AN EXAMINATION OF PYROGEN FROM VARIOUS SOURCES

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Whether bacteria in general produce the same pyretic substance or each bacterial species a specific pyrogen is not yet known. During our investigation of pyrogen we have discovered several variations, such as different types of fever curves following injection of modifications of the same culture and variations in stability of pyrogen, from different bacterial sources, when heated at 120°C., which suggest that different pyrogens exist.

Fever Curves

A study of published fever curves produced as the result of injection of pyrogen into rabbits[1,2,3,4,5,6] shows that these can 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), 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 rose 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[1,6].

None of the workers in this field appear to have observed any significant difference between the single- and double-peak curves which appeared in graphical form in their publications. This may have been due to the same culture, under what appeared to be exactly the same conditions, stimulating both the single- and double-peak types of curves. It has been shown here that this failure to observe significant differences in the shapes of the curves was probably caused by taking the temperature of the rabbits at hourly intervals only.

In the course of this work, the three types of fevers already discussed were frequently obtained. As the work proceeded, it became apparent that the differences in the curves were significant and were not caused by biological variation or by the method of temperature determination used, 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 to be associated not only with the delayed fever (type 3), as suggested in the literature, but to be due to a depressant substance produced by some organisms. After removal of this depressant substance, which can be driven off by gentle heat, the solutions stimulated the single- and double-peak types of fevers.

Typical examples of the three types of fevers are shown in Figure 1. In the single-peak curve (type 1) in which the rise in temperature is immediate, the peak temperature is reached in 70 to 120 minutes, the

average time being 86 minutes; in the double-peak curve (type 2) the first peak is identical with curve type 1, but this falls slightly, to be followed by a second rise, reaching its peak in 3 to 4 hours after the

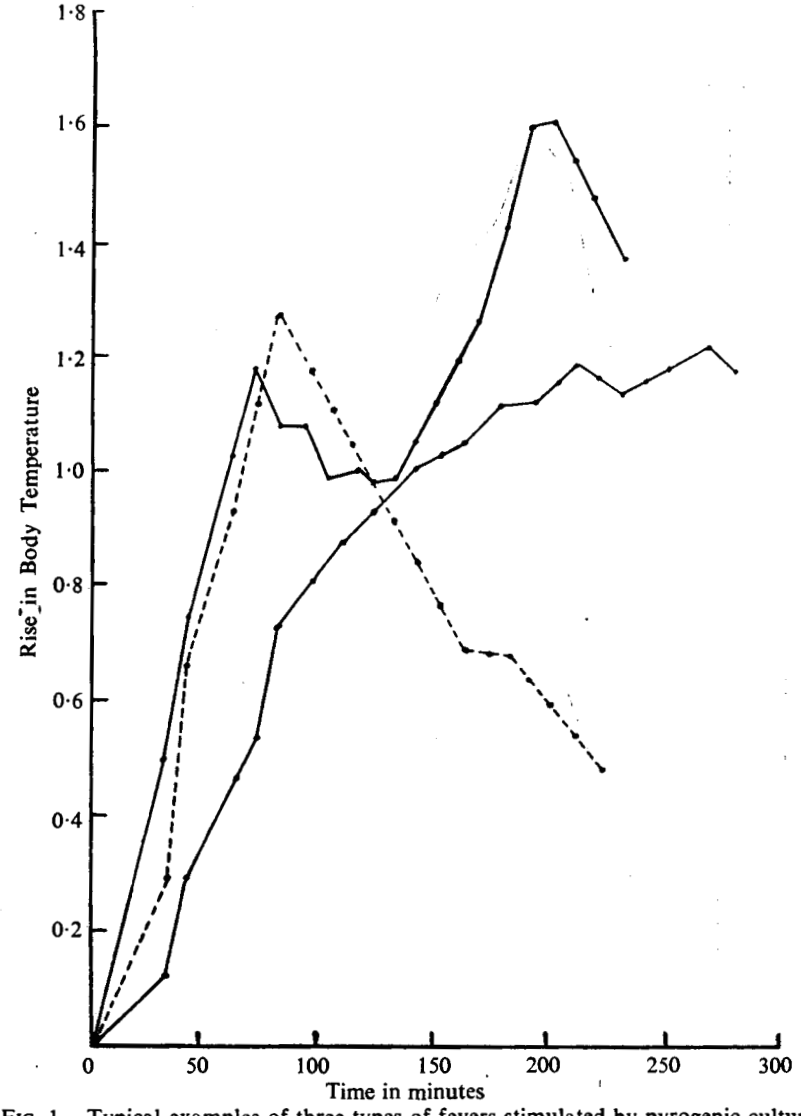

FIG. 1.—Typical examples of three types of fevers stimulated by pyrogenic cultures.

injection; in the delayed-peak fever 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 peak, it would seem that the double peak curve (type 2) is a combination of the two single-peak curves.

