The Properties of Pyrogenic Substances

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Introduction

All substances capable of producing a rise in body temperature may be described as pyrogenic but the term "pyrogen" has come to be used in a more restricted sense as the name for substances derived from micro-organisms which on injection into the blood stream produce a rise in temperature. These substances have attracted considerable attention and have been studied from two main aspects, firstly for their use as therapeutic agents to produce increases in temperature or in the production of fevers for experimental purposes, and secondly for their accidental occurrence in solutions for injection.

Methods of measuring pyrogens, both for clinical or experimental purposes and for detecting their presence as contaminants, have been studied by many workers during the last thirty years since pyrogens made their presence known in injection solutions by the production of undesired fevers.

The present work concerns their stability and two of their effects on rabbits. These were studied firstly with a view to producing a standard for use in biological assay and secondly in order to devise a quantitative assay. The assay devised depends on the effect of the pyrogen standard on the leucocytes of the rabbit.

REVIEW SECTION

Although effects of pyrogen other than rise in temperature have lately increased in importance this effect is still the most widely used both for therapeutic purposes and as a basis of assay.

Temperature variations in disease have attracted attention from very early times. Hippocrates is said to have written about fevers.[1,2] Accurate measurement of temperature was not however possible before the invention of the thermometer. Its first clinical use is generally attributed to Sanctorius of Padua, 1561 - 1635 [3], after which it seems to have been neglected or forgotten until Martine wrote about it in 1740 [4,5]. Despite the observation by Van Swieten in 1745 [6] that the estimation of temperature by hand was "uncertain", clinical thermometry has been practised only since about the beginning of the nineteenth century when a "portable" clinical thermometer was manufactured and sold to practitioners [7] and at the same time a thermocouple-galvanometer circuit was used for temperature recording [8]. About 1835 Becquerel and Breschet [6], measuring temperatures with thermometers and copper-steel thermocouples, collected much information about body temperature in man and in animals. Clinical

work on thermometry further advanced after Lord Kelvin's work in 1849. Probably the first scientific publication on clinical thermometry was that of Wünderlich [9] in 1868, reporting many clinical observations, recommending temperature measurement in disease and predicting that it would become routine.

The causes of temperature variations were now investigated. In 1865 Billroth [10] observed that a rise in temperature occurred after the administration of some injections and Roemer [11] in 1891 found that a rise in temperature occurred when extracts of Friedlander's Pneumobacillus and B. pyocyaneus were injected into healthy guinea-pigs whereas a fall in temperature occurred in tuberculous animals. Filtrates from E. typhosa were later found by Sanarelli [12] to give rise to fevers in rabbits.

Hammerschlag [13] suggested that injections giving rise to febrile reactions might contain not a pyrogenic substance but a substance which destroys blood corpuscles, whose breakdown products then produced the febrile reaction. It is surprising that this idea was not pursued by following workers, but it has now been shown that the injection of leucocytes can produce a rise in temperature [14].

Use was made of bacterial products as therapeutic agents capable of producing fever by Rumpf [15], who in 1893 treated typhoid infection with B. pyocyaneus, and by Cecil [16] in 1917 who treated rheumatic and toxic arthritis with typhoid vaccine. This type of work led to the well-known protein shock therapy. Petersen [17], the author of a standard work on this subject in 1922, believed that the beneficial results from foreign protein therapy were the increase in leucocytes and enzymes and the thermal reaction.

During the present century the increased use of injection as a method of administering medicaments made apparent the frequent occurrence of fevers and other undesirable symptoms following injections and many papers were published on the probable sources of these fevers[18-34]. Alleged sources of fevers were water, salvarsan, casein, acacia, inulin, haemolysis, salt, dextrose, urea, agar, gelatin, arsphenamine, peptone, greater difference between the pH of the injection and of the blood than the blood could buffer, bacteria and yeasts. Seibert showed conclusively that the occurrence of fever was associated with the microbiological contamination of the injection at some stage in its preparation.

The Removal of Pyrogen from Solutions for Injection.

Because of the difficulties in preventing the contamination of injections and their ingredients attempts have been made to remove pyrogens from injections by physical and chemical methods.

The physical methods are mainly adsorption methods. The fact that pyrogen can be removed by adsorption suggests that it is a large molecule. A gelatin filter was used successfully by Hort and Penfold [21] but they found that a Doulton filter did not remove pyrogen. Co Tui et al.[35-37] investigated the use of sintered glass, Berkefeld W, Zsigmondy and Seitz filters and found that Zsigmondy of small pore size and Seitz filters were of value in removing pyrogen. It appears therefore that the so-called filtration methods are really adsorption.

Various substances have been found to be useful adsorbents for pyrogen. Lees and Levvy [38] used carbon, aluminium oxide, kaolin, "Novasorb" (a magnesium trisilicate), kieselguhr and Fuller's Earth. Carbon was used also by Hudson [39], Brindle and Rigby [40,41] and Charonnat and Lechat [42]. Hudson pointed out the difficulty of removing traces of carbon and Brindle and Rigby avoided this difficulty by washing the colloidal particles out of the charcoal before adding it to the pyrogenic solution.

The problem of removing pyrogen from solutions of other large molecules has not yet been solved. Co Tui and Wright [43] found that Seitz pads adsorbed pyrogen from crystalloid solutions but not from colloidal sols, presumably due to saturation of the pad. Tønnesen and Vesterdal[44], using Seitz pads to remove pyrogen from solutions of penicillin, lowered the concentration of pyrogen relative to the penicillin. Zittle, Devlin, Rodney and Welcke [45], studying the removal of pyrogen from solutions of protein hydrolysates, found that the area of asbestos pad required for the removal of pyrogen from enzymic hydrolysate (63% hydrolysed) was about twenty times that required for the removal of the same amount from water. Smith and Pennell[46] while working on protein solutions found that the area of asbestos pad required was a function of the concentration of pyrogen and of protein. A similar result was obtained using "decalso", an ion-exchange agent described by its manufacturers as "a white sodium Alumino Silicate". Wylie [47] attempted without success to remove pyrogen from plasma using asbestos pads.

Another physical method claimed to have been successful is electrosmosis, which was used by Caillaud and Vincent[48].

Since pyrogen is not volatile stills have been designed to produce pyrogen-free water for injection both

on a small scale[24,26] and on a larger scale for hospitals[49-52]. The small scale stills are designed with baffles to avoid entrainment and the hospital designs include the use of condensate from the general steam system which is considered to be pyrogen-free since it has been maintained at a high temperature for a number of hours.

The chemical methods of removing pyrogen from solutions for injection are based on oxidation or hydrolysis. Taub and Hart[53] used hydrogen peroxide but emphasised that excess must be removed because of the danger of embolism on injection. Menczel[54] subsequently found hydrogen peroxide practicable on a hospital scale. The addition of sodium hypochlorite in conjunction with carbon, perhaps a dangerous addition to materials for injection, was found to be effective by Charonnat and Lechat[42] while Pingert and Ferry[55] used amylolytic enzymes to remove pyrogen from protein hydrolysates. Brindle and Rigby[40] found that chlorine, 30 parts per million, did not remove pyrogen.

Sources of Pyrogen

Since Seibert[24,26] and Bourn and Seibert[27] showed that pyrogen was associated with bacterial contamination much work has been done to investigate various micro-organisms to find out those which produced pyrogen.

The organisms investigated are shown in Appendix One, the table being divided into two sections - the pyrogenic and the apyrogenic organisms. The general conclusion from these investigations is that Gram-negative organisms are more pyrogenic than Gram-positive organisms. The fact that a number of organisms are in both sections of the table would indicate that the method of testing for pyrogen or the organisms or both require further investigation.

Testing for Pyrogen

Of the methods used to detect pyrogen's presence or to estimate the amount present those based on temperature response have attracted most attention. Other effects of pyrogen have been noted - for example the effect on the white blood cells - but do not form the basis of any current assay.

Among the earlier tests were those on which the test of the United States Pharmacopoeia was subsequently based. This work was carried out by Welch, Calvery, McClosky and Price[65] and McClosky, Price, Van Winkle, Welch and Calvery[66]. They gave specifications for the rabbits to be used, attempting to ensure that thay were healthy by measuring their weights and temperatures over a period before actual use in a pyrogen test. They also specified a rest period between tests of two days so that the effect

of a temperature rise in one test would not be felt in the next one. They later lengthened the rest period. During tests factors that would disturb the animals' temperatures were eliminated, such as excitement, digestion and variation of laboratory temperature. They suggested the use of five animals per test.

Practical improvements were made by Kuna, Edison and Butz[67] who described an arrangement of thermocouples and potentiometer which enabled many rabbits to be used simultaneously. The effect of this apparatus on the results was investigated by Molitor, Gundel, Kuna and Ott[68]. The rabbits had to be mildly restrained in boxes and this produced a slightly lower starting temperature. They suggested the injection of three dose levels, each increasing fourfold, into groups of five rabbits. Such a design is an advance in that a log.dose/response line may now be plotted.

The United States Pharmacopoeia[69] test embodies most of these recommendations, performing a preliminary test on three rabbits and using five only if the result is doubtful. It suggests a method of depyrogenising syringes and needles. They are heated in a muffle furnace at 250°C. for at least half an hour. The pyrogenic threshold is taken as 0.6°C. The test applies to solutions administered intravenously in large volumes,

dextrose, saline etc. and to heparin which is difficult to prepare pyrogen-free. The use of a reference standard for simultaneous injection is not mentioned.

The method of the British Pharmacopoeia[70] is similar but there is less warning about practical precautions such as investigation of the rabbits' usual temperature range or restraining the animals. The test applies to similar materials. There is no mention of testing for pyrogen in blood or blood products, which may easily become contaminated, or in solutions of calcium gluconate, which often seems to cause difficulty to manufacturers. The British Pharmaceutical Codex[71] test omits warming the material to 37° before injection - a matter which will presumably be amended in the next issue, since 10 ml./kg. of cold solution could lower a rabbit's temperature.

The test of the Codex Medicamentarius Gallicus[72] is similar to that of the United States Pharmacopoeia, applying to all injections of volume more than 125c.c. This test was criticised by Ardry[73] on grounds that the constant dose of 10 ml./kg. should be replaced by doses more related to the therapeutic dose of the material concerned. It is difficult to see how this could be done for want of knowledge of the relative sensitivity of man and the rabbit to pyrogen.

An improved test was designed in 1948 by Wylie and Todd[60]. A preliminary calibration of the entire stock of rabbits against a temporarily stable standard was carried out. The instability of the standard pyrogen solution necessitated this quick calibration and prevented storage of the solution for use as a standard on each occasion of assay. On the results of the calibration the population was divided into groups of five, each group having an equal mean response. Unknown samples were then tested by injection of 2 ml./kg. of isotonic solution at 37°C into a group of five rabbits. The test is an advance in that it gives some idea about sensitive and resistant rabbits, but its failing is in its assumption of linearity of response plotted against log.dose, of the constancy of gradient and position of such a line and of the stability of the standard solution throughout the period of calibration. Also recalibration of the entire population against a fresh standard was necessary as the original population became depleted due to rabbits' dying from natural causes.

Meantime further work had been done by Ott[74]. He now eliminated from his colony extremely resistant or extremely sensitive rabbits which had yielded results tending to show that rise was a function of basic temperature, and thus vitiating the use of a log.dose/response line, which he now uses. He calculated the error in the method

as " ± 60% of the estimated pyrogen unitage ". Planned assays were described by Tennent and Ott[75] in 1952. They calculated equations for regression lines of response on log.dose from 18 groups of three animals, having injected more than one dose level of sample and unknown into the groups. They found the equation varying, an argument for cross-over tests using a stable standard.

In a proposed new pharmacopoeial test[76] note is taken of the fact that restrained animals will have a lower starting temperature, and therefore the minimum is lowered below which an animal is considered abnormal and therefore rejected.

From all these tests it will be seen that two needs arise, one a theoretical need for a design of assay of calculable limits of error, and the other a practical need for the stable reference standard required by such an assay.

A collaborative study on pyrogen is about to begin[77], using two standards - a crude one obtained from Proteus vulgaris and a purer polysaccharide obtained by Shear[78] from Serratia marcescens. It is hoped that the use of these, along with local laboratory standards, will produce enough information for the design of a more reliable test and will eventually lead to the replacing of the biological test by a physical or chemical test.

The Temperature Responses of Various Animal Species to Pyrogen

Not a great deal of work has been done on establishing the relative sensitivity of different species of animal and little indeed on relating the responses of man to the responses of whatever animal was chosen as test animal, yet this would seem to be important in assaying pyrogen in injections for use in humans. The animals whose responses have been investigated are the mouse, rat, guinea-pig, rabbit, dog, cat, monkey and horse.

Mice have been found to react to pyrogen[26] but are too small for convenient temperature measurement. Rats too are too small for this[79], while guinea-pigs are unsuitable because of the considerable normal fluctuation in their temperatures[21,26].

Rabbits have been used by most workers on pyrogen but no real systematic work has been done on the variation of their responses to a fixed dose for want of a stable standard. They were first observed to react by Hort[18]. Seibert[26] used them also and was of the opinion that man reacted in the same way as the rabbit to comparable doses. Co Tui and Schrift[80] considered the rabbit less sensitive than the dog to the leucopenia following the injection of "reactive sera", and Lees and Levvy[38] considered 20c.c. per rabbit equivalent to 500c.c. per human. Co Tui and

Schrift[81] considered that rabbits frequently gave a false positive result and therefore a negative result would be more significant, meaning that rabbits were of more use in indicating the absence of pyrogen than in estimating how much was present. They considered the dog better for estimating this and suggested that the test therefore use both animals. Co Tui, Hope, Schrift and Powers[82] concluded that the rabbit was about a third as sensitive as man whereas the dog was about a twelfth as sensitive. They considered the ratio of minimum lethal dose to minimum pyrogenic dose to be of the order of hundreds in man and thousands in rabbits.

The dog has not been used a great deal - presumably for the same reason by other workers as in the present work - the greater expense in money and space required to keep dogs. Theoretically the dog would be a preferable animal to the rabbit, having a more highly evolved brain and therefore presumably a better temperature-regulating mechanism. On the other hand this could mean that the dog was less sensitive to small temperature changes, but there appears to be no work on this beyond the papers quoted. Co Tui and co-workers used dogs[37,57,80,83], finding the fever at its height two to three hours after injection and a leucopenia greatest three quarters of an hour after injection.

Cats were used by Ranson, Clark and Magoun[84] who showed that in these animals with fevers due to typhoid vaccine the temperature rise was not smooth but had several peaks with falls between them, and next morning was still above normal.

Weger[85] considered the activity of pyrogen to be of the same order of magnitude in man, rabbits and horses when the dose per weight was taken into account, while Windle, Chambers, Ricker, Ginger and Koenig[86] mentioned a descending order of sensitivity in the species dog, rabbit, cat, monkey, guinea-pig and rat.

The Properties of Pyrogenic Substances

A. Pharmacological properties

This is becoming a matter of increasing importance. There are now innumerable publications listing properties of pyrogen other than the temperature raising property, but they must at present be studied while bearing in mind that, for want of a pure substance, these properties are not necessarily properties of the substance which raises temperature.

The other properties are of importance clinically. If they do not belong to pyrogen they belong to other substances which occur in the bacterial contamination of injections and therefore their effects are equally likely

to arise as untoward symptoms. Another possibility is not of the occurrence simultaneously of more than one substance but of a"chain reaction", a substance in the injection causing the leucopenia and either decomposing into a substance causing fever or liberating from the broken down white blood corpuscles a substance causing the fever. Apparently this has not been investigated so far but it is a point the author considers worth investigating, as the leucopenia appears before the temperature peak is attained.

The other aspect of the matter is that raising the patient's temperature may be desirable, but before using pyrogen for this one would have to know any likely side-effects. If there were none, pyrogen would be very useful because there are drawbacks to the use of most hyperthermic chemical substances and to the physical heating of patients.

The other pharmacological property most frequently attributed to pyrogen is an effect on the white cell picture. Co Tui et al.[82] described a clinical example of a woman who received 750 times their "minimum pyrogenic dose" of pyrogen from E. typhosa. Her leucocyte count almost halved in about an hour after injection. Lonsen and Liebert[87] treated 48 neurosyphilitic patients with a proprietary brand of pyrogen from Pseudomonas

aeruginosa. They did total and differential blood counts and reported a fall in the number of white cells soon after injection followed by a great increase in total number, with a marked shift to the left in the Arneth count and with a decrease in the number of lymphocytes.

Besides these papers on the effect in man, there are several papers dealing with the white cell effect in guinea-pigs, rabbits and dogs.

Olitzki, Avinery and Bendersky[88] investigated the effect of various micro-organisms in the guinea-pig where the general effect was the production of a leucopenia and then a leucocytosis, but the type of white cell affected was not always the same. It would be an interesting but lengthy piece of work to investigate the type of cell change for each organism in man and various species of animal and see what correlations occurred in the findings. Bennett[89] compared the white cell changes produced in the guinea-pig by pyrogen and by anaphylaxis. He found them different, the antigen producing a rise in eosinophils and the pyrogens, from E.coli vaccine, typhoid vaccine and a Pseudomonas preparation, producing a fall in eosinophils. This is not strictly comparable with the findings of Olitzki et al.[88].

Rabbits were used by Chapman[90] to investigate the relative sensitivities of leucopenia and temperature re-

sponse for the estimation of pyrogen. Disturbance due to rectal bleeding was mentioned so it is probable that the temperature rises recorded would be inaccurate in a rabbit disturbed to this extent. She concluded that leucopenia was the simpler measure and required less in the way of handling precautions. On the other hand, Bose and Ahuja[91] found the fall in leucocytes after their injections to be less than the error in counting by means of a Neubauer chamber, and therefore rejected leucopenia as a measure of pyrogen. Bandelin[92] also set out to find if white cell count would be less variable than temperature response. He commented favourably on it, finding with decreasing doses that the temperature response was "washed out" before the white cell response, which he found more sensitive and more rapid. Beeson's paper[93], which concerned the behaviour of the circulating leucocytes throughout a course of injections, reported that after each daily injection a leucopenia occurred in one hour and a leucocytosis in four to eight hours, but that their extent lessened towards the end of the course. His pyrogen might of course have decomposed.

Dorche and Castaing[94] contributed a very useful paper towards this aspect of the work. It presented a clear diagram of frequencies of perecntages of polynuclears before injection and at intervals of one, three

and four hours after injection. However they considered the extent of the normal variation such that the sensitivity of this method was not greater than the sensitivity of the temperature method, but that the two in conjunction were useful.

The white cell and temperature effects due to typhoid vaccine were compared by Farr and Lequire[95], with particular reference to correlation between dose and magnitude of response. They found both responses varying with the dose, small doses producing no temperature rise but producing a white cell effect and larger doses producing both effects, from which they concluded that the white cell response was the more sensitive.

Windle et al.[86] included in their investigations histological changes relating to the blood picture. They found alterations in the spleen, lymph nodes and bone marrow indicative of increased phagocytosis and increased cell production.

A report on leucocytosis as a measure of pyrogen was published by Young and Rice[96]. They used dogs, considering them more reliable than rabbits but not saying why they thought this, and counting total white cell variation, neutrophil variation, maximum leucopenia, duration of leucopenia, maximum leucocytosis, duration of leucocytosis and temperature variation. Of these they

found leucocytosis the response demonstrable with the smallest dose.

Soylemezoglu and Wells[97], also using dogs, compared the white cell responses to ACTH and to pyrogen. They found them different, but noted that pyrogen could produce eosinopenia which therefore could no longer be used as a measure of ACTH.

Effects other than on the white cell picture have been much less extensively investigated. This is to be expected. Most of the earlier work on pyrogen was concerned with testing for its presence qualitatively only, and the white cell effect is easier to observe than most of the other effects. Now that pyrogen is being used therapeutically however the other effects will have to be elucidated. The lack of a pure substance may invalidate some of the properties attributed to pyrogen in the reports which follow.

The symptoms following large doses are generally described as prostration, vomiting, diarrhoea and refusing food. More specific symptoms have also been recorded, much of the work being stimulated by a commercial firm marketing a pyrogenic preparation obtained from Pseudomonas aeruginosa.

This pyrogen was observed to have an effect on blood pressure by Taylor, Corcoran, Fertig and Page[98], who found that the malignant phase of essential hypertension in some patients could be remitted by repeated injections,

and that patients tolerating the fevers badly could be made more comfortable with antipyretics such as aspirin or aminopyrine. Co Tui et al.[82] cited a clinical example of pyrogen treatment which was accompanied by a fall in blood pressure and Kobayashi, Fujitake and Yamada[99] recorded a fall in blood pressure in a rabbit.

A gastric effect in dogs or rats has been noted by three authors. Necheles, Dommers, Weiner, Olson and Rychel[100], after stimulating gastric motility in dogs by means of prostigmine, administered sub-pyrogenic doses of various pyrogens which were found to depress gastric motility and secretion. Dogs with gastric pouches were used by Blickenstaff and Grossman[101] in their quantitative study of the reduction of gastric acid secretion associated with pyrexia. They found a linear relationship between log. dose and temperature rise or inhibition. The ulcer-inhibiting action of pyrogens in pylorus-ligated rats was described by McGinty, Wilson and Rodney[102] who noted significantly fewer rats ulcerated in a group that had had pyrogen than in a group that had not had pyrogen.

A blood sugar rise accompanying temperature rise in rats was mentioned by Feldman and Gellhorn[79] who injected TAB vaccine intraperitoneally into normal rats.

An accelerating effect on respiration was described by Co Tui[82] and Kobayashi[99] too reported an increase in

depth and rate of respiration. Rodney and Welcke[103] studied the action of pyrogens on cellular oxidation of rat kidney and rabbit bone marrow slices in a Warburg bath, and found that the pyrogens had no effect on oxygen uptake.

Increased pulse rate was noted by Co Tui[82] in his clinical example and Takos and Moe[104] mentioned that pyrogen injected into dogs increased their renal plasma flow in about half an hour after injection. Windle[80] noted effects other than the haematological changes already mentioned. There were significant histological changes also in the kidneys and zona reticularis of the adrenals.

Pharmacological investigations are even more extensive if one includes both substances which their users do not call pyrogen but which are from micro-biological sources and are produced in the same ways as the 'pyrogens', and substances used in investigations not primarily concerned with pyrogenic effect. Such materials and their effects are listed in the appended table.

Auxiliary list of pharmacologiacl properties of pyrogen

Authors' name for material	Reference	Observed effect
A polysaccharide from pneumococcus	106	increase in blood sedimentation rate
Mycobacterium tuberculosum polysacchride	107	temp. rise, changing to temp. fall with a larger dose, and white cell change
Extracts, mostly protein, from bacteria	11	leucocytosis and temp. rise
Antipneumococcus serum chill-producing principle	108	chills and temp. rises, humans being more sensitive than dogs.
Antipneumococcic rabbit serum	109	temp. rise in rabbits, collapse in patients
Type-specific anti-pneumococcic rabbit serum	110	chills and temp. rises
Concentrated anti-pneumococcic serum	111	temp. rises and falls
Vaccines	112	fevers
Antigenic material from E. typhosa	113	leucopenia and temp. rise then leucocytosis and temp. fall
Serratia marcescens tumor-necrotizing polysaccharide	114	temp. rises
Ditto	115	temp. falls with large doses
Ditto	116	effect on blood non-protein nitrogen and ascorbic acid
Ditto	117	fever, leucocytosis, high blood sedimentation rate, blood pressure fall and tumour haemorrhage
Pyrogenic fraction of exudates	118	temp. rises
Gram-negative endotoxins	119	abortions
Infectious fevers	120	hyperglycaemia
E. typhosa culture filtrates	121	fall in neutrophils in vitro in heparinised rabbit and human blood
Muscle heated to 200°	122	temp. rises.

It would seem that these substances either are pyrogen, contain it or have it adsorbed on them. Some of the effects observed are effects of the temperature's being raised, through no matter what cause, and are not attributable specifically to pyrogen. As Bazett[105] says, "Temperature variations affect nearly every physiological process".

The conclusion is that the substance having the pyrogenic effect may have the other effects also but that this cannot be proved so far for want of a pure substance of reasonable stability. Nevertheless, when pyrogen occurs as a contaminant of injections, these injections tend to have also the other effects caused either by pyrogen itself or by associated substances also metabolised by the contaminating micro-organisms. If the pyrogenic material has the other properties they would repay investigation as a basis of assay, so unreliable is the rabbit temperature method of assay. From the clinical point of view too more knowledge of side-effects is required before widespread administration of pyrogen could be carried out. When a substance pure and stable enough for these pharmacological investigations is available the need for a biological assay as opposed to a chemical or physical assay will probably no longer exist.

B. Physical properties

None of the physical or chemical properties so far investigated seems a suitable basis of assay. Since the amount of pyrogen present in casually contaminated injection solutions appears to be very small this also may make a physical or chemical assay more difficult to apply than a biological test.

As a result of filtration studies pyrogen was considered by Co Tui et al.[36] to be a large molecule of size 50$\mu\mu$ - 1μ, and the material obtained by them[82] from E. typhosa was gummy and gave a colloidal, opalescent 'solution'.

The literature on the effect of heat on pyrogen is indeed contradictory. The general inference is however stability towards heat to the extent of withstanding sterilisation by autoclaving, contrary to the findings in the practical section of this work.

