretical | | | |
| **Standard I** | | | | | |
| 1.31,1.26,1.20<br>1.27,1.32,1.16 | 5.0 | 5.7,5.0,4.2,<br>5.2,6.0,3.6. | 14, 0, 16,<br>4, 20, 28. | 14 | |
| 0.98,0.98,1.05<br>1.02,0.97. | 2.0 | 2.0,2.0,2.5,<br>2.3,2.0. | 0, 0, 25,<br>15, 0. | 8 | Aver. %age 12 |
| 0.68,0.80. | 1.0 | 0.79,1.1. | 20, 10. | 15 | |
| 0.83,0.54,0.71 | 0.5 | 1.3,0.5,0.83. | 160, 0, 66 | 75 | |
| 0.72,0.54,0.61 | 0.33 | 0.87,0.5,0.6. | 162,50,82. | 98 | |
| **Standard II** | | | | | |
| 1.26,1.21,<br>1.15,1.20. | 0.08 | 0.093,0.079,<br>0.068,0.079. | 12, 1,<br>15, 1. | 7 | |
| 0.95,1.03,<br>1.02,0.94,<br>0.97,1.03. | 0.04 | 0.037,0.047,<br>0.046,0.035,<br>0.040,0.047. | 7, 17,<br>15, 12,<br>0, 17. | 11 | Aver. %age 6 |
| 0.74,0.75. | 0.02 | 0.02, 0.02. | 0, 0. | 0 | |
| 0.38,0.55,<br>0.40,0.54. | 0.01 | 0.0066,0.011<br>0.0069,0.011 | 34, 10<br>31, 10 | 21 | |
| **Standard V** | | | | | |
| 1.11,1.13,1.16<br>1.08,1.09,1.12<br>1.11,1.06,1.15<br>1.09,1.17. | 4.0 | 4.0,4.2,4.6,<br>3.6,3.7,4.1,<br>4.0,3.4,4.4,<br>3.7,4.7. | 0, 5, 15,<br>10, 7.5,2.5<br>0, 15, 10.<br>7.5, 17. | 8 | |
| 0.93,0.83,0.82<br>0.85,0.84,0.94 | 2.0 | 2.3,1.7,1.7,<br>1.8,1.8,2.3. | 15, 15, 15,<br>10, 10, 15. | 13 | Aver. %age 10.5 |

Maintenance of group response to the Standards.

Injection of pyrogen had little effect on the general health and condition of the rabbit and even while under the effect of pyrogen the animal continued to be quite active and was always eager to eat its food. Nor did continual use of a rabbit for pyrogen testing have any effect, 14 rabbits used over a period of one year showing no obvious lowering of general health and in no instance was pyrogen observed to cause undesirable after-effects.

The use of a standardised group of rabbits is limited, however, by such factors as:-

a) death or illness; this sometimes, although not often, necessitated the reconstruction and the restandardisation of a group,

b) the practical difficulties of injecting a much-used rabbit in which the ear-veins have become thickened and narrow in the lumen,

c) The possibility of the rabbits developing a resistance to pyrogen.

In practice the groups were reconstructed about once in every three months, new rabbits being introduced to replace these eliminated by factors 'a' and 'b', and the groups were then standardised. However the animals standardised against Standard I were used over a period of six months without regrouping and Table VIII shows the results obtained/

TABLE VIII

Constancy of response over a six-month period.

| Date of test on 5 rabbits | Dose in ml./kgm. bdy.wt. | Average maximum rise °C |
|---|---|---|
| 23/5/47 | 2.0 | 0.98 |
| 28/5/47 | 2.0 | 1.02 |
| 28/8/47 | 2.0 | 0.98 |
| 13/11/47 | 2.0 | 1.05 |
| 19/11/47 | 2.0 | 0.97 |

Throughout this six-month period the animals were used for pyrogen testing on an average of once in every five days but even with such prolonged use the animals did not show any change in response to the Standard. Thus with regrouping and standardisation of the new groups every three months the possibility of the groups changing in response to the Standard was considered remote. An analysis of the change in response of the 14 rabbits used over a period of a year is discussed in Appendix 4.

Effect of over-maximal doses. Subject to certain qualific-:ations, which will be shown later to have no effect on the comparison test, the highest average maximum rise reached by any of the constructed groups was 1.32°C., although individual rises as high 2.0°C. have been obtained. It must be emphasised that this maximum figure of 1.32°C. is for the/

the groups which were constructed to be equally reactive, groups chosen at random may produce average rises greater than this. Over-maximal doses have been tested and even with doses twenty-five times the maximal dose, the average reaction was never greater than that produced by the maximal dose. Thus, in quantitative testing, if the dose injected stimulates a reaction of 1.25°C. or more it is necessary to retest a smaller dose to find if the reaction of 1.25°C. is within the quantitative range.

<u>Summary of the quantitative test.</u>

Considering the fact that rabbits can be used repeatedly and can therefore be standardised in groups which do not appear to change in reaction by repeated use over three months nor show a very great day-to-day variation in reaction to a Standard pyrogen solution, the pyrogenicity of a solution can be compared with that of a Standard without simultaneously carrying out a duplicate test using the Standard, provided the comparison is made by means of the regression line constructed for that Standard when tested on the animals used in the comparison test. Although it is the usual proceedure in bioassays to carry out control tests at the same time, using a Standard, such a proceedure in this work was considered unnecessary.

The accuracy of the test would undoubtedly be increased by such control tests and more so by a test of a cross-over type in which the groups of animals are reversed in relation to standard and test, but the quantitative test without the accompanying control test was found to be sufficiently accurate for the purposes of this work and it was decided that any advantage of increased accuracy would be, under the circumstances, far outweighed by the disadvantage of the loss of time. Where it was essential to have a more completely reliable and more accurate estimate of the potency, the solution was tested at two or three different dose levels, but, in so far as the work has progressed up-to-date, such a proceedure has not been often necessary.

From the results obtained by use of the Standard solutions the average percentage error was calculated to be 10 per cent. with a Standard Deviation of 7.5 per cent.

## SOURCE OF PYROGEN.

Co Tui (1942) investigated the production of pyrogen by various bacteria and was unable to correlate pyrogen-production with pathogenicity, pigment production or reaction to gram stain. He only recorded the presence or absence of pyrogen, however, and did not attempt to estimate the amounts of pyrogen produced by the different species examined. Other workers such as Probey and Pittman (1945) and Hort & Penfold (1912c) have also investigated various bacterial sources and from such work it is clear that the property of pyrogen-production is very widespread among bacteria, but other classes of micro-organisms have not been examined. The following experiments were carried out with a view to determining which of the following bacteria and also which, if any, of common yeasts and moulds produced pyrogen and the relative quantities produced when grown at optimum temperature in synthetic medium. After growth of the organisms in fluid synthetic medium the whole cultures were tested in order to detect pyrogen which may have diffused from the organism into the surrounding medium and also pyrogen which might have remained within the cell or exist as part of the cell.

The bacteria and yeasts were grown in a medium composed/

composed of gelatin hydrolysate plus the missing essential amino-acids and other factors necessary for the growth of the micro-organisms tested. The gelatin hydrolysate was prepared by heating powdered gelatin with 10 per cent. sulphuric acid (5 ml. of acid per gm. of gelatin) at 125°C. for 8 hours. The excess acid was removed by precipitation as Berium sulphate and the final product, the gelatin hydrolysate concentrate, was sterilised by autoclaving at 115°C. for thirty minutes. The total nitrogen, estimated by the Kjeldahl method, was 1.64gm. per 100 ml. To prepare the final medium this concentrate was diluted, using pyrogen-free water, until it contained 0.27gm. $N_2$/100ml. and the following growth promoting substances added:

dl-tryptophane.......... 0.0108 gm./L.

$KH_2PO_4$.................. 4.5gm./L.

$FeSO_4(NH_4)_2SO_4$.......... 25ml./L.(0.5gm/100ml.of N/50 HCl)

$MgSO_4$.................. 0.02gm./L.

Glucose................. 2.25gm./L.

the pH was adjusted to 7.5, the solution heated to 80 to 90°C. and filtered. To the filtrate were added:

B-alanine............... 0.3mgm./L.

l-cystine...............10mgm./L.

Thiamin................. 1.0mgm./L.

Pyrodoxin............... 5.0mgm./L.

Ca-d-pantothenate....... 10mgm./L.

Lactoflavin.......................1.0mgm./L.

Nicotinamide......................5.0mgm./L.

Biotin...........................10µgm./L.

These growth factors were stored in stock solution from which the required quantity was added. The medium was finally sterilised by autoclaving at 115°C. for thirty minutes.

This medium was used for the growth of the bacteria listed in Table IX except C. diphtheriae which required the further addition of 2.5mgm./L. of oleic acid, and L.casei and the two yeasts which required the further addition 20 mgm./L. of Inositol.

The bacterial inoculum was small in all cases and all of the organisms tested showed evident growth. After incubation at optimum temperature for 5 weeks the cultures were distributed in 10 ml. ampoules and sterilised by autoclaving at 115°C. for thirty minutes.

The moulds were cultivated in Czapeck-Dox medium by incubation at 25°C. for three weeks. The medium was then distributed in 10 ml. ampoules and a little of the sporing felt added to each so that any pyrogen present within it would be detected. These cultures also were sterilised by autoclaving at 115°C. for thirty minutes.

The sterilised cultures were tested by injecting intra:venously 2.0ml./kgm. of body weight of a suitable dilution into 5 rabbits and determining the average maximum rise in body/

# TABLE IX

Pyrogen production by some micro-organisms.

| Organisms | Gram stain | Medium | Dose in ml./kg. body weight | Average rise in ° C. |
|---|---|---|---|---|
| *Bacillus subtilis* .. .. .. | positive | G.H. | 0·20 | 0·94 |
| *Bacillus megatherium* .. .. | positive | G.H. | 0·20 | 0·72 |
| *Bacillus anthracis* .. .. .. | positive | G.H. | 0·20 | 1·27 |
| *Bacillus aerosporus* .. .. .. | positive | G.H. | 0·20 | 0·83 |
| *Bacillus mycoides* .. .. .. | positive | G.H. | 0·10 | 0·82 |
| *Corynebacterium diphtheriæ* (*gravis*) | positive | G.H. | 0·20 | 0·68 |
| *Corynebacterium diphtheriæ* (*mitis*) | positive | G.H. | 0·20 | 0·48 |
| *Lactobacillus casei* .. .. .. | positive | G.H. | 0·40 | 0·86 |
| *Staphylococcus aureus* .. .. | positive | G.H. | 0·02 | 0·95 |
| *Micrococcus tetragenus* .. .. | positive | G.H. | 0·04 | 0·85 |
| *Streptococcus lactis* .. .. .. | positive | G.H. | 0·20 | 0·54 |
| *Azotobacter chroococcum* .. .. | positive | G.H. | 0·20 | 1·15 |
| *Actinomyces albus* .. .. .. | positive | G.H. | 0·40 | 0·87 |
| *Pseudomonas fluorescens* .. .. | negative | G.H. | 0·02 | 0·73 |
| *Pseudomonas æruginosa* .. .. | negative | G.H. | 0·0002 | 1·32 |
| *Serratia marcescens* .. .. .. | negative | G.H. | 0·006 | 1·16 |
| *Serratia kielensis* .. .. .. | negative | G.H. | 0·004 | 1·17 |
| *Proteus vulgaris* .. .. .. | negative | G.H. | 0·02 | 1·32 |
| *Proteus morganii* .. .. .. | negative | G.H. | 0·002 | 0·95 |
| *Eberthella typhosa* .. .. .. | negative | G.H. | 0·004 | 1·12 |
| *Escherichia coli* .. .. .. | negative | G.H. | 0·002 | 1·32 |
| *Alcaligenes fæcalis* .. .. .. | negative | G.H. | 0·002 | 1·06 |
| *Saccharomyces cerevisiæ* .. .. | | G.H. | 0·40 | 0·10 |
| *Saccharomyces ellipsoideus* .. | | G.H. | 0·40 | 0·26 |
| *Aspergillus glaucus* .. .. .. | | C.D. | 0·40 | 0·29 |
| *Aspergillus nidulans* .. .. | | C.D. | 0·40 | 0·26 |
| *Aspergillus niger* .. .. .. | | C.D. | 0·40 | 0·38 |
| *Penicillium patulum* .. .. | | C.D. | 0·40 | 0·36 |
| *Penicillium terrestre* .. .. | | C.D. | 0·40 | 0·32 |
| *Penicillium glaucus* .. .. | | C.D. | 0·40 | 0·10 |
| *Mucor* (species unknown) .. .. | | C.D. | 0·40 | 0·40 |
| Gelatin hydrolysate medium (G.H.) | | G.H. | 0·40 | 0·36 |
| Czapeck-Dox medium (C.D.) .. | | C.D. | 0·40 | 0·30 |

body temperature; the results are recorded in Table IX Control tests on the gelatin hydrolysate (G.H.) medium and the Czapeck-Dox (C.D.) medium carried out are also shown. A rise greater than 0.36°C. was regarded as a pyrogenic response. Under the conditions of the experiment all of the bacteria tested showed the production of pyrogen, whereas the yeasts and moulds showed no detectable quantities. The gram-negative bacilli showed much greater powers of pyrogen-production than the gram-positive types and it is interesting to note that C.diphtheriae, a highly pathogenic organism, stimulated very little pyrogenic response.

## THE RATE OF PRODUCTION OF PYROGEN

Although a systematic investigation of the rate of pyrogen production has not been carried out the following experiments show certain facts which are worth recording.

Serratia marcescens (B.prodigiosis) was grown at 37°C. in the gelatin hydrolysate synthetic medium already described. Samples were withdrawn at various intervals and viable counts made by the roll-tube method. The remainder of the sample was freed from cells by filtration through a Berkefeld filter and the filtrate sterilised by autoclaving at 115°C. for thirty minutes. A suitable dilution of each sample was then tested for pyrogen content, the results are shown in Table X. Unfortunately many of the samples were not sufficiently diluted to bring them within the quantitative range as is noted in the table and thus no direct comparison can be made between the batches. Nevertheless the results show that considerable quantities of pyrogen are produced in the first seventeen hours and therefore that at least some of the pyrogen is produced by the metabolic processes of the living organism. Due to the difficulty of keeping the dose within the quantitative range no other definite conclusions can be deduced from the results with the other samples.

TABLE X

The rate of production of pyrogen in a culture of Serratia marcescens.

| No. of hours at 37°C | Viable count in millions per ml. | Dose in ml./kgm. bdy.wt. | Average maximum rise °C | Remarks |
|---|---|---|---|---|
| 17 | 556 | 0.0027 | 0.80 | Within quantitative range |
| 65 | 1390 | 0.02 | 1.32 | Over-maximal dose |
| 115 | 472 | 0.004 | 1.06 | Over-maximal dose |
| 283 | 250 | 0.0027 | 1.15 | Over-maximal dose |
| 619 | --- | 0.0027 | 1.05 | Over-maximal dose |
| 840 | --- | 0.0007 | 1.20 | Over-maximal dose |

In another set of experiments carried out using Proteus vulgaris cultures of various ages it appeared that a seven-day culture contained approximately five times as much pyrogen, as a 28 day culture and in an investigation of the stability of old and fresh cultures of Proteus vulgaris, thirty-one day and two-day cultures, the thirty-one day culture was approximately fifteen times the strength of the two-day culture and approximately equal in strength to the twentyeight-day culture of the previous experiment. By combination of these results it would appear that a seven-day culture was approximately seventy-five times the strength of a two-day culture and five times the strength of a twenty-eight-day culture and although such figures cannot be taken as a true measurement of the relative strengths of the cultures they do show that the pyrogenic/

pyrogenic content increases until about the seventh day and thereafter seems to decrease.

## FEVER CURVES.

During a survey of previously published work it was noticed that the fever curves stimulated by the injection of pyrogenic solutions into rabbits could be placed in one or other of three main classes:

1. Curves in which the body temperature started to rise shortly after the injection, reached a peak and then returned to normal again.
2. Curves in which the body temperature started to rise shortly after the injection as in (1) then fell slightly but instead of returning to normal rose to a second peak which was then followed by a return to normal.
3. Curves in which there seemed to be a delay before the body temperature started to rise to its peak, this again being followed by a return to normal. In some of the fevers of this latter type the temperature fell distinctly during the initial period of no reaction, in a few cases this fall being so great that the animals collapsed and sometimes died (Seibert 1925 and Hort & Penfold 1912c)

None of the workers in this field appear to have observed any significant difference between the single-and double-peak types of fever curves which appeared in graphical form in their publications for there is no comment in the text of their papers, possibly because the same culture under what appeared to be exactly the same conditions could stimulate both/

both the single and double peak-types of fever. It will be shown later that this was due to the method used by these workers in determining the temperatures of the rabbits at hourly intervals only. In the cases in which a fall in body temperature led to collapse of the animal and sometimes to death, toxins present in the bacterial cultures rather than the pyrogen were stated by Seibert 1925 and Hort & Penfold 1912 (c) to be the probable cause.

In the course of this work the three types of fever curves already discussed were obtained, and, as the work proceeded it became apparent that the differences in the curves were significant and were not caused by either biological variation or by the method of temperature determination but by actual differences in the constitution of the solutions. In experiments in which a fall in body temperature occurred after injection of solutions the cause was found not to be associated only with the delayed type of fever as is suggested in the literature but to be due to a depressant substance produced by some organisms. After removal of this depressant substance the solutions stimulated the usual types of fevers.

Typical examples of the three types of fevers are given in Figure 6..