Figure 2 demonstrates how completely the shape of the curve is masked

by determining the rectal temperatures at hourly intervals only, the technique used by most of the investigators, and it is probably due to this that no significant conclusions were drawn from the shapes of the curves, as, when using this method, slight changes in the time to reach the peak could change what appeared to be a single-peak curve in one test to a

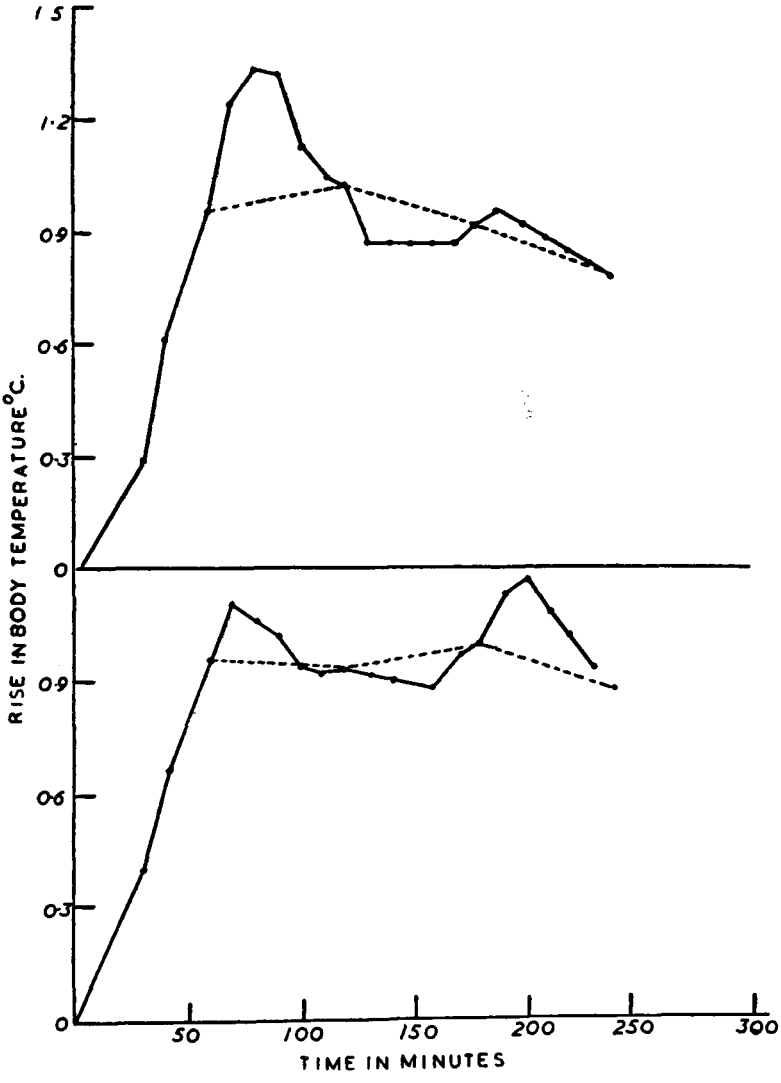

FIG. 2.—Showing effect of method of temperature determination on the shape of the fever curve.
———— Thermocouple method.
— — — — — Thermometer method.

double-peak curve in a repeat of that test. Four explanations can be advanced for the differences in the shapes of the curves.

1. That pyrogen in a whole culture containing the organism and its metabolic products (such a whole culture has been found to stimulate the double-peak reaction) is both dissolved in the medium and contained within the bacterial cell. Here the first rapid rise in temperature may be produced by the pyrogen dissolved in the medium, and the second fever by the slow liberation of pyrogen from the bacterial cells as they are broken down in the blood-stream. The cell-free filtrate therefore 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 immediate response fever is caused by one type of pyrogen, the delayed response by another type, and the double-peak response 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.

These explanations have been investigated in the experiments described below.

Experimental

A culture of *Proteus vulgaris* was grown for 4 weeks at 37°C. in gelatin hydrolysate synthetic medium and the following experiments carried out:

1. Samples of the whole culture were sterilised by autoclaving at 115°C. for 30 minutes and tested on two different groups of 5 rabbits at a dose level of 0·002 ml./kg. of body-weight. Both tests showed the double-peak type of fever, one of which is shown in Figure 3.

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 30 minutes. This filtrate was clear to the eye and no cells could be detected on microscopical examination. At a dose of 0·002 ml./kg. 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 3.

3. Samples of the autoclaved whole culture were centrifuged at 4,000 r.p.m. for 45 minutes, and the supernatant liquid decanted and retained. The cells were then washed 3 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 liquid the double-peak fever (Figure 3).

From the results of this experiment it is seen that the same culture can produce all three types of fever depending on the state of the sample when injected, that 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 the combination of the

two fractions, i.e., the whole culture, causes the double-peak type of fever. 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