The United States Pharmacopoeia[69] quotes the highest 'safe' temperature in the literature for the destruction of pyrogen, requiring heating in a muffle furnace at 250°C. for the destruction of pyrogen on glassware. Banks[50] found that autoclaved vaccines gave greater rises than they had given before autoclaving but that 140° destroyed pyrogen, while Brindle and Rigby[40] found that half an hour at 15 lb. per sq. in. or one hour at 10 lb. per sq. in. did not destroy pyrogen. Weger[85] found

60 - 70° destroyed pyrogen but Hort and Penfold[20] found that 120° "for a long time" did not destroy pyrogen. Differences between Gram-positive and Gram-negative organisms were observed by Probey and Pittman[61] and Wylie and Todd[60] found the rate of destruction at a given temperature to be a function of concentration and to vary in different organisms, thus accounting for the divergent results.

The effect of pH value appears to have been little studied. Banks[50] carrying out experiments on autoclaving at various pH values found that autoclaving at pH 8.4 and 9.2 produced no effect while pH 3.8 lessened the pyrogen after one hour and destroyed it all in two hours.

The question of decomposition on storage was studied by Collier and Paris[123] who considered that the initial concentration might affect the rate of decomposition on storage. Dorche and Castaing[94] obtained variable results from storage of different pyrogens in different vehicles and concluded that storage would not ensure the depyrogenising of solutions for injection.

C. Chemical properties

Several papers are available on the chemical properties of pyrogen. The analyses are not identical, varying with the source and the method of preparation. Instability in air of the pure substance is a difficulty in this part of the work. The preponderance of opinion favours a polysaccharide substance, not a protein as was formerly believed.

Evidence in confirmation of this was obtained by Jona[122] who found that protein reagents gave negative results with his material and that sulphur was absent from it.

Co Tui et al.[82] isolated 0.5g. pyrogenic material from one litre suspensions of E. typhosa containing 50-88 million organisms per c.c. The analyses were -

Strain	C	H	N	S	P	Ash
1	39.4	7.1	1.5	1.25	0.61	4.4
	39.3	7.0	1.5	1.29	0.67	4.5
2	46.7	6.7	1.5			4.2

They said that the atomic ratios suggested one glucosamine per five or six hexose units. Hydrolysis yielded sugars whose osazones were made but not identified.

Materials of the following composition were isolated by Robinson and Flusser[124] -

Source	C	H	N	S	P
P.vulgaris	35.83	6.06	0	0.29	8.33
E.typhosa	39.28	6.95	0	0.38	4.43
Ps.aeruginosa	38.75	6.53	0	2.38	12.18

Rodney and Welcke[103] isolated material from Ps.aeruginosa, E.typhosa and B.subtilis, finding it to be non-protein, to have 2% glucosamine and to hydrolyse to hexoses. It was difficult to separate from thymus nucleic acid.

Weger[85] recorded the following analyses -

Source	C	H	N	O	S	P	Ash
typhus	35.83	6.42	2.22	47.8	<0.3	-	-
coli	36.64	6.40	1.84	49.9	<0.3	-	-
abortus equi	37.66	6.58	2.12	46.6	<0.3	-	-

A pyrogenic material was isolated from Pseudomonas sp. by means of tryptic digestion by Nesset et al.[125], and was found to consist of

C	H	S	N	P	Ash
35.76	6.46	-	6.68	3.29	14.73

It too was tightly bound to nucleic acids. Hydrolysis yielded reducing sugars and hexosamine was also found.

Finally, Ginger et al.[126] examined pyrogen from E.coli, S.typhi, B.subtilis, S.marcescens and P.vulgaris. They found differences in composition, total polysaccharide produced, reducing sugar produced, non-reducing carbohydrate and hexosamine, which could not be correlated with differences in biological activity. They therefore concluded that this must be due to something as yet unknown. How much the results on biological activity suffered from variations in the responses in the tests used cannot be calculated on the available data.

Thus it will be seen that much work remains to be done on isolating pure substances and identifying them. The yields of substances will be small, therefore experiments will have to start with very large volumes of cultures, solvents and precipitants. This fact, as well as the instability in air of the matreials obtained by the author, have prevented her so far from taking the theoretically best next step in pyrogen work - the isolation of a pure substance.

Standard Pyrogens

The need for a standard has been indicated by reviewing the types of tests hitherto used and the contradictory findings of different workers. It will be more clearly seen from the results in the practical section of this work. Other workers who also have expressed their need for a standard have prepared various standards. These were mostly for their own convenience in only comparatively short-term investigations of a particular aspect of the work, whereas the current need is for an international standard for use in a reasonable assay process. The drawback to the use of current standards, including the author's material, is lack of knowledge of the validity of comparing pyrogens from different organisms. Once pyrogen is sufficiently well chemically defined to know this, the need for a biological assay and a standard may well have passed or lessened for that very reason.

Among standards made by other workers are these - Welch et al.[65] made a standard from Ps.aeruginosa, using the supernatant liquid from cultures, made isotonic and diluted to a fixed concentration of nitrogen. The stability of such a solution is, in the author's opinion doubtful.

Wylie[60] used supernatant liquids from cultures of various organisms. Their instability depended roughly on their concentration.

Paton[59] emphasised the need for a standard for purposes of assay and said he obtained quantitative results with preparations from four organisms and that the slopes of dose/response curves for the four were the same. This would be a useful point if confirmed by further trials with other organisms, from the point of view of being able to use pyrogen from one organism for estimating that from another.

Dare[127] made a standard from an acetone-acetic acid precipitate of supernatant liquid of culture, drying it over calcium chloride and storing it in sealed ampoules. This is probably the most stable material so far, having been stable now for five years.

Tennent and Ott[75] also made a dry standard. An alcohol precipitate from Pseudomonas culture was dried with ether, assayed and diluted as required with solid sodium chloride.

Details of the preparation of the commercial pyrogens, "Pyromen" [128] and "Pyrifer" [129] are not available and so far requests for samples have not been met.

It is seen that the trend of standards is towards a

dry solid which is likely to be more stable than a solution.

Many stable, pure, chemical substances produce hyperthermia, for example, phenol, p-cresol, 2-amino-tetraline, 2-4-dinitrophenal, 2-4- dinitro-α-naphthol[130]; cocaine[131]; methylene blue, atropine, caffeine, convulsants such as santonin, picrotoxin or strychnine, cyclopentylphenol[132]; antipyretics and turpentine[133].

The drawback to their use is lack of knowledge of pyrogen's constitution and mode of action and therefore comparability with them.

The need for pyrogen from a clinical point of view is mentioned in an Editorial of the American Journal of Clinical Pathology[134], and by Windle et al.[86], who mentioned the drawbacks in physically induced fevers - the expense of the equipment and the need for the trained personnel to use it - and by Lonsen and Liebert[87] who find "Pyromen" less dangerous and exhausting to their patients than malaria and less toxic than typhoid vaccine.

P R A C T I C A L S E C T I O N

PRELIMINARY INVESTIGATIONS ON METHODS OF PREPARING, STORING AND CONCENTRATING PYROGEN

A - Escherichia coli as source of pyrogen

Preparation of pyrogenic solutions

The evidence accumulated by other workers and tabulated in Appendix One shows that there is a wide choice of organisms as a source of pyrogen. The features desirable in the organism to be chosen are copious production of pyrogen, non-pathogenicity to man and to rabbits and abundant growth in a simple medium. The organism considered best to fulfil these requirements was E.coli. According to Wylie and Todd[60] it produces pyrogen relatively abundantly, and it is also relatively non-pathogenic and easily grown. A strain was obtained on agar and it was subcultured on agar as long as E.coli was used as a source of pyrogen.

A simple medium was chosen so that a pure or at least a concentrated pyrogen would be the more easily separated from it, and also to lessen the probability of side-reactions when it was injected. The composition of the medium was -

Ammonium phosphate	80 grammes
Sodium chloride	20 g.
Potassium acid phosphate	20 g.
Magnesium sulphate	14 g.
Ferrous sulphate	trace
Glucose	200 g.
Water, freshly distilled, to	20 litres

The medium was distributed in one litre volumes in Thomson bottles and sterilised by autoclaving at 115°C. for one hour. In this work all temperatures are measured in Centigrade degrees. The pH value after autoclaving was 7.2. Two changes were observed to occur during autoclaving - the glucose caramelised and a precipitate formed. To avoid caramelisation the glucose was replaced with lactic acid but E.coli failed to grow in this, whereas it grew in the original medium despite the caramelisation. To try to avoid the precipitate which tended to obscure the presence of growth during incubation, batches were made without magnesium, without iron and replacing phosphate with chloride. In no case did E.coli grow, therefore the medium described above was used without any modification.

To obtain E.coli pyrogen, Thomson bottles were inoculated each with a loopful of culture from the agar slope and incubated at 37°. Most of the organisms were separated from the medium with the aid of a Sharples continuous

centrifuge working at 30,000 r.p.m. The centrifuge was previously washed out with freshly distilled water, complete sterilisation of it being impracticable, and the local water supply being very pure. It was considered that the amount of foreign pyrogen thus introduced would be small compared with the amount of coli pyrogen already present. The centrifuged liquid was sterilised by filtration through sterile Doulton candles and stored in sterilised ampoules.

The method of estimating pyrogen used at this stage was that of Wylie and Todd[60]. The liquid was diluted with a sufficient quantity of apyrogenic saline to give a response on their quantitative range and was injected, after being heated to 37°, into groups of five rabbits in volumes of 2 ml.per kg. of body weight. The population had been grouped into fives by Wylie and Todd so that the mean rise of all groups in response to an apparently temporarily stable standard was the same. Throughout all the experiments in this work the varying doses of pyrogen were all contained in a final volume of 2 ml. per kg. body weight. The apyrogenic saline was a 0.9% solution of A.R. sodium chloride in freshly distilled water, distributed in 500 c.c. blood bottles and immediately sterilised by autoclaving. On no occasion were samples from batches thus made found to be pyrogenic.

Attempts to concentrate these pyrogenic solutions

a) By evaporation under reduced pressure

There was no available evidence about the effect of heat on coli pyrogen but the general inference to be drawn from other pyrogens was that the least possible heat should be used to drive off the water.

Four experiments were carried out from which it was concluded that evaporation under reduced pressure would not be a profitable way to concentrate the pyrogen in the supernatant liquid of a culture of E.coli. The results of these experiments are shown in Table 1.

Experiment 1 - The organism was subcultured on agar for 24 hours, in 10 ml. of the synthetic medium for 24 hours and then incubated in a litre of medium for 48 hours at 37°. The organisms were centrifuged off and the liquid passed through sterile Doulton candles. This liquid was now evaporated at 70° to a quarter of its original volume and passed through sterile Doulton candles. Samples of original and of concentrated liquids were tested for pyrogen and it was found that pyrogen had been lost.

Experiment 2 - A check experiment was performed. The organism was incubated for three days, then centrifuged and filtered as above. The liquid was evaporated from 800 ml. to 25 ml. in two hours at 55° and the concentrate

sterilised by filtration. Again pyrogen was lost.

Experiment 3 - Since the first two experiments showed that pyrogen was being lost to a greater extent than had been generally reported by other workers on pyrogens, the effect of the production of acid by E.coli during its growth was investigated. Although the medium after autoclaving had a pH value of 7.2 the growth of E.coli reduced this to 4.6-4.9.

The organism was incubated six days and the growth centrifuged and filtered as above. One volume of 100 ml. was retained at the pH of the growth, 4.8, and a second volume of 100 ml. was adjusted to pH 6.9. These were evaporated to 25 ml. at 40° for 20 minutes. Pyrogen was still lost.

Experiment 4 - A check experiment was performed in which the incubation was for seven days and the heating was at 50° for three quarters of an hour to reduce 100 ml. to 25 ml. Again pyrogen was lost.

Table 1

Loss of pyrogen from E.coli supernatant liquid on evaporation under reduced pressure

Exp't. No.	Dilution for injection	Material injected	Temperature rises					Average
1	1 in 500	supernatant	0.74	0.61	0.56	0.46	0.08	0.49
	1 in 100	concentrate	0.68	0.54	0.11	0.16		0.37
2	1 in 500	supernatant	1.12	1.12	0.95	0.62		0.95
	1 in 1000	concentrate	0.46	0.42				0.44
3	1 in 500	supernatant	1.09	1.58	1.06	1.40	0.82	1.19
	1 in 1000	concentrate (pH 4.8)	0.60	0.85	0.42			0.62
	1 in 100	concentrate (pH 6.9)	0.93	0.55	0.15	0.82	0.46	0.58
4	1 in 500	supernatant	1.37	0.82	1.15	0.97	1.34	1.13
	1 in 1000	concentrate (pH 4.9)	0.32	0.54	0.46	0.72	0.32	0.47
	1 in 1000	concentrate (pH 6.7)	0.77	1.11	1.62	0.62	0.55	0.93

b) By adsorption

It has been shown by many workers that asbestos pads of the grades used to free solutions from micro-organisms freed them also from pyrogens (pp. 5,6). The possibility of eluting pyrogen from these pads was now investigated, also the possibility of removing it into a smaller volumes of liquid than that which had originally contained it. The pH of the original growth from which the pyrogen was adsorbed was 4.6-4.9. Buffer solutions for eluting the pyrogen were made according to Britton[135], and their pH values measured after autoclaving. Four experiments were carried out and their results are shown in Table 2. 1 - A seven days' growth was centrifuged and filtered through sterile candles as above and 100 ml. volumes passed through Ford's Sterimats, 3.6cm. diameter, bacterial grade. The filtrate from the candles was pyrogenic and the filtrate from the asbestos mats non-pyrogenic. Each mat containing the pyrogen from 100 ml. solution was transferred to 2 litres of sterilised buffer solutions, in which it remained for the times shown in Table 2. Since the original volume of 100 ml. had become 2000 ml. the dilution of 1 in 500 became 1 in 25 so that comparable doses were administered. The experiment indicated that alkaline buffer removed the pyrogen the more effectively.

2 - A similar experiment was carried out, leaving the pads in the buffer solutions for periods of one, one and a half and two and a half hours, with intermittent shaking. It appeared that more pyrogen came off the pads than had gone on. This odd finding was reversed on making the supernatant liquid alkaline before heating to 37° for injection. It is probable that the first anomalous result for supernatant liquid was due to depressant[60] and that this was driven off in the warming of the alkaline solution.

3 - The experiment was as above except that the pad was broken up with flamed forceps before transfer to the buffer solution. An acid buffer was used but the solution was made just alkaline before heating as above. However no pyrogen was eluted.

4 - In this experiment a buffer less acid than the above was used.

Four more experiments were performed, eluting by means of two sixty ml. volumes of buffer solution and collecting the filtrates separately. The results are shown in Table 3. The eight experiments showed that alkaline buffers could effectively elute the pyrogen but that it was so unstable in alkaline solution that the method was of no practical value.

Table 2

Elution of coli pyrogen from asbestos pads by means of two-litre volumes of buffer solutions

Exp't. No.	Day	Dilution for injection	Material	Time of contact of pad & buffer	pH of buffer soln.	Average temp. rise
1	1	1 in 500	supernatant			1.03
	2	1 in 500	asbestos pad filtrate			0.29
	3	1 in 25	eluate	½ hr.	6.2	0.15
	3	1 in 25	eluate	7¼ hr.	6.2	0.55
	4	1 in 25	eluate	½ hr.	11.5	0.93
	4	1 in 25	eluate	2½ hr.	11.5	1.26
	20	1 in 25	eluate	3 hr.	11.5	0.95
2	1	1 in 500	supernatant			0.73
	7	1 in 25	eluate	1 hr.	11.9	0.73
	3	1 in 25	eluate	1½ hr.	11.9	0.50
	1	1 in 25	eluate	2½ hr.	11.9	0.99
	10	1 in 25	eluate	1 hr.	9.5	0.94
	8	1 in 25	eluate	1½ hr.	9.5	0.99
	2	1 in 25	eluate	2½ hr.	9.5	1.08
	3	1 in 25	eluate	1 hr.	9.3	1.12
	4	1 in 25	eluate	1½ hr.	9.3	0.69
	7	1 in 25	eluate	2½ hr.	9.3	0.66
	2	1 in 500	supernatant (acid)			0.49
	4	1 in 500	supernatant (acid)			0.58
	8	1 in 500	supernatant (alkaline)			1.25
	9	1 in 500	supernatant (acid)			0.28
	9	1 in 500	supernatant (alkaline)			1.16
3	1	1 in 500	supernatant			0.72
	1	1 in 500	asbestos pad filtrate			0.23
	4	1 in 25	eluate	½ hr.	2.0	0.20
	4	1 in 25	eluate	1 hr.	2.0	0.31
	3	1 in 25	eluate	1½ hr.	2.0	0.33
	3	1 in 500	supernatant			0.70
4	1	1 in 500	supernatant			0.51
	6	1 in 25	eluate	1 hr.	5.0	0.44
	7	1 in 25	eluate	1½ hr.	5.0	0.41

Table 3

Elution of coli pyrogen from asbestos pads by means of two 60 ml. volumes of buffer solution per pad

Exp't. No.	Day	Material	Fraction	pH	Aver. temp. rise
1	1	supernatant			1.56
	2	eluate	1	2.4	0.47
	2	eluate	2	2.4	0.61
	9	eluate	1	3.7	0.54
	9	eluate	2	3.7	0.63
	8	supernatant			0.83
2	1	supernatant			0.67
	2	eluate	1	6.6	0.38
	2	eluate	2	6.6	0.58
3	1	supernatant			0.73
	6	eluate	1	7.4	0.26
	1	eluate	2	7.4	0.86
4	1	supernatant			0.69
	1	eluate	1	10.0	0.88
	3	eluate	2	10.0	0.46

Table 4

Investigation of the effect of the age of the culture used to inoculate the Thomson bottles and of the time of incubation on the amount of pyrogen produced

Age of culture (days)	Time of incubation (days)	Average rise in temperature produced by a 1 in 500 dilution	
?	2	0.59	
?	3	0.95	
12	4	0.51	
16	4	1.56	Av. = 0.91
14	4	0.67	
?	6	1.19	
?	7	1.13	
?	7	1.03	
?	7	0.73	
1	7	0.72	
?	7	0.74	Av. = 0.77
3	7	1.24	
14	7	0.69	
21	7	0.43	
?	7	0.23	

The general trend of the relation between the pH value of the eluting buffer solution and the percentage of pyrogen eluted is shown in Figure 1. This however must be regarded as no more than a trend because of the extent of the error found in this method of estimating pyrogen when eventually a stable pyrogen was obtained and used to investigate this error. The graph also assumes that the rise in temperature is proportional to log. dose as claimed by Wylie and Todd[60] but not as yet established with a stable pyrogen.

Throughout these experiments it was observed that the amount of pyrogen produced varied. The age of culture used to inoculate the medium and the period of incubation had varied, therefore these were examined to see if they had any bearing on the amount of pyrogen produced. The results are shown in Table 4. Within the periods examined their length seems to be unimportant.

The finding of greatest immediate and practical importance in these experiments was the instability of coli pyrogen on storage in solution. This fact was observed during the attempts to make from such solutions a standard to use in grouping the rabbits into fives whose mean response would be equal. The extent of this instability is shown in Table 5.

Figure 1. Relation between pH value of the eluting solution and percentage of pyrogen eluted.

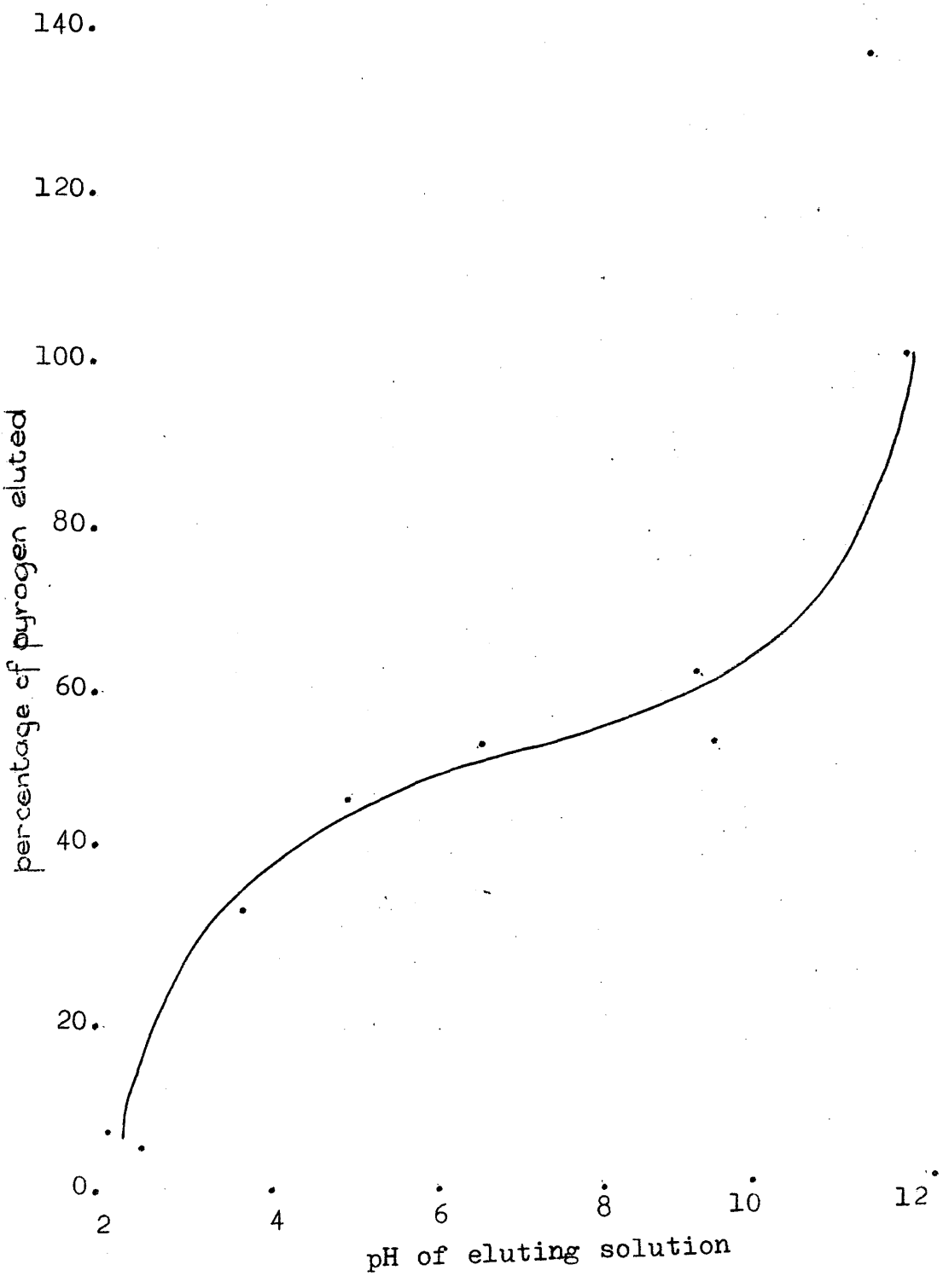

Table 5

Loss of E.coli pyrogen from solution on storage

Time of storage (days)	Material	Mean temperature rise	
		before	after
1	*supernatant	0.73	0.62
1	supernatant	0.72	0.70
1	supernatant made alkaline	1.25	1.16
4	*supernatant	0.73	0.58
5	supernatant stored diluted 1.5 in 100	0.64 (1 in 100 on day of preparation)	0.31
8	supernatant	1.56	0.83
9	*supernatant	0.73	0.28
10	supernatant	0.67	0.14

* same starting solution

B - Proteus vulgaris as source of pyrogen

Preparation of pyrogenic solutions

Because of the instability of E.coli pyrogen in solution it was decided to examine another organism. The same criteria, pyrogen production, non-pathogenicity and ease of growth, were applied. The organism chosen was Proteus vulgaris. Because it was found that the organism would not grow in the caramelised medium, the glucose was sterilised separately to avoid caramelising it and then added aseptically to the other sterilised ingredients along with a sterile solution of nicotinic acid which was present to a final concentration of 2×10^{-5}M nicotinic acid. This was necessary as the strain of Proteus available was evidently one for which inorganic nitrogen was not sufficient. An inoculum of a loopful per litre was found to be too small to grow, although it had been enough in the case of E.coli, but growth readily took place when a whole slope was washed into one litre of medium.

Attempts to concentrate the pyrogenic solutions by adsorption

The same general conclusions were reached as for coli pyrogen - that it could be adsorbed from acid solution and eluted with alkaline solution (Table 6) and that solutions were unstable on storage (Table 7).

The stability of the pyrogen in a dry state was also investigated. A pad in which it was adsorbed was stored in a desiccator over calcium chloride. The eluate, immediately on elution with alkaline buffer solution, was compared with eluate which had been collected immediately after adsorption and stored for the same period as such. It was found that the pyrogen stored dry had been the more stable (Table 8). This is of interest only in indicating that a dry standard would be preferable to a liquid standard. This form of dry standard would however be inconvenient in practice because of the need for elution immediately before performing a pyrogen test.

The inconstancy of results in Table 7 would seem to indicate that pH difference did not completely account for the differing rates of loss of pyrogen from solution. The effect of keeping the pH constant but changing the osmotic pressure was now investigated as the solutions of the same pH value had had different osmotic pressures. From the four experiments performed it appears that the rate of loss of pyrogen is greater from solutions of

greater osmotic pressure. The application of this is in the dialysis of any solution to be used over a period as a standard. The results of the osmotic pressure experiments are shown in Table 9. Details of the dialyses are given in Appendix Two.