In the single-peak curve (type 1) in which the rise in/

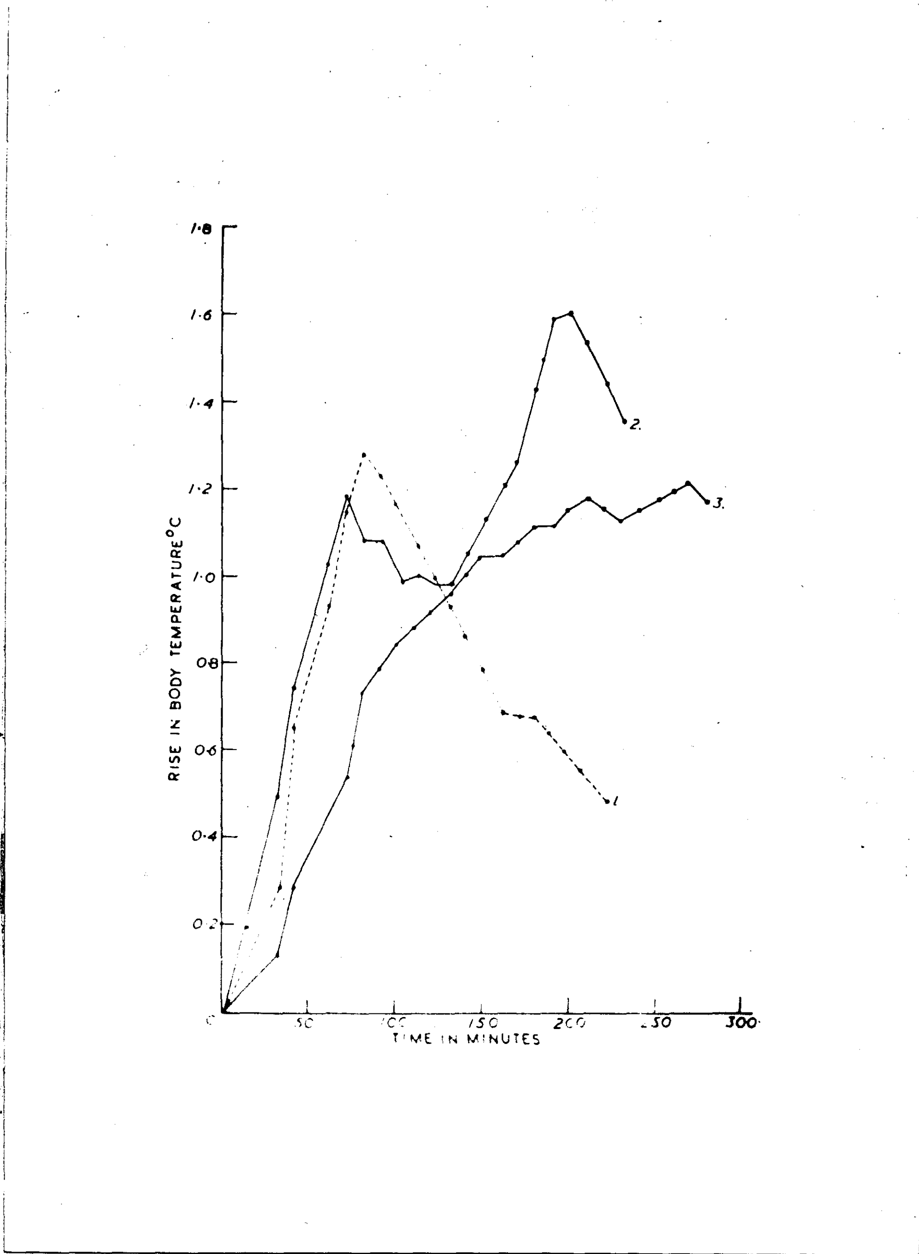


FIG.6. Typical examples of three types of fever curves stimulated by pyrogenic cultures.

in temperature is immediate, the peak temperature is reached in 70 to 120 minutes after the injection with an average of 86 minutes. In the double-peak fever curve (type 2) the first peak is identical to the previously described curve, but falls slightly to be followed by a second rise reaching its peak in three to four hours after the injection. In the delayed-peak curve (type 3) the time to reach the peak temperature is similar to that of the second peak in the double-peak curve.

From a study of the time to reach the peaks it would seem that the double-peak curve is a combination of the two single-peak curves.

The effect on the shape of the curve caused by determining the rectal temperature at hourly intervals by means of a clinical thermometer, the technique used by most of the investigators, is very marked. Because irritation is set up by two frequent insertion of a thermometer into the rectum most workers advocated the determination of the temperature at hourly intervals only. Fig.7 demonstrates how completely the shape of the curve is masked by this method and it is probably due to this that no significant conclusions were drawn from the shape of the curves because slight changes in the times to reach the peaks could change what appeared to be a single-peak curve in one test to a double-peak curve in a repeat of that test.

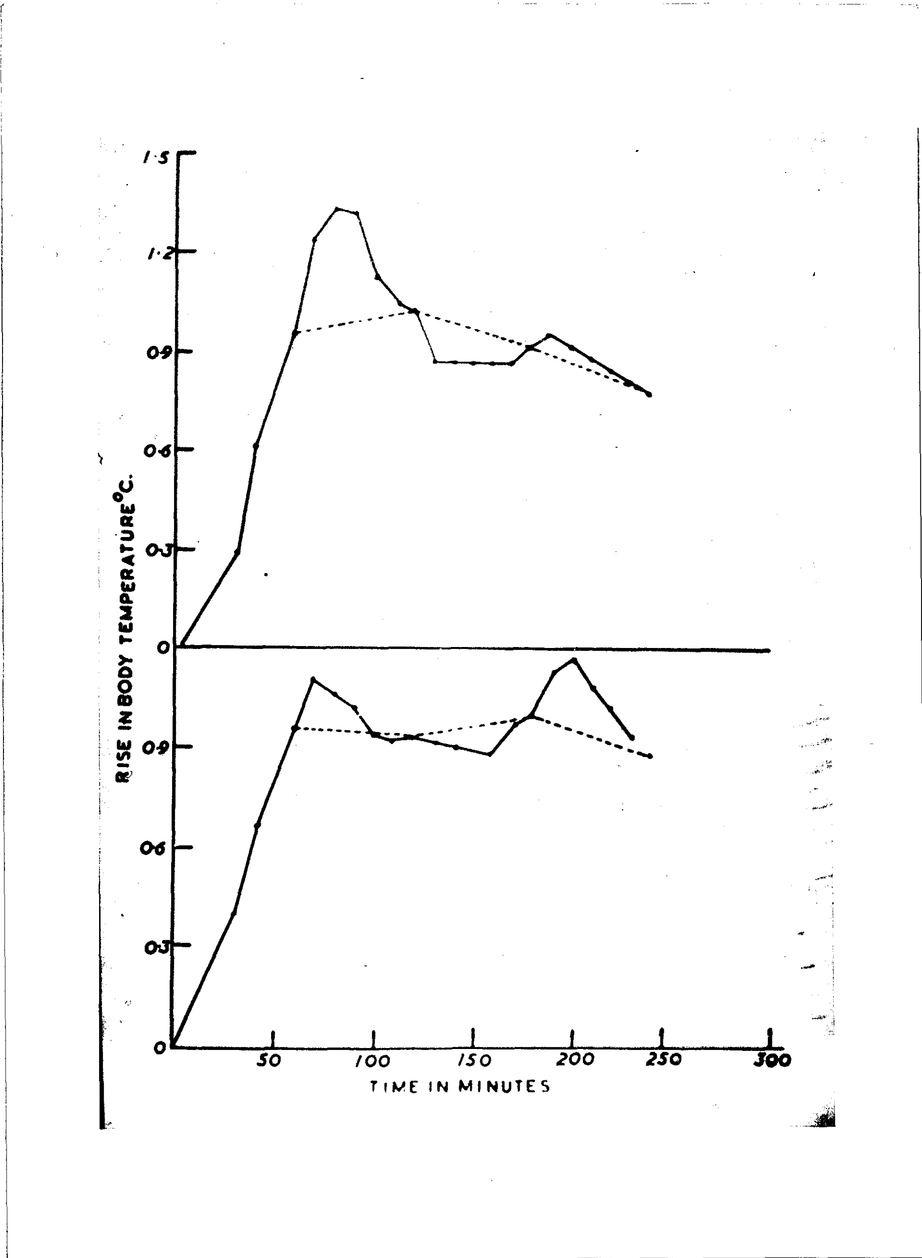


FIG.7. Showing effect of method of temperature determination on shape of fever curves.

Thermocouple method ——•——•——•——

Thermometer method - - -•- -•- -•- -

On theoretical grounds there are four possible explanations for the differences in the shapes of the fever curves stimulated by pyrogenic cultures:

1. That pyrogen in a culture containing the organism and its metabolic products is dissolved both in the medium and within the bacterial cell. Injection of such a whole culture stimulates the double-peak type of reaction, the first rapid rise in temperature being stimulated by the pyrogen dissolved in medium and the second fever stimulated by the slow liberation of pyrogen from the cells as they are broken down in the blood-stream. Therefore the cell-free filtrate should cause the immediate reaction only, and a suspension of the cells the delayed reaction.
2. That each of the three types of fevers is stimulated by a different substance.
3. That the fever showing the immediate response is stimulated by one type of pyrogen, that the delayed response fever is stimulated by another type and that the double-peak response is caused by a mixture of the two substances.
4. That the actual dosage of pyrogen is the controlling factor and that by varying the dose the shape of the curve can be altered.

It is hoped to show which of these explanations is the most reasonable by the experiments which are described below.

Experimental.

A culture of Proteus vulgaris was grown for four weeks at 37°C. in the gelatin hydrolysate synthetic medium and the following experiments carried out:

1. Samples of the whole culture were sterilised by autoclaving at 115°C. for thirty minutes and tested on two different groups of five rabbits at a dose level of 0.002 ml./kgm. of body weight. Both tests showed the double-peak type of fever curve, the average maximum rises being,

Test 1 first peak 1.04°C. second peak 1.46°C.
Test 2 " " 1.24°C. " " 1.64°C.

The fever curve representing one of these tests is shown in Figure 8..

2. Samples of the viable culture were filtered free of bacterial cells by passage through Berkefeld filter candles and the filtrate sterilised by autoclaving at 115°C. for thirty minutes. This filtrate was clear to the eye and no cells could be detected on microscopical examination. At a dose of 0.002 ml. per kgm. of body weight, similar to the dose of the whole culture, this filtrate stimulated only the single-peak fever of the immediate response type as is shown in Figure 8..

3. Samples of the whole culture were sterilised by autoclaving and the cells then separated by centrifuging at/

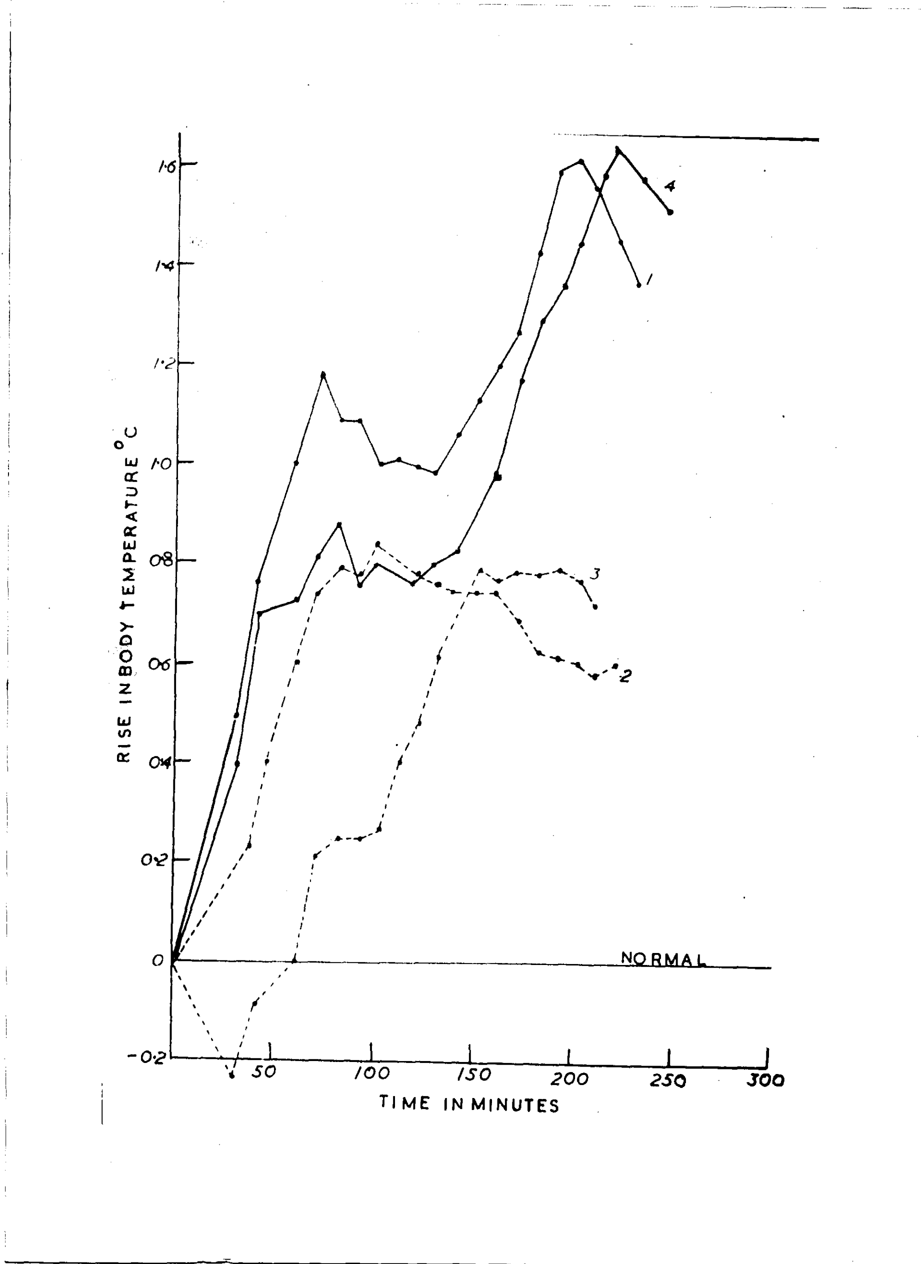


FIG.8. Showing different fever curves stimulated by modifications of a culture of Proteus vulgaris.

Curve 1 - Whole culture
Curve 2 - cell-free filtrate
Curve 3 - Washed bacterial cells.
Curve 4 - Centrifuged supernatant of sterilised culture.

at 4,000 r.p.m. for 45 minutes, the supernatant was decanted off and retained. The cells were then washed three times with pyrogen-free saline, separated by centrifuging each time and the washings discarded. The washed cells were suspended in pyrogen-free saline and the cell-count adjusted to approximately the same as that of the original culture as determined by Brown's Opacity Tubes. Both the supernatant and the suspension of washed cells were tested. The suspension of cells stimulated the single-peak fever of the delayed type and the supernatant the double-peak fever.

From the results of this experiment it is seen that the same culture can stimulate all three types of fever depending on the state of the sample when injected, the fraction stimulating the immediate response, the cell-free filtrate of the viable culture, can be separated from that causing the delayed response (the washed cells) and that combination of the two fractions, i.e. the whole culture, causes the double-peak type of curve. This then tends to discount the theory that there are three separate substances and also the theory that the dosage of pyrogen is the important controlling factor, further proof of this latter point is that removal of the cells has little effect on the first peak but removes the second peak.

The fact that the supernatant liquid from the

centrifuged sample caused a double-peak type of reaction was unexpected, since, if this supernatant were really cell-free, as it appeared to be when examined, then the hypothesis that the double-peak fever curve is attributable to the same pyretic substance being present both dissolved in the medium and in the bacterial cells, the pyrogen in the bacterial cells stimulating the second peak, was not tenable. Since the other results of the experiment supported the hypothesis it was possible that the centrifuged supernatant still contained sufficient cells to cause the second peak. A careful examination of all previous experiments using a variety of organisms showed that some bacterial-free solutions obtained by Berkefeld filtration did in fact stimulate the double-peak type of curve and therefore it became necessary to repeat the above experiments with additional refinements, the most important one being to find how many cells, if any, were necessary to stimulate the second peak and from this whether the double-peak fever which is stimulated by supposedly cell-free solutions could be due to the presence of a few cells or, whether it was really possible to stimulate the double-peak type of curve by injection of a completely cell-free solution.

Escherichia coli was grown on gelatin hydrolysate medium at 37°C. for five weeks and the following tests were/

were carried out on the culture:

1. Samples of the whole culture were sterilised by autoclaving at 115°C. for thirty minutes, these were tested on groups of five rabbits in doses of 0.02, 0.004 0.002 and 0.0002 ml./kgm. of body weight, the fevers stimulated are shown in Figure 9 , the larger doses of 0.02 and 0.004 ml./kgm. of body weight stimulated the double-peak fever but those of 0.002 and 0.0002 ml./kgm. of body weight stimulated only the single peak.