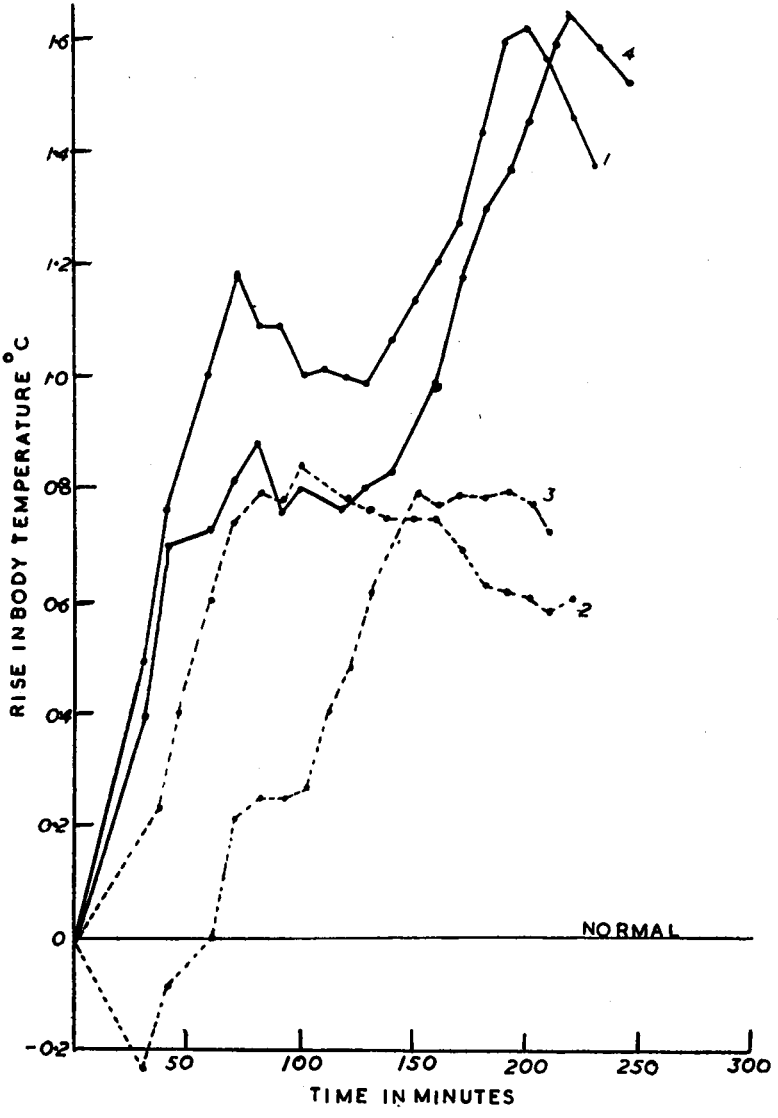

FIG. 3.—Showing different fever curves stimulated by modifications of the same culture of *Proteus vulgaris*.
Curve 1.—Whole culture. Curve 2.—Cell-free filtrate.
Curve 3.—Washed bacterial cells.
Curve 4.—Centrifuged supernatant of sterlised culture.

the double-peak type of reaction was unexpected, if this supernatant liquid were really cell-free, as it appeared to be when examined, the hypothesis that the double-peak fever is attributable to the same pyretic substance being present in the medium and in the bacterial cells is not tenable. Since the other results of the experiment supported the hypothesis it was possible that the centrifuged supernatant liquid still contained sufficient cells to cause the second peak. The above experiments were therefore repeated with additional refinements, mainly to find how many cells, if any, were necessary to stimulate the second peak, or

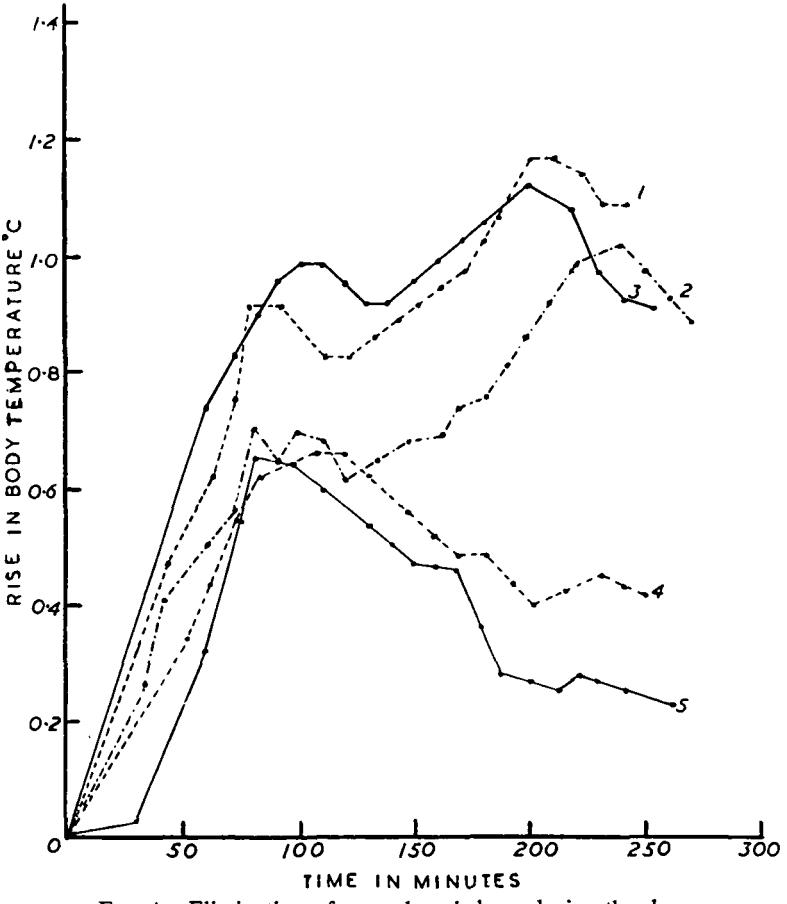

FIG. 4.—Elimination of second peak by reducing the dose.
Curve 1.—Dose in ml./kg. of body-weight.
Curve 1.—0·02 Curve 2.—0·02 Curve 3.—0·004
Curve 4.—0·002. Curve 5.—0·0002.

whether it was possible to stimulate the double-peak type of fever by injection of a completely cell-free solution.