The effect of time of incubation on the amount of pyrogen produced was investigated here as for E. coli and it was concluded that the periods investigated did not produce amounts whose differences were measurable by the present test. The results are shown in Table 10.

Table 6

Elution of Proteus pyrogen from asbestos pads

Exp't. No.	Day	Materials	Dilution	pH	Aver. temp. rise
1	1	supernatant	1 in 100	6.0	0.38
	1	eluate		2.3	0.30
	5	supernatant	1 in 10	6.0	0.95
	5	eluate		2.3	0.42
2	1	supernatant	1 in 20	5.9	0.51
	1	eluate		6.9	0.31
	5	supernatant	1 in 5	5.9	0.75
	5	eluate		6.9	0.49
3	7	supernatant	1 in 10	5.7	1.10
	6	eluate (stored in a refrigerator six days before being tested)		9.5	0.67

Table 8

Comparison of the loss of pyrogen in the adsorbed and eluted states on storage for five days

Average rise in temperature in groups of five rabbits due to		
Pyrogen adsorbed, immediately eluted and immediately injected	Pyrogen stored on a pad for five days, eluted and immediately injected	Pyrogen adsorbed, immediately eluted and eluate stored for five days
0.77	0.80	0.31
0.95	0.75	0.49

Table 7

Loss of Proteus pyrogen during storage from solutions of various pH values

Time of storage (days)	Material	pH	Dilution	Mean temperature rises	
				before	after
5	supernatant untouched	4.7	1 in 100	0.81	0.71
6	supernatant +NaOH	7.1	1 in 10	1.48	1.31
6	supernatant +NaOH	6.1	1 in 100	0.81	0.80
6	supernatant +HCl	2.3	1 in 100	0.81	0.30
6	supernatant untouched	4.4	1 in 10	1.09	1.01
6	supernatant +HCl	2.4	1 in 10	1.09	0.47
6	supernatant +HCl	3.5	1 in 10	1.09	0.48
6	supernatant +NaOH	8.4	1 in 10	1.09	1.02
5	supernatant untouched	5.5	1 in 10	1.38	1.42
5	supernatant +NaOH	9.7	1 in 10	1.38	0.70
76	supernatant untouched	3.8	1 in 500	0.93	0.93
174	supernatant stored diluted 2-10l	3.8	-	1.03	0.24
9	supernatant stored diluted 1-10	6.1	-	1.04	1.03
13	ditto			1.04	0.36
16	ditto			1.04	0.64
26	ditto			1.04	0.06

Table 9

Effect of osmotic pressure on the rate of loss of Proteus pyrogen from solutions

Exp't. No.	Day	Materials	Dilution	pH	Aver. temp. rise
1	1	supernatant +4% water	1 in 10	5.5	1.38
	5	ditto	ditto		1.42
	5	supernatant +4% of soln. of NaOH to a final concentration of N/150 + HCl to a final concentration of N/15	ditto	5.3	0.99
2	1	supernatant +4% water	1 in 10	4.7	1.10
	4	ditto	ditto		1.19
	4	supernatant + NaOH + HCl as above	ditto	4.8	0.92
	5	supernatant + NaCl ≡ above	ditto	4.8	1.28
	5	supernatant + NaCl ≡ 9 x isotonicity	ditto	4.7	0.34
3	1	supernatant	1 in 10		1.59
	1	supernatant	1 in 100		0.81
	7	supernatant	1 in 100		0.87
	21	supernatant	"		0.89
	30	"	"		0.23
	135	"	"		0.38
	138	"	"		0.20
	244	"	"		0.55
	389	"	"		0.30
	1	dialysate	"		0.87
	7	"	"		0.80
	21	"	"		0.90
	30	"	"		0.94
	41	"	"		0.93
	99	"	"		1.06
	135	"	"		1.21
	138	"	"		1.30
	244	"	"		1.14
	389	"	"		1.22

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Exp't. No.	Day	Materials	Dilution	pH	Aver. temp. rise
4	1	supernatant	1 in 100		0.92
	14	"	"		1.07
	28	"	"		0.97
	49	"	"		1.10
	71	"	"		0.98
	3	dialysate	"		0.85
	14	"	"		1.00
	28	"	"		0.94
	49	"	"		1.18
	71	"	"		1.15

Table 10

Influence of the time of incubation on the amount of pyrogen produced by Proteus vulgaris

Time of incubation (days)	Dilution	Average rise in temperature	
2	0.96 - 10	1.38	
5	1 - 10	1.17	
		0.86	
		0.96	Av. = 1.18
		1.59	
		1.30	
	1 - 100	0.76	
		0.84	
		1.03	Av. = 0.87
		0.81	
		0.92	
6	1 - 10	1.04	
		1.48	Av. = 1.20
		1.09	
	0.96 - 10	1.37	
	1 - 100	0.81	Av. = 0.56
		0.31	
	1 - 500	0.93	
7	1 - 10	0.86	
		1.33	Av. = 1.10
		1.10	
	1 - 20	0.51	
	1 - 100	0.47	Av. = 0.43
		0.38	

THE PRODUCTION AND USE OF PYROGEN STANDARDS

Production of standards

During the course of the work as older rabbits died and new members joined the population it became necessary to produce some sort of standard to divide the rabbits into groups of five to continue the investigation of the properties of pyrogen using the test of Wylie and Todd[60]. The first of these standards were from E.coli and were in liquid form. They were very unstable. Solutions of Proteus vulgaris pyrogen were also found to be unstable. Since the adsorption experiments had shown a dry standard to be desirable and since a freeze-drying apparatus became available at this time the next standard made consisted of freeze-dried supernatant liquid of P.vulgaris. After it had been shown that this process could be carried out without loss of pyrogen an attempt was made to prepare a purer standard by freeze-drying eluate. This however was not a success, but it was found that dialysate could be successfully dried. This is currently in use as standard.

Details of standards

A - Liquids

(i) A solution of pyrogen from E.coli

This was grown as usual for 7 days at 37° in the medium described on p.34, the organisms centrifuged off and the liquid sterilised by filtration through Doulton candles. The liquid, after being tested to find the dilution giving a rise on the part of the dose/response curve reported quantitative[60], was diluted 1 up to 100 in apyrogenic saline, distributed in screw-capped bottles and autoclaved. The bottles were stored at room temperature. All the pyrogen was found to have been lost on autoclaving (table 11).

(ii) A solution of pyrogen from E.coli

A method similar to the above was used, except that the dilution was 1.5 up to 100, and autoclaving was replaced by sterilisation by filtration. The results (Table 11) again show loss of pyrogen.

(iii) A solution of pyrogen from P. vulgaris

This was grown for 6 days in the medium described on p.46, the organisms centrifuged off and the liquid filtered through Doulton candles, diluted 2 up to 10l and autoclaved.

(iv) A solution of pyrogen from P.vulgaris

A similar standard was made, the dilution being 1 up to 10, and sterilisation being not by autoclaving but by filtration. Both solutions lost pyrogen on storage, (Table 12).

Table 11

Instability of pyrogen prepared from E.coli

Std. No.	Material	Dilution	Aver. temp. rise
1	supernatant	1 - 100	0.85
	ditto	1 - 500	0.43
	supernatant diluted 1-100 & autoclaved	-	0.31
2	supernatant	1 - 100	0.64
	ditto	1 - 500	0.23
	supernatant diluted 1.5-100 aseptically & stored 4 days	-	0.25
	ditto stored 5 days	-	0.31

Table 12

Instability of pyrogen prepared from P.vulgaris

Std. No.	Material	Dilution	Aver. temp. rise	
3	supernatant	1 - 100	0.75	anomalous
	supernatant	1 - 500	0.93	
	supernatant diluted 2-101 & autoclaved	-	1.03	
	supernatant stored for 66 days diluted	1 - 500	0.93	
	supernatant stored for 249 days	1 - 500	0.31	
4	supernatant	1 - 10	1.04	
	supernatant diluted 1-10, stored 8 days	-	1.03	
	do.,12 days	-	0.36	
	do.,15 days	-	0.64	
	do.,25 days	-	0.06	

B - Solids

Freeze-dried standards were now prepared from the supernatant liquid of cultures of P.vulgaris. The organism was grown for five days at 37°, the growth centrifuged, the liquid sterilised by filtration and freeze-dried.

The apparatus used was an Edwards' Freeze-Drying Unit, Model No.3PS. The general principle is to freeze the liquid and sublime off the ice, thus avoiding removing water from a progressively concentrating solution, and so lessening the destruction of labile materials. Sublimation is made easier by freezing the liquid in a shell round the walls of the containers, this being achieved by spinning them as their contents freeze. In the model used, tubes capable of holding 2.5 ml. of spinning liquid (Figure 2) are placed in a disc bored to take 120 such tubes and this disc is spun at about 400 r.p.m. on a central spindle while the liquid freezes. When the material has frozen the spinning is stopped and suction applied to remove most of the ice. This process is referred to as primary drying. The process of secondary drying removes final traces of water. The ampoules are connected individually via rubber nozzles to a manifold in which the pressure is reduced and which contains

phosphorus pentoxide to absorb the removed water. Final sealing takes place with the contents still in vacuo. In order to prevent the tendency to implosion the ampoule necks are constricted before secondary drying at the point where they are to be sealed off. The ampoules are allowed to stand for half an hour in a draught-free atmosphere and then tested for sealing with an Edwards' Betoray High Frequency Glow Discharge Tester.

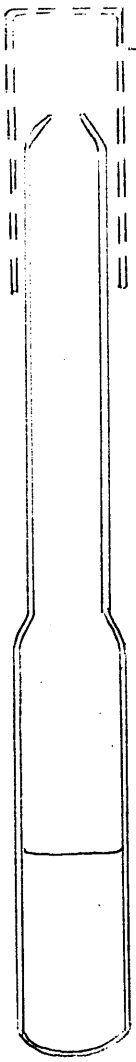
First freeze-dried standard

The culture supernatant liquid was tested for pyrogenicity. Volumes of 2.5 ml. were transferred by sterile syringes to sterile, gauze-capped ampoules and these placed in the primary-drying chamber at a refrigerator temperature of -35° and dried for six hours. They were left overnight in the closed apparatus. Meantime plugs of cotton wool to fit the necks had been sterilised in petri dishes by autoclaving. These were inserted aseptically with flamed forceps two and a quarter inches down the necks of the ampoules and then the necks, one and a half inches down, were constricted in a blowpipe flame to 1mm. internal diameter and 2½mm. external diameter. This capillary was kept as short as possible so that it was not a weak point during the secondary drying. During this insertion and constriction the partially dried

material was seen to be absorbing moisture from the atmosphere and was therefore put in a desiccator with silica gel. This was stored overnight in a refrigerator at -10° and next day the ampoules were attached to the rubber nozzles of the secondary headers and suction applied. The pressure quickly fell to 0.02mm. of mercury and then more slowly to 0.01mm. where it remained for 24 hours. The ampoules were sealed off under vacuum and tested for sealing.

The material was a creamy, porous, hygroscopic solid and was easily reconstituted. The responses obtained with it are shown in Table 13. The loss during drying presumably occurred during the deliquescence between primary and secondary drying. Some material was stored at 0°, some at room temperature in the light, some at room temperature in the dark and some in an incubator at 37°. Unfortunately the seals of all these latter subsequently cracked, presumably due to stresses in the glass, and the resulting non-sterile material deliquesced with caramelisation and so was not available for examination.

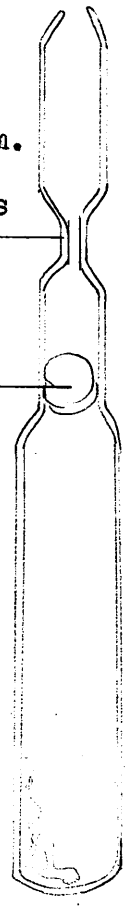
Figure 2



sterile gauze cap, 8 thicknesses stapled together. Cotton wool plugs are too impermeable for efficient removal of the moisture

Constriction to prevent implosion, internally 1mm. diameter and externally 2½mm. Walls of thickness equal to original

Sterilised cotton wool plug



Ampoule to hold 2.5 ml., x 1

Ampoule ready for secondary drying

Freeze-drying Ampoules

The constriction is sufficiently far away from the wool to avoid charring it, which would make sealing more difficult. At the same time it is not so near the mouth of the ampoule that it has to bear more weight than absolutely necessary when the amp ule is in the horizontal position of secondary drying.

Table 13

First freeze-dried standard

Material	Dilution	Rise
supernatant	1 - 10	1.17
supernatant	1 - 100	0.76
reconstituted freeze-dried material	1 - 10	1.01
freeze-dried material stored 143 days at room temp. in the dark	1 - 10	0.87
ditto in the light	1 - 10	0.93
ditto 152 days at 0°	1 - 10	1.00
ditto 260 days at 0°	1 - 10	1.24

Table 14

Second freeze-dried standard

Material	Dilution	Rise
supernatant	1 - 10	0.86
supernatant	1 - 100	0.84
reconstituted supernatant	1 - 10	0.84
ditto, stored dry 240 days at room temp. in the light	1 - 10	0.90

Table 15

Third freeze-dried standard

Material	Dilution	Rise	
supernatant	1 - 10	0.96	anomalous
supernatant	1 - 100	1.03	
reconstituted material	1 - 10	1.04	
ditto	1 - 100	0.52	

Second freeze-dried standard

In drying this batch volumes of 2.5 ml. were distributed in ampoules as before and these were put in the primary-drying chamber when its temperature was 14° and the refrigerator temperature was -35°. As the liquid froze the refrigerator temperature rose to -12.5° and the chamber pressure fell to 1mm. The refrigerator temperature now slowly fell until in 35 minutes it was -32° and the pressure was 0.35mm. The centrifuge was switched off after half an hour and the primary drying continued for six hours. The tubes were plugged and constricted as before and stored in a desiccator overnight. Again some absorption of water had occurred during the plugging and constricting even although the tubes were removed from the desiccated atmosphere for the shortest possible time. The tubes were therefore again snap-frozen in the primary chamber before secondary drying lest liquid was lost by bubbling. Secondary drying was continued for 24 hours, and the tubes sealed as before. The results obtained with this material are shown in Table 14. The precautions taken to lessen absorption of moisture between primary and secondary drying appear to have been successful.

Third freeze-dried standard

During the making of this the refrigerator temperature was -40° and the time of primary drying was increased to 24 hours to ensure that the material was as dry as possible before it was exposed to atmospheric moisture in the plugging and constricting. This was carried out as quickly as possible and the tubes at once attached to the secondary drier. Unfortunately no figures are available for pressures as the Pirani gauge was out of order, the water content of the materials and the pressures being roughly estimated throughout the process by the note of the pumps and by the glow-discharge tester.

During the making of this batch it was observed that it was desirable to check the ampoules for fit in the holes in the primary-drying disc after thay had been sterilised by dry heat at 150° for one hour as this tended to change their size permanently. If they fitted loosely they shattered in the rotation, slow as it was, and if they had expanded of course they were not usable.

Responses to this standard are shown in Table 15. This material was used to investigate variations of the whole population, the results and calculations being shown in Appendix Five.

Fourth freeze-dried standard

In an attempt to make a purer standard, pyrogenic eluate was produced by passing buffer solution pH 9.9 through asbestos pads on which pyrogen was adsorbed, and this eluate dried. All the pyrogen was however lost on drying. The experiment was repeated, in this case half of the eluate being dried as such and half being adjusted to pH 6.7 before being dried. Nevertheless pyrogen was lost during drying, as seen from Table 16. The loss of pyrogen was not therefore caused by either the pH value or by an increased osmotic pressure, as might have been thought from the storage experiments, and remains unknown. The relevant calculations are shown in Appendix Three.

Fifth freeze-dried standard

This is the current standard and was made by dialysing the supernatant liquid through cellophane until it gave no reaction for chloride but was still pyrogenic. This material dried without loss of pyrogen and remained stable throughout its use in investigating white blood cell change as an assay of pyrogen. The figures for its responses before and after drying are shown in Table 17.

Table 16

Loss of pyrogen during the freeze-drying of eluate

Materials	Dilution	pH Rise
supernatant	1 - 10	0.86
supernatant	1 - 100	0.47
reconstituted eluate	1 - 10	0.34
supernatant	1 - 10	1.33
reconstituted eluate	1 - 10	0.75
reconstituted eluate which had been adjusted to pH 6.7 before drying	1 - 10	0.69

Table 17

Freeze-drying of dialysate of supernatant liquid

Material	Dilution	Rise
supernatant	1 - 10	1.14
supernatant	1 - 100	0.92
dialysate	1 - 10	0.94
dialysate	1 - 100	0.85
reconstituted material	1 - 10	1.17
reconstituted material	1 - 100	0.63

Use of standards

The two supernatant liquids from E.coli intended to be standards were too unstable for use. The fourth liquid standard, from Proteus vulgaris, was used only for storage experiments, as were the first two freeze-dried standards, and the results of these have already been tabulated on pages 55 and 60. The dried eluate, also intended as a standard, decomposed during the drying as shown in Table 16, thus the materials used for the subsequent investigations are the first Proteus vulgaris liquid standard (Appendix four), the third freeze-dried standard (Appendix five) and the fifth freeze-dried standard (Appendix six).

Standard no. iii - the first P.vulgaris liquid standard

The object in making this standard was to group the population in order to investigate the properties of the pyrogenic preparations made by various processes and to assay the yields at various stages using the assay method of Wylie and Todd[60]. However after the series of injections required for the grouping was completed and the results examined it was seen that this method of assay was not sufficiently accurate for this purpose. In fact, the idea of isolating a pure material and investigating its properties has had to be postponed until the yields at

stages in the isolation could be more accurately compared.

The volume of standard prepared was enough for 123 injections into the population of 27 rabbits. The rises in temperature produced by these injections are shown in Appendix four. The mean response was 0.87° and the standard deviation was 0.36°. The magnitude of this deviation in relation to the size of the response was a matter for concern in an assay process and therefore causes of it were sought. The results were first examined for normality of distribution to ensure that the standard statistical methods were in fact strictly applicable. The distribution was found to be normal.

The first examination of the variance was by a formal analysis of variance calculation, breaking the total variance into two components - variance in the responses of individual rabbits on successive occasions and variance existing among different rabbits. The latter was found to exist significantly, a finding confirmed with later standards. The application of this knowledge would be that a preferable design of assay would incorporate the testing of sample and standard on the same animals, i.e. in a cross-over type of assay. In such an assay of course there would need to be an

interval of a few days between injections into the same animals and this would restrict its use to stable samples of pyrogen.

Further information was extracted from the results with a view to assessing the reliability of the current pharmacopoeial tests and with a view to lessening the extent of the variance relative to the response. The highest value for the response is limited to about 1.3° according to Wylie and Todd[60], increasing doses thereafter failing to produce proportionately increasing responses. Thus to lessen the relative value of variance to response, i.e. coefficient of variation, the variance must be decreased as the response cannot be increased.

The following queries are relevant in assessing the official tests -

What is the chance of getting a false negative, i.e. of failing to find pyrogen when it is in fact present? What is this chance when the average result of a group of three is used? What size must a sample be to have a mean deviating from the "true" mean by 0.1° with a probability of 5% ? These latter figures for deviation and probability were arbitrarily chosen as being representative of a reasonably accurate test.

The conclusions drawn from the answers are that the proportion of responses below 0.36° (the threshold of Wylie and Todd) when the "true" mean is 0.87° is about 8% and the proportion below 0.6° (the pharmacopoeial threshold) is about 23%; that the chance of a false negative from groups of three in Wylie and Todd's test is about 1% and in the pharmacopoeial test about 10%; that the size of a sample complying with the required conditions is about 50.

The conclusion to which these answers point is that more than the usual three or five rabbits should be used if their reactions are completely unknown, i.e. if they are truly random samples.

The accuracy of the test of Wylie and Todd cannot be fully investigated from these results. Their test assumes constancy of position and gradient of log.dose/response line and for the investigation of this injections of more than one dose level are required, at least three levels being desirable. Only one dose level of this standard was injected as the original purpose of the injections was only to group the rabbits.

Before going on to the injection of other dose levels of other standards, this material now being used up, it was decided to make further examination of the results to seek the cause of the magnitude of the variance.

The responses of the male and female rabbits to the injections were compared and found not to differ significantly. The effect of breed on the response was also examined. The population consisted of 12 Dutch, 6 Fox, 2 Ermine, 1 Rex, 1 Beveran and 5 cross-bred rabbits, this enabling a comparison of only the Dutch and Fox breeds to be made. While miscellaneousness of population is desirable in preliminary work to prevent eccentricity in response of any one breed leading to false conclusions, it is probable that the use of a uniform strain might lessen the variance, and a uniform strain is now being bred for this purpose. The two breeds examined did not differ significantly in response.

Emmens[136] claimed that the state after experiment was as valid a measure as the difference between before and after test states if the first is variable and the two are correlated. That the first was variable was obvious during the carrying out of the experiments. The mean temperature of the population before injection was 38.39° with a standard deviation of 0.49°. That the maximum temperature attained after injection was related to the temperature before injection (referred to as the normal temperature) was not obvious from scatter diagrams and therefore Pearson's Correlation Coefficient

was calculated. No correlation was however found, either with the calculations based on individual responses or on mean responses per animal. Nevertheless the variance in the maximum temperatures attained after injection was calculated and found to be .3521, i.e. greater than the variance in temperature rise. The F test showed it to be significantly greater. Thus it would seem that temperature rise is a better estimate of pyrogen than maximum temperature attained after injection.

Differently coloured rabbits were used in these experiments. Rabbits lose heat through the skin of their ears rather than from all over their bodies, and therefore it was thought that rabbits having differently coloured ears might radiate heat to different extents and so introduce variation into their responses if pyrogen acts by increased heat production. However the differently coloured rabbits did not show significant difference in response.

The existence of correlation between weight and normal temperature, between colour and normal temperature and between weight and rise in temperature was also investigated. It was concluded that there might be some slight correlation between weight and normal temperature, the heavier rabbits tending to have higher temperatures,

but that there was none between the other pairs of factors investigated.

It had been observed during the experiments that different rabbits took different lengths of time to reach their maximum temperatures after injection and so the existence of a relation between height of rise and time taken to reach that rise was investigated, but none was found.

The most profitable step to be taken now in the investigation of temperature response in the rabbit as a measure of pyrogen was the injection of more than one dose level of material and this was accordingly done.

Standard no. vii - the third freeze-dried standard

The responses to this material are shown in Appendix five. The doses injected were such as to give temperature rises greater than the normal fluctuation while still not giving maximal rises, according to the evidence of Wylie and Todd who quoted a quantitative range of 0.75-1.30°. The reconstituted material had been brought back to its original volume of 2.5 ml.per ampoule with apyrogenic water and was then diluted with apyrogenic saline so that the dose was contained in 2 ml. per kilogram body weight. The three doses were chosen so that they would be of equal intervals when converted into logarithms to the

base ten since it was desired to have three points as equally as possible spaced along the line, if there were a straight line, and since there are many more substances having a straight line for log.dose/response than for dose/response. The capacity of the freeze-drying apparatus limited the amount of standard that could be prepared in one batch. It was expended on the maximum number of injections at the minimum number of dose levels capable of giving a line, i.e. three, rather than vice versa, it being reasonable that this would give the 'best line'. The base ten was chosen for the logarithms as it is commonly tabulated and for no other reason.

Similar statistics were investigated for these results as for the previous group of results and further calculations were also carried out.

The responses to the three dose levels were -

Dose ml./kg.	Response	Standard deviation
0.2	1.20	0.39
0.06324	1.14	0.34
0.02	0.90	0.36

The variances at the three dose levels were found not to differ significantly, indicating that in this respect temperature rise would form a useful basis of assay. The range of the responses at each of the three levels was

normally distributed - another factor favouring this measure, but the extent of the variance again was too great for an accurate assay.

The chances of false negatives were calculated here as for the previous standard and similar results obtained. A calculation on the size a sample of rabbits should be to give a tolerable degree of accuracy in the results was again worked out and the same answer obtained - about 50.

Investigations of the causes of the size of the variance also led to the same conclusions, namely that different sexes, different breeds and different colours of rabbits showed no significant difference in response to the injections.

A correlation between basic temperature and weight was again found, the heavier rabbits tending to have higher normal temperatures. No real correlation was found between weight and response, indicating that the use of volume per body weight as a means of administering comparable doses to the different animals was valid. Correlations were also worked out for basic temperature and response. Using the three hundred individual results obtained, some correlation was found. Since there appears to be no formal method of analysis of correlation analogous to analysis of variance, an

empirical method of analysing the correlation into within rabbit and between rabbit correlation was used. The mean values for basic temperature and for response per animal were calculated and a correlation calculation ~~calculation~~ based on these was carried out, i.e. a calculation of between rabbit correlation. No between rabbit correlation was found which would indicate that a considerable amount of within rabbit correlation exists. This was not a matter easily demonstrated by r calculations as only four injections per animal per dose level had been administered. The correlation was therefore illustrated by scatter diagrams, i.e. the visual foundation of the r calculation. Observation of these 75 diagrams, which are not reproduced here because of their bulk, shows that there is within-rabbit correlation of a negative type, i.e. greater response in any one rabbit occurring on a day when he showed lower normal temperature. This finding may account for the contradictory findings on the relation between normal temperature and response reported in the literature[74].

The normal temperature was again found to be independent of the colour of the animal, and once again the variance in final temperature attained after injection was significantly greater than the variance in rise.