2. Samples of the viable culture were filtered free of cells by filtration through a Berkefeld filter candle, and the filtrate sterilised by autoclaving at 115°C. for thirty minutes. A dose of 0.02 ml./kgm. of body weight was tested on groups of five rabbits on three separate occasions, only the single-peak curves of the immediate response type were stimulated as shown in Fig. 10. However in the previous set of experiments on Proteus vulgaris the supposedly cell-free supernatant which unexpectedly stimulated the double-peak reaction was prepared by sterilising the culture before the cells were removed by centrifugation and to bring the filtration experiments into line with this, samples of the whole culture of E. coli were first sterilised by autoclaving/

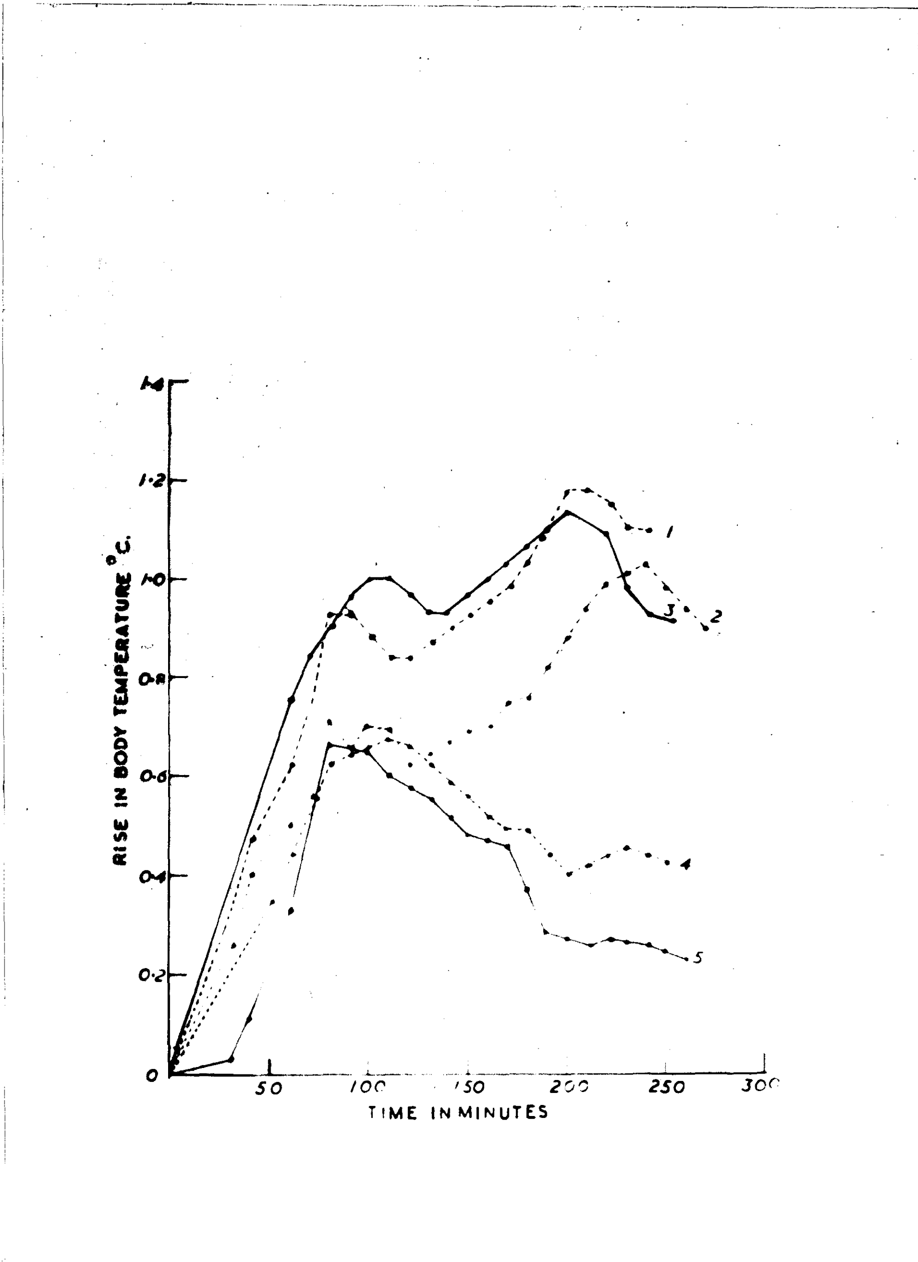


FIG.9. Ellimination of second peak by reducing the dose.

Dose in ml./kgm. of body weight.

Curve 1 0.02 Curve 2 0.02 Curve 3 0.004

Curve 4 0.002 Curve 5 0.0002

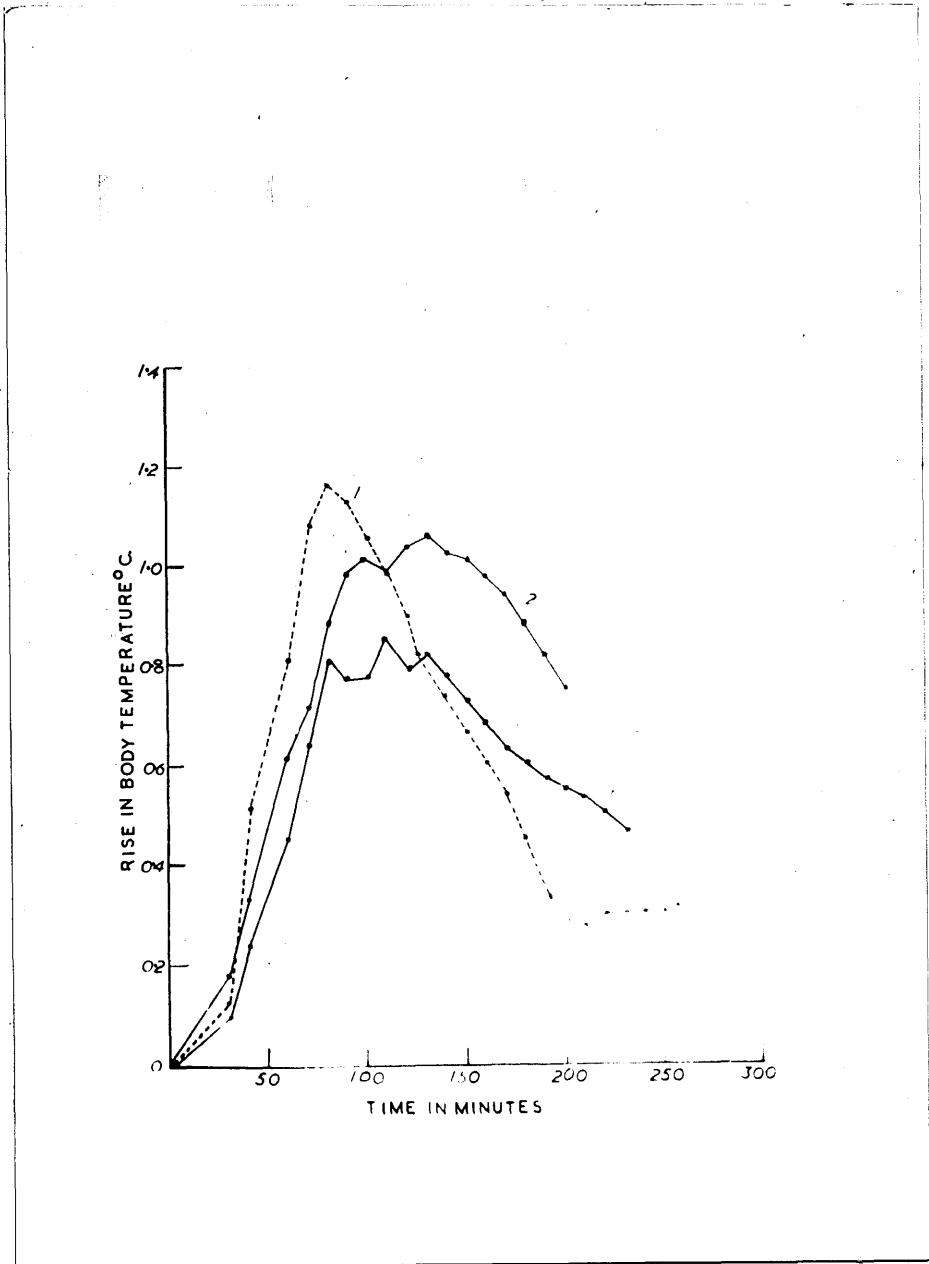


FIG.10. Single-peak fever curves stimulated by cell-free filtrates of a viable culture of Escherichia coli. Dose in ml./kgm.of body weight.

Curve 1 0.04 Curve 2 0.02 Curve 3 0.02

autoclaving and then filtered free of bacterial cells. A dose of 0.02 ml./kgm. of body weight of this filtrate was tested on groups of five rabbits on two separate occasions and the double-peak type of fever was stimulated, Figure 11. Now a dose of 0.002 ml./kgm. of body weight of the whole culture would contain more cells than a dose of 0.02 ml./kgm. of body weight of the filtered culture and yet the whole culture does not stimulate the double-peak fever and the filtrate does, it is therefore obvious that any cells present in the filtrate would not cause the double-peak, thus it would seem that the only possible explanation is that the solutions stimulating the double-peak type of re-action contain two pyretic substances, one causing the immediate rise in body temperature and the other the delayed rise, a mixture of the two causing the double-peak type of fever, and by the fact that the second peak is either eliminated or reduced by removal of the cells, it would seem that most of the substance stimulating the second peak is within the cell, whereas that stimulating the first peak is mainly dissolved in the medium.

3. The tests on the centrifuged supernatant were also carried out using the supernatant of both the viable and the sterilised whole culture. In all cases the centrifuging/

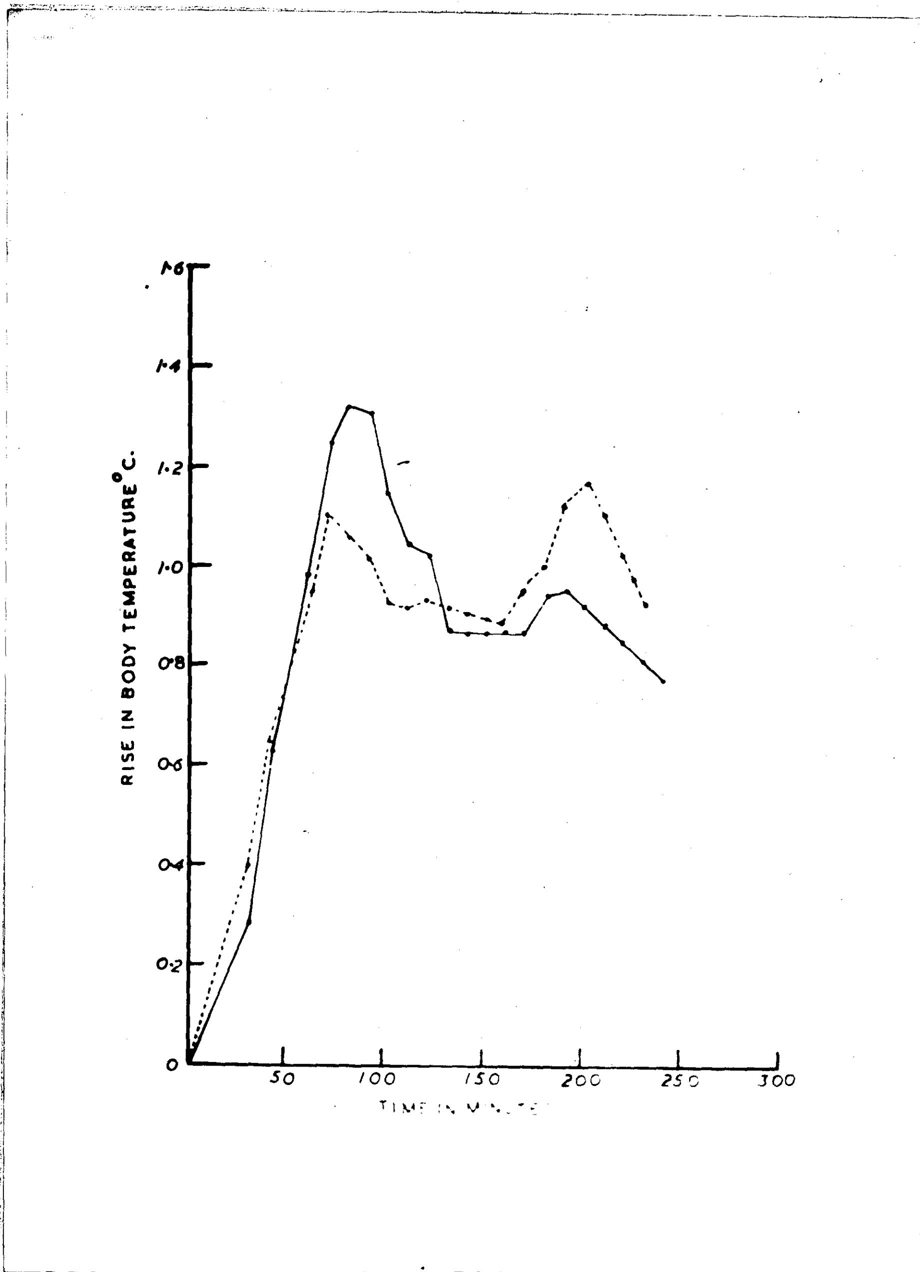


FIG.11.Double-peak fever curves stimulated by cell-free filtrates of an autoclaved culture of Escherichia coli. Dose - 0.02 ml./kgm.of body weight.

centrifuging was at 3750 r.p.m. for $3\frac{1}{2}$ hours in an attempt to produce complete deposition of the cells and no cells could be detected on microscopical examination. On four separate occasions a dose of 0.02 ml./kgm. of body weight of the centrifuged supernatant of the previously sterilised whole culture was tested on groups of five rabbits, the fever curves for these tests are shown in Fig. 12, all of the curves exhibited the double-peak phenomenon although in one case the second peak was rather low, but even in this instance the curve showed more tendancy to conform to the double-peak type than those stimulated by the supernatant from the viable culture. The viable culture was subjected to the conditions of centrifuging and examination described above but in this case the centrifuged supernatant stimulated only the single-peak fevers, none of them showing even a tendancy to rise to a second peak, Figure 13. These results are therefore in direct support of those obtained for the filtrate. The cells obtained by centrifugation of the sterilised whole culture were washed three times with pyrogen-free saline and finally diluted with the required amount of saline to bring the cell-count back to approximately that of the original culture and tested at a dose of 0.02 ml./kgm. of body weight. The fever stimulated was of the delayed/

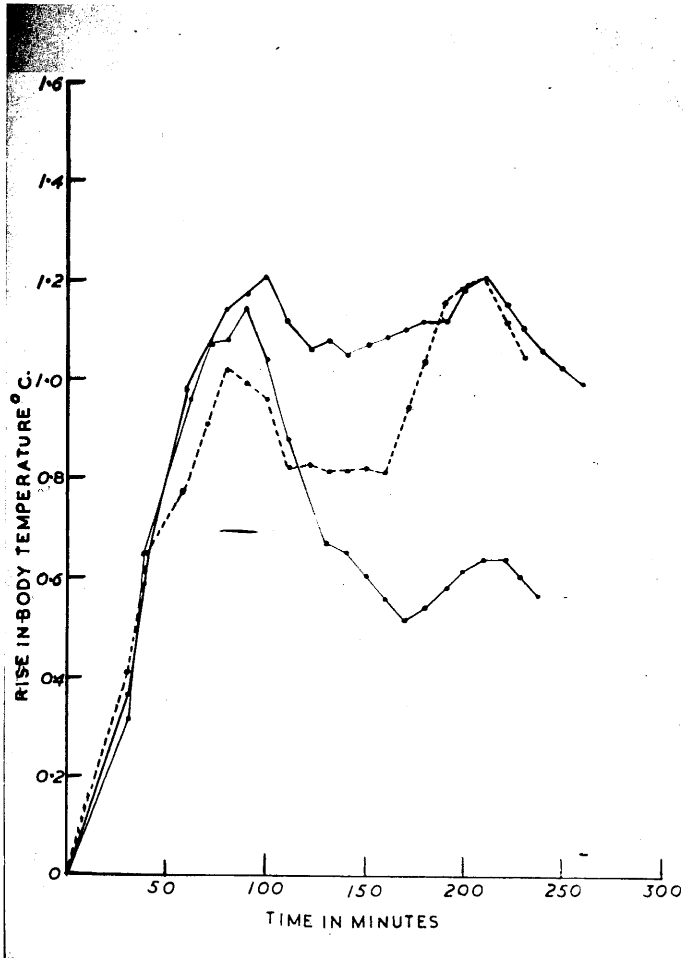


FIG.12. Double-peak fever curves stimulated by the cell-free centrifuged supernatant of the autoclaved culture of Escherichia coli.

Dose 0.02 ml./kgm. of body weight.