Escherichia coli was grown on a gelatin hydrolysate medium at 37°C. for 5 weeks and samples of this culture tested as follows:—

1. Samples of the whole culture were sterilised by autoclaving at

115°C. for 30 minutes; these were tested on groups of five rabbits in doses of 0·02, 0·004, 0·002 and 0·0002 ml./kg of body weight. The curves for these experiments are shown in Figure 4. The larger doses of 0·02 and 0·004 ml./kg. of body-weight stimulated the double-peak fever but those of 0·002 and 0·0002 ml./kg. of body-weight 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 30 minutes. A dose of 0·02 ml./kg. of body-weight was tested on groups of 5 rabbits on 3 separate occasions, only the single-peak fevers of the immediate response type were stimulated. The curve for

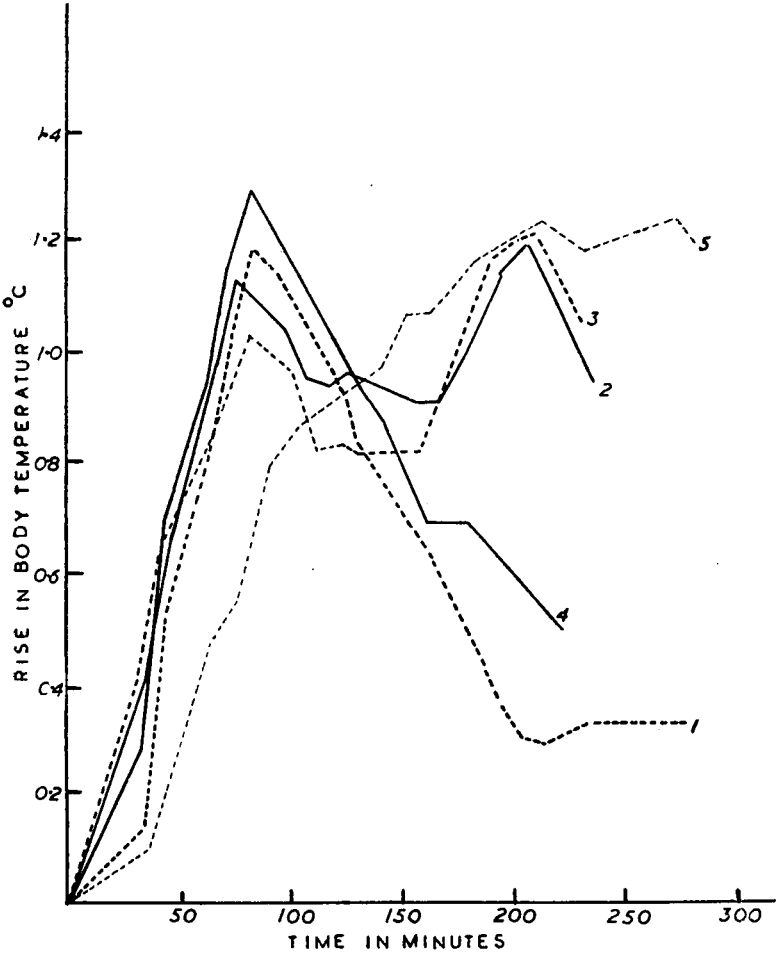

FIG. 5.—Showing different fevers stimulated by modifications of the same culture of *Escherichia coli*.

Curve 1.—Filtrate of viable culture.
Curve 2.—Filtrate of autoclaved culture.
Curve 3.—Centrifuged supernatant of autoclaved culture.
Curve 4.—Centrifuged supernatant of viable culture.
Curve 5.—Washed bacterial cells.

one test is shown in Figure 5 (curve 1). However, in the previous set of experiments on *P. vulgaris,* the supposedly cell-free supernatant which unexpectedly gave rise to the double-peak reaction was prepared by sterilising the culture before the cells were removd 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 and then filtered free of bacterial cells. A dose of 0·02 ml./kg. of body-weight of this filtrate was tested on groups of 5 rabbits on 2 separate occasions and the double-peak type of fever was obtained (Figure 5, curve 2). A dose of 0·002 ml./kg. of body-weight of the whole culture would contain more cells than a dose of 0·02 ml./kg. of body-weight of the filtered culture, but such a dose of the whole culture does not stimulate the double-peak fever and the filtrate does; it would appear, therefore, that, the few cells possibly present in the filtrate would not cause the double-peak. Therefore of the 4 theories advanced, the most probable one is that the solutions stimulating the double-peak of reaction contain two pyretic substances, one causing the immediate rise in body temperature and the other a delayed rise, a mixture of the two causing the double rise. 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 liquid were also carried out using the supernatant liquid of both the viable and the sterilised whole culture. In all cases the centrifuging was at 3,750 r.p.m. for 3½ hours in an attempt to produce complete deposition of the cells, and no cells could be detected on microscopical examination. In four tests, each at a dose of 0·02 ml./kg. of body-weight, the supernatant liquid of the previously sterilised whole culture caused the double-peak fever, one of which is shown in Figure 5 (curve 3). The supernatant of the viable culture at the same dose stimulated only the single-peak fever (Fig. 5, curve 4). These results therefore support those obtained for the filtrate. The cells obtained by centrifugation of the sterilised whole culture were washed 3 times with pyrogen-free water and finally diluted with the required amount of saline solution to bring the cell count back to approximately that of the original culture, and tested at a dose of 0·02 ml./kg. of body-weight. The fever developed was of the delayed type as was found in the previous experiment (Figure 5, curve 5).