It was observed while carrying out the experiments that all the rabbits did not reach their peak temperatures after injection at the same time but it could not easily be seen if the times varied with the dose level. The times taken to reach the peaks were calculated and the mean times at the three dose levels in descending order of these were 130, 110 and 120 minutes respectively, showing no significant difference with dose.

The existence of a relation between height of rise and time taken to reach it was investigated and a very slight negative correlation was found with the middle and lower doses, i.e. greater height was associated with shorter time. This type of calculation however took no account of the fact that some rabbits' temperatures rose quickly at first and then more slowly to their maximum, whereas others showed a steady ascent to the peak. To investigate the use in estimating pyrogen of a measure taking into account time to reach peak, height of peak and rate of temperature rise, three hundred graphs were plotted with time in minutes along the abscissa and rise in temperature on the ordinate. They are not included in this thesis as they are very bulky. From the point where the temperature began to fall a perpendicular was dropped to the abscissa and the enclosed area was

measured with a planimeter. The results recorded in planimeter units are shown in Appendix five, and show that the areas were not normally distributed and were more variable than temperature rise alone. Areas measured in this way were therefore not a useful estimate of pyrogen. The error in the method of measuring was not calculated as the variance shown in the areas could hardly be expected to be reduced even to a size comparable with that in temperature rise no matter how area was measured. No experiments were carried out measuring temperature until it regained its normal level. It is not known if the areas enclosed by such graphs would show less variability but it is probable that the accuracy of the temperatures recorded as being due to pyrogen would be impaired by the variation due to diurnal fluctuation which would then become apparent.

Finally the temperature responses were considered from the point of view of the existence of a straight log.dose/response line as had been suggested by several workers. The high and middle responses were found however not to be significantly different although they were within the range previously considered quantitative. The equation of a line could therefore be based only on the middle and low points which did differ significantly.

Thus, although a line representing the log. dose/response could be found its linearity could not be checked. Assuming the two points to be joined by a straight line, that line's equation was found to be $y = 0.48x + 1.72$. This line shows very different gradient from that of Wylie and Todd[60] for their Pseudomonas standard. This shows the need for extreme caution in using a standard from one organism in the estimation of pyrogen from another.

The general conclusion from these experiments and calculations is that, due to the proportion of false negatives obtainable from the smallest dose used and to the fact that a dose ten times this size is no longer quantitative, rabbit temperature rise is not an accurate measure of pyrogen. Also pyrogens from different organisms are of doubtful comparability.

Standard ix - Fifth freeze-dried standard

The assay might be improved in accuracy and constancy if based on some response other than rabbit temperature rise and consideration was now given to this.

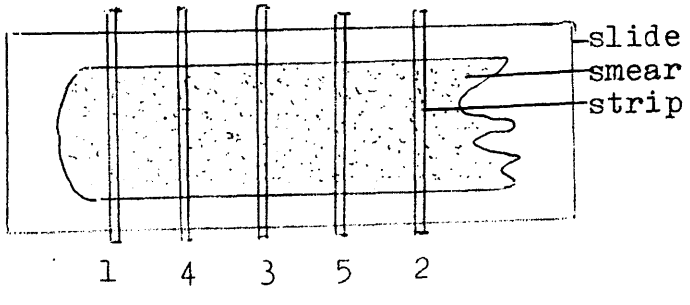
The first choice lay between using a different animal or using a different response in the same animal. Practical considerations of space and economics eliminated

the former possibility. On pp. 18 - 23 other possible responses of the rabbit are listed. The response considered probably the most useful basis of assay was the change in the white cell picture, and therefore standard six was used to investigate this quantitatively.

The literature is scant, vague and contradictory on the normal blood picture of the rabbit[137 - 143]. This picture was examined under the conditions of the experiments about to be performed, and using the same rabbits. Of the two standard methods of counting white cells, total and differential counts, the latter was chosen as it did not entail the accurate measuring of small volumes which might introduce error.

After trials of various minor modifications of stains and times the following method of carrying out the differential counts was adopted. Blood from the marginal ear-vein of the rabbit was made into a smear on a microscope slide, 25 rabbits being examined thus weekly for six weeks. The cells were stained with Leishmann's stain for 90 seconds, then twice the amount of water was added and this left for 25 minutes. Glasgow tap water, used for washing the smears, was found pure enough to give results indistinguishable from those obtained with distilled water or buffer solution.

The cells counted were large lymphocytes, small lymphocytes, monocytes, eosinophils, basophils and neutrophils. It is known that in the rabbit the neutrophils are very eosinophilic. Care was therefore taken to distinguish them accurately. The ease in distinguishing seemed to vary from batch to batch of stain. In making the smears of blood on the slides the different types of white cell became distributed unevenly throughout the smear. This was found to occur also in the cover-slip technique for making smears, where they are placed between two cover-slips which are then pulled apart, and which was developed to lessen this unequal distribution. In the slide technique, the small lymphocytes, being smallest, remain at the beginning of the smear or are dragged along the middle whereas the neutrophils tend to lie along the sides or to be dragged to the end. By means of a microscope with a mechanical stage and at a magnification of 600 the smears were counted in strips as in the diagram to lessen the error due to this uneven distribution.



Three, five or more strips were counted symmetrically until about 300 cells had been counted. Counting was stopped after the first symmetrical pattern of strips totalled over 300. Thus the actual number of cells counted varied for each smear - for example 312, 320, 325 etc. For ease in comparison of the smears the actual numbers were reduced to percentages. The mean percentage of small lymphocytes over these six weeks in the 25 rabbits was 79 with a standard deviation of 11. Analysis of variance showed that the weekly fluctuation among rabbits was not significantly greater than that within a rabbit. Most of the other cells were neutrophils, the percentages of large lymphocytes, monocytes, eosinophils and basophils being too small for any change in them to be used as a basis of assay. The cells were also counted as total mononuclears (small lymphocytes, large lymphocytes and monocytes) and total granulocytes (neutrophils, eosinophils, and basophils) only. The variances in the percentages of small lymphocytes and total mononuclears were not significantly different.

It was established that the error involved in counting the cells was less than the normal week-to-week fluctuation. This was done by comparing the variance in repeated counts of one smear with the variance in counts of

successive weekly smears from the same animal, using the F-test. This was repeated in other animals until it was established that the variances were in fact different.

Some of the rabbits were being used for the first time, but others had had the previous series of injections of pyrogen and had been rested in the interval for about six weeks. A comparison was made of the percentages of small lymphocytes in these two groups and no significant difference was found. Therefore six weeks is a long enough rest period for a rabbit to recover from the effects of pyrogen so far as its white cell picture is concerned.

The effect on the local white cell picture of repeated puncture of the ear vein was examined because the ear veins often looked dilated after a series of punctures. It was found that repeated hourly punctures might have a slight effect on the picture. In any case they were to be avoided in experiments where temperature was being recorded simultaneously as they made the rabbits restless and this in itself raised their temperatures.

The effect of pyrogen on this white cell picture was now investigated, preliminary work being carried out to find the time after injection when the change was greatest so that the effect of pyrogen could be investigated from one withdrawal of blood at that time in preference to a series of with-

drawals for the reason stated above. This time was found to be about three hours. It was observed that some rabbits regained their normal temperatures in 4½ hours whereas others still showed the effect of pyrogen in 24 hours, but the duration of the effect of pyrogen has not been systematically investigated in the present work.

The systematic experiments on the effect of pyrogen on the white cell picture in the rabbit were now begun. The rabbits were put in restraining boxes and thermocouples inserted rectally. Syringes and other apparatus were sterilised. The standard was reconstituted in apyrogenic saline and diluted appropriately. The rabbits' "normal" temperatures were recorded after they had fallen and then had become steady, i.e. after about an hour in the boxes. Blood smears were made. The pyrogen was injected during the 25 minutes while they were staining. Temperatures were recorded at 10 minute intervals during three hours. Blood smears were again made. Temperature recording was stopped when the second lot of blood was being withdrawn as the disturbance, even of its being withdrawn from other animals, raised the animals' temperatures. Each of the 19 available rabbits had 12 injections, four at each of the three dose levels as before. The temperature and white cell results are recorded in Appendix six . These experiments could be carried out

on 10 rabbits simultaneously but unfortunately the whole population could not be handled at once single-handed even although a set of 20 thermocouples was constructed for this purpose.

The general trend in the white cell change was a fall in the percentage of small lymphocytes and a corresponding rise in the percentage of neutrophils. From the general appearance of the smears it was considered that this net effect was due to a rise in the total number of neutrophils, but no systematic work on total counts has yet been carried out.

The temperature rises shown in response to the three levels were 1.18, 1.18 and 0.93° with variances of .2157, .1699 and .2540 respectively. At each level the rises were normally distributed and again analysis of variance showed between rabbit variance to exist significantly. The time taken to reach the peak after injection was again found not to vary with the dose.

The United States Prarmacopoeia precludes the use of rabbits of normal temperature greater than 39.8° or less than 38.9°. The justification for this was examined as no correlation between normal temperature and subsequent rise had been observed with the previous standard. Unfortunately on only two occasions out of the total 228 was there an

animal of normal temperature more than 39.8° so only the middle and low groups could be examined. Most of the animals were in fact in the group whose normal temperature was less than 38.9°, presumably due to the fact that the experiments were carried out in an atmospheric temperature lower than the usual United States indoor temperature and also due to the fact that the rabbits were restrained. Only at the low dose level was a difference in the groups found. Similar calculations were carried out based on a proposed pharmacopoeial test[76] excluding restrained rabbits of normal temperature less than 38.3° and similar results were obtained.

The white cell change was first of all calculated as difference between the percentages of small lymphocytes before and after injection. Examination of the falls in the percentage of small lymphocytes showed that their distribution was normal but the extent of their variance was too great for this measure to be a basis of assay. It was seen that the percentage of small lymphocytes before injection was very variable, both within and among rabbits, and in some cases was so low that, in conjunction with the higher falls occurring in some other rabbits, a negative value for the percentage after injection would have been obtained. The percentage of small lymphocytes before injection was distinctly correlated to the subsequent fall, both within

and between rabbits. Therefore it was decided to relate the fall to the percentage of small lymphocytes before injection by calculating it as a percentage of this percentage. This is referred to as the percentage fall. No correlation was found between the percentage of small lymphocytes before injection and the percentage fall.

The variance in percentage fall was found to be much smaller, making it a preferable basis of assay. However the distribution of the responses to the high dose was no longer normal but formed a negative skew curve. Its shape suggested that the cube roots of the percentage falls might be normally distributed but on calculation this was found not to be the case. No further transformations were investigated and no attempt was made to fit an equation to the curve obtained as there are no tables of statistics referring to curves other than normal. The distributions at the other dose levels were normal and that at this dose level was not so far from normal that the figures in the standard tables were not applicable. All subsequent calculations were therefore based on percentage fall. The percentage falls at the three levels were 76,59 and 44 with standard deviations of 15,20 and 24 respectively.

The variance in this response was analysed into between and within rabbit variance and in this case the

variance between rabbits was not found significantly greater than the variance within rabbits. It appeared also that the percentage fall in the new members of the population was indistinguishable from that in the group of rabbits which had had the previous series of pyrogen injections.

The coefficients of variation of temperature rise and small lymphocyte percentage fall at each level were compared and it was seen that at the high dose level the coefficient of variation of the small lymphocyte percentage fall was about half that of the temperature rise, while at the other two dose levels they were almost the same. This means that that at the high dose level the white cell method is the more accurate but at the other two dose levels equal accuracy is obtainable with either method. Similar results were obtained with coefficients of variation based on mean response per animal to eliminate variance within a rabbit.

Comparison by the F-test of the variances at the three dose levels showed that the variance in temperature rise and in small lymphocyte fall did not vary with the dose but that the variance in small lymphocyte percentage fall seemed to rise as the dose fell. Temperature rise expressed as a percentage of temperature before injection

showed no significant difference of variance with dose level, nor was it less variable than temperature rise alone. In view of this finding, the lessened variance in the expression of white cell change in terms of original level seems less of a mathematical artefact but this is not conclusively proven.

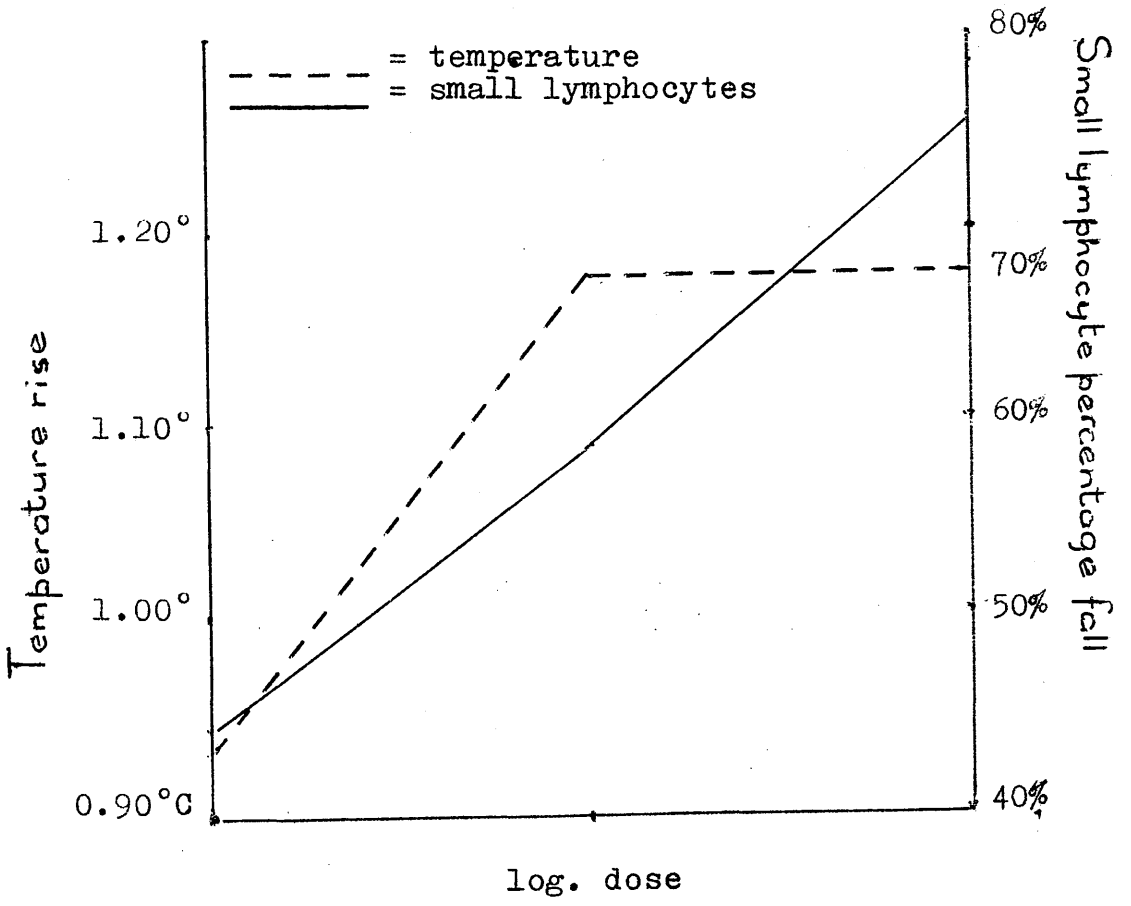
Calculations based on total mononuclears gave results similar to those based on small lymphocytes and no significant difference was found in the variance of the two measures.

The chance of the normal fluctuation in small lymphocytes being as great as that due to the doses of pyrogen used was found to be very remote, a point in favour of the white cell method.

The small lymphocyte percentage falls were significantly different at the three dose levels whereas the temperature rises due to the middle and high doses were obviously not different although the middle and low doses produced significantly different rises. This is illustrated in Figure 3. Thus the small lymphocyte percentage fall remained quantitative at a higher dose as well as being less variable there.

Figure 3

Comparison of rise in temperature and percentage fall in small lymphocytes as responses in the same experiments. Each point is the mean of 76 injections. Points for total mononuclears coincide with points for small lymphocytes



The correlation between temperature rise and small lymphocyte percentage fall was examined, using both the individual results and the mean results per animal. In no case was definite correlation found. The conclusion is that although rabbits of high sensitivity and rabbits of low sensitivity are encountered in the responses to both methods, the magnitude of the response by one method is not necessarily related to the magnitude of the response by the other method.

The percentage of small lymphocytes over the six weeks before the injections was compared with the percentage during the twelve weeks of injection and it was considered that the latter was slightly lower. Therefore probably more than one week's rest between injections is required for recovery of the normal white cell picture.

The line joining the low and middle temperature rises was assumed straight and its equation calculated to be $y = 0.5x + 1.78$, i.e. very similar to the equation for the previous standard.

Since in the small lymphocyte method three distinct results were obtained, not only could an equation be calculated but the responses could be tested for linearity. A straight line was found to fit the data and the equation of the line was $y = 32x + 98$. The general conclusion from this series of experiments is therefore that at the high dose level the white cell method had the advantage of remaining quantitative and of being less variable.

Summary

The literature on pyrogen has been reviewed, the review dealing with the early phase when materials not designated pyrogen were used specifically to produce fever, with the next phase when the possibility of pyrogen as a contaminant in intravenous fluids was recognised and with the current phase when pyrogen is again being used to produce fevers. A landmark in the literature is the association of pyrogenic effect with bacterial contamination, thus enabling logical work to be begun on eliminating pyrogen from solutions for injection and on attempting to isolate it for clinical or experimental purposes.

The various pharmacopoeias are concerned with ensuring that their intravenous fluids do not produce untoward reactions on injection, and to this end they contain limit tests for pyrogen. These serve their purpose as limit tests but were found not accurate enough for quantitative work and also not of sound design for this. Removal of pyrogen from injection solutions forms a considerable part of the literature, the most successful method being adsorption on asbestos or charcoal.

The physical, chemical and pharmacological properties of pyrogen have been reviewed, but the accuracy of the reports is obscured for want of knowledge of the exact nature

of the material or materials being described. Chemically pyrogen seems polysaccharide in nature, not protein as was formerly believed. A physiological effect often described in the literature and caused either by pyrogen or by something closely associated with it is the production of a leucopenia before the temperature rise and a leucocytosis after it. It is not known if the substance causing the temperature rise causes the white cell change too, if different substances occurring together produce the changes or if a substance from the broken down white cells causes the temperature rise.

Use of current pyrogen tests indicates the need for an international standard substance, and standards prepared by other workers have been reviewed. It appears difficult to produce a stable, pure material and it is not known if it is valid to compare pyrogen from one organism with that from another.

In the present work preliminary investigations were carried out on methods of preparing pyrogenic solutions and concentrating them for eventual extraction of a pure substance. Solutions from cultures of E.coli and P.vulgaris were made. E.coli pyrogen was found to decompose on heating under reduced pressure and on storage. It could be adsorbed on asbestos pads from acid solution and eluted with alkaline

solution but these eluates lost pyrogen rapidly. P.vulgaris pyrogen showed similar properties on storage of both eluate from asbestos pads and supernatant liquid from cultures. Dialysed supernatant liquid, free from the salts of the medium, appeared more stable.

Freeze-dried materials were now produced, firstly from whole supernatant liquid and then from dialysed supernatant liquid. Eluate decomposed on drying. These materials have so far been stable on storage in vacuo, both at room temperature and at 0°C.

Three batches of material were used for surveys of their effect in rabbits. The first was supernatant liquid from cultures of Proteus vulgaris, the second was the same freeze-dried and the third was the same dialysed and freeze-dried.

The principal observation made during the use of the first material was of the great variability in temperature response to the same dose, a factor which would diminish the reliability of temperature rise in the rabbit as a measure of pyrogen. Attempts were made to find the cause of the variation and lessen it. Variation among rabbits contributed to the total variance more than variation in any one animal in successive tests, but this variation could not be ascribed to sex, breed, weight, colour or normal temperature of the rabbits. It was concluded that

more than three rabbits per test would be an advantage if their reactions were completely unknown. Use of final temperature attained instead of rise as a measure of pyrogen was no advantage.

The freeze-dried supernatant liquid was injected at three dose levels, to investigate the nature of a log.dose/response line as well as to investigate variance in temperature response. The range of doses over which the line remained quantitative was found inconveniently short for temperature rise to be used as a basis of assay. The magnitude of the variance once again could not be attributed to any single factor. Attempts were made to use as an index of pyrogen a measure taking into account time required to reach peak as well as height of peak. This was even more variable than height of peak alone.

The freeze-dried, dialysed material was similarly injected at three dose levels. Simultaneously with temperature recording, white cell changes were measured by differential counts. First of all the normal white cell picture of the rabbits and the time of greatest change after injection were established. The error in reading a smear was found to be less than the normal fluctuation, which in turn was less than the fluctuation due to the doses of pyrogen used.

The pyrogen caused a fall in the relative percentage of small lymphocytes and this was expressed as a percentage of the initial level. At the high dose level injected the small lymphocyte percentage fall had a coefficient of variation of 20% and the temperature rise a coefficient of 39%. At the middle and low dose levels the responses were of equal variability. The small lymphocyte response remained quantitative over the three doses but the temperature response failed to distinguish between the high and middle doses.

Thus, so far as the present material is concerned, white cell change appears to be a basis of assay preferable to temperature rise, but much work remains to be done on the pyrogens from other organisms before a preparation from any one can validly be used as a reference standard in an assay process.

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A P P E N D I X O N E

ORGANISMS INVESTIGATED AS SOURCES OF PYROGEN

A - Organisms found pyrogenic

Nomenclature according to Bergey (56)	Reference	Authors' nomenclature
*Achromobacter candicans	27 59	Achromobacter candicans
*Achromobacter lacticum	27 59	Achromobacter lacticum
*Achromobacter pinnatum	27 59	Achromobacter pinnatum
*Achromobacter refractans	27 59	Achromobacter refractans
*Achromobacter solitarium	27 59	Achromobacter solitarium
*Achromobacter tiogense	27 59	Achromobacter tiogense
?	57	Achromobacter W 14 B
?	57	Achromobacter W 14 C
?	57	Achromobacter 2 W S
Aerobacter cloacae	59	B. cloacae
Alcaligenes faecalis	58	B. alcaligenes
Alcaligenes faecalis	59 60	Alcaligenes faecalis
Azotobacter chroococcum	59 60	Azotobacter chroococcum
Bacillus anthracis	59 60	Bacillus anthracis
Bacillus cereus var. mycoides	59 60	B. mycoides
Bacillus megatherium	59 60	B. megatherium
Bacillus polymyxa	59 60	B. aerosporus
Bacillus subtilis	21 57 59 60 61	Bacillus subtilis
?	57	Bacteriaceae
Brucella abortus	59	Brucella abortus
Brucella melitensis	21 59	Brucella melitensis
Corynebacterium acnes	59	Corynebacterium acnes
Corynebacterium acnes	21	B. acne
Corynebacterium diphtheriae	59	Corynebacterium diphtheriae
Corynebacterium diphtheriae (gravis)	60	Corynebacterium diphtheriae (gravis)
Corynebacterium diphtheriae (mitis)	60	Corynebacterium diphtheriae (mitis)
?	61	Diphtheroids
Escherichia coli	21 59	B. coli
Escherichia coli	57 58 60	Escherichia coli
?	61	coli-aerogenes types
*Escherichia formica	27 57	Escherichia formica
*Escherichia formica	59	B. formica
Escherichia freundii	61	Escherichia freundii
Gaffkya tetragena	59 60	Micrococcus tetragenes
Hemophilus bronchisepticus	59	H. bronchisepticus

Hemophilus influenzae	59	H.influenzae
Hemophilus influenzae	21	B. influenzae
Lactobacillus casei	59 60	Lactobacillus casei
Malleomyces mallei	59	Pfeiferella mallei
Micrococcus pyogenes var. albus	21 59	Staphylococcus albus
Micrococcus pyogenes var. aureus	57 59 60	Staphylococcus aureus
Micrococcus citreus	21 59	Staphylococcus citreus
Neisseria catarrhalis	21 59	M. catarrhalis
Neisseria gonorrhoeae	21	Gonococcus
Neisseria gonorrhoeae	59	Neisseria gonorrhoeae
Neisseria meningitidis	57	N. intracellularis
Neisseria meningitidis	59	N. meningitidis
Pasteurella pestis	21	B. pestis
Pasteurella pestis	59	P. pestis
Pseudomonas aeruginosa	21	B. pyocyaneus
Pseudomonas aeruginosa	59 60	Pseudomonas aeruginosa
Pseudomonas fluorescens	59 60	Pseudomonas fluorescens
Pseudomonas ovalis	61	Pseudomonas ovalis
Pseudomonas punctata	27	Achromobacter punctatum
Pseudomonas scissa	50	Pseudomonas scissa
Pseudomonas urea	50	Pseudomonas ureae
?	50	Pseudomonas 31
Proteus morganii	59	Proteus morganii
Proteus vulgaris	57 59 60	Proteus vulgaris
Salmonella typhosa	21	B.typhi
Salmonella typhosa	57	E.typhi
Salmonella typhosa	60	E.typhosa
Salmonella typhosa	59	S.typhi
Serratia kilensis	59 60	Serratia kielensis
Serratia marcescens	59 60	Serratia marcescens
Streptococcus lactis	59 60	Streptococcus lactis
Streptococcus pyogenes	57 59	Streptococcus pyogenes
Vibrio comma	21 59	Vibrio cholera
Vibrio comma	57 59	Vibrio comma
not in Bergey	60	Actinomyces albus
" " "	60	Actinomyces griseus
" " "	62	Aspergillus spp.
" " "	64	Penicillium notatum
" " "	57	Pink Wild Yeast
" " "	62	Saccharomyces spp.
" " "	59	The following viruses:- Influenza A(PR8), B(LEE), Newcastle Disease(B Strain)

B - Organisms found non-pyrogenic

Nomenclature according to Bergey	Reference	Authors' nomenclature
Bacillus anthracis	21	B. anthracis
Bacillus subtilis	50	Bacillus subtilis (delayed responses observed)
Clostridium tetani	21	B. tetanus
Corynebacterium diphtheriae	21	B. diphtheriae
Corynebacterium diphtheriae	57	Corynebacterium diphtheriae
Diplococcus pneumoniae	21	Pneumococcus
*Flavobacter arborescens	27	Flavobacter arborescens
*Flavobacter ochraceum	27	Flavobacter ochraceum
*Flavobacter radiatum	27	Flavobacter radiatum
Micrococcus pyogenes var. aureus	21	S. aureus
Micrococcus pyogenes var. aureus	50	S. aureus (delayed responses observed)
Micrococci	61	Staphylococci and micrococci (delayed responses observed)
Mycobacterium tuberculosum	57	M. tuberculosum
Neisseria gonorrhoeae	57	N. gonorrhoeae
Pasteurella multocida	57	P. cuniculocida
Pasteurella pestis	57	P. pestis
*Serratia rubra (?)	27	Serratia ruber
Streptococci	61	Streptococci (delayed responses observed)
not in Bergey	60	Aspergillus Glaucus
" " "	60	Aspergillus nidulans
" " "	60	Aspergillus niger
" " "	58	Monilia albicans
" " "	63	Moulds
" " "	60	Mucor sp.
" " "	60	Penicillium glaucum
" " "	60	Penicillium patulum
" " "	60	Penicillium terrestre
" " "	60	Saccharomyces cerevisiae
" " "	60	Saccharomyces cerevisiae, var. ellipsoideus

* In appendix to genus

A P P E N D I X T W O

Details of the Dialyses of Supernatant Liquids.