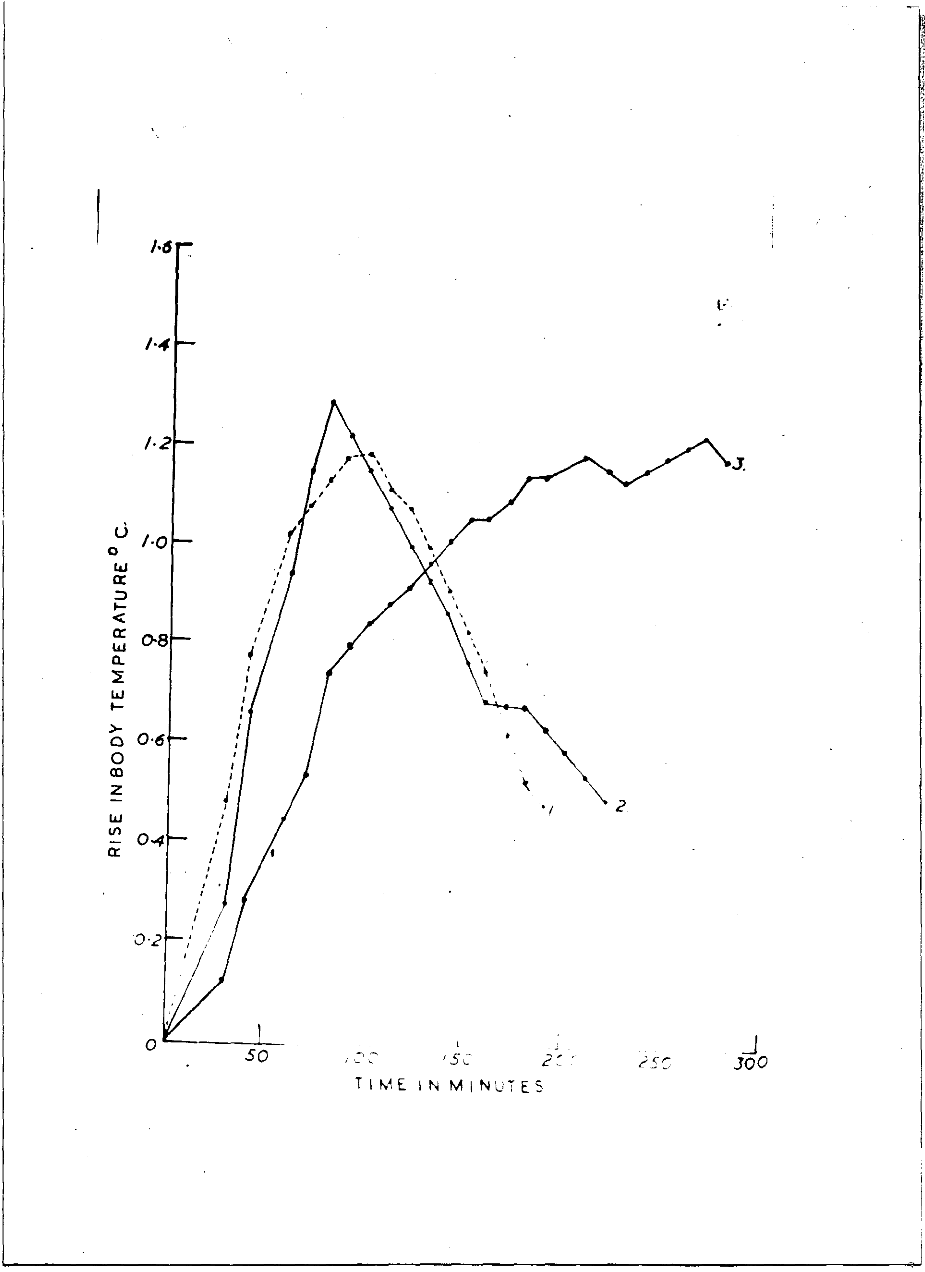


FIG.13. Single-peak fevers stimulated by the cell-free centrifuged supernatant of the viable culture (1&2) and a delayed fever stimulated by the washed cells of the autoclaved culture.

delayed type as was found in the previous experiment.

A third series of experiments were carried out, again on Proteus vulgaris, using a seven-day culture:

1. Three doses of the whole culture, 0.02, 0.002 and 0.0002 ml./kgm. of body weight were tested in groups of five rabbits. Figure 14 shows again that the second peak can be diluted out more readily than the first.
2. Filtrates from both the viable and the autoclaved cultures were tested. The filtrate of the autoclaved culture stimulated a definite double-peak response in agreement with the results of the previous experiment. In the case of the viable culture however, one sample gave the expected single peak but the other stimulated a double peak. (Fig.15).

Summarising these results we have that the second peak of a double-peak fever is more readily diluted out than the first, that the cell-free filtrate or the centrifuged supernatant stimulates the single-peak type of curve if the cells are removed before autoclaving and the double-peak type of curve if the whole culture is autoclaved before removal of the cells. The washed cells of the sterilised culture stimulate the delayed fever. It would appear from the results therefore that there are two pyretic substances, one which stimulates the immediate fever, the other the delayed fever, and a mixture of both stimulating the double-peak/

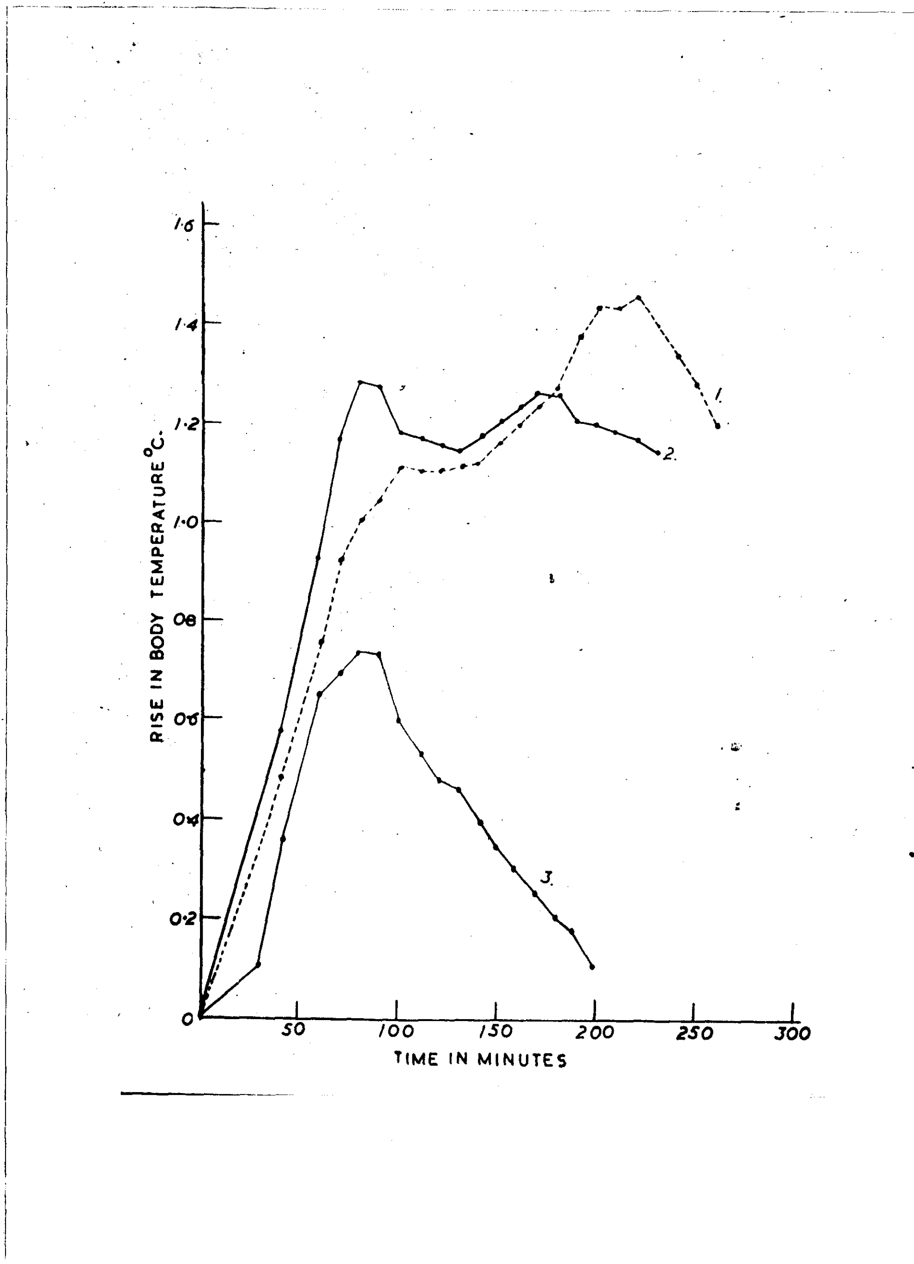


FIG.14. Ellimination of second peak by reducing the dose.

Dose in ml./kgm. of body weight.

Curve 1 0.02 Curve 2 0.002 Curve 3 0.0002

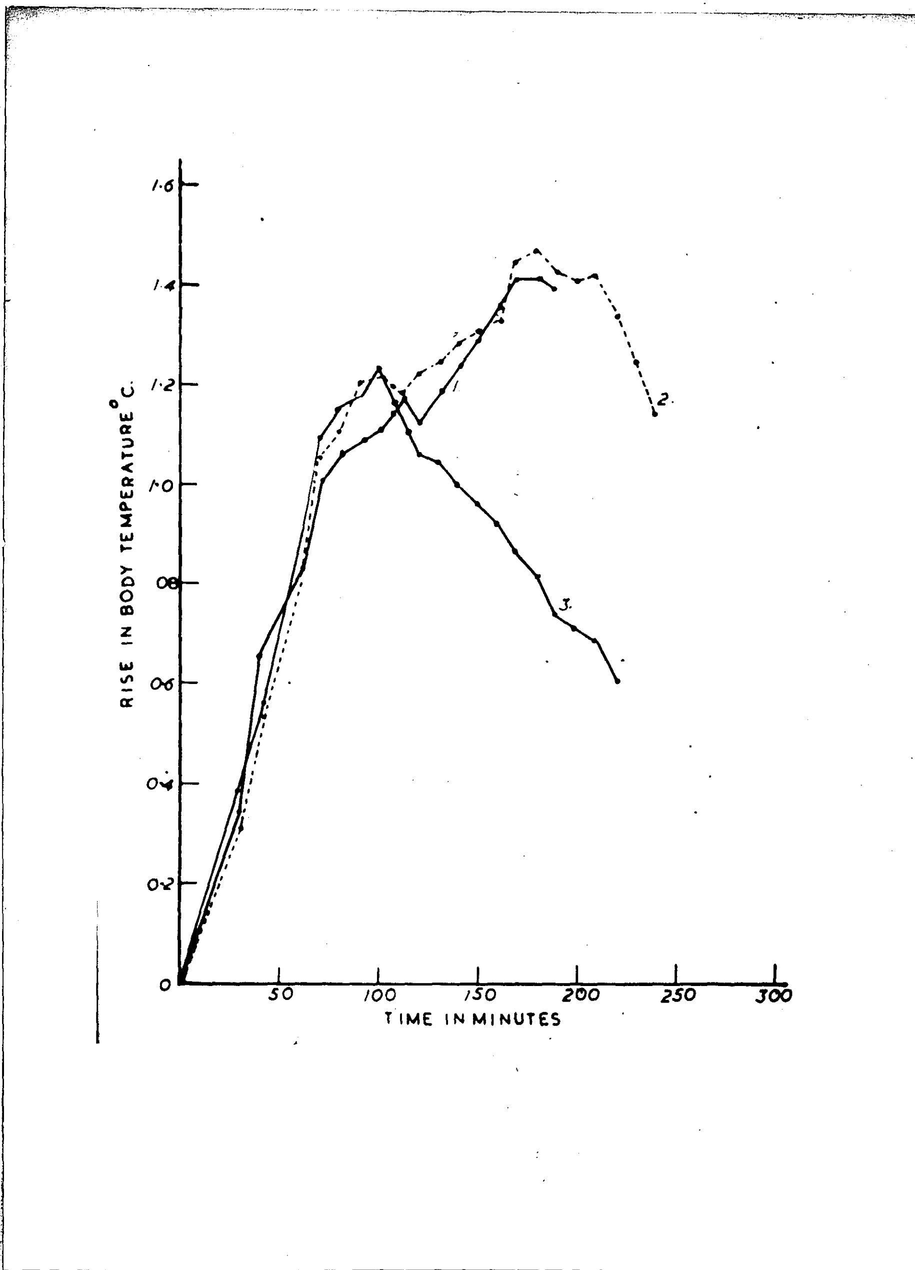


FIG.15. Showing fever curves stimulated by cell-free filtrates of both the visble (1&3) and the autoclaved culture (2).

peak fever. However it would also appear from the results that the substance causing the second peak might be formed by the autoclaving of the cells, if the culture is autoclaved while the cells are present this substance is found in the medium, whereas if the cells are first removed before autoclaving the substance is usually absent. To investigate this point a further series of experiments were carried out on a seven-day culture of Proteus vulgaris, the whole culture was divided into three parts, the first was sterilised by autoclaving at 115°C. for thirty minutes, the second by heating at 60°C. for one hour and the third was sterilised by addition of 0.3 per cent. chlorocresol. All three solutions were tested for sterility and were found to be sterile and were then injected into groups of five rabbits at a dose of 0.02 ml./kgm. of body weight and all three stimulated the double-peak type of fever curve. The actual quantity of chlorocresol injected into the rabbits was 0.1 mgm. and would have little effect. Thus the substance stimulating the double-peak fever is not formed by autoclaving the cells. The only other explanation which is offered is that in the viable culture the substance causing the first peak is mainly dissolved in the medium and that stimulating the second peak mainly within the cell and if there is any of this latter substance/

substance in the medium it is not usually present in sufficient quantity to stimulate the second peak (this is possible as it has been shown to be readily diluted out). Autoclaving of this viable culture however increases the quantity of this substance dissolved in the medium sufficiently that the cell-free solution can stimulate the double peak type of curve. This explanation is further supported by the fact that another organism, Serratia marcescens (B. prodigiosus), stimulated the double-peak type of re-action whether the culture was sterilised before or after the removal of the cells, Fig. 16, showing that the second substance is not an artifact produced by autoclaving the cells, it is assumed that in the case of S. marcescens the second substance passes more readily from the cell into the medium that it does in either E.coli or Proteus vulgaris.

The organisms examined which have stimulated the double-peak fevers are Proteus vulgaris, Proteus morganii, Ps. fluorescens, S. marcescens, S.keilensis, E. coli, Eb.typhosa, B.mycoides and Staphylococcus aureus. On the other hand it cannot be claimed that the other organisms tested in the course of this work which did not stimulate the double-peak type of re-action do not produce the substance which causes the second peak, as it may not have been present in sufficient quantity.

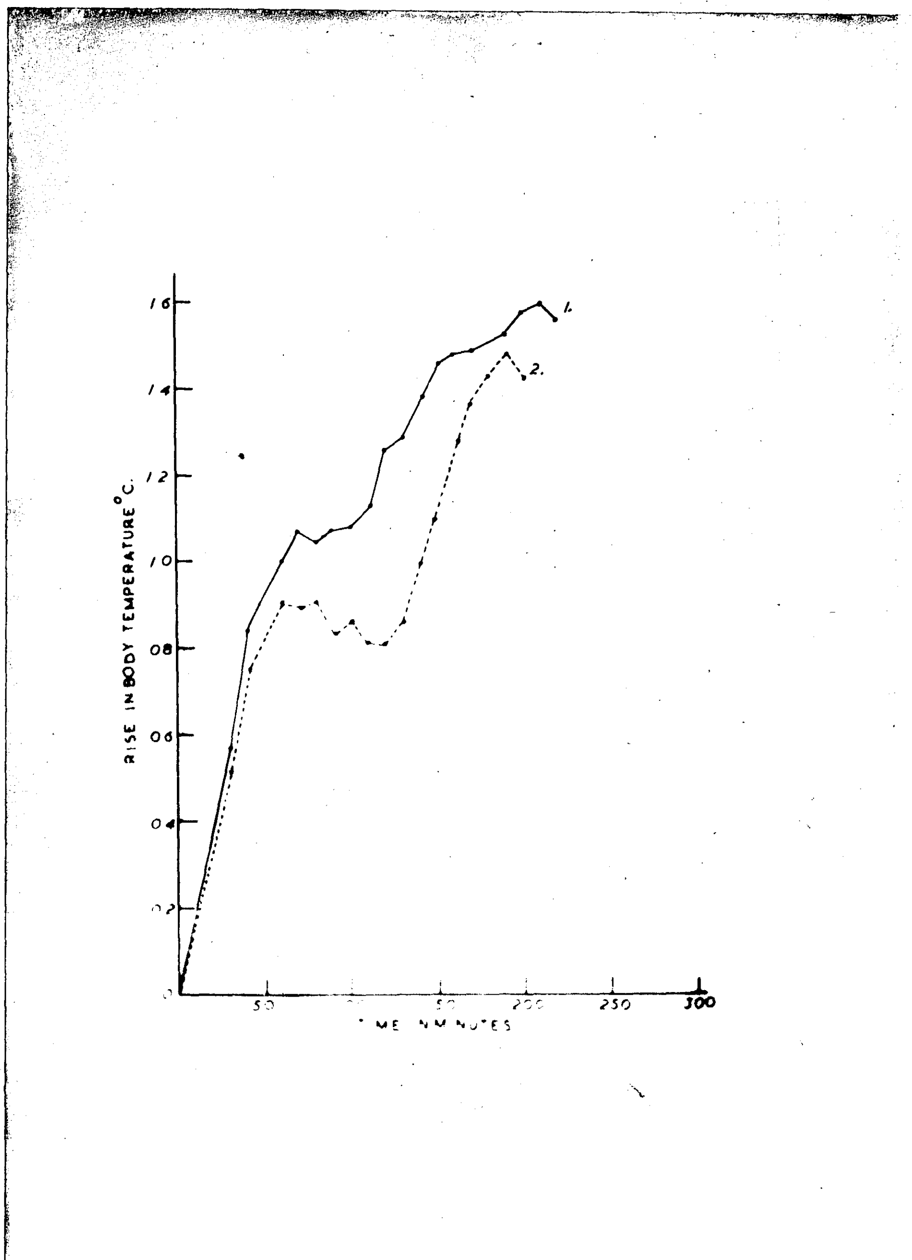


FIG.16. Double-peak fever curves stimulated by a whole culture (1) and by a cell-free filtrate of the viable culture (2) of S.marcescens.

Production of a depressant substance by some organisms and the effect that this has on the fever curve.