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

1. Three doses of the whole culture, 0·02, 0·002 and 0·0002 ml./kg. of body-weight, were tested. The two higher doses stimulated the double-peak reaction and the dose of 0·0002 ml./kg. of body-weight the single-peak reaction, showing again that the second peak can be diluted out more readily than the first.

2. Filtrates from both the viable and the sterilised cultures were tested, that of the autoclaved culture stimulated a definite double-peak response and that of the viable culture the single-peak reaction.

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 centrifuged supernatant stimulates the single-peak type of fever if the cells are removed before autoclaving, and the double-peak type if the whole culture is autoclaved before removal of the cells, and that the washed cells of the centrifuged culture cause the production of the delayed fever. From the results therefore it appears that there are two pyretic substances, one causing the immediate fever and the other the delayed fever, and a mixture of both the double-peak fever. However, it also appears 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 clarify this point, a further set 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 30 minutes, the second by heating at 60°C. for 1 hour and the third by addition of 0·3 per cent. of chlorocresol. At a dose of 0·02 ml./kg. of body-weight all three solutions caused the double-peak type of reaction. The actual quantity of chlorocresol injected into the rabbits was 0·1 mg. and would have little effect. Thus the substance stimulating the double-peak fever is not formed by autoclaving the cells. Another possible explanation 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 in the medium it is not usually present in sufficient quantity to stimulate the second peak. Autoclaving however increases the quantity of this substance in the medium sufficiently that the cell-free solution can stimulate the double-peak type of fever. This explanation is further supported by the fact that *Serratia marcescens (Bacillus prodigiosus)* stimulated the double-peak fever whether the culture was sterilised before or after removal of the cells, showing that the second-peak substance is not an artifact produced by autoclaving the cells. It may be assumed that in this case the second substance passes more readily from the cell into the medium than it does in either *Escherischia coli* or *P. vulgaris*.

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

Production of Depressant Substance by some Organisms and the Effect this has on the Fever Curve

Reactions, following injection of cultures of *P. vulgaris* and *Ps. fluorescens*, were obtained from time to time which differed from the

usual type of fever reaction. In these cases 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 five rabbits was 1·34°C. below the initial temperature (Figure 6), one of the rabbits actually showing a fall of 3·4°C. within 1½ hours of the injection. In the more severely affected 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

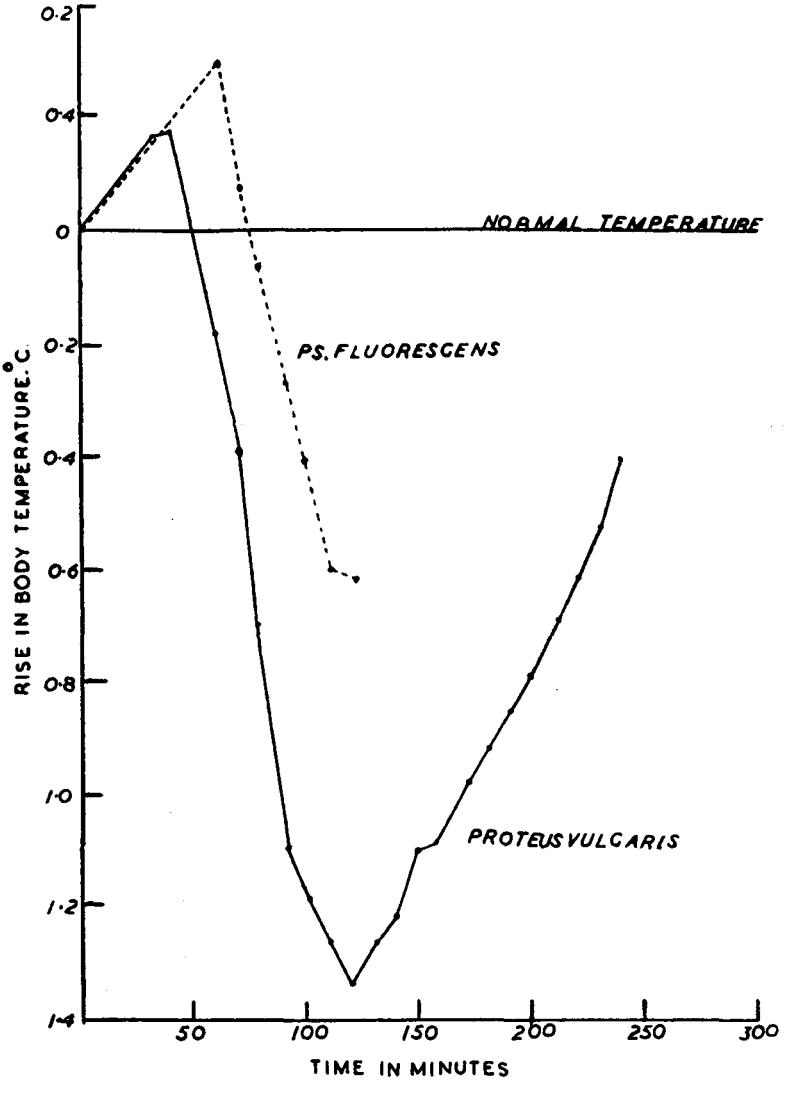

FIG. 6.—Fall in body temperature caused by a depressant substance present in cultures of *Ps. fluorescens* and *Proteus vulgaris*.