The object of the dialyses was to remove the salts of the medium in which the Proteus vulgaris had been grown, leaving a purer solution of pyrogen.

1. Corresponding to Experiment 3, Table 9.

The dialysing membrane used was Cellophane of porosity "300 PT". It was found that sterilisation by heating in an autoclave at 115° for thirty minutes made the cellophane very porous and that sterilisation by dry heat at 150° for one hour made it brittle. The cellophane was washed with freshly distilled water and made into a bag. Two hundred ml. of supernatant liquid were put into the bag and dialysed against running tap-water for one and a half hours and then left overnight in contact with static tap-water. Next day dialysis was carried out against running tap-water for six hours and the material again left overnight in static water. After half an hour of dialysis against running water on the third day the liquid was free from chloride. The volume of liquid in the bag had increased to 230 ml.

2. Corresponding to Experiment 4, Table 9 and to Table 17.

Two hundred ml. volumes of supernatant liquid were dialysed against three successive eight hundred ml. volumes of static apyrogenic water for half an hour each. After this dialysis of one and a half hours

the liquid was free from chloride. The volumes in the bags remained unchanged. It would appear that the cellophane was of different porosity although from the same batch.

A P P E N D I X T H R E E

Total Solids in Medium and in Eluting Buffer Solution.

The concentrations of solids in the medium in which Proteus was grown and in the buffer solutions used to elute pyrogen from asbestos pads were calculated to see if they had any bearing on the stability of pyrogen during freeze-drying. The supernatant liquid from a culture could be dried successfully but the eluate lost pyrogen on drying. It had already been observed that pyrogen in dialysed solution remained stable for longer than pyrogen in solutions of higher osmotic pressure. The calculations show that the higher osmotic pressure was not the cause of the decomposition of the pyrogen during the freeze-drying of the eluate. The cause is unknown.

1. Supernatant liquid.

Ammonium phosphate	80 g.
Sodium chloride	20 g.
Potassium acid phosphate	20 g.
Magnesium sulphate	14 g.
Ferrous sulphate	trace
Glucose	200 g.
Nicotinic acid	2×10^{-5} M
Water to	20 litres
Total solids	1.54%

2. Buffer solution, pH 9.9 after autoclaving.

Sodium carbonate	50 ml. of 0.1 M
Hydrochloric acid	25 ml. of 0.1 N
Water to	100 ml.

Total solids = 0.55%

3. Buffer solution, pH 10.4 after autoclaving.

0.1 M Glycine + 0.1 M Sodium chloride	60 ml.
0.1 M Sodium hydroxide	40 ml.

Total solids = 0.96%

APPENDIX FOUR

Rises in temperature in 27 rabbits in response to standard number three .

Rabbit No.	Responses to injections						
	1	2	3	4	5	6	7
1	0.71	0.41	0.11	0.30	0.00		
2	0.90	1.27	0.63	0.99	0.45		
3	1.01	0.83	1.17	1.15			
4	0.82	1.15	0.79	1.06			
5	1.77	0.90	1.35	1.11			
6	1.26	1.36	1.20	0.55	1.41		
7	0.46	0.90	1.32	0.86	0.49		
8	0.64	0.69	1.05	0.95	1.00		
9	0.79	1.15	1.01	0.82	1.05		
10	0.52	1.06	1.12	0.84			
11	0.68	0.92	0.50	0.40			
12	0.77	1.12	1.27	0.70	1.20		
13	0.58	1.26	0.63	0.98			
14	0.59	0.60	0.47	0.91			
15	0.75	0.78	0.37	0.23	0.79		
16	0.45	0.59	0.55	0.90	0.84		
17	1.26	0.74	0.83	0.77	1.07		
18	0.44	0.47	0.27	0.31			
19	0.50	0.80	0.86	0.77	0.19	0.76	0.53
20	0.95	1.23	1.53	1.38			
21	1.71	0.65	1.10	0.97			
22	1.25	0.81	0.73	0.24	0.80		
23	1.08	1.52	0.90	0.83			
24	0.92	0.63	1.45	0.62			
25	0.96	0.97	1.25	0.98			
26	1.27	1.95	1.39	0.41	1.21		
27	1.51	0.76	0.24	1.12			

Mean of 123 rises $= \frac{107.10}{123} = 0.87°$

Calculation of standard deviation in responses.

Rabbit No.	Deviations of responses from 0.87° (disregarding sign)						
1	0.16	0.46	0.76	0.47	0.87		
2	0.03	0.50	0.24	0.12	0.42		
3	0.14	0.04	0.40	0.28			
4	0.05	0.28	0.08	0.19			
5	0.90	0.03	0.48	0.24			
6	0.39	0.49	0.33	0.32	0.54		
7	0.41	0.03	0.45	0.01	0.38		
8	0.23	0.18	0.18	0.08	0.13		
9	0.08	0.28	0.14	0.05	0.18		
10	0.35	0.19	0.25	0.03			
11	0.19	0.05	0.37	0.47			
12	0.10	0.25	0.40	0.17	0.33		
13	0.29	0.39	0.24	0.11			
14	0.28	0.27	0.40	0.04			
15	0.12	0.09	0.50	0.64	0.08		
16	0.42	0.28	0.32	0.03	0.03		
17	0.39	0.13	0.04	0.10	0.20		
18	0.43	0.40	0.60	0.56			
19	0.37	0.07	0.01	0.10	0.68	0.11	0.34
20	0.08	0.36	0.66	0.51			
21	0.84	0.22	0.23	0.10			
22	0.38	0.06	0.14	0.63	0.07		
23	0.21	0.65	0.03	0.04			
24	0.05	0.24	0.58	0.25			
25	0.09	0.10	0.38	0.11			
26	0.40	1.08	0.52	0.46	0.34		
27	0.64	0.11	0.63	0.24			

$\Sigma d^2 = 16.2222$

$\sigma^2 = 0.1330$

$\sigma = 0.36$

d = deviation of response from mean

σ^2 = variance = $\frac{\Sigma d^2}{n - 1}$

n = 123

σ = standard deviation

Check by χ^2 method for normality of distribution of temperature responses, to ensure that the standard statistical methods are strictly applicable.

The 123 responses were divided into groups of not less than eight and χ^2 calculated as below:-

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o - f_t)^2}{f_t}$
0.00 - 0.40	11	11	0	0.0000
0.41 - 0.60	19	17	2	0.2354
0.61 - 0.80	25	24	1	0.0417
0.81 - 1.00	26	26	0	0.0000
1.01 - 1.20	19	22	3	0.4091
1.21 - 1.40	15	13	2	0.3077
1.41 - 2.00	8	9	1	0.1110
				$\chi^2 = 1.105$

Degrees of freedom = 6

From χ^2 tables, there is a probability of 98 - 99% of having by chance discrepancies between observed and theoretical frequencies as great as the above. Therefore the responses are normally distributed.

Analysis of the variance in the responses into variance between rabbits and variance within rabbits

1 Sum of squares of all the responses = 109.4264
2 Square of total responses for each animal divided by the number of responses per animal, summed for all animals = 100.6682
3 Grand total of all responses squared and divided by grand total number of responses = 93.2554

Number of rabbits = m = 27
Number of responses per column = n

σ_A^2 = variance between rabbits

σ_B^2 = variance within rabbits

F = ratio of variances, where n_1=26 and n_2=96.

Table of Analysis of Variance

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Components of variance
Between rabbits	2-3 = 7.4128	m-1 = 26	.2851	$n\sigma_A^2 + \sigma_B^2$
Within rabbits	1-2 = 8.7582	mn-m = 96	.0912	σ_B^2
Total	1-3 = 16.1710	mn-1 = 122	.1325	

Therefore $n\sigma_A^2 + \sigma_B^2$ is greater than σ_B^2, i.e. there is variance between rabbits. To see if it exists significantly -

$$F = \frac{.2851}{.0912} \triangleq 3.1$$

This is greater than the level of significance for any figure tabulated, that is, the variance between rabbits does exist significantly.

Calculation of the chances of getting false negative results.

1. Solutions for injection are considered apyrogenic by the method of Wylie and Todd if they produce rises in temperature of not more than 0.36°. The proportions of answers below 0.36 were therefore calculated as below:-

Proportion below 0.36 when the "true" mean is 0.87 -

Mean response of the population of 123 = 0.87
Standard deviation = 0.36
Percentage of answers between 0.87 and 0.36 =

$$\frac{0.87 - 0.36}{0.36}$$

$$= 1.417$$

$$= 42.18\%$$

(These figures were obtained from tables of areas under the normal curve.)
Therefore percentage of answers below 0.36 = 7.82%

2. Proportion below 0.36 when means of groups of three responses are considered.

Standard deviation of groups of three $= \frac{0.36}{\sqrt{3}}$

Percentage of answers below 0.36, calculated as above = 0.75%

3. The pyrogenic threshold according to the British, French and United States Pharmacopoeias is 0.6°. The proportions of answers below 0.6 were calculated as above, for individual responses. The percentage = 22.66%

4. The percentage of answers below 0.6 when groups of three are considered = 9.92%

To find the size of a group of rabbits which would have a mean differing from the "true" mean by not more than 0.1° with a probability of 95%.

Let the size of the group be equal to n.

Mean response of the population = 0.87°

Therefore 95% of the area under the normal curve has to lie between 0.77° and 0.97°.

Therefore area between mean and either limit = 47.5%

From tables of area under the normal curve, 47.5% is equivalent to a value of 1.96 for $\frac{\text{deviation}}{\text{standard deviation}}$.

Therefore:- $$\frac{0.1}{\frac{0.36}{\sqrt{n}}} = 1.96$$

$$n = 50$$

Thus to have a 95% probability of the mean of a sample lying not more than 0.1° from the "true" mean the size of the sample would have to be 50.

To compare the responses of male and female rabbits to the pyrogen injected.

Bucks			Does		
Mean response	d	d^2	Mean response	d	d^2
1.25	+0.41	.1681	1.28	+0.35	.1225
1.11	+0.27	.0729	1.27	+0.34	.1156
1.04	+0.20	.0400	1.16	+0.23	.0529
1.04	+0.20	.0400	1.08	+0.15	.0225
1.01	+0.17	.0289	0.96	+0.03	.0009
0.96	+0.12	.0144	0.93	0.00	.0000
0.91	+0.07	.0049	0.91	-0.02	.0004
0.89	+0.05	.0025	0.87	-0.06	.0036
0.86	+0.02	.0004	0.81	-0.12	.0144
0.85	+0.01	.0001	0.64	-0.29	.0841
0.77	-0.07	.0049	0.63	-0.30	.0900
0.67	-0.17	.0289	0.63	-0.30	.0900
0.58	-0.26	.0676			
0.37	-0.47	.2209			
0.31	-0.53	.2809			
Mean 0.84			0.93		
Variance		.0697			.0543

$t \triangleq .9$. Degrees of freedom = 25. Probability = 30-40%

Therefore there is no significant difference in the responses of bucks and does to these injections.

Comparison of the responses of the two breeds predominating in the population.

The population consisted of 12 Dutch, 6 Fox, 2 Ermine, 1 Rex, 1 Beveran and 5 cross-bred rabbits. Therefore only the Dutch and Fox were examined in this connection.

Dutch			Fox		
Mean response	d	d^2	Mean response	d	d^2
1.28	+0.31	.0961	1.08	+0.24	.0576
1.27	+0.30	.0900	0.96	+0.12	.0144
1.25	+0.28	.0784	0.91	+0.07	.0049
1.11	+0.14	.0196	0.91	+0.07	.0049
1.04	+0.07	.0049	0.85	+0.01	.0001
0.96	-0.01	.0001	0.31	-0.53	.2809
0.89	-0.08	.0064			
0.87	-0.10	.0100			
0.86	-0.11	.0121			
0.81	-0.16	.0256			
0.64	-0.33	.1089			
0.63	-0.34	.1156			
Mean 0.97			0.84		
Variance		.0516			.0726

t ≐ 1. Degrees of freedom = 16. Probability = 30%

Therefore there is probably no significant difference in the responses of the two breeds to these injections.

Temperatures of rabbits immediately before injection.

Rabbit No.	Temperatures in Centigrade degrees							
1	37.12	38.00	37.85	37.65	38.92			
2	38.49	38.47	38.75	38.60	38.50			
3	38.12	38.06	38.58	38.55				
4	38.94	38.97	39.13	38.74				
5	37.27	38.00	37.95	37.87				
6	39.17	38.94	39.30	39.10	39.17			
7	38.31	38.08	37.67	38.38	38.11			
8	38.68	38.38	38.39	38.72	38.64			
9	37.90	37.59	37.96	37.98	37.86			
10	38.67	38.13	37.41	37.86				
11	38.84	38.56	38.45	38.25				
12	38.46	37.29	37.92	38.34	38.70			
13	39.00	38.18	38.19	38.48				
14	38.61	37.61	37.96	38.80				
15	37.45	38.40	37.61	38.80	38.79			
16	38.18	38.38	37.78	37.43	37.56			
17	38.07	38.31	38.50	38.58	38.72			
18	38.80	38.53	38.42	38.49				
19	38.88	38.46	38.40	38.58	38.36	38.54	38.31	
20	38.36	38.18	38.74	38.67				
21	37.73	39.30	38.95	38.13				
22	38.30	38.91	38.85	38.70	38.80			
23	38.65	38.91	38.72	38.60				
24	38.62	38.96	39.24	39.19				
25	38.04	38.19	37.93	38.38				
26	37.99	37.93	38.16	38.03	37.97			
27	38.97	39.44	38.35	39.35				

Sum of temperatures = 4722.14
Mean temperature = 38.39
Variance = 0.2417
Standard deviation = 0.49

Maximum temperatures reached after injection.

Rabbit No.	Temperatures						
1	37.83	38.41	37.96	37.95	38.92		
2	39.39	39.74	39.38	39.59	38.95		
3	39.13	38.89	39.75	39.70			
4	39.76	40.12	39.92	39.80			
5	39.04	38.90	39.30	38.98			
6	40.43	40.30	40.50	39.65	40.58		
7	38.77	38.98	38.99	39.24	38.60		
8	39.32	39.07	39.44	39.67	39.64		
9	38.69	38.74	38.97	38.80	38.91		
10	39.19	39.19	38.53	38.70			
11	39.52	39.48	38.95	38.65			
12	39.23	38.41	39.19	39.04	39.90		
13	39.58	39.44	38.82	39.46			
14	39.20	38.21	38.43	39.71			
15	38.20	39.18	37.98	39.03	39.58		
16	38.63	38.97	38.33	38.33	38.40		
17	39.33	39.05	39.33	39.35	39.79		
18	39.24	39.00	38.69	38.80			
19	39.38	39.26	39.26	39.35	38.55	39.30	38.84
20	39.31	39.41	40.27	40.05			
21	39.44	39.95	40.05	39.10			
22	39.55	39.72	39.58	38.94	39.60		
23	39.73	40.43	39.62	39.43			
24	39.54	39.59	40.69	39.81			
25	39.00	39.16	39,18	39.36			
26	39.26	39.88	39.55	38.44	39.18		
27	40.48	40.20	39.59	40.47			

Sum of the temperatures = 4829.24
Mean temperature = 39.26
Variance = 0. 3521
Standard deviation = 0.59

Mean values per rabbit of normal temperature and maximum temperature reached after injection.

Rabbit No.	Normal temp.	Final temp.
1	37.91	38.21
2	38.56	39.41
3	38.33	39.37
4	38.95	39.90
5	37.77	39.06
6	39.14	40.29
7	38.11	38.92
8	38.56	39.43
9	37.86	38.82
10	38.02	38.90
11	38.53	39.15
12	38.14	39.15
13	38.46	39.33
14	38.25	38.89
15	38.21	38.79
16	37.87	38.53
17	38.44	39.37
18	38.56	38.93
19	38.50	39.13
20	38.49	39.76
21	38.50	39.64
22	38.71	39.48
23	38.72	39.80
24	39.00	39.91
25	38.14	39.18
26	38.02	39.26
27	39.03	40.19

Sum of final temperatures = 1060.80
Mean final temperature = 39.29
Standard deviation = 0.44

Sum of normal temperatures = 1036.78
Mean normal temperature = 38.40
Standard deviation = 0.38

Investigation of correlation between normal temperature and rise in temperature on injection.

Figures for normal temperature (X) and rise in temperature (Y) as before.

xy
+0.2793
-0.0048
-0.0112
+0.0440
-0.2520
+0.2072
+0.0203
-0.0016
-0.0432
-0.0038
-0.0325
-0.0338
-0.0012
+0.0360
+0.0570
+0.1113
+0.0020
-0.0816
-0.0250
+0.0351
+0.0230
-0.0341
+0.0640
+0.0180
-0.0416
-0.1406
+0.0189

$\Sigma x^2 = 3.7136$ $\Sigma y^2 = 1.6257$ $\Sigma xy = +0.2091$

$$r = \frac{+0.2091}{\sqrt{3.7136 \times 1.6257}}$$

$$= +0.08509$$

$n = 25$

Probability of r's being as grest as this by chance is very great. Therefore there is no correlation between normal temperature and rise in temperature.

Investigation of correlation between normal temperature and rise on injection, considering individual results.

Deviations from mean normal temperature(X) = x						
-1.27	-0.39	-0.54	-0.74	+0.53		
+0.10	+0.08	+0.36	+0.21	+0.11		
-0.27	-0.33	+0.19	+0.16			
+0.55	+0.58	+0.74	+0.35			
-1.12	-0.39	-0.44	-0.52			
+0.78	+0.55	+0.91	+0.71	+0.78		
-0.08	-0.31	-0.72	-0.01	-0.28		
+0.29	-0.01	0	+0.33	+0.25		
-0.49	-0.80	-0.43	-0.41	-0.53		
+0.28	-0.26	-0.98	-0.53			
+0.45	+0.17	+0.06	-0.14			
+0.07	-1.10	-0.47	-0.05	+0.31		
+0.61	-0.21	-0.20	+0.09			
+0.22	-0.78	-0.43	+0.41			
-0.94	+0.01	-0.78	+0.41	+0.40		
-0.21	-0.01	-0.61	-0.96	-0.83		
-0.32	-0.08	+0.11	+0.19	+0.33		
+0.41	+0.14	+0.03	+0.10			
+0.49	+0.07	+0.01	+0.19	-0.03	+0.15	-0.08
-0.03	-0.21	+0.35	+0.28			
-0.66	+0.91	+0.56	-0.26			
-0.09	+0.52	+0.46	+0.31	+0.41		
+0.26	+0.52	+0.33	+0.21			
+0.23	+0.57	+0.85	+0.80			
-0.35	-0.20	-0.46	-0.01			
-0.40	-0.46	-0.23	-0.36	-0.42		
+0.58	+1.05	-0.04	+0.96			

Deviations from mean rise(Y) = y						
-0.16	-0.46	-0.76	-0.47	-0.87		
+0.03	+0.50	-0.24	+0.12	-0.42		
+0.14	-0.04	+0.40	+0.28			
-0.05	+0.28	-0.08	+0.19			
+0.90	+0.03	+0.48	+0.24			
+0.39	+0.49	+0.33	-0.32	+0.54		
-0.41	+0.03	+0.45	-0.01	-0.38		
-0.23	-0.18	+0.18	+0.08	+0.13		
-0.08	+0.28	+0.14	-0.05	+0.18		
-0.35	+0.19	+0.25	-0.03			
-0.19	+0.05	-0.37	-0.47			
-0.10	+0.25	+0.40	-0.17	+0.33		
-0.29	+0.39	-0.24	+0.11			
-0.28	-0.27	-0.40	+0.04			
-0.12	-0.09	-0.50	-0.64	-0.08		
-0.42	-0.28	-0.32	+0.03	-0.03		
+0.39	-0.13	-0.04	-0.10	+0.20		
-0.43	-0.40	-0.60	-0.56			
-0.37	-0.07	-0.01	-0.10	-0.68	-0.11	-0.34
+0.08	+0.36	+0.66	+0.51			
+0.84	-0.22	+0.23	+0.10			
+0.38	-0.06	-0.14	-0.63	-0.07		
+0.21	+0.65	+0.03	-0.04			
+0.05	-0.24	+0.58	-0.25			
+0.09	+0.10	+0.38	+0.11			
+0.40	+1.08	+0.52	-0.46	+0.34		
+0.64	-0.11	-0.63	+0.24			

xy				
+.2032	+.1794	+.4104	+.3478	-.4611
+.0030	+.0400	-.0864	+.0252	-.0462
-.0378	+.0132	+.0760	+.0448	
-.0275	+.1624	-.0592	+.0665	
-1.0080	-.0117	-.2112	-.1248	
+.3042	+.2695	+.3003	-.2272	+.4212
+.0328	-.0093	-.3240	+.0001	+.1064
-.0667	+.0018	0	+.0264	+.0325
+.0392	-.2240	-.0602	+.0205	-.0954
-.0980	-.0494	-.2450	+.0159	
-.0855	+.0085	-.0222	+.0658	
-.0070	-.2750	-.1880	+.0085	+.1023
-.1769	-.0819	+.0480	+.0099	
-.0616	+.2106	+.1720	+.0164	
+.1128	-.0009	+.3900	-.2624	-.0320
+.0882	+.0028	+.1952	-.0288	+.0249
-.1248	+.0104	-.0044	-.0190	+.0660
-.1763	-.0560	-.0180	-.0560	
-.1813	-.0049	-.0001	-.0190	+.0204 -.0165 +.0272
-.0024	-.0756	+.2310	+.1428	
-.5544	-.2002	+.1288	-.0260	
-.0342	-.0312	-.0644	-.1953	-.0287
+.0564	+.3380	+.0099	-.0084	
+.0115	-.1368	+.4930	-.2000	
-.0315	-.0200	-.1748	-.0011	
-.1600	-.4968	-.1196	+.1656	-.1428
+.3712	-.1155	+.0252	+.2304	

$$r = \frac{-8.1913 + 6.9249}{\sqrt{29.4829 \times 16.2222}} = -0.0579$$

Degrees of freedom = 121

Therefore there is no correlation between normal temperature and rise on injection, considering individual results.

Comparison of variance in temperature rise with variance in maximum temperature attained after injection.

Variance in temperature rise = 0.1325

Variance in maximum temperature = 0.3521

Number of injections = 123

Therefore $n_1 = n_2$ = 122

Therefore $F = \frac{.3521}{.1325}$ = 2.66

This is greater than any tabulated value in Tables of F. Therefore the variance in maximum temperature after injection is significantly greater than the variance in temperature rise.

To compare the responses of three groups of rabbits of different colours.

Black-eared			White-eared			Grey-eared		
Mean rise	d	d^2	Mean rise	d	d^2	Mean rise	d	d^2
0.31	0.55	0.3025	1.04	0.21	0.0441	0.96	0.01	0.0001
0.85	0.01	0.0001	0.67	0.16	0.0256	0.81	0.14	0.0196
1.28	0.42	0.1764	0.77	0.06	0.0036	0.87	0.08	0.0064
0.63	0.23	0.0529				0.96	0.01	0.0001
1.01	0.15	0.0225				0.89	0.06	0.0036
0.64	0.22	0.0484				0.86	0.09	0.0081
0.93	0.07	0.0049				1.27	0.32	0.1024
0.63	0.23	0.0529				1.08	0.13	0.0169
1.11	0.25	0.0625				0.91	0.04	0.0016
1.25	0.39	0.1521				0.91	0.04	0.0016
Number in group = 10			Number in group = 3			Number in group = 10		
Mean rise = 0.86			Mean rise = 0.83			Mean rise = 0.95		
Variance =.0972			Variance = .0367			Variance = .0178		

Comparison of white and grey groups. (These groups show the greatest difference in means)

$$t = \frac{.12}{\sqrt{\frac{.0367}{3} + \frac{.0178}{10}}} = 1.014$$

Degrees of freedom = 11

Probability of t as great as this by chance = 30-40%

Comparison of black and white groups.