In the course of testing various micro-organisms two reactions were obtained which differed from the usual type of fever reaction, one which was stimulated by a culture of Proteus vulgaris and the other by a culture of Pseudomonas fluorescens. In these reactions the body temperature commenced to rise sharply in the first hour, as it would in the usual fever reaction, but this was followed by a rapid fall in body temperature to well below normal, in one of the tests the average fall for the five rabbits was 1.34°C. below the initial temperature (Fig. 17) one of the rabbits actually showing a fall in body temperature of 3.4°C. within 1½ hours of the injection. In the more severely effected rabbits of the groups the other symptoms were signs of general collapse, the hind quarters were paralysed and there was no control over urination, the animals could not stand and the head could only be moved slightly, these symptoms appeared in 1¼ to 1¾ hours after the injection. Two hours after the injection the animals usually began to recover from this state, the temperature began to rise slowly and the animal was able to stand and move about. The symptom which was apparent in all of the animals injected, whether severely effected as above or not, was the loss of/

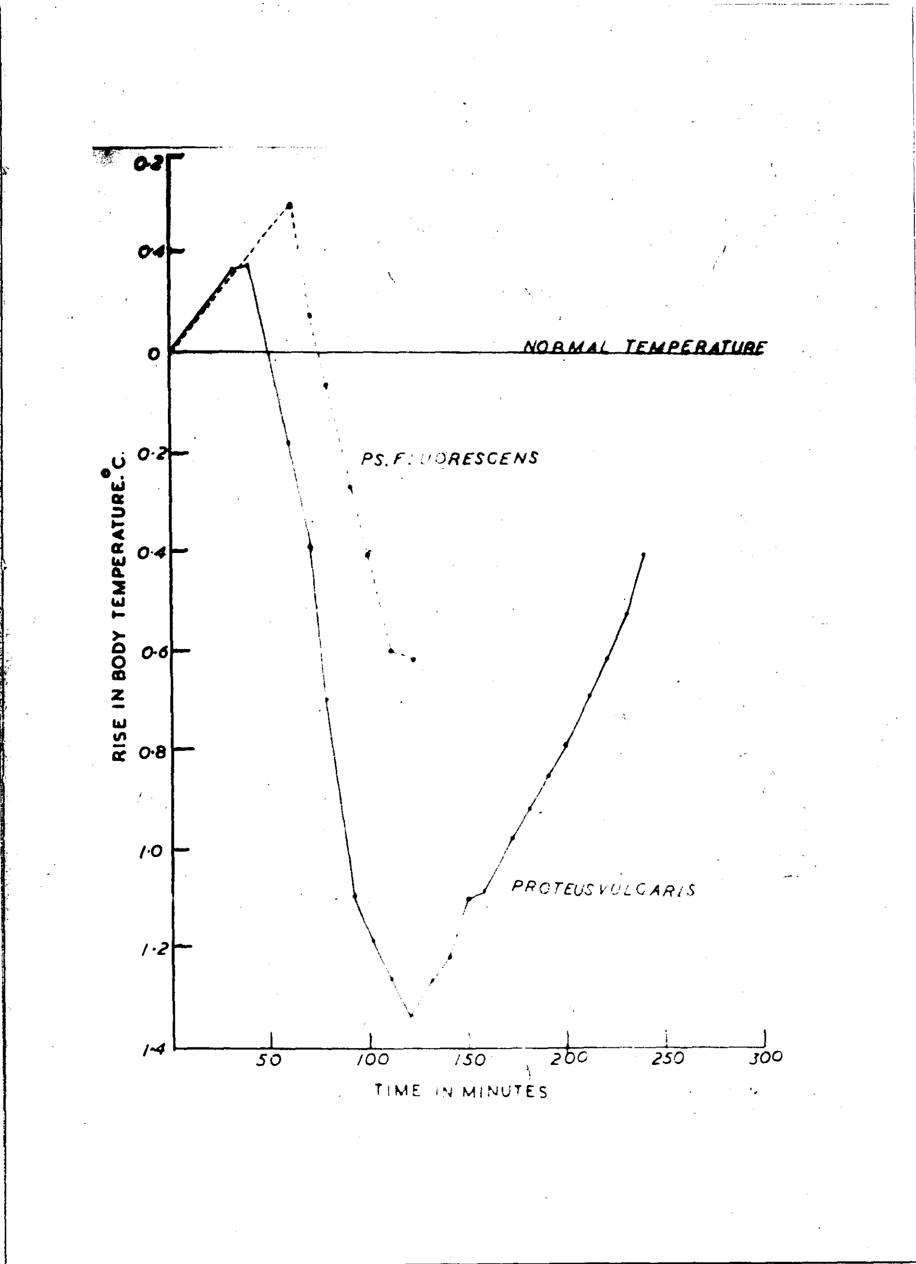


FIG.17. Fall in body temperature caused by a depressant substance present in cultures of Ps.fluorescens and Proteus vulgaris.

of appetite, the animal refusing food for a number of days after the experiment. In one rabbit which showed signs of collapse the heart rate was 270/minute and the respiratory movements 150/minute.

The depressant effect was not repeated when further samples of the same cultures mentioned above were retested, and it was also observed that when the effect occurred the first rabbit in each group to be injected was usually the most severely effected. As the solution to be injected is heated to approximately body temperature before it is injected it was realised that any very volatile substance might be slowly driven off and, therefore, the solution injected into the first rabbit might contain a higher concentration of the depressant substance than that injected into the following rabbits. On this assumption the following experiments were carried out on a seven-day culture of Proteus vulgaris. Samples of the whole culture in sealed ampoules were sterilised by autoclaving at 115°C. for thirty minutes, and tested in doses of 0.02 and 0.002 ml./kgm. of body weight, in these tests the solution was diluted, placed in an open beaker and heated to 30 to 40°C. for ten to fifteen minutes before being injected, this solution stimulated the normal fever curve reaction (Fig.18) no depressant action or loss in appetite being observed. Similar samples of this culture/

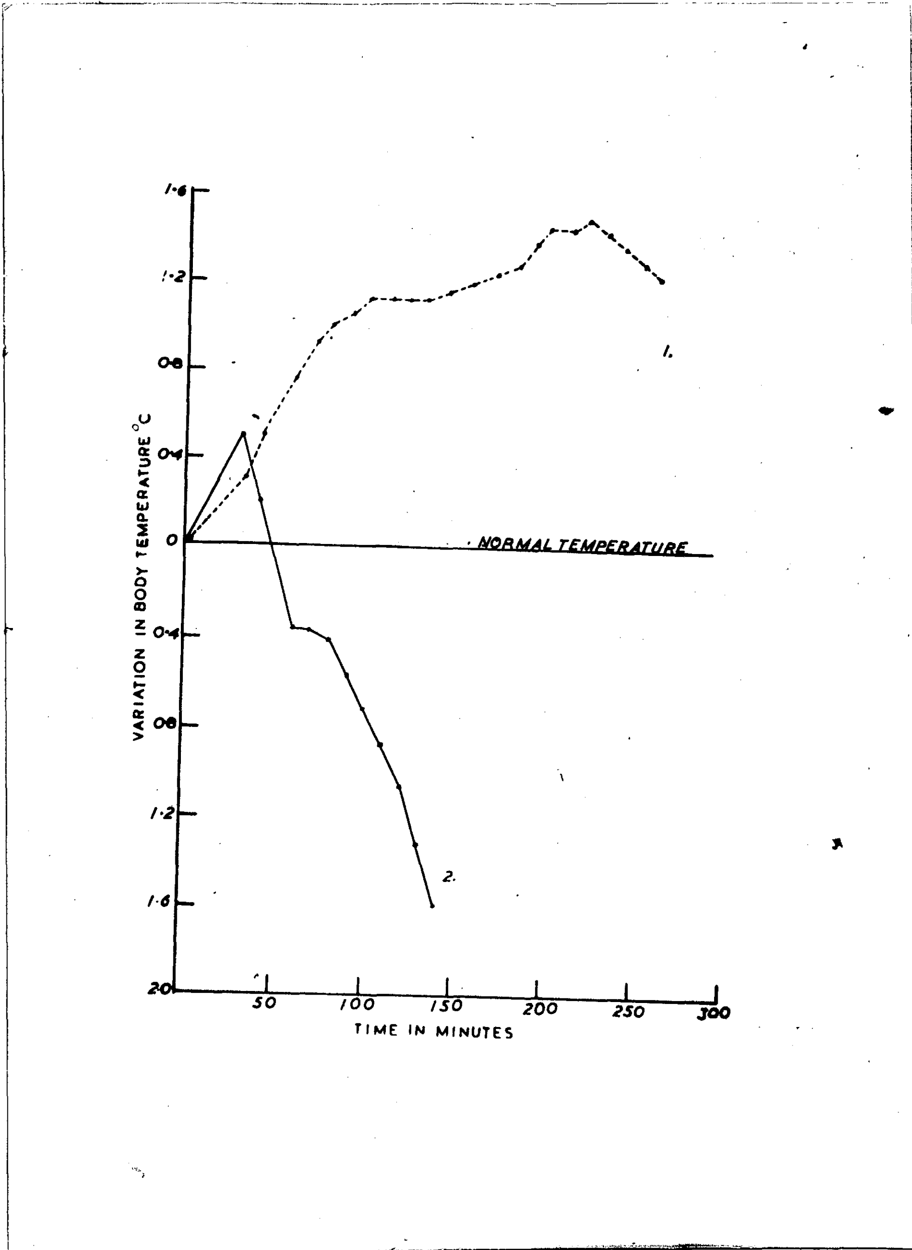


FIG.18. Showing effect of heating solution before injection.

Curve 1 - solution heated before injection.

Curve 2 - solution not heated before injection.

culture were tested in doses of 0.04, 0.02 and 0.01 ml./kgm. of body weight, this time taking care during the preparation of the dilution to prevent loss of any volatile substance and the solution was not heated before injection. Here the depressant action was apparent at a dose of 0.04 ml./kgm. of body weight there being a distinct fall in body temperature and a development of paralysis (Fig.18). The rabbits receiving the other two lower doses although not showing the fall in body temperature nor paralysis lost appetite for food for a few days. Thus the substance causing this fall in body temperature and loss in appetite is volatile, and since it is present in the autoclaved sealed cultures it appears to be relatively stable towards heat.

Modifying effect of depressant on the fever curve.

Fig.19 shows how the height of the fever stimulated by a culture may be modified by this depressant substance. When the solution was heated before injection the usual type of fever was stimulated; the modifying effect of the depressant is seen in the fevers stimulated by the solutions injected without previous heating, the temperature rises sharply in the first hour but instead of continuing up to the usual peak in 1½ hours it either falls sharply or tends to remain at the point reached, such a reaction in which the depressant effect is not obvious might easily lead/

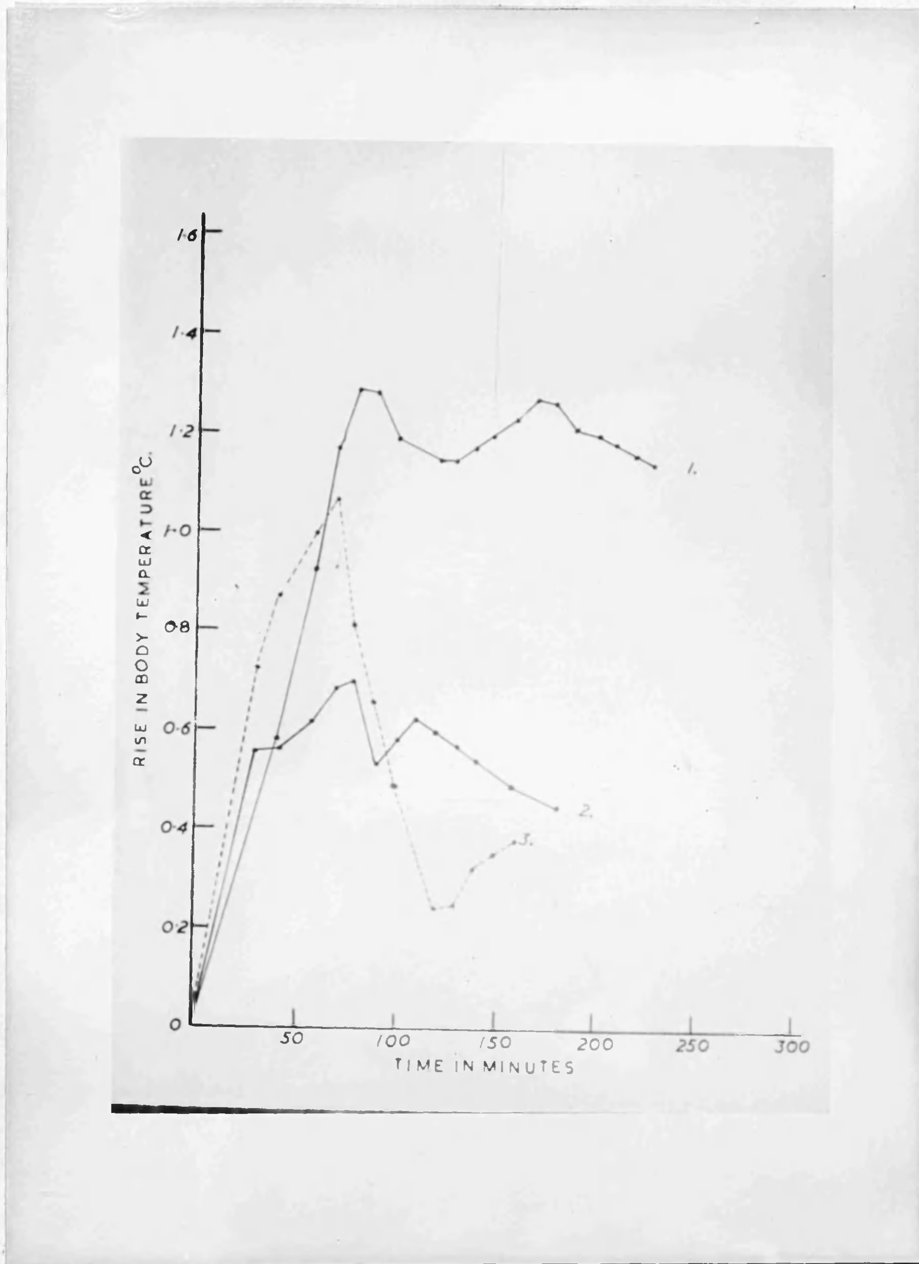


FIG.19.Modifying effect of depressant on the fever curve stimulated by a whole culture of Proteus vulgaris.

Curve 1 Dose - 0.002 ml./kgm.bdy.wt.,no depressant effect.

Curve 2 Dose - 0.02 ml./kgm.bdy.wt., depressant effect

Curve 3 Dose - 0.005 ml./kgm.bdy.wt., depressant effect.

lead to the wrong conclusions as to the pyrogenic activity of the solution. The depressant was inferred to be present in these solutions, for although the temperature of the animals did not fall below normal, the animals refused food for a number of days following the experiment. It is clear that when testing cultures of bacteria it is advisable to heat the solution before injection to drive off any volatile substance and the results of any experiment which is followed by the animals refusing to eat should be examined for signs of the modifying effect of the depressant and the experiment repeated if necessary.

This depressant substance might also account for the distinct fall in body temperature following the injection of the washed cells of Proteus vulgaris as is seen in Fig.7, in this delayed type of curve the temperature fell slightly before beginning to rise and this reaction seems to be similar to the type of reaction referred to by both Seibert (1925) and Hort & Penfold (1912c).

<u>Summary</u>

Three distinct types of fever curves can be stimulated by the intravenous injection of cultures of certain organisms and it has been shown that all three fevers may be stimulated by certain modifications of the same culture. Proof of the possible existence of two pyretic substances in the same culture has been given, in the cases of E. coli and Proteus vulgaris/

vulgaris one substance is mainly dissolved in the medium and the other within the cell.

How a volatile depressant substance is produced by some organisms and the effect that this may have on the fever curve is also discussed.

## PROPERTIES OF PYROGEN

The necessity of finding means of eliminating pyrogen from solutions to be used for intravenous administration directed much of the past work on the properties of pyrogen into certain channels. Such means of elimination as adsorption by activated carbon (Lees & Levy 1940 and Todd et al. 1941 and 1946) adsorption by the seitz filter pad (Co Tui et al. 1936) oxidation by hydrogen peroxide (Campbell and Cherkin 1945) and destruction by storage (Collier and Paris 1947) and by heating (Banks 1934) have been investigated. However the conclusions which could be drawn from such investigations were limited by the lack of a satisfactory method of comparing the pyrogenic activity of two solutions. The workers could only state that the solutions were pyrogenic before and were or were not pyrogenic after the treatment. Although such results admirably suited the requirements at the time they do not give a true indication of the properties of pyrogen, for this it is necessary to investigate the rate of removal of pyrogenic activity by the various processes.

It was necessary to investigate a few properties of pyrogen in order that the proceedures used in other sections of the work could be controlled. Even although it has been possible in this work to measure the rate of removal by the various methods the results are still of little value

in/

in adding to the knowledge of the properties of pure pyrogen as the work was not carried out using the pure substance, on the contrary the source of pyrogen was, in each case, the cell-free filtrate of a culture. The results, however, satisfied our needs in that they showed the degree of elimination which could be expected by sterilising a solution by autoclaving at 115°C for thirty minutes, the effect of storage and Seitz filtration, and also gave an indication of the methods permissible in attempts to isolate the active substances.

Effect of storage.