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 rose slowly and the animal was soon able to stand and move about. The symptom which was apparent in all of the animals injected, whether severely affected or not, was the loss of appetite, the animal refusing food for a number of days after the experiment.

The depressant effect was not repeated when further samples of the same cultures were retested, and it was also observed that when the reaction occurred, the first rabbit in each group to be injected was usually the most severely affected. As the solution to be injected is heated to approximately body temperature before injection, it was realised that if the depressant were very volatile it 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 experiments were carried out on a seven-day culture of *P. vulgaris*. Samples of the whole culture, in sealed ampoules, were sterilised by autoclaving at 115°C. for 30 minutes, and tested in doses of 0·02 and 0·002 ml./kg. of body-weight. In these tests the solution was diluted, placed in an open beaker and heated to 30° to 40°C. for about 10 minutes before injection. This solution produced the usual fever reaction (Figure 7), no depressant action or loss of appetite being observed. Similar samples of this culture were tested in doses of 0·04, 0·02 and 0·01 ml./kg. of body-weight, this time taking care during the preparation of the solution 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./kg. of body-weight, there being a distinct fall in body temperature and development of paralysis (Figure 7). The rabbits receiving the other two doses, although not showing the fall in body temperature nor the paralysis, lost appetite for food for a few days. Thus the substance causing this fall in body temperature and loss of appetite is volatile, and since it is present in the cultures autoclaved in sealed ampoules, it appears to be relatively stable towards heat.

Modifying effect of depressant on the fever.—Figure 8 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 it caused the usual type of fever, the modifying effect of the depressant is seen in the fevers stimulated by the solution injected without previous heating, the temperature rose sharply in the first hour but instead of continuing to the usual peak in 1½ hours, it either fell sharply, or tended to remain at the point reached. Such a reaction, in which the depressant effect is not very obvious, might lead to the wrong conclusions concerning 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, they 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 solutions before injection and the result of any

experiment which is followed by the refusal of the animals to eat should be examined for signs of the modifying effect of the depressant, and the experiment repeated if necessary.

This depressant might also account for the distinct fall in body temperature following the injection of the washed cells of *P. vulgaris*, as is seen in Figure 3. In this delayed fever the temperature fell before beginning to rise, and this reaction seems to be similar to the reactions referred to by Seibert[3] and Hort and Penfold[6].

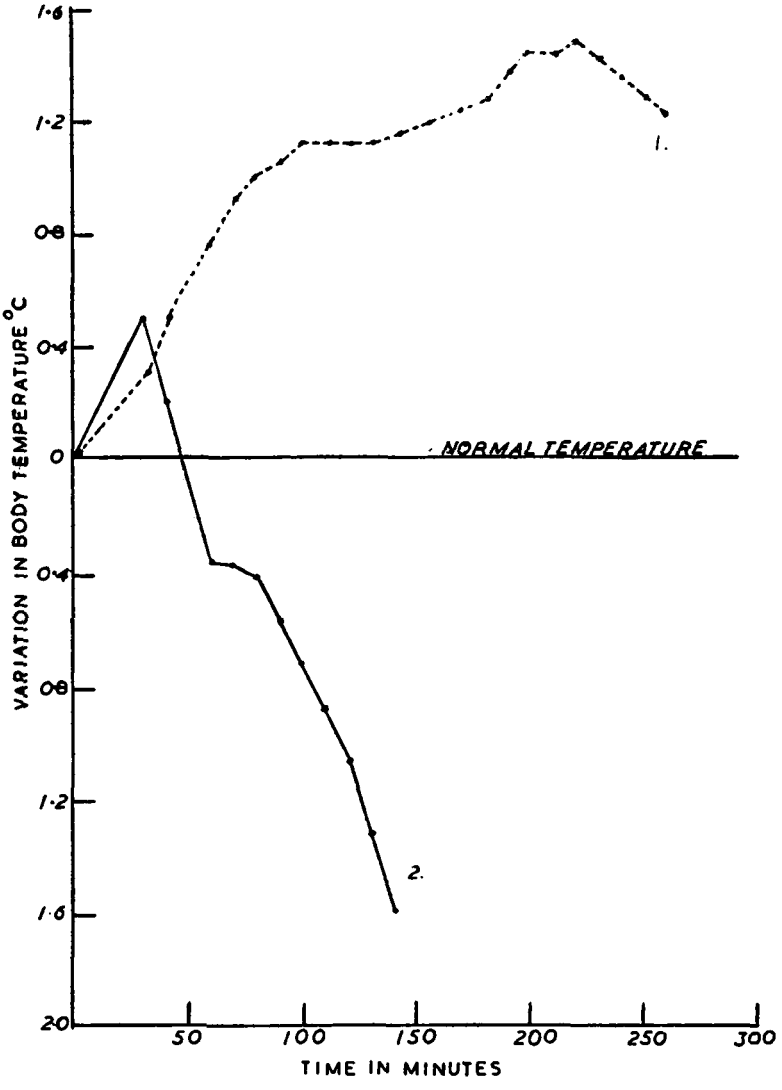

FIG. 7.—Showing effect of heating solution before injection.
Curve 1.—Solution heated before injection.
Curve 2.—Solution not heated before injection.