This gives a value of t = .2025, which, with 11 degrees of freedom is equivalent to a probability of 80-90%.

Comparison of black and grey groups.

This gives a value of t = .8390, which, with 18 degrees of freedom is equivalent to a probability of 40-50%.

Therefore the groups may belong to the same population, i.e. the responses are not affected by differing abilities, if any, of differently coloured rabbits to radiate heat.

Check that the three white rabbits are representative of the whole.

This was checked because the group was small, even although t-tests are designed for small numbers.

Mean response of the whole population (considering only the mean responses per animal) = 0.88

Variance = 0.0625

Mean response of group of white rabbits = 0.83

Variance = 0.0367

$$t = \frac{0.88 - 0.83}{\sqrt{\frac{0.0625}{27} + \frac{0.0367}{3}}} = 0.4153$$

Degrees of freedom = 28

Probability of t as great as this by chance = 50 - 60%

Therefore the three white rabbits may be taken as repressntative of the whole population.

Investigation of correlation between rabbit weight and normal temperature.

Weight in kg. Y	Dev. from $\bar{Y} = y$	y^2	Normal temp. X	Dev. from $\bar{X} = x$	x^2	xy
2.25	+0.30	0.0900	37.91	-0.49	0.2401	-0.1470
1.70	-0.25	0.0625	38.56	+0.16	0.0256	-0.0400
2.05	+0.10	0.0100	38.33	-0.07	0.0049	-0.0070
2.50	+0.55	0.3025	38.95	+0.55	0.3025	+0.3025
1.70	-0.25	0.0625	37.77	-0.63	0.3969	+0.1575
1.70	-0.25	0.0625	39.14	+0.74	0.5476	-0.1850
1.55	-0.40	0.1600	38.11	-0.29	0.0841	+0.1160
1.95	0	0	38.56	+0.16	0.0256	0
1.85	-0.10	0.0100	37.86	-0.54	0.2916	+0.0540
1.65	-0.30	0.0900	38.02	-0.38	0.1444	+0.1140
1.65	-0.30	0.0900	38.53	+0.13	0.0169	-0.0390
2.45	+0.50	0.2500	38.14	-0.26	0.0676	-0.1300
2.05	+0.10	0.0100	38.46	+0.06	0.0036	+0.0060
1.65	-0.30	0.0900	38.25	-0.15	0.0225	+0.0450
1.80	-0.15	0.0225	38.21	-0.19	0.0361	+0.0285
2.15	+0.20	0.0400	37.87	-0.53	0.2809	-0.1060
2.25	+0.30	0.0900	38.44	+0.04	0.0016	+0.0120
2.05	+0.10	0.0100	38.56	+0.16	0.0256	+0.0160
2.45	+0.50	0.2500	38.50	+0.10	0.0100	+0.0500
2.00	+0.05	0.0025	38.49	+0.09	0.0081	+0.0045
1.65	-0.30	0.0900	38.50	+0.10	0.0100	-0.0300
1.95	0	0	38.71	+0.31	0.0961	0
2.20	+0.25	0.0625	38.72	+0.32	0.1024	+0.0800
2.15	+0.20	0.0400	39.00	+0.60	0.3600	+0.1200
1.90	-0.05	0.0025	38.14	-0.26	0.0676	+0.0130
1.60	-0.35	0.1225	38.02	-0.38	0.1444	+0.1330
2.00	+0.05	0.0025	39.03	+0.63	0.3969	+0.4315

$\bar{Y} = 1.95$ $\bar{X} = 38.40$

$\Sigma y^2 = 2.0250$ $\Sigma x^2 = 3.7136$ $\Sigma xy = 0.5995$

$$r = \frac{+0.5995}{\sqrt{3.7136 \times 2.0250}}$$

$$= +0.2186$$

$n = 25$

Therefore there is little, if any, correlation between weight and normal temperature.

To compare the normal temperatures of three groups of rabbits of different colours.

Black-eared			White-eared			Grey-eared		
Temp.	d	d^2	Temp.	d	d^2	Temp.	d	d^2
37.91	0.35	0.1225	38.33	0.03	0.0009	38.95	0.43	0.1849
38.56	0.30	0.0900	37.87	0.43	0.1849	38.11	0.41	0.1861
37.77	0.49	0.2401	38.71	0.41	0.1681	38.56	0.04	0.0016
38.53	0.27	0.0729				37.86	0.66	0.4356
38.14	0.12	0.0144				38.02	0.50	0.2500
38.25	0.01	0.0001				38.46	0.06	0.0036
38.44	0.18	0.0324				38.49	0.03	0.0009
38.50	0.24	0.0576				38.72	0.20	0.0400
38.50	0.24	0.0576				39.00	0.48	0.2304
38.02	0.24	0.0576				39.03	0.51	0.2601
Mean temp.		38.26			38.30			38.52
Variance		.0828			.1770			.1750

1. Comparison of black and grey groups.

$$t = \frac{0.26}{\sqrt{\frac{.0828}{10} + \frac{.1750}{10}}} = 1.619$$

Degrees of freedom = 18

Probability of t as great as this by chance = 10-20 %

2. Comparison of black and white groups.

$$t = \frac{0.04}{\sqrt{\frac{.0828}{10} + \frac{.1770}{3}}} = .1543$$

Degrees of freedom = 11

Probability = 80-90 %

3. Comparison of white and grey groups.

$$t = \frac{.22}{\sqrt{\frac{.1770}{3} + \frac{.1750}{10}}} = .4547$$

Degrees of freedom = 11

Probability = 60-70 %

Therefore the groups may be from the same population, i.e. the normal temperatures of the rabbits are not affected by their colours.

Investigation of correlation between rabbit weight and rise in temperature.

Temp. rise X	Dev. from $\bar{X}$ = x	x^2	Weight in kg. Y	Dev. from $\bar{Y}$ = y	y^2	xy
0.31	-0.57	0.3249				-0.1710
0.85	-0.03	0.0009				+0.0075
1.04	+0.16	0.0256				+0.0160
0.96	+0.08	0.0064				+0.0440
1.28	+0.40	0.1600	See p.129			-0.1000
1.16	+0.28	0.0784				-0.0700
0.81	-0.07	0.0049				+0.0280
0.87	-0.01	0.0001				0
0.96	+0.08	0.0064				-0.0080
0.89	+0.01	0.0001				-0.0300
0.63	-0.25	0.0625				+0.0750
1.01	+0.13	0.0169				+0.0650
0.86	-0.02	0.0004				-0.0020
0.64	-0.24	0.0576				+0.0720
0.58	-0.30	0.0900				+0.0450
0.67	-0.21	0.0441				-0.0420
0.93	+0.05	0.0025				+0.0150
0.37	-0.51	0.2601				-0.0510
0.63	-0.25	0.0625				-0.1250
1.27	+0.39	0.1521				+0.0195
1.11	+0.23	0.0529				-0.0690
0.77	-0.11	0.0121				0
1.08	+0.20	0.0400				+0.0500
0.91	+0.03	0.0009				+0.0060
1.04	+0.16	0.0256				-0.0080
1.25	+0.37	0.1369				-0.1295
0.91	+0.03	0.0009				+0.0015
ΣX = 23.79			ΣY = 52.85		Σxy	= -0.3610
$\bar{X}$ = 0.88			$\bar{Y}$ = 1.95			
Σx^2 = 1.6257			Σy^2 = 2.0250			

$$r = \frac{-.3610}{\sqrt{1.6257 \times 2.0250}}$$

$$= -0.1990$$

$$n = 25$$

The probability of r's being as great as this by chance is very great. Therefore there is little if any correlation between rabbit weight and temperature rise. This is a check on the validity of administering a dose proportional to body weight.

Times taken for temperatures to reach a peak after injection.

Rabbit No.	Times - in minutes							Mean time
1	180	120	80	30	*			100
2	130	160	120	130	100			130
3	140	150	90	100				120
4	200	120	130	100				140
5	160	130	130	140				140
6	80	80	80	80	80			80
7	130	70	140	120	120			120
8	120	90	80	90	100			100
19	140	80	130	80	140			110
10	80	110	100	120				100
11	80	100	80	130				100
12	170	40	140	100	110			110
13	110	80	50	120				90
14	120	110	110	100				110
15	140	150	30	30	90			90
16	140	100	110	70	110			110
17	60	170	70	110	90			100
18	70	120	80	60				80
19	180	180	130	130	100	130	130	140
20	170	120	70	90				110
21	160	100	120	180				140
22	120	100	120	70	100			100
23	110	110	100	150				120
24	90	90	100	100				100
25	110	130	80	160				120
26	100	90	90	100	70			90
27	90	100	110	120				110
	Mean of all the times =							110

* The rabbit's temperature showed no rise on this occasion. The mean shown in the final column is the mean of the other four values.

Investigation of the correlation between height of rise in temperature and time taken to reach it.

Dev.from $\bar{X}$ = x	Time Y	Dev.from $\bar{Y}$ = y	y^2	xy
-0.57	100	-10	100	-5.7
-0.03	130	+20	400	-0.6
+0.16	120	+10	100	+1.6
+0.08	140	+30	900	+2.4
+0.40	140	+30	900	+12.0
+0.28	80	-30	900	-8.4
-0.07	120	+10	100	-0.7
-0.01	100	-10	100	+0.1
+0.08	110	0	0	0
+0.01	100	-10	100	-0.1
-0.25	100	-10	100	+2.5
+0.13	110	0	0	0
-0.02	90	-20	400	+0.4
-0.24	110	0	0	0
-0.30	90	-20	400	+6.0
-0.21	110	0	0	0
+0.05	100	-10	100	-0.5
-0.51	80	-30	900	+15.3
-0.25	140	+30	900	-7.5
+0.39	110	0	0	0
+0.23	140	+30	900	+6.9
-0.11	100	-10	100	+1.1
+0.20	120	+10	100	+2.0
+0.03	100	-10	100	-0.3
+0.16	120	+10	100	+1.6
+0.37	90	-20	400	-7.4
+0.03	110	0	0	0

$\Sigma x^2 = 1.6257$ $\Sigma y^2 = 7700$ $\Sigma xy = +20.7$

$$r = \frac{+20.7}{\sqrt{1.6257 \times 7700}} = .185$$

n = 25

Probability = >.1

Therefore there is no correlation between height of rise in temperature and time taken to reach it.

A P P E N D I X F I V E

Rises in temperature shown by 25 rabbits to four injections each at three dose levels.

Rabbit * No.	Responses to 0.02 ml. per kilogram			
1	0.96	1.46	0.91	1.02
2	0.66	0.97	1.20	0.94
3	0.64	0.84	1.00	1.30
4	0.88	0.78	1.01	1.10
5	1.03	0.85	0.83	0.82
6	0.75	0.28	0.05	0.46
7	1.25	1.16	0.87	1.77
8	0.32	1.02	0.95	0.95
9	0.56	0.56	1.07	0.94
10	1.00	1.22	0.71	0.65
11	0.00	0.48	0.57	0.87
12	0.48	1.15	0.71	0.56
13	0.70	0.79	1.03	1.75
14	1.71	1.53	1.58	1.43
15	0.47	0.77	0.20	0.31
16	0.61	1.29	1.26	0.81
17	0.92	0.65	0.41	1.93
18	1.08	0.71	0.74	1.64
19	0.50	0.36	0.48	1.20
20	1.57	1.21	1.27	0.94
21	0.88	0.95	1.29	1.06
22	0.77	0.96	1.06	1.52
23	1.13	0.28	0.70	0.97
24	0.45	1.07	0.83	1.07
25	0.66	0.95	0.47	0.72

Mean of 100 rises = 0.90°

* These numbers do not represent the same rabbits as numbers 1 - 25 in Appendix Four .

Rabbit No.	Responses to 0.06324 ml./kg.			
1	1.01	1.52	1.34	1.27
2	0.34	1.19	0.95	0.85
3	1.29	0.86	0.66	1.09
4	1.35	1.73	1.24	1.07
5	1.63	1.22	1.15	1.00
6	1.47	1.38	1.30	1.43
7	0.92	1.15	0.48	0.44
8	1.23	1.05	1.17	0.98
19	0.77	1.11	1.14	1.44
10	1.28	1.10	1.21	1.33
11	0.67	0.41	0.48	1.05
12	0.95	1.07	1.03	1.26
13	1.63	1.65	1.23	1.44
14	1.76	1.19	1.29	1.79
15	0.70	0.75	0.77	1.22
16	1.55	1.45	1.15	1.08
17	1.77	1.09	1.86	1.39
18	1.33	1.41	1.70	1.13
19	0.80	0.91	0.64	0.62
20	1.19	1.46	1.44	0.92
21	1.09	1.15	1.06	1.09
22	1.61	1.66	1.28	1.14
23	0.96	1.42	1.17	0.38
24	0.86	1.05	0.95	1.18
25	0.88	0.53	1.15	0.83

Mean of 100 rises = 1.14°

Rabbit No.	Responses to o.2 ml. per kilogram			
1	0.67	0.60	1.80	1.09
2	1.14	1.11	0.34	1.06
3	1.10	1.27	0.96	0.90
4	1.30	1.38	1.62	1.62
5	0.99	1.00	1.33	1.48
6	0.82	1.31	1.11	1.20
7	0.83	0.80	1.26	0.78
8	0.70	1.00	0.83	0.53
9	1.70	1.67	1.16	1.70
10	1.32	1.15	1.68	0.88
11	0.62	0.77	0.91	0.62
12	1.14	1.17	1.12	0.81
13	1.04	1.26	1.58	0.83
14	1.69	1.66	1.95	1.57
15	0.99	1.20	1.70	0.37
16	1.22	2.08	1.14	1.21
17	1.74	1.90	2.20	1.28
18	0.85	0.95	1.35	1.41
19	1.08	1.25	0.86	0.73
20	1.33	1.32	1.43	0.93
21	1.80	1.71	1.90	1.76
22	1.43	1.29	2.05	1.32
23	0.86	1.09	1.31	1.14
24	1.04	1.19	0.95	0.89
25	1.07	1.17	0.65	1.60

Mean of 100 rises = 1.20

Standard deviations in the responses at the three dose levels.

Deviations											
0.2 ml./kg.				0.06324 ml./kg.				0.02 ml./kg.			
.53	.60	.60	.11	.13	.38	.20	.13	.06	.56	.01	.12
.06	.09	.86	.14	.80	.05	.19	.29	.24	.07	.30	.04
.10	.07	.24	.30	.06	.28	.48	.05	.26	.06	.10	.40
.10	.18	.42	.42	.21	.59	.10	.07	.02	.12	.11	.20
.21	.20	.13	.28	.49	.08	.01	.14	.13	.05	.07	.08
.38	.11	.09	.00	.33	.24	.16	.29	.15	.62	.25	.44
.37	.40	.06	.42	.22	.01	.66	.70	.35	.26	.03	.13
.50	.20	.37	.67	.09	.09	.03	.16	.68	.12	.05	.05
.50	.47	.04	.50	.37	.03	.00	.30	.34	.34	.17	.04
.12	.05	.48	.32	.14	.04	.07	.19	.10	.32	.19	.25
.58	.43	.29	.58	.47	.73	.66	.09	.90	.42	.33	.03
.06	.03	.08	.39	.19	.07	.11	.12	.42	.25	.19	.34
.16	.06	.38	.37	.49	.51	.09	.30	.20	.11	.13	.85
.49	.46	.75	.37	.62	.05	.15	.65	.81	.63	.68	.53
.21	.00	.50	.83	.44	.39	.37	.08	.43	.13	.70	.59
.02	.88	.06	.01	.41	.31	.01	.06	.29	.39	.36	.09
.54	.70	1.00	.08	.63	.05	.72	.25	.02	.25	.49	1.03
.35	.25	.15	.21	.19	.27	.56	.01	.18	.19	.16	.74
.12	.05	.34	.47	.34	.23	.50	.52	.40	.54	.42	.30
.13	.12	.23	.27	.05	.32	.30	.22	.67	.31	.37	.04
.60	.51	.70	.56	.05	.01	.08	.05	.02	.05	.39	.16
.23	.09	.85	.12	.47	.52	.14	.00	.13	.06	.16	.62
.34	.11	.11	.06	.18	.28	.03	.76	.23	.62	.20	.07
.16	.01	.25	.31	.28	.09	.19	.04	.45	.17	.07	.17
.16	.01	.25	.31	.28	.09	.19	.04	.45	.17	.07	.17
.13	.03	.55	.40	.26	.61	.01	.31	.24	.05	.43	.18
Variance		.1517				.1132				.1331	
Standard deviation		.39				.34				.36	

Comparison of the variances at the three dose levels.

Dose level (ml./kg.)	Variance
0.02	0.1331
0.06324	0.1132
0.2	0.1517

Greatest ration of variances $= \frac{.1517}{.1132} = 1.340$

$n_1 = n_2 = 99$

Therefore F is equivalent to a probability of 1 - 5%.

Therefore the variances do not differ significantly at the three dose levels.

Check by χ^2 method for normality of distribution of temperature responses.

1. Dose = 0.2 ml./kg.

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o - f_t)^2}{f_t}$
0.00 - 0.80	13	16	3	0.5625
0.81 - 1.00	21	15	6	2.4000
1.01 - 1.20	22	19	3	0.4737
1.21 - 1.40	18	19	1	0.0526
1.41 - 1.60	7	15	8	4.2667
1.61 - 1.80	13	9	4	1.7778
1.81 - 2.20	6	6	0	0.0000

$\chi^2 = 9.5333$
Degrees of freedom = 6
Probability = 10-20%

2. Dose = 0.06324 ml./kg.

Range of temperature rise of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o - f_t)^2}{f_t}$
0.00 - 0.80	16	17	1	0.0588
0.81 - 1.00	14	19	5	1.3158
1.01 - 1.20	30	22	8	2.9091
1.21 - 1.40	18	21	3	0.4286
1.41 - 1.60	11	13	2	0.3077
1.61 - 2.00	11	8	3	1.1250

$\chi^2 = 6.1450$
Degrees of freedom = 5
Probability = 20-30%

3. Dose = 0.02 ml./kg.

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o - f_t)^2}{f_t}$
0.00 - 0.60	20	20	0	0.0000
0.61 - 0.80	20	19	1	0.0526
0.81 - 1.00	25	20	5	1.2500
1.01 - 1.20	17	19	2	0.2105
1.21 - 1.40	8	12	4	1.3333
1.41 - 2.00	10	8	2	0.5000

χ^2 = 3.3464
Degrees of freedom = 5
Probability = 50-70%

Therefore at each dose level the responses are normally distributed.

Calculation of the chances of getting false negative results.

1. Percentage of answers below 0.36° when the "true" mean is 1.20° and σ is 0.39 = 1.56%

2. Percentage of answers below 0.36° when the "true" mean is 1.14° and σ is 0.34 = 1.09%

3. Percentage of answers below 0.36° when the "true" mean is 0.90° and σ is 0.36 = 6.68%

4. Percentage of answers below 0.60° when the "true" mean is 1.20° and σ is 0.39 = 6.20%

5. Percentage of answers below 0.60° when the "true" mean is 1.14° and σ = 0.34 = 5.61%

6. Percentage of answers below 0.60° when the "true" mean is 0.90° and σ is 0.36 = 20.25%

Calculation of the chances of getting false negatives using the means of samples of three.

1. Percentage of answers below 0.36 when the "true" mean is 1.20 and σ is 0.39 = 0.01%

2. Percentage of answers below 0.36 when the "true" mean is 1.14 and σ is 0.34 = 0%

3. Percentage of answers below 0.36 when the "true" mean is 0.90 and σ is 0.36 = 0.47%

4. Percentage of answers below 0.60 when the "true" mean is 1.20 and σ is 0.39 0.39%

5. Percentage of answers below 0.60 when "true" mean is 1.14 and σ is 0.34 0.22%

6. Percentage of answers below 0.60 when the "true" mean is 0.90 and σ is 0.36 = 7.31%

To find the size of a group of rabbits which would have a mean differing from the "true" mean by not more than 0.1° with a probability of 95% .

Let the size of the group be equal to n

Percentage of answers lying within the area between mean and mean + or - 0.1 = 47.5%

From tables of area under the normal curve this is equivalent to $\frac{x}{\sigma} = 1.96$

1. Dose = 0.2 ml./kg. $\sigma = 0.39$

$$\text{Therefore } \frac{0.10}{\left(\frac{0.39}{\sqrt{n}}\right)} = 1.96$$

$$n = 58$$

2. Dose = 0.06324 ml./kg. $\sigma = 0.34$

$$\text{Therefore } \frac{0.10}{\left(\frac{0.34}{\sqrt{n}}\right)} = 1.96$$

$$n = 44$$

3. Dose = 0.02 ml./kg. $\sigma = 0.36$

$$\text{Therefore } \frac{0.10}{\left(\frac{0.36}{\sqrt{n}}\right)} = 1.96$$

$$n = 50$$

To compare the responses of male and female rabbits.

1. Dose level 0.2 ml./kg,

Bucks			Does		
Temp. rise	d	d^2	Temp. rise	d	d^2
1.04	0.13	.0169	1.06	0.18	.0324
0.91	0.26	.0676	1.48	0.24	.0576
1.20	0.03	.0009	1.26	0.02	.0004
1.11	0.06	.0036	1.06	0.18	.0324
0.92	0.25	.0625	1.18	0.06	.0036
0.77	0.40	.1600	1.72	0.48	.2304
1.56	0.39	.1521	1.41	0.17	.0289
0.73	0.44	.1936	0.98	0.26	.0676
0.82	0.35	.1225	1.02	0.22	.0484
1.78	0.61	.3721			
1.14	0.03	.0009			
1.25	0.08	.0064			
1.79	0.62	.3844			
1.52	0.35	.1225			
1.10	0.07	.0049			
1.12	0.05	.0025			
Mean rise	1.17		1.24		
σ^2	.1116		.0627		

$$t = \frac{1.24 - 1.17}{\sqrt{\frac{.1116}{16} + \frac{.0627}{9}}} = .5928$$

degrees of freedom = 23

Therefore the probability of a value of t as great as this by chance is 50 - 60 %

2. Dose level 0.06324 ml./kg.

Bucks			Does		
Temp. rise	d	d^2	Temp. rise	d	d^2
1.29	0.18	.0324	0.95	0.22	.0484
0.83	0.28	.0784	1.35	0.18	.0324
1.25	0.14	.0196	1.23	0.06	.0036
1.40	0.29	.0841	1.08	0.09	.0081
0.75	0.36	.1296	1.49	0.32	.1024
1.11	0	0	1.51	0.34	.1156
1.12	0.01	.0001	1.21	0.14	.0196
0.65	0.46	.2116	0.74	0.43	.1849
0.86	0.25	.0625	0.85	0.32	.1024
1.53	0.42	.1764			
1.25	0.14	.0196			
1.39	0.28	.0784			
1.10	0.01	.0001			
1.42	0.31	.0961			
0.98	0.13	.0169			
0.85	0.26	.0676			
Mean rise	1.11		1.17		
Variance	.0716		.0772		

$$t = \frac{1.17 - 1.11}{\sqrt{\frac{.0716}{16} + \frac{.0772}{9}}} = .5252$$

degrees of freedom = 23

Therefore the probability of a value of t as great as this by chance is 60 - 70%

3. Dose level 0.02 ml./kg.

Bucks			Does		
Temp. rise	d	d^2	Temp. rise	d	d^2
1.09	0.23	.0529	0.95	0.01	.0001
0.94	0.08	.0064	0.94	0.02	.0004
0.88	0.02	.0004	0.90	0.06	.0036
0.54	0.32	.1024	0.73	0.23	.0529
1.01	0.15	.0225	1.07	0.11	.0121
0.81	0.05	.0025	1.56	0.60	.3600
0.78	0.08	.0064	0.99	0.03	.0009
0.43	0.43	.1849	0.64	0.32	.1024
0.44	0.42	.1764	0.86	0.10	.0100
0.98	0.12	.0144			
1.04	0.18	.0324			
1.25	0.39	.1521			
1.05	0.19	.0361			
1.08	0.22	.0484			
0.77	0.09	.0081			
0.70	0.16	.0256			
Mean rise	0.86		0.96		
Variance	.0581		.0678		

$$t = \frac{0.96 - 0.86}{\sqrt{\frac{.0581}{16} + \frac{.0678}{9}}} = .9466$$

degrees of freedom = 23

Therefore the probability of a value of t as great as this by chance is 30 - 40%

Therefore there is no significant difference in the rises of temperature of bucks and does at the three dose levels used.

To compare the responses of the two breeds predominating in the population.

Rises in temperature					
0.2 ml./kg.		.06324 ml./kg.		0.02 ml./kg.	
Dutch	Fox	Dutch	Fox	Dutch	Fox
1.48	1.04	1.35	1.29	0.94	1.09
1.20	1.06	1.25	0.95	0.88	0.95
1.11	1.41	1.40	1.31	0.54	0.99
0.92	1.14	0.75	1.39	1.01	1.04
1.56		1.12		0.78	
1.26		1.23		0.90	
1.72		1.51		1.56	
1.78		1.53		0.98	
0.98		0.74		0.64	
1.25		1.25		1.25	
1.79		1.10		1.05	
1.52		1.42		1.08	
1.10		0.98		0.77	
1.12		0.85		0.70	

t = 1.540 t = .4976 t = 1.182

degrees of freedom = 16

Probability = 10-20% Probability = 60-70% Probability = 20-30%

Therefore there is no significant difference in the responses of the two breeds predominating in the population.