After six months storage at room temperature and in darkness, Standard I, from pseudomonas aeruginosa, still retained its original potency. After fourteen months, however, under the same conditions it had lost about two-thirds of its original potency but was still distinctly pyrogenic, 2.0 nl./kgm. stimulating an average maximum rise of 0.66°C. in five rabbits. In contrast to this relative stability the very much stronger solution, Standard II, also prepared from Ps. aeruginosa, rapidly decreased in potency losing half of its original strength on storage at room temperature for five days and 75 per cent. on storage at just above freezing temperature for a further ten days. Three tests/

tests during the following eight months, in which time the solution was still stored at just above freezing temperature, showed that the solution lost little further potency. Thus there was an initial rapid loss in activity until about 80 per cent. was lost and thereafter the rate of destruction was very much slower. It has been stated * that pyrogen is adsorbed by glass and as Standard II had been stored in glass ampoules this rapid initial loss might have been due to such adsorption. Eight ampoules, in which the Standard had been stored for three months were emptied of the solution by vigorous shaking, ground up in a mortar with 50 ml. of pyrogen-free saline (about six times the original volume of Standard present in the eight ampoules) and the mixture heated at about 60°C. for a few minutes. A dose of 2.0 ml./kgm. of body weight of the filtrate of this solution stimulated an average maximum rise of 0.55°C. in five rabbits, the Standard taken from these same ampoules and tested on the same day stimulated a response of 1.21°C. at a dose of 0.16 ml./kgm. of body weight. It was estimated that such results represented an adsorption of roughly 2.5 per cent of the pyrogen present in the original solution, thus adsorption does not account for the 70 to 80 per cent loss of activity.

* No reference available - stated in "Penicillin, its properties, uses and preparations". The Pharmaceutical Press 1946, page 116.

The apparent contradiction of the results of the stability of the two Standards may be explained by the differences in their concentration as it will be shown later that heating a solution at 120°C. produced an initial rapid loss of 80 per cent of the strength after which the rate of destruction was much slower. Collier and Paris (1947) drew attention to the fact that some pyrogenic glucose solutions rapidly lost pyrogenic potency on storage and this seems to agree with the above results, as Collier's solutions were relatively strong, but the rapid loss is initial only when the pyrogen is that produced by Ps. aeruginosa.

Effect of Seitz filtration.

Seibert (1923 a) found that it was extremely difficult to clean Berkefeld filter candles free from pyrogen after use, and for this reason it would appear that Seitz filtration offers certain advantages as the filter pads can be discarded after use. However, as it had been shown by Co Tui (1936) that pyrogen was adsorbed by Seitz filter pads, it was thought necessary to find how complete this removal was and whether the adsorbing powers of the pad were readily exhausted, before such a method of filtration could be used.

400 ml. of pyrogenic saline was passed through a 4 cm.

4 cm. Seitz filter pad using negative pressure. As each 25 ml. quantity of filtrate passed through it was removed aseptically and in this way sixteen 25 ml. quantities were filtered so that the first sample, probably pyrogen-free, would not dilute the following samples. Table XI shows the results of the pyrogen tests on the solutions before and after filtration. As sample 7 is pyrogen-free it shows that even after the passage of 150 ml. the pad is still adsorbing pyrogen from the solution, at the fifteenth sample small quantities of pyrogen were passing through.

TABLE XI

Showing the removal of pyrogen from solution by Seitz filtration.

| | | Dose in ml./kgm. bdy.wt. | Average maximum rise °C | Remarks |
|---|---|---|---|---|
| Unfiltered solution | | 0.5 | 0.88 | Pyrogenic |
| Filtered solution | Sample 7 | 0.5 | 0.36 | Non-pyrogenic |
| | Sample 15 | 0.5 | 0.54 | Slightly pyrogenic |

Thus pyrogen may be adsorbed from solution to an appreciable extent by Seitz filtration and the adsorbing powers of the pad are not readily exhausted and therefore Seitz filtration of bacterial cultures is of little value where the solution is to be tested for pyrogen.

Contrary to Seibert's findings the filter candles used in/

in this work were found to be quite easily cleaned by thorough scrubbing in hot soapy water followed by passage through them of 80 to 100 ml. of pyrogen-free water. In this way seven filter candles used for the filtration of cultures of Proteus vulgaris, Ps. aeruginosa and B. coli were cleaned free of pyrogen as indicated by the non-pyrogenicity of 30 ml. of water passed through them. These filters did not exhibit any power in adsorbing pyrogen, and this is in agreement with the results found by Seibert.

Effect of heat.

The stability of pyrogen to heat has been investigated by Seibert (1925) and Banks (1934) and both concluded that it was a thermostable substance. Banks stated that to remove the pyrogenic activity from a solution required either; refluxing under atmospheric conditions for 7 hours, or, heating at a 120°C. for four hours using steam under pressure, or, heating at 145°C. for 30 minutes using steam under pressure.

In this work the stability of pyrogen from a number of bacterial sources has not yet been exhaustively examined as the results would be of little value if carried out on the impure pyrogen available at the moment. In determining the rate of destruction of pyrogen by heat/

heat the cell-free solutions obtained by filtration or centrifugation of the viable cultures were used and only one of the solutions exhibited a tendency to stimulate the double-peak type of fever and even this was very slight, therefore any conclusions arrived at from the results are limited to the pyrogen which stimulates the immediate response. In view of these limitations it was decided to postpone a systematic examination of the properties of pyrogen until a relatively pure sample of pyrogen could be isolated from different bacterial sources.

Experimental.

Three different cultures of Proteus vulgaris grown in gelatin hydrolysate medium were subjected to heating at 120°C. and the rate of destruction of their pyrogenic activities examined. Two of these cultures were 48 hour cultures and the other a 31-day culture. The bacterial cells were removed by filtration of the viable culture and the filtrate distributed in 10-ml. ampoules which were sealed, divided into five batches, and subjected to various degrees of heating as is shown in Table XII. The temperature of 120°C. was obtained by the use of steam under pressure. To facilitate the comparison of the strengths of the batches a unit system was devised/

devised and as tests were carried out on the rabbits standardised against Standard II, the regression line for that Standard was used and the unit devised was the amount of pyrogen present in 0.04 ml. of Standard II.

TABLE XII.

Showing the rate of destruction of pyrogen produced by Proteus vulgaris when heated at 120°C.

| Heat treatment | | 48 hour culture | | 48 hour culture | | 31 day culture | | Aver. %age dest-ryed |
|---|---|---|---|---|---|---|---|---|
| Temp °C. | Duration hours | Conc units per ml | %age dest-royed | Conc units per ml | %age dest-royed | Conc units per ml | %age dest-royed | |
| 60 | 1 | 46 | 0 | 87.5 | 0 | 825 | 0 | 0 |
| 120 | ½ | 31 | 33 | 42.5 | 38 | 362 | 56 | 42 |
| 120 | 1 | 6.75 | 85 | 21.9 | 76 | 400 | 52 | 71 |
| 120 | 2 | 2.0 | 96 | 6.25 | 93 | 41 | 95 | 94 |
| 120 | 4 | 0.75 | 98 | 1.9 | 98 | 14 | 98 | 98 |

For purposes of simplification it was assumed that no pyrogen was destroyed by heating at 60°C. for one hour. By calculating the percentage destruction for each batch of the three cultures the trend in the destruction of the pyrogen in the three cultures could be compared. Although the results for the 31-day culture deviate greatly from those of the other two cultures the trend seems to be similar.

The average percentage destruction at each stage for the three cultures was calculated and the curve obtained by plotting this against time is shown in Fig. 20. The apparent deviation among the three sets of results led to the conclusion that the conditions of the experiment were not properly controlled to be absolutely reproducable and it was decided that in further experiments on this section the reaction of the solution should be adjusted to pH 7 and that the heating should be done by immersion of the ampoule containing the solution into oil in an oil-bath which was thermostatically controlled at 120°C. It was not considered necessary to add buffers to the solution which was really the buffered medium in which the organism had been grown.

Ps. aeruginosa, B.subtilis and M. tetragenes were grown in the gelatin hydrolysate medium for three, two/

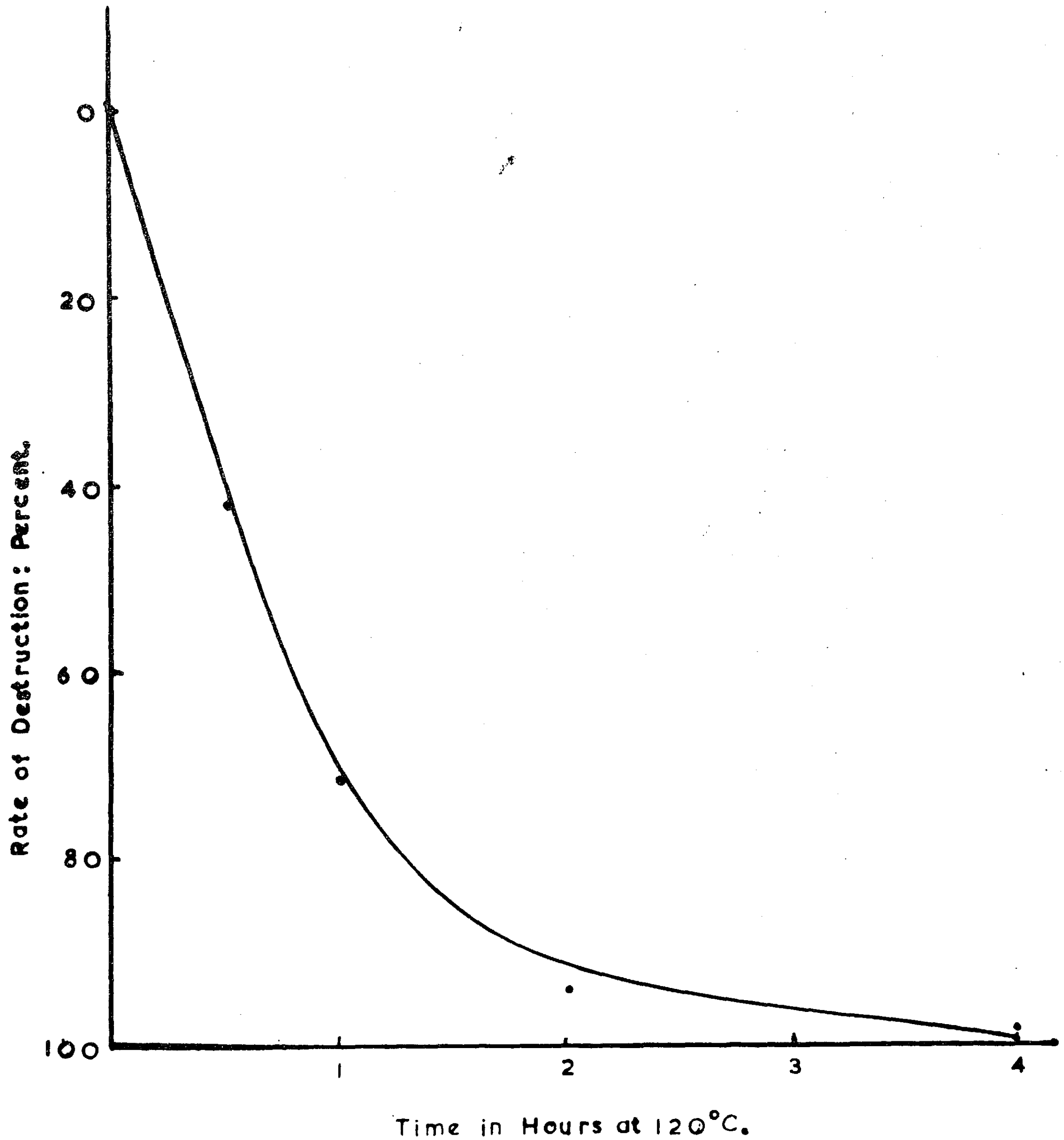


FIG.20. Rate of destruction of pyrogen in a culture of Proteus vulgaris when heated at 120°C.

two and one week respectively. The cells were centrifuged off and the reaction of the supernatant adjusted to pH7. This was distributed in clean thick glass ampoules which were sealed and batches of each immersed in the oil at 120°C. for thirty minutes, 1 hour, 1½ hours and 2 hours.

As it was possible that some destruction of pyrogen might take place on heating at 60°C. for 1 hour as was done in the experiment on Proteus vulgaris it was decided to calculate the original strength by extrapolation of the logarithm of the concentration against time curve which will later be shown to be a straight line. The results of these experiments are shown in Table XIII

TABLE XIII

Showing the rate of destruction of the pyrogen from Ps. aeruginosa, B.subtilis and M. tetragenes.

| Heat treatment | | Pseudomonas aeruginosa | | Bacillus subtilis | | Micrococcus tetragenes | |
|---|---|---|---|---|---|---|---|
| Temp °C | duration hours | Conc units per ml | %age dest-royed | Conc units per ml | %age dest-royed | Conc units per ml | %age dest-royed |
| —— (Extrapolation) | —— | 214 | 0 | 19.0 | 0 | 9.1 | 0 |
| 120 | ½ | 75 | 65 | 9.0 | 52.6 | 8.75 | 3.8 |
| 120 | 1 | 51 | 76 | 4.4 | 76.8 | 8.00 | 12.6 |
| 120 | 1½ | 26.9 | 87 | 2.4 | 87.4 | 7.5 | 17.6 |
| 120 | 2 | 10.6 | 95 | 0.95 | 95 | 7.3 | 19.8 |

It would seem from the results that the rate of loss of activity in cultures of Proteus vulgaris, Ps. aeruginosa and B. subtilis is somewhat similar in that approximately 50 per cent. is lost after 30 minutes at 120°C. and 95 per cent. after 2 hours. The activity of the M. tetragenes culture on the other hand was much more difficult to reduce, only 20 per cent. of the activity being destroyed after 2 hours at 120°C. How much of this difference in stability is due to other substances in the solution is not known.

<u>Linear relationship between log. of concentration and time</u>.

As the curve in Fig. 20 is suggestive of the logarithmic type of curve the results were examined for a linear relationship between the logarithm of the concentration and time. In the experiments on the Proteus cultures this relationship was only found to exist for that solution with the initial concentration of 87.5 units/ml. the other two deviating too greatly from any one line to say that a straight line best represented the results. To take the average of the concentrations of the three cultures at each stage was of no additional benefit due to the great divergence of one set of results from the other two and the fact that it was this set of results which deviated most from the straight line. By converting the unit concentration of pyrogen/

pyrogen left at each stage into a percentage concentration of the original strength, i.e. 100 minus the percentage destruction, the concentration of the three sets of results were brought to the same numerical order and by averaging this percentage concentration for each batch a linear relationship was found to exist between the logarithm of the percentage concentration and time which satisfied all points within the experimental error of the quantitative test. The point which represented the concentration of the solution after heating at 120°C. for four hours however deviated from this line in all three sets of individual results and in the averaged value. For the cultures of Ps. aeruginosa, B. subtilis and M. tetragenes a linear relationship was found to exist between the logarithm of the actual concentration in units and time, this is in agreement with one set of results for Proteus vulgaris and is probably due to the increase in the accuracy of the later methods used. However to bring the results of the other three organisms into line with those for Proteus vulgaris the percentage residual concentration was calculated and the results are shown in Fig. 21. This figure shows that the same straight line, the calculated 'line of best fit' in this case, can be drawn to represent the rate of destruction of the pyrogen produced by Proteus vulgaris, Ps. aeruginosa and B.subtilis/

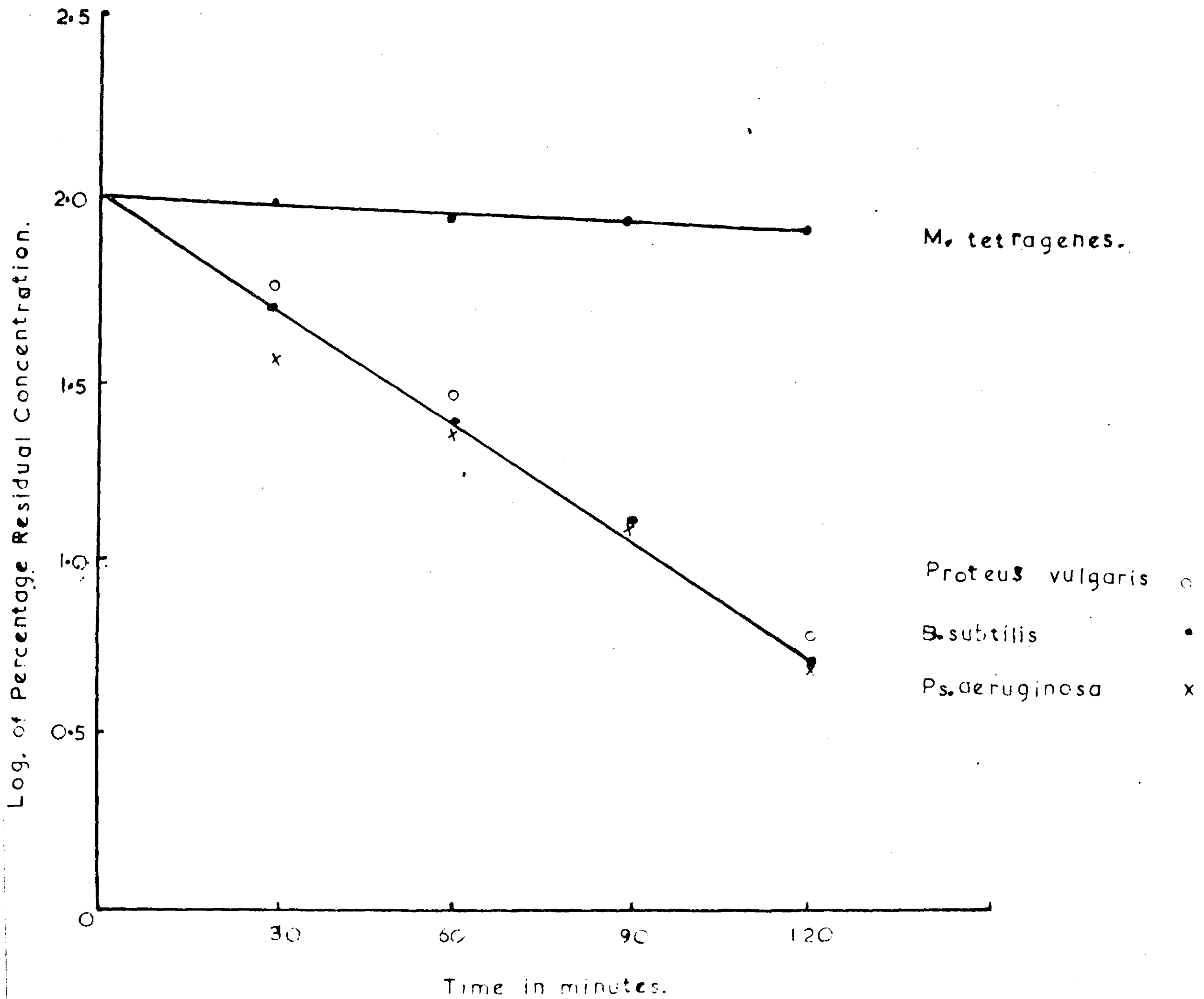


FIG.21. Showing linear relationship between logarithm of residual concentration and duration of heating at 120°C.