Variations in stability to heat.—The stability of pyrogen to heat has been investigated by Seibert[3] and Banks[7], both concluded that it was a thermostable substance. In a previous paper, however, we showed that a cell-free filtrate of a 48-hour culture of *P. vulgaris* showed an initial rapid loss in activity when heated at 120°C., but that the solution was still pyrogenic after 4 hours at 120°C.

A further 48-hour and a 31-day culture of *P. vulgaris* and also cultures of *Ps. æruginosa, B. subtilis* and *M. tetragenes* of 3, 2 and 1 weeks incubation at 37°C. respectively, have since been examined for stability

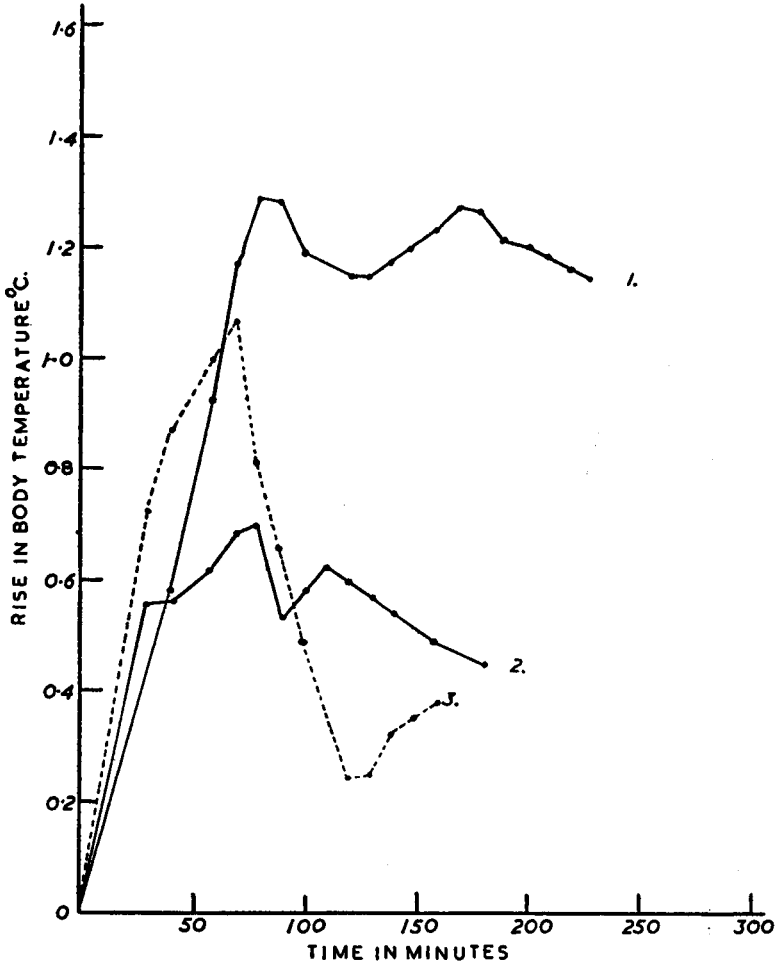

FIG. 8.—Modifying effect of depressant on the fever stimulated by a whole culture of *Proteus vulgaris*.

Curve 1.—Dose—0·002 ml./kg. of body weight, no depressant effect.
Curve 2.—Dose—0·02 ml./kg. of body weight, depressant effect.
Curve 3.—Dose—0·005 ml./kg. of body weight, depressant effect.

to heat at 120°C. As the method of heating at 120°C. by autoclaving, as described in the previous paper, is not easily controlled or reproduced, the cultures were filtered free of bacterial cells, sealed in thick-glass ampoules and immersed in an oil-bath maintained at 120 ± 1°C. The reaction of each solution was previously adjusted to *p*H7, it was not considered necessary to add buffering agents to the solution, as it was the buffered medium in which the organisms had been grown. Samples of each of the cultures were heated at 120°C. for 30 minutes, 1 hour, 1½ hours and 2 hours. The strengths of the unheated cultures were calculated by extrapolation of the log. concentration against time curve, which is a straight line. Table I shows the results for this set of experiments.

It would seem, from the results, that the rate of loss inactivity of cultures of *P. vulgaris, B. subtilis* and *Ps. æruginosa* is somewhat similar, in that approximately 50 per cent. is destroyed after 30 minutes 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 original activity being destroyed after 2 hours at 120°C.

TABLE I

SHOWING THE RATE OF DESTRUCTION OF THE PYROGEN FROM *PS. ÆRUGINOSA, B. SUBTILIS AND M. TETRAGENES*

Heat Treatment		*Pseudomonas æruginosa*		*Bacillus subtilis*		*Micrococcus tetragenes*	
Temp. °C.	Duration hours	Units/ml.	Percentage destroyed	Units/ml.	Percentage destroyed	Units/ml.	Percentage destroyed
Extrapolation		214	0	19	0	9·1	0
120	½	75	65	9	52·6	8·75	3·8
120	1	51	76	4·4	76·8	8·0	12·6
120	1½	26·9	87	2·4	87·4	7·5	17·6
120	2	10·6	95	0·9	95	7·3	19·8

		Proteus vulgaris						
		48-hour culture		48-hour culture		31-day culture		Average percentage destroyed
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	6·2	93	41	95	94
120	4	0·75	98	1·9	98	14	98	98

Linear relationship between the log. of concentration and time.—By determining the percentage of the original concentration left at each stage, and plotting the logarithm of this against time, a linear relationship was found to exist for all four cultures, which satisfied all points within the experimental error of the quantitative test, i.e. about 15 per cent. Wylie and Todd[8]. In the case of *Proteus vulgaris*, a batch was heated at 120°C. for 4 hours, but this point deviated in all three experiments from the linear relationship of the other points.