To compare the responses of two groups of rabbits of different colours.

Only the black-eared and grey-eared groups were compared. The white rabbits in the population consisted of two ermine and one rex rabbit and they were shown by t-test not to be a representative sample of the population. The group of grey includes brindle-grey. The calculations are based on mean response per animal.

Rises in temperature					
0.2 ml./kg.		.06324 ml./kg.		0.02 ml./kg.	
Black	Grey	Black	Grey	Black	Grey
1.04	1.06	1.29	0.95	1.09	0.95
0.92	1.48	0.75	1.35	1.01	0.94
0.77	1.20	1.11	1.25	0.81	0.88
1.26	1.11	1.23	1.40	0.90	0.54
1.06	1.56	1.08	1.12	0.73	0.78
1.18	1.72	1.49	1.51	1.07	1.56
1.78	1.41	1.53	1.31	0.98	0.99
1.25	1.14	1.25	1.39	1.25	1.04
1.79	0.98	1.10	0.74	1.05	0.64
1.12	1.52	0.85	1.42	0.70	1.08
	1.10		0.98		0.77
	1.02		1.01		0.86

t = .47 .2862 .4337

degrees of freedom = 20

Probability = 60-70% Probability = 70-80% Probability = 60-70%

Therefore there is no significant difference in the responses of the two groups of rabbits of different colours.

Check to see if the temperatures of the three white rabbits are representative of the whole.

Mean temperature of whole population of 25 = 38.59
Variance = .1053
Mean tempertaure of three white rabbits = 38.52
Variance = .0817
Degrees of freedom = 26

$$t = \frac{.07}{\sqrt{\frac{.1053}{25} + \frac{.0817}{3}}}$$

$$= .3948$$

$$\equiv \text{Probability of } 60\text{-}70\%$$

Therefore the basic temperature of the three white rabbits is representative of that of the whole population.

Temperatures of the rabbits immediately before injection , ("normal temperatures").

1. Dose level subsequently injected = 0.2 ml./kg.

38.70	39.15	38.70	38.65
39.34	37.93	39.89	38.23
39.21	38.51	39.33	38.86
38.95	38.37	38.48	38.25
38.52	38.57	37.91	37.52
38.97	37.90	38.39	38.14
39.21	39.17	38.86	38.10
38.75	38.54	38.68	38.84
38.50	39.39	38.49	38.14
38.09	38.47	38.60	38.86
38.00	38.41	37.65	38.25
39.16	38.98	38.77	38.91
38.96	38.34	38.72	39.23
38.91	38.38	39.00	38.61
39.01	37.55	38.39	38.66
38.30	38.16	38.82	39.18
38.63	38.52	39.01	38.13
39.35	38.10	38.80	39.00
38.65	38,84	38.75	38.85
38.27	38.49	38.39	38.34
38.14	38.06	38.52	38.66
38.14	37.95	38.05	37.87
38.86	38.88	38.65	38.54
38.66	38.91	39.00	39.07
38.89	39.06	39.21	39.01

2. Dose subsequently injected = 0.06324 ml./kg.

38.93	38.57	38.68	38.73
38.47	38.69	38.38	39.50
38.95	39.40	39.74	39.38
38.37	38.45	38.88	38.99
37.73	37.99	37.78	37.76
38.48	37.64	38.13	38.43
38.81	38.59	39.39	38.99
38.69	38.86	38.68	38.87
38.84	38.52	39.37	37.83
38.42	38.54	38.32	38.03
37.79	38.47	38.79	38.63
38.98	39.31	39.05	39.15
39.01	38.98	38.77	38.89
38.68	39.01	38.87	38.47
38.58	38.90	39.02	38.85
39.06	39.07	38.95	39.07
38.37	39.22	38.31	38.26
39.45	38.53	38.87	38.90
38.62	38.89	38.89	38.92
37.73	38.09	37.78	38.45
38.51	38.59	38.47	38.19
37.05	37.24	37.14	37.60
38.69	38.47	38.45	38.15
38.83	38.87	39.19	38.51
38.92	39.22	38.51	38.58

3. Dose subsequently injected = 0.02 ml./kg.

37,79	38.13	38.62	38.65
38.71	38.46	38.35	38.35
39.40	38.90	38.99	38.41
38.49	38.72	37.94	38.35
37.98	38.03	38.10	38.03
38.35	38.50	37.99	38.32
39.11	38.40	39.00	38.55
39.00	38.85	38.64	38.97
39.24	38.44	38.18	38.45
37.89	37.63	38.37	38.65
38.85	37.99	38.20	37.27
37.90	38.64	39.17	38.93
39.07	38.95	38.92	39.25
38.76	38.20	38.65	38.36
39.42	38.60	38.44	38.76
39.33	38.89	38.52	39.11
38.33	38.04	38.74	38.61
38.07	38.77	39.00	39.23
38.51	39.11	38.80	38.83
37.87	38.59	38.19	38.83
38.29	38.64	38.53	38.23
38.40	38.00	37.88	37.42
38.50	38.81	38.94	38.60
39.34	39.17	38.50	38.85
38.85	38.09	38.96	38.95

Maximum temperatures attained after injection.

1. .2 ml./kg.

39.37	39.75	40.50	39.74
40.48	39.04	40.23	39.29
40.31	39.78	40.29	39.76
40.25	39.75	40.10	39.87
39.51	39.57	39.24	39.00
39.79	39.21	39.50	39.34
40.04	39.97	40.12	38.88
39.45	39.54	39.51	39.37
40.20	40.06	39.65	39.84
39.41	39.62	40.28	39.54
38.62	39.18	38.56	38.87
40.30	40.15	39.89	39.72
40.00	39.60	40.30	40.06
40.60	40.04	40.95	40.18
40.00	38.75	39.09	39.03
39.52	40.24	39.96	40.39
40.37	40.42	41.21	39.41
40.20	39.05	40.15	40.41
39.73	40.09	39.61	39.58
39.60	39.81	39.82	39.27
39.94	39.77	40.42	40.42
39.57	39.24	40.10	39.19
39.72	39.97	39.96	39.68
39.70	40.10	39.95	39.96
39.96	40.23	39.86	40.61

Mean = 39.80

2. .06324 ml./kg.

39.94	40.09	40.02	40.00
38.81	39.88	39.33	39.35
40.15	40.26	40.40	40.47
39.72	40.18	40.12	40.06
39.36	39.21	38.93	38.76
39.95	39.02	39.43	39.86
39.73	39.74	38.87	39.43
39.92	39.91	39.85	39.85
39.61	39.63	40.51	39.27
39.70	39.64	39.53	39.36
38.46	38.88	39.27	39.68
39.93	40.38	40.08	40.41
40.64	40.63	40.00	40.33
40.44	40.20	40.16	40.26
39.28	39.65	39.79	40.07
40.61	40.52	40.10	40.15
40.14	40.31	40.17	39.65
40.78	39.94	40.57	40.03
39.42	39.80	39.53	39.54
38.92	39.55	39.22	39.37
39.60	39.74	39.53	39.28
38.66	38.90	38.42	38.74
39.65	39.89	39.62	38.53
39.69	39.92	40.14	39.69
39.80	39.75	39.66	39.41

Mean = 39.73

3. .02 ml./kg.

38.75	39.59	39.53	39.67
39.37	39.43	39.55	39.29
40.04	39.74	39.99	39.71
39.37	39.50	38.95	39.45
39.01	38.88	38.93	38.85
39.10	38.78	38.64	38.78
40.36	39.56	39.87	39.32
39.32	39.87	39.59	39.92
39.80	39.00	39.25	39.39
38.89	38.85	39.08	39.30
38.85	38.47	38.77	38.14
38.38	39.79	39.88	39.49
39.77	39.74	39.95	41.00
40.47	39.73	40.23	39.79
39.89	39.37	38.64	39.07
39.94	40.18	39.78	39.92
39.25	38.69	39.15	40.54
40.15	39.48	39.74	40.87
39.01	39.47	39.28	40.03
39.44	39.80	39.46	39.77
39.17	39.59	39.82	39.29
39.17	38.96	38.94	38.94
39.63	39.09	39.64	39.57
39.79	40.24	39.33	39.92
39.51	39.04	39.43	39.67

Mean = 39.46

Mean temperatures responses of the population at the three dose levels.

Dose		
0.2 ml./kg.	0.06324 ml./kg.	0.02 ml./kg.
1.04	1.29	1.09
0.91	0.83	0.94
1.06	0.95	0.95
1.48	1.35	0.94
1.20	1.25	0.88
1.11	1.40	0.54
0.92	0.75	1.01
0.77	1.11	0.81
1.56	1.12	0.78
1.26	1.23	0.90
0.73	0.65	0.48
1.06	1.08	0.73
1.18	1.49	1.07
1.72	1.51	1.56
0.82	0.86	0.44
1.41	1.31	0.99
1.78	1.53	0.98
1.14	1.39	1.04
0.98	0.74	0.64
1.25	1.25	1.25
1.79	1.10	1.05
1.52	1.42	1.08
1.10	0.98	0.77
1.02	1.01	0.86
1.12	0.85	0.70
Mean of each column = 1.20	1.14	0.90

Mean normal temperatures of the rabbits during the series of injections.

Mean of the 4 values per rabbit immediately before injection of .2 ml./kg.	.06324 ml./kg.	.02 ml./kg.	Mean of 12 values
38.80	38.73	38.30	38.61
38.85	38.76	38.47	38.69
38.98	39.37	38.93	39.09
38.51	38.67	38.38	38.52
38.13	37.82	38.04	37.99
38.35	38.17	38.29	38.27
38.84	38.70	38.77	38.77
38.70	38.78	38.87	38.78
38.38	38.64	38.58	38.53
38.46	38.33	38.14	38.31
38.08	38.42	38.08	38.19
38.96	39.12	38.66	38.91
38.81	38.91	39.05	38.92
38.73	38.76	38.49	38.66
38.40	38.84	38.81	38.68
38.62	39.04	38.96	38.87
38.57	38.54	38.43	38.51
38.81	38.94	38.77	38.84
38.77	38.83	38.81	38.81
38.35	38.01	38.37	38.24
38.35	38.44	38.42	38.40
38.00	37.26	37.93	37.73
38.73	38.44	38.71	38.63
38.91	38.85	38.97	38.91
39.04	38.81	38.71	38.85
Means 38.61	38.61	38.56	

Mean of the 300 values for normal temperature 38.59

Investigation of the correlation between normal temperature and rise, using the mean values per animal.

1. Dose = 0.2 ml./kg.

Dev. from normal temp. = x	x^2	xy
+.19	.0361	-.0304
+.24	.0576	-.0696
+.37	.1369	-.0518
-.10	.0100	-.0280
-.48	.2304	0
-.26	.0676	+.0234
+.23	.0529	-.0644
+.09	.0081	-.0387
-.23	.0529	-.0828
-.15	.0225	-.0090
-.53	.2809	+.2491
+.35	.1225	-.0490
+.20	.0400	-.0040
+.12	.0144	+.0624
-.21	.0441	+.0798
+.01	.0001	+.0021
-.04	.0016	-.0232
+.20	.0400	-.0120
+.16	.0256	-.0352
-.26	.0676	-.0130
-.26	.0676	-.1534
-.61	.3721	-.1952
+.12	.0144	-.0120
+.30	.0900	-.0540
+.43	.1849	-.0344

$$r = \frac{-.5433}{\sqrt{2.0408 \times 2.2023}}$$

$$= -.2563$$

Degrees of freedom = 23

Level of significance for this value of r is greater than any tabulated. Therefore there is no correlation.

2. Dose = .06324 ml./kg.

Dev. from normal temp. = x	x^2	xy
+.12	.0144	+.0180
+.15	.0225	-.0465
+.76	.5776	-.1444
+.06	.0036	+.0126
-.79	.6241	-.0869
-.44	.1936	-.1144
+.09	.0081	-.0351
+.17	.0289	-.0051
+.03	.0009	-.0006
-.28	.0784	-.0252
-.19	.0361	+.0931
+.51	.2601	-.0306
+.30	.0900	+.1050
+.15	.0225	+.0555
+.23	.0529	-.0644
+.43	.1849	+.0731
-.07	.0049	-.0273
+.33	.1809	+.0825
+.22	.0484	-.0880
-.60	.3600	-.0660
-.17	.0289	+.0068
-1.35	1.8225	-.3780
-.17	.0289	+.0272
+.24	.0576	-.0312
+.20	.0400	-.0580

$$r = \frac{-.7279}{\sqrt{4.6987 \times 1.6437}}$$

$$= -.2619$$

Degrees of freedom = 23

Level of significance for this value of r is greater than any tabulated. Therefore there is no correlation.

3. Dose = .02 ml./kg.

Dev. from normal temp. = x	x^2	xy
-.16	.0256	-.0304
-.09	.0081	-.0036
+.37	.1369	+.0185
-.18	.0324	+.0072
-.52	.2704	+.0104
-.29	.0841	+.1044
+.21	.0441	+.0231
+.31	.0961	-.0279
+.02	.0004	-.0024
-.42	.1764	0000
-.48	.2304	+.2016
+.10	.0100	-.0170
+.49	.2401	+.0833
-.07	.0049	+.0462
+.25	.0625	-.1150
+.40	.1600	+.0360
-.13	.0169	-.0104
+.21	.0441	+.0294
+.25	.0625	-.0650
-.19	.0361	-.0665
-.14	.0196	-.0210
-.63	.3969	-.1134
+.15	.0225	-.0195
+.41	.1681	-.0164
+.15	.0225	-.0300

$$r = \frac{-.0390}{\sqrt{2.3716 \times 1.4254}}$$

$$= -.02121$$

Degrees of freedom = 23

Level of significance for this value of r is greater than any tabulated. Therefore there is no correlation.

Therefore when only among rabbit correlation is considered, and within rabbit correlation disregarded, there is no correlation between normal temperature and rise. Only within a rabbit does correlation occur. This was found to be negative in type, i.e. the higher the normal temperature the lower the subsequent rise.

Investigation of the correlation between normal temperature and rise on injection.

Dose = 0.02 ml./kg.

Products of the deviations from normal temperature mean and rise in temperature mean = xy			
-.0462	-.2408	+.0006	+.0108
-.0360	-.0070	-.0630	-.0084
-.2184	-.0204	+.0430	-.0600
+.0014	-.0192	-.0682	-.0420
-.0754	+.0265	+.0322	+.0424
+.0315	+.0372	+.1425	+.1056
+.1925	-.0416	-.0132	+.0013
-.2992	+.0348	+.0040	+.0205
-.2312	+.0408	-.0646	-.0044
-.0670	-.2976	+.0361	-.0225
-.1710	+.2394	+.1188	+.0387
+.2772	+.0200	-.1159	-.1258
-.1020	-.0429	+.0468	+.5865
+.1620	-.2268	+.0612	-.1060
-.3698	-.0052	+.0840	-.1180
-.2233	+.1287	-.0144	-.0495
-.0046	+.1300	-.0882	+.0618
-.0882	-.0399	-.0704	+.4958
+.0200	-.2970	-.1008	+.0810
-.4623	+.0093	-.1369	+.0108
+.0054	+.0040	-.0117	-.0528
+.0208	-.0336	-.1088	-.7068
-.0138	-.1550	-.0760	+.0028
-.3510	+.1037	+.0042	+.0493
-.0696	-.0235	-.1720	-.0720

$$r = \frac{-3.1814}{\sqrt{19.6419 \times 13.1814}}$$

$$= -.1979$$

Degrees of freedom = 98

Tabulated level of significance for this value of r is .05 - .02. Therefore there may be slight correlation.

Investigation of the correlation between normal temperature and rise on injection.

2. Dose = 0.06324 ml./kg.

xy			
-.0416	-.0152	+.0140	+.0156
+.1120	+.0040	+.0437	-.2581
+.0204	-.2212	-.5424	-.0385
-.0504	-.0944	+.0270	-.0266
-.4312	-.0496	-.0083	+.1190
-.0429	-.2328	-.0768	-.0522
-.0440	-.0002	+.1452	-.2660
+.0072	-.0225	+.0021	-.0416
-.0851	+.0027	0000	-.2340
-.0266	+.0028	-.0203	-.1102
+.3854	+.1022	-.1188	-.0018
-.0703	-.0490	-.0484	+.0684
+.1960	+.1887	+.0144	+.0840
+.0434	+.0200	+.0390	-.0910
+.0132	-.1131	-.1517	+.0192
+.1845	+.1426	+.0034	-.0276
-.1512	-.0305	-.2160	-.0875
+.1596	-.0216	+.1456	-.0029
-.0034	-.0644	-.1400	-.1612
-.0440	-.1664	-.2490	+.0352
+.0050	-.0002	+.0112	+.0210
-.7332	-.7124	-.2058	0000
-.0144	-.0392	-.0048	+.3496
-.0616	-.0234	-.1102	-.0040
-.0806	-.3721	-.0010	+.0093

$$r = \frac{-4.6524}{\sqrt{25.7409 \times 11.2112}}$$

$$= -.2739$$

Degrees of freedom = 98

Tabulated level of significance for this value of r is .01 - .001. Therefore there is some correlation.

Investigation of the correlation between normal temperature and rise on injection.

3. Dose = 0.2 ml./kg.

xy			
-.0477	-.3240	+.0540	-.0044
-.0438	+.0612	-1.1008	+.0532
-.0600	-.0070	-.1728	-.0750
+.0340	-.0432	-.0546	-.1512
+.0189	+.0080	-.0910	-.3052
-.1368	-.0781	+.0198	0000
-.2220	-.2240	+.0150	+.2142
-.0700	+.0140	-.0259	-.1541
-.0550	-.1034	+.0048	-.2350
-.0624	+.0070	-.0048	-.0160
+.3538	+.0860	+.2784	+.2088
-.0330	-.0111	-.0128	-.1170
-.0560	-.0162	+.0418	-.2294
+.1470	-.1058	+.2925	0000
-.0840	0000	+.1100	-.0415
-.0062	-.3960	-.0126	-.0057
+.0108	-.0630	+.4000	-.0384
-.2590	+.1275	+.0285	+.0819
-.0048	+.0115	-.0476	-.1128
-.0442	-.0144	-.0506	+.0729
-.2820	-.2805	-.0630	+.0280
-.1081	-.0594	-.4760	-.0888
-.0850	-.0297	+.0044	+.0042
-.0080	-.0030	-.0975	-.1426
-.0364	-.0135	-.3300	+.1600

$$r = \frac{-4.7703}{\sqrt{15.0181 \times 18.7516}}$$

$$= -.2843$$

Degrees of freedom = 98

Tabulated level of significance for this value of r is .01 - .001. Therefore there is some correlation.

Variance in the basic temperatures of the rabbits.

The variance was calculated separately for each dose level so that the results could be used for calculations of the correlation between normal temperature and response at each level. The deviations tabulated below are therefore the deviations from the mean of the 100 results obtained at each level.

1. Dose subsequently injected = 0.2 ml./kg.

Deviations			
+.09	+.54	+.09	+.04
+.73	-.68	+1.28	-.38
+.60	-.10	+.72	+.25
+.34	-.24	-.13	-.36
-.09	-.04	-.70	-1.09
+.36	-.71	-.22	-.47
+.60	+.56	+.25	-.51
+.14	-.07	+.07	+.23
-.11	-.22	-.12	-.47
-.52	-.14	-.01	+.05
-.61	-.20	-.96	-.36
+.55	+.37	+.16	+.30
+.35	-.27	+.11	+.62
+.30	-.23	+.39	0
+.40	-1.06	-.22	+.05
-.31	-.45	+.21	+.57
+.02	-.09	+.40	-.48
+.74	-.51	+.19	+.39
+.04	+.23	+.14	+.24
-.34	-.12	-.22	-.27
-.47	-.55	-.09	+.05
-.47	-.66	-.56	-.74
+.25	+.27	+.04	-.07
+.05	+.30	+.39	+.46
+.28	+.45	+.60	} >40

$$\sigma^2 = 0.1894$$

2. Dose subsequently injected = .06324 ml./kg.

Deviations			
+.32	-.04	+.07	+.12
-.14	+.08	-.23	+.89
+.34	+.79	+1.13	+.77
-.24	-.16	+.27	+.38
-.88	-.62	-.83	-.85
-.13	-.97	-.48	-.18
+.20	-.02	-.22	+.38
+.08	+.25	+.07	+.26
+.23	-.09	+.76	-.78
-.19	-.07	-.29	-.58
-.82	-.14	+.18	+.02
+.37	+.70	+.44	+.54
+.40	+.37	+.16	+.28
+.07	+.40	+.26	-.14
-.03	+.29	+.41	+.24
+.45	+.46	+.34	+.46
-.24	+.61	-.30	-.35
+.84	-.08	+.26	+.29
+.01	+.28	+.28	+.31
-.88	-.52	-.83	-.16
-.10	-.02	-.14	-.42
-1.56	-1.37	-1.47	-1.01
+.08	-.14	-.16	-.46
+.22	+.26	+.58	-.10
+.31	+.61	-.10	-.03

Variance = 0.2600

3. Dose subsequently injected = 0.02 ml./kg.

Deviations			
-.77	-.43	+.06	+.09
+.15	-.10	-.21	-.21
+.84	+.34	+.43	-.15
-.07	+.16	-.62	-.21
-.58	-.53	-.46	-.53
-.21	-.06	-.57	-.24
+.55	-.16	+.44	-.01
+.44	+.29	+.08	+.41
+.68	-.12	-.38	-.11
-.67	-.93	-.19	+.09
+.19	-.57	-.36	-1.29
-.66	+.08	+.61	+.37
+.51	+.39	+.36	+.69
+.20	-.36	+.09	-.20
+.86	+.04	-.12	+.20
+.77	+.33	-.04	+.55
-.23	-.52	+.18	+.06
-.49	+.21	+.44	+.67
-.05	+ .55	+.24	+.27
-.69	+.03	-.37	+.27
-.27	+.08	-.03	-.33
-.16	-.56	-.68	-1.14
-.06	+.25	+.38	+.04
+.78	+.61	-.06	+.29
+.29	-.47	+.40	+.39

Variance = 0.1984

Investigation of the use of final temperature attained as a measure of pyrogen.

Dose level ml./kg.	Mean final temperature	Variance in final temp.	Variance in temp. rise
0.2	39.80	.2450	.1517
0.06324	39.73	.2534	.1132
0.02	39.46	.2691	.1331

F test to see if variances in rise are significantly less than variances in final temperature -

1. $F = \frac{.2450}{.1517} = 1.615$ $n_1=n_2=99$. Probability ≐ 5%

2. $F = \frac{.2534}{.1132} = 2.24$ Probability = .1-1%

3. $F = \frac{.2691}{.1331} = 2.02$ Probability ≐ 1%

Therefore the variances in rise are significantly less than the variances in final temperature.

Weights of rabbits throughout the experiments, to the nearest 0.05 kg.

1. Over period when 0.2 ml./kg. was injected.

2.40	2.40	2.25	2.25
2.30	2.30	2.05	2.05
2.60	2.60	2.50	2.50
2.05	2.05	1.95	1.95
2.05	2.05	1.85	1.85
1.75	1.75	1.65	1.65
1.90	1.65	1.65	1.65
2.60	2.45	2.45	2.45
2.20	2.05	2.05	2.05
1.80	1.65	1.65	1.65
2.35	2.15	2.15	2.15
2.50	2.25	2.25	2.25
2.70	2.70	2.45	2.45
2.15	2.00	2.00	2.00
2.10	2.10	1.95	1.95
2.35	2.20	2.20	2.20
1.70	1.70	1.60	1.60
2.10	2.00	2.00	2.00
1.80	1.80	1.80	1.80
1.95	1.95	1.95	1.95
1.35	1.35	1.35	1.35
1.60	1.60	1.60	1.70
1.35	1.35	1.35	1.60
2.00	2.00	2.00	2.00
1.70	1.70	1.70	1.90

2. Over period when .06324 ml./kg. was injected.

2.25	2.15	2.15	2.15
2.05	2.05	2.05	2.05
2.50	2.50	2.50	2.45
1.95	1.95	1.95	1.95
1.95	1.95	1.95	1.95
1.65	1.65	1.80	1.80
1.65	1.65	1.65	1.65
2.45	2.55	2.55	2.25
2.05	2.05	2.05	2.05
1.70	1.70	1.70	1.75
2.15	2.15	2.30	2.30
2.25	2.25	2.25	2.35
2.45	2.40	2.40	2.40
2.00	2.00	2.05	2.05
1.95	1.95	1.95	1.90
2.20	2.20	2.20	2.20
1.60	1.60	1.60	1.60
2.00	2.00	2.00	2.05
1.90	1.90	2.00	2.00
2.00	2.00	2.00	2.10
1.55	1.55	1.55	1.65
1.70	1.70	1.70	1.75
1.60	1.60	1.80	1.80
1.95	1.95	1.95	2.00
1.90	1.90	2.10	2.10

3. Over period when .02 ml./kg. was injected.

2.25	2.25	2.25	2.25
2.05	2.05	2.05	2.05
2.50	2.50	2.50	2.50
1.95	1.95	1.95	1.95
1.85	1.85	1.85	1.85
1.65	1.65	1.65	1.65
1.90	1.65	1.65	1.65
2.60	2.45	2.45	2.45
2.20	2.05	2.05	2.05
1.80	1.65	1.65	1.65
2.35	2.15	2.15	2.15
2.50	2.25	2.25	2.25
2.45	2.45	2.45	2.45
2.15	2.00	2.00	2.00
1.95	1.95	1.95	1.95
2.35	2.20	2.20	2.20
1.60	1.60	1.60	1.60
2.10	2.00	2.00	2.00
1.80	1.80	1.80	1.80
1.95	1.95	1.95	2.00
1.35	1.35	1.35	1.35
1.60	1.60	1.55	1.70
1.35	1.35	1.35	1.80
2.00	2.00	2.00	1.95
1.70	1.70	1.70	1.70

Mean weights of the rabbits throughout the injection of each dose level and mean throughout the period of the twelve injections.