B. subtilis within the experimental error of the quantitative test and also that the results for M.tetragenes, although still linear, are quite different.

Summarising these results we find that the pyrogen present in the cell-free filtrate of the cultures of Ps. aeruginosa, Proteus vulgaris and B. subtilis show very similar rates of destruction, M.tetragenes on the other hand seems to produce a pyretic substance of totally different stability. How far these results would agree with those obtained by use of pure pyrogen is unknown and continuation of the work on these lines for purposes of comparing the stability of pyrogen produced by different organisms would be of little value unless carried out on the purified substance. The results fulfil the desired requirements in that they give an indication that much pyrogen may be lost during any heating process and also of the degree of severity of treatment permissible in the isolation of the substance.

Summary.

On storage the loss of activity of a concentrated solution of the pyrogen produced by Ps. aeruginosa is very rapid even when stored at just above freezing point, the rate of destruction becomes less as the solution becomes weaker. Initially weak solutions show little loss over a period of six months.

The pyrogen produced by Ps. aeruginosa is readily adsorbed from aqueous solution by the Seitz filter pad.

Pyrogenic cultures of Proteus vulgaris, Ps. aeruginosa and B. subtitis show an initial rapid loss of pyrogen on heating at 120°C. with again the rate of destruction becoming less as the solution becomes weaker, 90 per cent of the original activity is lost by heating at 120°C. for 1½ hours and therefore all pyrogens are not as stable as Seibert and Banks maintained. The pyrogen prepared by M. tetragenes is apparently more stable but it is emphasised that the conclusions regarding the similarity of the nature of the pyrogen produced by the organisms tested are limited by the impurity of the solutions used.

# APPENDICES

## Appendix 1. Preparation of pyrogen-free saline.

The water used was freshly distilled water collected from a Manesty still fitted with a baffle to prevent entrainment, 0.90 gm./100 ml. of pure sodium chloride was dissolved in this water and the saline distributed in 500 ml. quantities in bottles and sterilised by autoclaving using steam under pressure at 115°C. for thirty minutes. The bottles used were of the blood flask type used by the Blood Transfusion services for distributing physiological saline solutions and blood. Before use the bottles were cleaned by washing in soap and water, rinsing thoroughly with tap water and finally rinsing three times with freshly-distilled water. The time elapsing between the distillation of the water and the sterilisation of the final product was never greater than seven hours. A large number of salines have been prepared in this way and no instance of pyrogenic activity has yet been encountered.

Appendix II. Details of test-box.

To retain the thermocouple in the rectum throughout the entire experiment it was necessary to immobilise the rabbits. After the investigation of various methods such as the use of hip-stocks and straps to retain the rabbit in a rigid position (Kuna *et al*.1946) the box finally evolved consisted merely of two sides and a floor and was fitted only with a neck-stock. The distance between the floor and the hole in the neck-stock could be adjusted to suit the size of the rabbit to be immobilised and the edges of the hole were covered with rubber tubing slit longtitudinally. These measures were adopted to enable the immobilised animal to remain quite comfortable for many hours. Fig. 22 is a diagram of the box in general use in this work.

Three main difficulties occurred in the use of a test-box:

1. Certain rabbits, when immobilised in the box, developed spasms of sneezing and no modification of the box was effective in preventing this; Fortunately it was confined to a few rabbits only.

2. In struggling to free itself a rabbit occasionally paralysed its hind-quarters, probably by crushing the inter vertebral discs with consequent injury to the spinal/

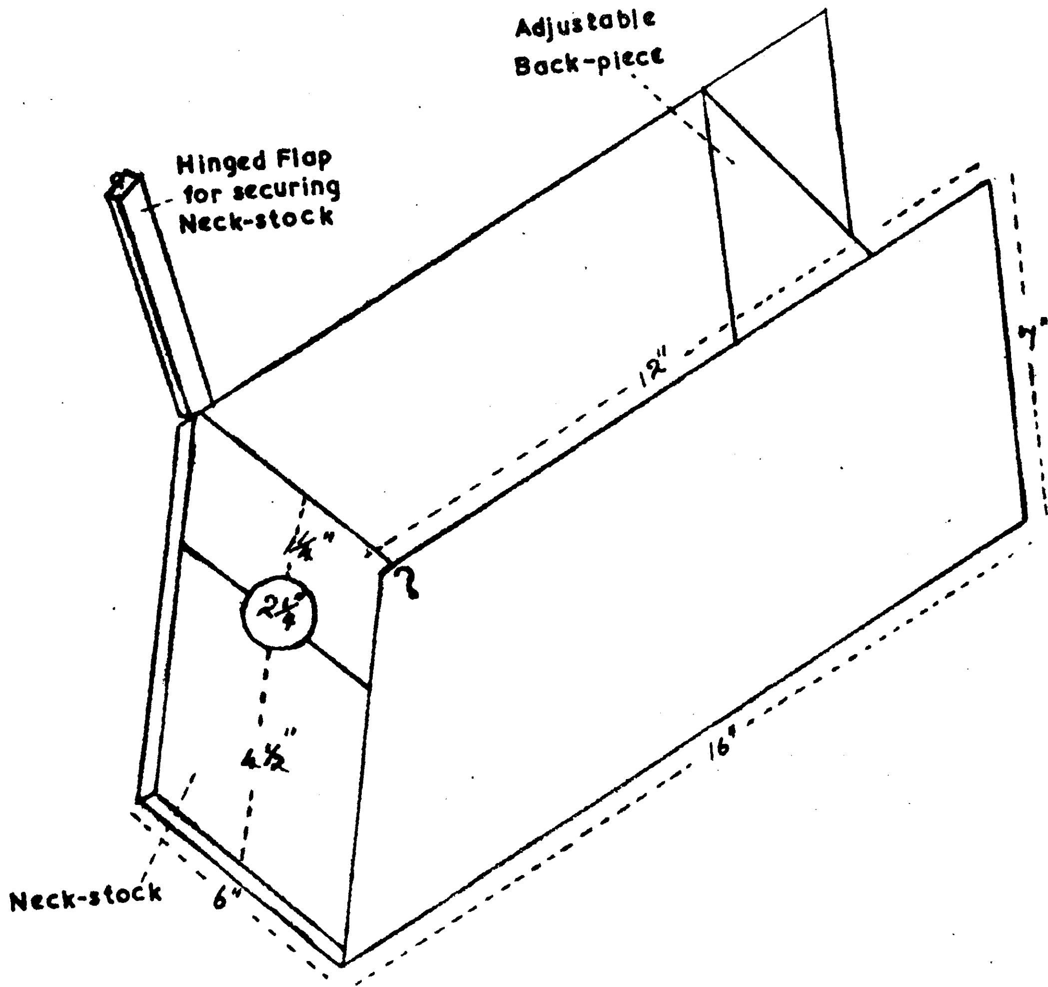


FIG.22. Diagram of rabbit test-box.

spinal cord, this happened in only a few of the experiments.

3. Occasionally a rabbit relaxed by completely stretching out and lying on its side in the box, this always caused a fall in body temperature or reduced the fever but could be prevented by slipping in a back-wall to keep the rabbit in a sitting position, the distance between the back-wall and the neck-stock was adjustable.

## Appendix III. Details of the Thermocouple circuit.

The complete circuit is shown diagramatically in Fig. 23.

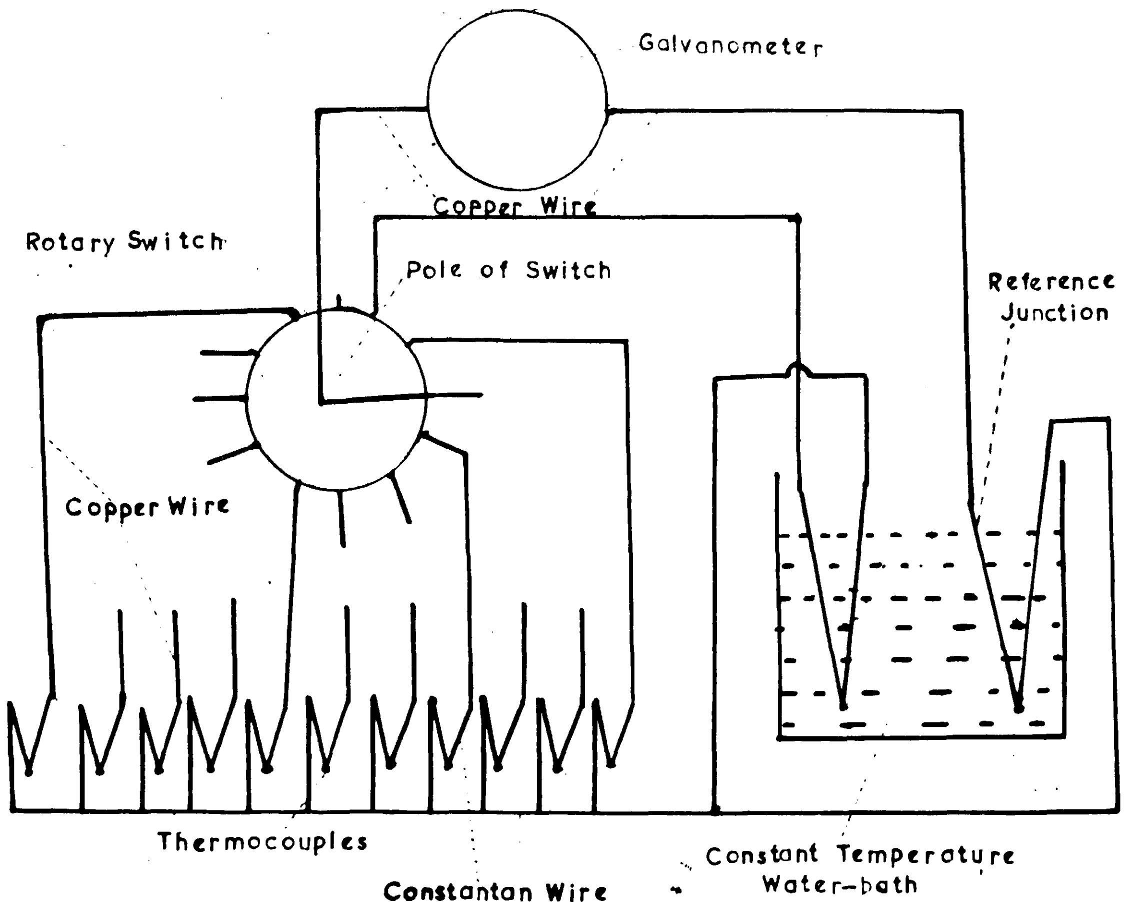


Fig. 23. Details of Thermocouple Circuit.

The circuit consisted of ten thermocouples each of which could be switched into the circuit by means of a rotary switch. Each thermocouple when in the circuit was in opposition to a reference junction, which was a thermocouple similar in all respects to the other ten and was immersed in water in a constant-temperature water-bath maintained at about/

about 35°C. the difference in temperature between the rabbit (about 38°C.) and the reference junction being measured by a mirror galvanometer.

<u>Details of the thermocouple</u>. Fig. 24 shows the details of the part of the thermocouple inserted rectally. The wire (Durawire) consists of Copper-constantan wires of S.W.G. 26. Each wire is rubber covered and both are enclosed in a common outer sheathing. The part of the thermocouple to be inserted in the rectum was prepared by stripping off about 6 cm. of the common outer sheathing, twisting the two wires together, baring the tips of the wires and welding them together by striking an arc between the thermocouple and a carbon rod.

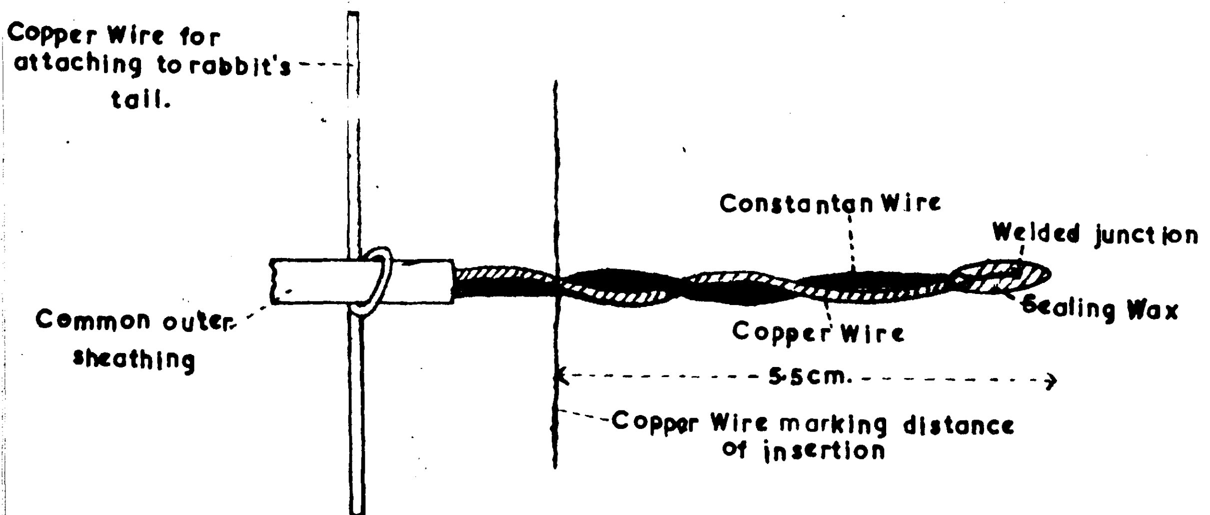


Fig. 24. Diagram of part of thermocouple inserted in rectum.

The distance of insertion, 5.5 cm., was marked on the thermocouples by a piece of cotton-covered copper wire twisted round it. The copper wire used to fix the thermocouple to the rabbit's tail is shown in this figure. The bare tip of the thermocouple was covered with red sealing wax to prevent damage of the rectal tissues, the resulting diameter of this tip rarely exceeded $\frac{1}{8}$ inches.

The circuit was so constructed, as can be seen from Fig. 23 that the connections both to the rotary switch and to the galvanometer terminals were made with copper wire only in an attempt to prevent the formation of secondary thermocouples which might be effected by variations in the room temperature. The constantan component of each thermocouple was joined to a common constantan wire by welding. A further thermocouple, inserted in the same water-bath as the reference junction, was incorporated in the circuit. This thermocouple was at the same temperature as the reference junction, and as it was in opposition to it, the galvanometer registered zero when it was switched into the circuit, this proceedure was used to fix the zero of the instrument. The two thermocouples in the water-bath were also covered in sealing wax to prevent corrosion, this waxing caused no detectable increase in the time required to respond to a change in temperature.

<u>The constant temperature water-bath</u>. This consisted of a 15 litre capacity copper bath, lagged with fibre-glass blanket and regulated by means of a mercury-toluol regulator in conjunction with a proportioning head and electronic relay as supplied by Sunvic Controls Ltd., The proportioning head and electronic relay were refinements used in an attempt to eliminate the temperature fluctuations inherent in the 'on-off' regulation of a mercury-toluol regulator and they accomplished this very effectively. The heater used was a 60 watt elongated electric bulb which was partially immersed in the water and mixing was accomplished by means of an electric stirrer. When at approximately 35°C. the temperature fluctuation was 0.01°C. about the mean and thus the temperature of the reference junction was taken as constant.

<u>Details of the galvanometer</u>. The instrument used was a 'Spot' galvanometer of 50 ohms resistance as supplied by Cambridge Instruments Co. A full scale deflection of 16 cm. represented approximately 8°C. and it could be read accurately to one-twentieth of a cm.i.e. to about 0.025°C. therefore reading of the deflection is associated with an error of ±0.01°C. This instrument was found to be exceedingly reliable for the purposes.