Figure 9 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 *P. vulgaris, Ps. æruginosa* and *B. subtilis* within the

experimental error of the quantitative test. 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 cultures of *Ps. æruginosa, P. 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 the 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 substances. The results obtained here fulfil the desired requirements, in that they give an indication of how much pyrogen may be lost during any heating process, and also of the degree of severity of treatment permissible in the isolation of pyrogen.

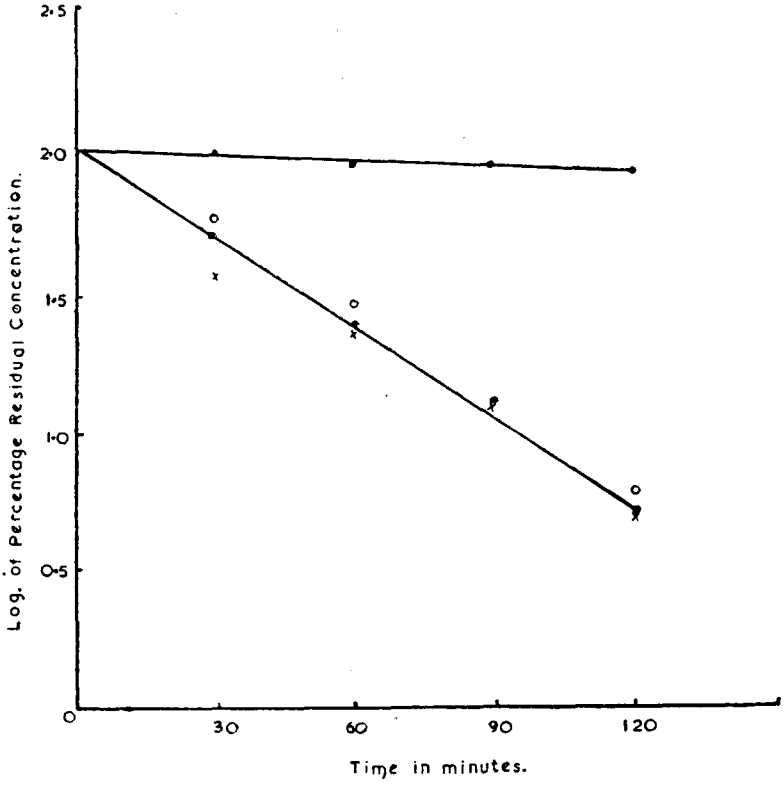

FIG. 9.—Showing linear relationship between logarithm of residual concentration and duration of heating at 120°C.

Upper graph *Micrococcus tetragenes.*
Lower graph o *Proteus vulgaris.*
● *Bacillus subtilis.*
× *Pseudomonas æruginosa.*

SUMMARY

1. Three distinct types of fever curves can be stimulated by injection of modifications of the same bacterial culture.

2. Injection of a sterilised whole culture causes a double-peak fever; the cell-free filtrate or supernatant liquid of a culture stimulates a single-peak fever, rising to a peak in an average time of 86 minutes if the cells are removed before autoclaving, and a double-peak reaction if the culture is autoclaved before removal of the cells; the fever caused by injection of a suspension of washed bacterial cells is a single-peak fever of the delayed reaction type, reaching its peak in 3 to 4 hours.

3. The results show that certain bacteria probably produce two pyretic substances, one of which, in the viable culture, is dissolved in the medium and stimulates the single-peak fever of the immediate reaction type, and the other is contained mainly in the bacterial cell and stimulates the single-peak fever of the delayed reaction type, a mixture of both causing the double-peak fever.

4. *Proteus vulgaris* and *Ps. fluorescens* can produce a volatile depressant substance, which either lowers the body temperature to below normal, or prevents the pyrogen from causing a rise in body temperature. This depressant causes general paralysis if injected in sufficiently large doses, in smaller quantities the most obvious effect is the loss of appetite. It has been shown that any pyrogen test followed by the rabbits refusing to eat for a few days should be examined for other effects of the depressant substance, and should be repeated, taking care to heat the solution to between 30° and 40°C. for about 10 minutes before injection.

5. An investigation of the stability of pyrogen to heat at 120°C. showed that *Ps. æruginosa, B. subtilis* and *P. vulgaris* produce pyretic substances of similar stability, whereas *M. tetragenes* produces a much more thermostable substance.

6. The results from the investigation of the stability showed that the pyrogens of *P. vulgaris, Ps. æruginosa* and *B. subtilis* are sufficiently labile to require care when using heat during isolation, as approximately 95 per cent. is destroyed after 2 hours at 120°C. Finally it must be emphasised that further investigation into these points would be of little value unless carried out on the pure pyretic substances isolated from various bacterial sources.

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