<u>Calibration of the thermocouple.</u> The thermocouple to be calibrated/

calibrated was immersed in water contained in a Dewar flask, the temperature of this water remained steady sufficiently long to allow the various readings to be taken. The number of units deflection per degree centigrade for each thermocouple was calculated by dividing the galvanometer deflection by the difference in temperature between the water in the Dewar flask and the water in the bath. The average value for each thermocouple was calculated from 15 to 20 such results and was found to differ from one thermocouple to another as is shown in Table XIV.

TABLE XIV

The calibrated values of the ten thermocouples in use.

| Thermo-couple No. | No.of units deflection per °C. | Thermo-couple No. | No.of units deflection per °C. |
|---|---|---|---|
| 1 | 2.10 | 6 | 2.04 |
| 2 | 2.10 | 7 | 2.00 |
| 3 | 2.10 | 8 | 2.00 |
| 4 | 2.08 | 9 | 1.95 |
| 5 | 2.04 | 10 | 1.95 |

As the calibration curve of the galvanometer was a straight line (Fig. 25) it was unnecessary to refer to it when converting deflection to temperature.

Accuracy of the temperature determinations. It was thought desirable to determine the accuracy of the thermocouple/

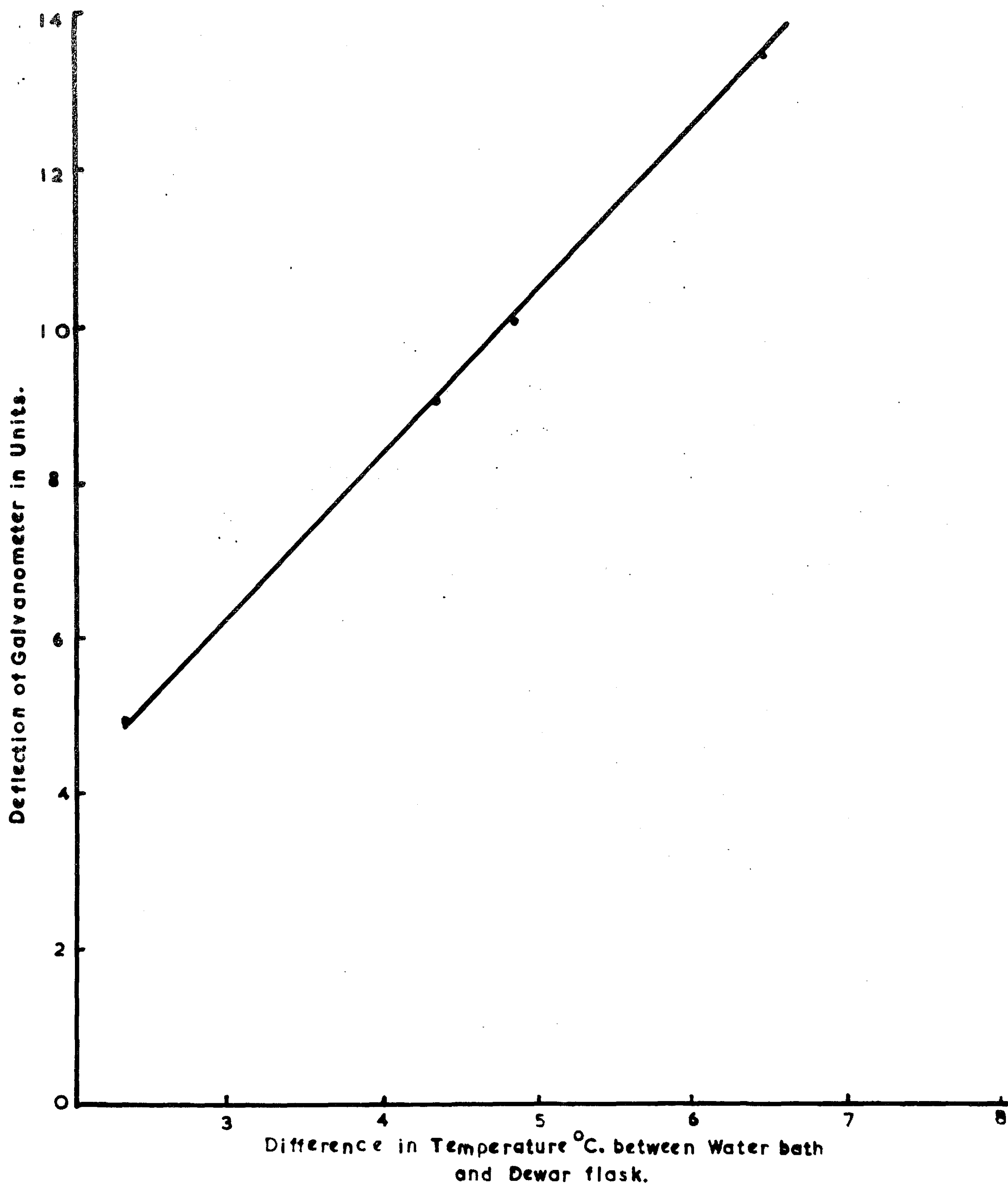


FIG.25. Calibration curve of 'Spot' Galvanometer.

thermocouple set-up by a method identical to that used in determining the temperature of the rabbit, the thermocouple being immersed in water of known temperature contained in a Dewar flask instead of being inserted in the rectum of the rabbit. The temperature of the water in the Dewar flask was measured by the thermocouple and compared with the temperature as determined by a thermometer. The thermocouple reading deviated from the thermometer reading by an average of $0.02 \pm 0.003^{o}C$. (Standard error of 37 results). As the thermometer in the Dewar flask was graduated in $0.1^{o}C$. it was necessary to magnify the scale and to wait until the temperature reading was directly on a graduation mark before being read, the thermometers in the Dewar flask and in the water-bath were standardised against each other. This accuracy is only claimed when dealing with relative temperatures, i.e. change in temperature throughout an experiment.

Appendix 4. Change in sensitiveness of 14 rabbits when used over a period of one year.

In the discussion of the quantitative test it was stated (page 46) that over a period of six months three groups of rabbits maintained a constant reaction to Standard I. Fourteen rabbits have been used over a period of one year, however, and their responses over that period of use have been examined for development of resistance towards pyrogen. It was not really possible to state accurately whether the animals changed in response or not as they were switched from one group to another throughout that time. Although these various groups were standardised using Standard solutions they were not all necessarily equally responsive for in no case was any one of the Standards used throughout a year nor was it possible to compare one Standard against another on the same animals. However, since the regression lines for the three Standards, Fig. 5, were almost parallel, it could be assumed that the groups standardised against one Standard were equivalent in response to the groups standardised against the other standards and on such an assumption it is possible to state that even although a rabbit was changed from group to group its response as a percentage of the group response would remain the same. The method used for comparing the sensitiveness/

sensitiveness of various rabbits was to calculate the response of a rabbit in a group of five as a percentage of the average response of the group. By doing this over a large number of experiments the average percentage response for each rabbit could be calculated. Table XV gives such averages for 14 rabbits and the Standard Deviations for each average. By calculating the average over the two six-month periods it was possible to find if there was any change in response. Using Fisher's 't' test it was found, as is shown in the table, that only two rabbits, rabbit 4 and rabbit 26 showed a significant difference between the means of the two periods. However although the 't' test showed that, in general, no significant difference existed between the means for the two six-month periods, the impression given by the results when considered collectively was that the animals did show lessening in response, it was therefore decided to weight all the results in each six-month period and then test the weighted means for significant difference. The weighted means of the percentage responses for the 14 rabbits in each six months and the combined standard deviation of all these reactions from the weighted means were calculated.

# Table XV

Sensitiveness to pyrogen over a period of one year.

| No. of rabbit | First 6 months | | | Second 6 months | | | t | Total period of use | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Average %age reaction | Stand devn. | No. of expts | Average %age reaction | Stand devn. | No. of expts | | Average %age reaction | Stand devn. | No. of expts |
| 4 | 107 | 24 | 17 | 87 | 9 | 20 | 3.38 | 96 | 21 | 37 |
| 7 | 96 | 26 | 17 | 84 | 27 | 29 | 1.44 | 89 | 25 | 46 |
| 11 | 127 | 21 | 23 | 119 | 19 | 28 | 1.41 | 122 | 23 | 51 |
| 12 | 105 | 8 | 11 | 93 | 27 | 27 | 1.42 | 97 | 21 | 38 |
| 13 | 128 | 23 | 20 | 113 | 34 | 20 | 1.54 | 120 | 32 | 40 |
| 23 | 133 | 26 | 11 | 128 | 35 | 16 | 0.39 | 130 | 31 | 27 |
| 26 | 109 | 22 | 18 | 95 | 17 | 18 | 2.07 | 102 | 21 | 36 |
| 27 | 129 | 26 | 13 | 132 | 26 | 38 | 0.35 | 131 | 27 | 51 |
| 28 | 94 | 34 | 18 | 104 | 20 | 15 | 0.97 | 98 | 31 | 33 |
| 30 | 94 | 26 | 16 | 78 | 16 | 12 | 1.82 | 87 | 24 | 28 |
| 31 | 154 | 38 | 20 | 152 | 21 | 20 | 0.20 | 153 | 30 | 40 |
| 33 | 96 | 27 | 11 | 109 | 30 | 15 | 1.08 | 103 | 31 | 26 |
| 35 | 82 | 24 | 16 | 81 | 13 | 20 | 0.16 | 81 | 20 | 36 |
| 37 | 77 | 17 | 11 | 75 | 13 | 15 | 0.33 | 76 | 14 | 26 |
| Weighted Averages | 111.2 | | | 105.7 | | | | | | |
| Total No. of expts. | | | 222 | | | 293 | | | | 515 |

$\sigma$ (combined standard deviation)

$$= \sqrt{\frac{\sum d_1^2 + \sum d_2^2}{n_1 + n_2 - 2}}$$

$$= \sqrt{\frac{244,333 + 308,939}{513}}$$

$$= \sqrt{1078.5}$$

$$= 32.8$$

Where $d_1$ is the deviation of the percentage response of a rabbit in any one test during the first six-month period from the weighted average of all the responses of the 14 rabbits in the first six-month period, and $d_2$ similarly for the second six-month period.

The difference in the weighted means was then tested using the 't' test, where,

$$t = \frac{\text{Difference in means}}{\text{Standard deviation (combined)}} \cdot \sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$$

$$= \frac{5.5}{32.8} \cdot \sqrt{\frac{222 \times 293}{222 + 293}}$$

$$= 0.16 \cdot \sqrt{126.3}$$

$$= 1.88$$

Fisher's tables of 't' give P. corresponding to this as 0.07 and therefore the 't' test does not show that the differences between the means is very significant. The number of results in each group, however, 222 and 293, are sufficient to give confidence in the accuracy of the means and as these means are numerically, so different and different in the expected direction, i.e. a lessening of response by continual use, the results are definitely suggestive of a significant lessening in response throughout a year of use. However, as has already been emphasised, the groups were re-:constructed about every three months and re-standardised, and thus any change in sensitiveness in a rabbit over a year will have no untoward effect on the quantitative test.

## DISCUSSION.

When starting this work it was hoped to investigate the nature, sources and properties of pyrogenic substances. It was proposed to examine first the various bacteria, yeasts and moulds for the ability to produce pyrogen and then investigate the quantities produced by these organisms, whether it was produced by the metabolic processes of the living organism or by autolysis of the dead organism, and how long a culture required to reach maximum pyrogenic activity. These points were considered essential before finally attempting to isolate the pyretic substances. Having isolated and purified the active substances it was intended to carry out an examination of their chemical nature and their physical, chemical and pharmacological properties.

Previous workers, however, have not sufficiently investigated the ground work to permit such advanced research. The test for detection by the thermometer method, used by most workers, for example, is unreliable and inconvenient. Also, in examining the sources and properties, and to find whether a given extraction process used during the isolation of the substance increases the pyrogenic activity of the solution, or not, it is necessary to have some means of comparing the pyrogenic activity of one solution with another, but such a quantitative test had not been evolved.

The two methods for testing for the presence of pyrogen were examined. These methods differ in that the rectal temperature is measured in one by a clinical thermometer and in the other by a thermocouple, the thermocouple method was found to be both more reliable and convenient than the thermometer method and was used throughout the remainder of the work. The thermocouple apparatus was constructed and the test for detection using this apparatus was found to be very sensitive, an average maximum rise in rectal temperature of 0.36°C. or more in 5 rabbits being indicative of pyrogenic activity of the solution tested. This test was then further dev-:eloped to provide a method of comparing the pyrogenic activity of various solutions, which was sufficiently accurate and reliable for the work and yet was convenient and not too time-consuming. The various questions associated with the construction of a bioassay, such as preparation of Standards, the relationship between the dose and the reaction produced and the accuracy of any determination, were solved in a manner which was considered satisfactory for the requirements of the work. This problem of detection and assaying, however, proved so complex that a major portion of the time was spent in solving it.

Following this it was decided to proceed on the originally intended lines of investigation which, it was hoped, would ultimately lead to the isolation and purification of the active substance, this step being an essential precursor to further work. Thus various species of bacteria, yeasts and moulds were tested for pyrogen-producing ability when grown in synthetic medium and it was concluded that of the three types of micro-organisms the bacterial group was probably the only group to produce pyrogen and also that the Gram-negative organisms produced greater quantities than the Gram-positive types.

It was essential to investigate during the course of the work a few properties of pyrogen in solution. It was found, (1) that Berkefeld filtration had little effect on the pyrogenic activity of a solution but that Seitz filtration removed pyrogen from solution very efficiently, (2), that on storage the rate of loss of potency of a Standard solution depended on the concentration, the initial loss being rapid in a concentrated solution and becoming slower as the solution became weaker, and (3), that the effect of heating a pyrogenic solution at 120°C. was similar to storage, the destruction being initially rapid and decreasing as the solution became weaker, the results of this last set of experiments also suggested that M. tetragenes produced a different, and much more stable, pyrogen from Proteus vulgaris, Ps. aeruginosa and B. subtilis.

Differences in the fever curves stimulated by different modifications of the one culture were observed and the research was necessarily directed into an investigation of this point. The results suggested that some cultures contained two different pyretic substances, one which stimulated an immediate rise in body temperature and the other a delayed rise, a mixture of both stimulating a double-peak fever, and, while investigating this, it was discovered that some organisms produced a volatile depressant substance which had, when present in sufficient quantities, a definite modifying effect on the fever curve and which also caused general paralysis, fall in body temperature and a loss of appetite, the latter lasting for a few days.

It is the intention of the author, having thus developed methods for the accurate assay of solutions and having obtained information on the stability and properties of the active substances concerned, to utilise these in the isolation of pyrogen from large volumes of solution in the hope that it will be isolated in sufficient quantity to throw some light on its chemical nature.

further

## SUMMARY.

1. The test for the detection of pyrogen by the thermometer method was examined and found to be unreliable although the results obtained were within the limits of the test as described in the United States Pharmacopoeia, thirteenth revision, and the British Pharmacopoeia, 1948.

2. A thermocouple circuit was constructed which could accommodate ten rabbits and a test for detection using this apparatus devised. The sensitivity of the test was such that an average maximum rise in rectal temperature of 0.36°C. in five rabbits was indicative of pyrogenic activity of the solution being tested. The thermocouple method was found to be more accurate, reliable and convenient than the thermometer method.

3. A quantitative test for pyrogen was devised whereby an unknown solution could be compared with a Standard. The average error of the test was calculated to be ± 10 per cent. with a Standard Deviation of ± 7 per cent., thus 21 out of every 22 results would probably show an error of less than 24 per cent.

4. The relationship between the dose of pyrogen and the reaction stimulated was found to be logarithmic.

5. Bacteria, yeasts and moulds were tested for ability to produce pyrogen; only the bacteria produced pyrogen in detectable quantities, and of these the gram-negative organisms/

organisms produced much greater quantities than the gram-positive types.

6. In a preliminary investigation of the rate of production of pyrogen by an organism when grown in synthetic medium at optimum temperature for growth, it was found that considerable quantities of pyrogen were produced in the first 24 hours, and that pyrogen production continued after that time. Although the time to reach maximum pyrogenicity was not accurately assessed it was found that a seven-day culture contained much more pyrogen than either a two-day or a 28 day culture.

7. The fact that modifications of the one culture could stimulate three distinct types of fever curves was examined. It was found that cultures of certain bacteria contained two pyretic substances, one which stimulated an immediate response and the other a delayed response, a mixture of both stimulating a double-peaked response.

8. A volatile depressant substance was produced by Proteus vulgaris and Ps. fluorescens which caused general paralysis, fall in body temperature often to well below normal, and a loss of appetite. This substance had a definite modifying effect on the fever curve.

9. Storage for six months at room temperature and in darkness had little effect on a relatively weak solution of pyrogen. The more concentrated solution, Standard II, however, rapidly lost/

lost potency even when stored at just above freezing temperature, but the rate of loss decreased as the concentration of the solution decreased.

10. Little pyrogen is removed by Berkefeld filtration. Seitz filtration on the other hand removed considerable quantities of pyrogen from aqueous solution and the absorbing powers of the pad were very difficult to exhaust.

11. Heating at 120°C. caused an initial rapid loss in pyrogenic activity of cell-free filtrates of cultures of Proteus vulgaris, Ps. aeruginosa and B. subtilis until about 80 per cent. was destroyed, thereafter the rate decreased, after 2 hours at 120°C. about 95 per cent. of the original concentration was destroyed. M. tetragenes seemed to produce a more stable pyrogen as heating at 120°C. for 2 hours destroyed only about 20 per cent. of the activity. How much the stability of the pyrogen in the M. tetragenes culture is due to the presence of other substances in the culture is not known. In all four organisms the rate of destruction was found to be logarithmic i.e. the relationship between the time at 120°C. and the logarithm of the concentration was linear.

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