Mean weights during injection of			
.2 ml./kg.	.06324 ml./kg.	.02 ml./kg.	all doses
2.35	2.20	2.25	2.25
2.20	2.05	2.05	2.10
2.55	2.50	2.50	2.50
2.00	1.95	1.95	1.95
1.95	1.95	1.85	1.90
1.70	1.75	1.65	1.70
1.70	1.65	1.70	1.70
2.50	2.45	2.50	2.50
2.10	2.05	2.10	2.10
1.70	1.70	1.70	1.70
2.20	2.25	2.20	2.20
2.30	2.30	2.30	2.30
2.60	2.40	2.45	2.50
2.05	2.05	2.05	2.05
2.05	1.95	1.95	1.95
2.25	2.20	2.25	2.25
1.65	1.60	1.60	1.60
2.05	2.00	2.05	2.00
1.80	1.95	1.80	1.85
1.95	2.05	1.95	2.00
1.35	1.60	1.35	1.45
1.65	1.70	1.60	1.65
1.40	1.70	1.45	1.55
2.00	1.95	2.00	2.00
1.75	2.00	1.70	1.80

Investigation of the correlation between normal temperature and weight, using the means of the 12 values of each.

Dev. from $\overline{X}$ = x	x^2	Dev. from $\overline{Y}$ = y	y^2	xy
+.02	.0004	+.25	.0625	+.0050
+.10	.0100	+.10	.0100	+.0100
+.50	.2500	+.50	.2500	+.2500
-.07	.0049	-.05	.0025	+.0035
-.60	.3600	-.10	.0100	+.0600
-.32	.1024	-.30	.0900	+.0960
+.18	.0324	-.30	.0900	-.0540
+.19	.0361	+.50	.2500	+.0950
-.06	.0036	+.10	.0100	-.0060
-.28	.0784	-.30	.0900	+.0840
-.40	.1600	+.20	.0400	-.0800
+.32	.1024	+.30	.0900	+.0960
+.33	.1089	+.50	.2500	+.1650
+.07	.0049	+.05	.0025	+.0035
+.09	.0081	-.05	.0025	-.0045
+.28	.0784	+.25	.0625	+.0700
-.08	.0064	-.40	.1600	+.0320
+.25	.0625	0	0	0
+.22	.0484	-.15	.0225	-.0330
-.35	.1225	0	0	0
-.19	.0361	-.55	.3025	+.1045
-.86	.7369	-.35	.1225	+.3010
+.04	.0016	-.45	.2025	-.0180
+.32	.1024	0	0	0
+.26	.0676	-.20	.0400	-.0520

$$r = \frac{\Sigma xy}{\sqrt{\Sigma x^2 . \Sigma y^2}}$$

$$= \frac{+1.1280}{\sqrt{2.5280 \times 2.1625}}$$

$$= +.4822$$

$$n = 23$$

Tabulated level of significance for this value of r is .02-.01. Therefore there is positive correlation, i.e. the heavier rabbits have higher normal temperatures.

Investigation of the correlation between weight and temperature response, using the means of the four values for each.

1. Dose = .2 ml./kg.

Dev.from $\bar{X} = x$	x^2	Dev.from $\bar{Y} = y$	y^2	xy
+.35	.1225	-.16	.0256	-.0560
+.20	.0400	-.29	.0841	-.0580
+.55	.3025	-.14	.0196	-.0770
0	0	+.28	.0784	0
-.05	.0025	0	0	0
-.30	.0900	-.09	.0081	+.0270
-.30	.0900	-.28	.0784	+.0840
+.50	.2500	-.43	.1849	-.2150
+.10	.0100	+.36	.1296	+.0360
-.30	.0900	+.06	.0036	-.0180
+.20	.0400	-.47	.2209	-.0940
+.30	.0900	-.14	.0196	-.0420
+.60	.3600	-.02	.0004	-.0120
+.05	.0025	+.52	.2704	+.0260
+.05	.0025	-.38	.1444	-.0190
+.25	.0625	+.21	.0441	+.0525
-.35	.1225	+.58	.3364	-.2030
+.05	.0025	-.06	.0036	-.0030
-.20	.0400	-.22	.0484	+.0440
-.05	.0025	+.05	.0025	-.0025
-.65	.4225	+.59	.3481	-.3835
-.35	.1225	+.32	.1024	-.1120
-.60	.3600	-.10	.0100	+.0600
0	0	-.18	.0324	0
-.25	.0625	-.08	.0064	+.0200

$$r = \frac{-.9455}{\sqrt{2.69 \times .2023}}$$

$$= -.3885$$

$$n = 23$$

Tabulated level of significance for this value of r is .02 - .05. Therefore there is negative correlation, the heavier rabbits having lower responses.

2. Dose = .06324 ml./kg.

Dev. from $\bar{X} = x$	x^2	Dev. from $\bar{Y} = y$	y^2	xy
+.20	.0400	+.15	.0225	+.0300
+.05	.0025	-,31	.0961	-.0155
+.50	.2500	-.19	.0361	-.0950
-.05	.0025	+.21	.0441	-.0105
-.05	.0025	+.11	.0121	-.0055
-.25	.0625	+.26	.0676	-.0650
-.35	.1225	-.39	.1521	+.1365
+.45	.2025	-.03	.0009	-.0135
+.05	.0025	-.02	.0004	-.0010
-.30	.0900	-.09	.0081	+.0270
+.25	.0625	-.49	.2401	-.1225
+.30	.0900	-.06	.0036	-.0180
+.40	.1600	+.35	.1225	+.1400
+.05	.0025	+.37	.1369	+.0185
-.05	.0025	-.28	.0784	+.0140
+.20	.0400	+.17	.0289	+.0340
-.40	.1600	+.39	.1521	-.1560
0	0	+.25	.0625	0
-.05	.0025	-.40	.1600	+.0200
+.05	.0025	+.11	.0121	+.0055
-.40	.1600	-.04	.0016	+.0160
-.30	.0900	+.28	.0784	-.0840
-.30	.0900	-.16	.0256	+.0480
-.05	.0025	-.13	.0169	+.0065
0	0	-.29	.0841	0

$$r = \frac{-.0905}{\sqrt{1.6425 \times 1.6437}}$$

$$= -.05507$$

$$n = 23$$

Level of significance for this value of r is greater than any tabulated. Therefore there is no correlation between weight and response at this dose level.

3. Dose = 0.02 ml./kg.

Dev. from $\bar{X}$ = x	x^2	Dev. from $\bar{Y}$ = y	y^2	xy
+.30	.0900	+.19	.0361	+.0570
+.10	.0100	+.04	.0016	+.0040
+.55	.3025	+.05	.0025	+.0275
0	0	+.04	.0016	0
-.10	.0100	-.02	.0004	+.0020
-.30	.0900	-.36	.1296	+.1080
-.25	.0625	+.11	.0121	-.0275
+.55	.3025	-.09	.0081	-.0495
+.15	.0225	-.12	.0144	-.0180
-.25	.0625	0	0	0
+.25	.0625	-.42	.1764	-.1050
+.35	.1225	-.17	.0289	-.0595
+.50	.2500	+.17	.0289	+.0850
+.10	.0100	+.66	.4356	+.0660
0	0	-.46	.2116	0
+.30	.0900	+.09	.0081	+.0270
-.35	.1225	+.08	.0064	-.0280
+.10	.0100	+.14	.0196	+.0140
-.15	.0225	-.26	.0676	+.0390
0	0	+.35	.1225	0
-.60	.3600	+.15	.0225	-.0900
-.35	.1225	+.18	.0324	-.0630
-.50	.2500	-.13	.0169	+.0650
+.05	.0025	-.04	.0016	-.0020
-.25	.0625	-.20	.0400	+.0500

$$r = \frac{+.102}{\sqrt{2.44 \times 1.4254}}$$

$$= +.0547$$

$$n = 23$$

Level of significance for this value of r is greater than any tabulated. Therefore there is no correlation between weight and response at this dose level.

To compare the basic temperatures of the three differently coloured groups.

	Black			Grey			White		
	Temp.	d	d^2	Temp.	d	d^2	Temp.	d	d^2
	38.61	.02	.0004	39.09	.52	.2704	38.69	.17	.0289
	38.77	.14	.0196	38.52	.05	.0025	38.19	.33	.1089
	38.78	.15	.0225	37.99	.58	.3364	38.68	.16	.0256
	38.31	.32	.1024	38.27	.30	.0900			
	38.91	.28	.0784	38.53	.04	.0016			
	38.92	.29	.0841	38.66	.09	.0081			
	38.51	.12	.0144	38.87	.30	.0900			
	38.24	.39	.1521	38.84	.27	.0729			
	38.40	.23	.0529	38.81	.24	.0576			
	38.85	.22	.0484	37.73	.84	.7056			
				38.63	.06	.0036			
				38.91	.34	.1156			
Mean	38.63			38.57			38.52		
σ^2	.0639			.1595			.0817		
No.	10			12			3		

Comparison of black and white groups.

$$t = \frac{.11}{\sqrt{\frac{.0639}{10} + \frac{.0817}{3}}} = .5999$$

Degrees of freedom = 11
Probability of t as great as this by chance = 50-60%

Comparison of black and grey groups.

$t = .4278$

Degrees of freedom = 20. Therefore probability = 60-70%

Comparison of grey and white groups.

$t = .2484$

Degrees of freedom = 13. Therefore probability = 80-90%

Therefore there is no significant difference between the basic temperatures of the three groups.

Times, in minutes, required to reach maximum temperature after injection.

.2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
120	100	220	140	110	160	100	170	120	250	110	150
80	180	70	90	200	110	130	150	180	150	110	170
90	160	140	110	110	100	80	100	150	120	130	130
80	80	70	80	90	90	100	80	90	150	110	130
80	90	240	120	120	120	110	120	130	170	100	120
80	190	110	130	70	90	70	90	90	160	140	170
70	80	230	120	130	120	80	140	70	100	130	80
100	150	230	100	120	140	180	130	80	210	200	140
210	70	90	220	110	100	180	140	120	120	150	120
90	70	190	80	110	70	90	160	100	120	130	100
70	190	110	140	200	100	110	120	*	140	150	90
190	80	180	80	60	60	80	80	100	100	100	140
100	170	210	100	70	80	90	80	100	130	220	80
60	190	170	80	70	70	70	180	80	120	80	90
70	140	200	160	140	100	100	80	150	120	210	150
180	220	90	90	90	70	70	90	110	130	150	120
80	60	60	90	80	180	80	90	80	120	90	80
90	80	80	90	90	110	100	130	100	130	120	70
80	170	90	80	130	70	110	100	90	70	90	160
60	200	220	120	90	90	100	130	90	80	100	110
200	190	170	140	80	70	60	90	180	80	100	70
120	170	230	100	70	90	110	100	110	80	140	160
100	200	70	190	100	70	90	90	70	120	140	60
100	220	120	90	130	120	110	150	100	90	180	110
80	180	70	200	110	70	120	100	90	120	130	140

Mean of all results at each dose level -

0.2 ml./kg. = 130 minutes
0.06324 ml./kg. = 110 minutes
0.02 ml./kg. = 120 minutes

* This was counted as zero because no peak was reached.

Average times required to reach maximum temperatures.

.2 ml./kg.	.06324 ml./kg.	.02 ml./kg.
150	140	130
110	150	150
130	100	130
80	90	120
130	120	130
130	80	140
130	120	100
150	140	160
150	130	130
110	110	110
130	130	100
130	70	110
150	80	130
130	100	90
140	110	160
150	80	130
70	110	90
90	110	110
110	100	100
150	100	100
180	80	110
160	90	130
140	90	100
130	130	120
130	100	120

Investigation of differences in times required to reach maximum temperatures.

Dose level ml./kg.	Mean time	Variance
.2	130	621
.06324	110	467
.02	120	454

Comparison of .2 and .06324 groups

$t \triangleq 2.93$. Degrees of freedom = 48. Probability = .1-1%

Comparison of .02 and .2 groups

$t \triangleq 1.47$ Probability = 10-20%

Comparison of .02 and .06324 groups

$t \triangleq 1.65$ Probability = 10-20%

Therefore there is no significant difference in the times required to reach maximum temperatures after injection .

Investigation of correlation between height of response and time taken to reach it.(calculation based on mean response per animal - between rabbit correlation only)

1. Dose level .2 ml./kg.

Deviation from temp. rise = x	x^2	Deviation from time = y	y^2	xy
-.16		+20	400	-3.2
-.29		-20	400	+5.8
-.14		0	0	0
+.28	See p.173	-50	2500	-14.0
0		0	0	0
-.09		0	0	0
-.28		0	0	0
-.43		+20	400	-8.6
+.36		+20	400	+7.2
+.06		-20	400	-1.2
-.47		0	0	0
-.14		0	0	0
-.02		+20	400	-0.4
+.52		0	0	0
-.38		+10	100	-3.8
+.21		+20	400	+4.2
+.58		-60	3600	-34.8
-.06		-40	1600	+2.4
-.22		-20	400	+4.4
+.05		+20	400	+1.0
+.59		+50	2500	+29.5
+.32		+30	900	+9.6
-.10		+10	100	-1.0
-.18		0	0	0
-.08		0	0	0

$$r = \frac{-2.9}{\sqrt{2.2023 \times 14900}}$$

$$= 1/60$$

$n = 23$

Level of significance greater than any tabulated value. Therefore there is no correlation.

2. Dose level 0.06324 ml./kg.

Deviation from temp. rise = x	x^2	Deviation from time = y	y^2	xy
+.15		+30	900	+4.5
-.31		+40	1600	-12.4
-.19		-10	100	+1.9
+.21	See p.174	-20	400	-4.2
+.11		+10	100	+1.1
+.26		-30	900	-7.8
-.39		+10	100	-3.9
-.03		+30	900	-0.9
-.02		+20	400	-0.4
+.09		0	0	0
-.49		+20	400	-9.8
-.06		-40	1600	+2.4
+.35		-30	900	-10.5
+.37		-10	100	-3.7
-.28		0	0	0
+.17		-30	900	-5.1
+.39		0	0	0
+.25		0	0	0
-.40		-10	100	+4.0
+.11		-10	100	-1.1
-.04		-30	900	+1.2
+.28		-20	400	-5.6
-.16		-20	400	+3.2
-.13		+20	400	-2.6
-.29		-10	100	+2.9

$$r = \frac{-46.8}{\sqrt{1.6437 \times 11700}}$$

$$= -.34$$

$$n = 23$$

Level of significance for this value of r is approximately .1. Therefore there may be very slight correlation.

3. Dose level .02 ml./kg.

Deviation from temp. rise = x	x^2	Deviation from time = y	y^2	xy
+.19		+10	100	+1.9
+.04		+30	900	+1.2
+.05		+10	100	+0.5
+.04	See p.175	0	0	0
-.02		+10	100	-0.2
-.36		+20	400	-7.2
+.11		-20	400	-2.2
-.09		+40	1600	-3.6
-.12		+10	100	-1.2
0		-10	100	0
-.42		-20	400	+8.4
-.17		-10	100	+1.7
+.17		+10	100	+1.7
+.66		-30	900	-19.8
-.46		+40	1600	-18.4
+.09		+10	100	+ 0.9
+.08		-30	900	-2.4
+.14		-10	100	-1.4
-.26		-20	400	+5.2
+.35		-20	400	-7.0
+.15		-10	100	-1.5
+.18		+10	100	+1.8
-.13		-20	400	+2.6
-.04		0	0	0
-.20		0	0	0

$$r = \frac{-39.0}{\sqrt{1.4254 \times 9400}}$$

$$= -.34$$

$$n = 23$$

Level of significance for this value of r is approximately 0.1. Therefore there may be very slight correlation.

Areas, in planimeter units, under height of rise/time graph.

.2 ml./kg				.06324 ml./kg				.02 ml./kg.			
130	164	1358	407	312	807	400	662	399	880	237	611
225	591	138	314	110	342	376	553	448	582	497	490
288	916	309	238	411	235	178	391	278	323	512	524
322	440	419	457	363	489	260	384	152	330	442	495
237	230	1158	649	530	658	609	472	625	501	310	377
148	988	315	425	257	305	251	415	151	87	159	122
197	188	980	291	289	406	131	120	287	358	342	160
200	293	926	85	428	456	702	424	210	743	580	585
1249	372	463	1505	235	356	762	739	71	114	510	343
223	204	1170	178	381	272	371	668	198	584	214	227
149	346	146	273	226	104	130	323	218	216	256	0
925	316	820	232	1522	193	205	370	144	328	157	207
271	830	1603	248	354	458	407	441	166	215	656	462
472	1403	1385	427	430	296	277	1317	338	680	440	439
193	671	134	595	327	210	257	357	193	289	51	54
814	1648	311	320	429	395	208	381	155	428	622	368
410	342	400	267	526	608	540	414	188	264	107	408
432	128	241	344	306	548	533	516	217	187	255	370
277	727	323	147	407	217	223	122	45	152	129	794
296	957	978	240	350	423	539	315	531	282	370	264
1505	1142	1318	951	279	258	265	363	229	352	496	145
716	786	1742	519	308	616	587	368	324	499	440	1593
169	661	372	757	355	316	450	64	188	59	112	760
325	1112	310	170	414	411	315	499	69	321	433	1259
220	867	85	730	246	104	304	187	187	521	40	1135
Mean	549			394				359			
Variance	176205.5			46830				66726			

Investigation of normality of distribution of areas

1. Dose level .2 ml./kg.

Mean area = 549. Variance = 420^2. Degrees of freedom = 6

Groups of areas	Observed frequency	Theoretical frequency	(f_o-f_t)	$(f_o-f_t)^2$	χ^2
0- 200	18	14	4	16	1.1
201- 300	19	7	12	144	20.6
301- 400	16	8	8	64	8.0
401--500	10	9	1	1	0.1
501- 800	11	27	16	256	9.5
801-1100	12	18	6	36	2.0
1101-1800	14	9	5	25	2.8

$\chi^2 = 44.1$

Probability of ^{2} as large as this by chance is very remote. Therefore the areas are not normally distributed.

2. Dose level .06324 ml./kg.

Mean area =394. Variance = 216^2. Degrees of freedom = 4

Groups of areas	Observed frequency	Theoretical frequency	$(f_o- f_t)$	$(f_o-f_t)^2$	χ^2
0- 200	11	15	4	16	1.1
201- 300	20	15	5	25	1.7
301- 400	28	18	10	100	5.6
401- 500	20	18	2	4	0.2
501- 600	[illegible]	14	5	25	1.8
601-1600	12	?			

$\chi^2 = ?$

Therefore the areas are not normally distributed.

3. Dose level .02 ml./kg.

Mean area = 359. Variance = 258^2. Degrees of freedom = 5

Groups of areas	Observed frequency	Theoretical frequency	$(f_o - f_t)$	$(f_o - f_t)^2$	χ^2
0- 100	9	8	1	1	0.1
101- 200	21	11	10	100	9.1
201- 300	18	14	4	16	1.1
301- 400	16	15	1	1	0.1
401- 500	14	14	0	0	0
501- 600	10	12	2	4	0.3
601-1600	12	?			

$\chi^2 = ?$

Therefore the areas are not normally distributed.

Comparison of the responses obtained at the three dose levels.

Dose level ml./kg.	Mean response	Variance
.2	1.20	.1517
.06324	1.14	.1132
.02	0.90	.1331

Comparison of .2 and .06324 groups

$t = 1.166$. Degrees of freedom = 198. Probability = 20-30%

Comparison of .06324 and .02 groups

$t = 4.84$. Degrees of freedom = 198. Probability $< .1$

Therefore the responses to the high and middle doses do not differ significantly but the responses to the middle and low doses differ significantly.

Equation of line joining lower and middle responses plotted against log. dose.

General equation = $y = mx + c$, where m is the gradient and c is the point of intersection of line and y-axis.

$$m = \frac{\text{difference of y's}}{\text{difference of x's}} = \frac{1.14 - 0.90}{\log.0.06324 - \log.0.02} = .48$$

c = or

$(1.14 = .48 \times -1.1990 + c)$ $(0.90 = .48 \times -1.6990 + c)$

$c = 1.71552$ $c = 1.71552$

Therefore equation of log. dose / response line is

$$y = 0.48x + 1.72$$

Use of this equation to find theoretical response to high dose -

In the equation $y = .48x + 1.72$, if $x = -0.6990$, $y = 1.38$

This is significantly different from the observed value

($t = 3.268$).

A P P E N D I X S I X

Percentages of small lymphocytes counted at weekly intervals.

	Not followed by injection						Followed by injection of .2 ml./kg.				Followed by injection of .06324 ml./kg.				Followed by injection of .02 ml./kg.			
	66	81	96	86	78	88	35	27	49	38	63	63	45	33	59	69	45	38
	81	46	88	83	74	56	36	29	24	60	61	49	49	41	44	60	65	44
	82	50	78	83	83	58	69	71	72	65	79	78	63	72	61	55	75	62
	82	62	80	82	75	73	39	59	70	71	47	82			60	64		
	98	73	56	73	95	58	44	61	56	57	68	70	61	65	71	71	46	76
	75	79	74	97	90	89	81	81	80	80	79	89	79	79	82	83	85	68
	86	86	88	74	91	85	53	66	72	80	65	77	73	80	66	87	74	81
	54	69	86	89	77	86	78	72	73	73	78	82	73	69	85	86	83	79
	81	68	67	77	88	83	59	62	78	71	82	68	81	76	78	62	44	81
	86	82	84	83	67	72	75	63	74	71	54	66	47	67	63	68	66	55
	87	94	93	88	75	85	54	75	79	79	79	84	85	78	74	91	72	80
	84	86	62	80	73	75	41	34	65	71	69	74	73	68	61	82	71	69
	85	79	74	94	85	90	74	82	84	83	76				75			
	78	66	67	55	93	78	57	55	69	67	59	70	60	66	48	57	67	66
	82	64	87	45	77	82	61	58	85	64	72	50	50	68	77	75	69	64
	85	83	86	91	90	85	68	66	68	61	83	77	68	86	80	79	68	77
	86	71	85	72	67	69	53	53	77	62	46	52	80	61	64	51	84	55
	79	78	92	85	73	78	75	62	82	61								
	92	80	79	92	82	56												
	76	90	74	77	92	81	54	74	74	66	79				65			
	87	74	86	94	81	87	79	83	86	87	91	76	88	92	79	89	90	89
*	55	85	75	71	66	63	65	62	57	66	54	63	52	79	67	62	69	67
*	87	91	76	89	77	83	74	75	43	79	79	81	61	81	68	85	80	68
*	69	73	87	80	50	54	56	64	59	55	86	76	71	74	79	73	85	75
*	74	80	96	86	77	78	71	66	52	56								

Mean of all the above = 72%
Mean of values for first six weeks = 79%

* Rabbits not previously injected.

Calculation of the variance in percentages of small lymphocytes over six weeks when no pyrogen was injected.

$$\text{Variance} = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n - 1}$$

where x = percentage of small lymphocytes and n = number of percentages under consideration.

$\Sigma x = 11797$

$(\Sigma x)^2 = 139169209$

$\Sigma(x)^2 = 946263$

$n = 150$

$$\text{Therefore variance} = \frac{946263 - \frac{139169209}{150}}{149}$$

$$= 124$$

Therefore standard deviation = 11

Analysis of variance of normal percentage of small lymphocytes into between rabbit and within rabbit variance

Let the percentage of small lymphocytes = x

1. $\Sigma(x)^2 = \underline{946263}$

2. Total for each rabbit squared, squares added and the sum divided by the number of values for each rabbit

$= \underline{932187}$

3. $\frac{(\Sigma x)^2}{150} = \underline{927795}$

Number of rabbits = 25 = m

Number of values per rabbit = 6 = n

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Components
Between rabbit	2-3 = 4392	m-1 = 24	$\frac{4392}{24} = 183$	$n\sigma_A^2 + \sigma_B^2$
Within rabbit	1-2 = 14076	mn-m = 125	$\frac{14076}{125} = 113$	σ_B^2
Total	1-3 = 18468	mn-1 = 149	$\frac{18468}{149} = 124$	

Therefore $n\sigma_A^2$ exists. To see if it exists significantly -

$$F = \frac{183}{113} \quad \begin{matrix} n_1 = 24 \\ n_2 = 125 \end{matrix} \quad \pm 5\%$$

Therefore $n\sigma_A^2 + \sigma_B^2$ is probably not significantly greater than σ_B^2.

Therefore it may be said that the fluctuation of the normal percentage of small lymphocytes within a rabbit is as great as the fluctuation between rabbits.

Calculation of variance among all values of percentages of small lymphocytes.

Deviations from the mean																	
6	9	24	14	6	16	37	45	23	34	9	9	27	39	13	3	27	14
9	26	16	11	2	16	36	43	48	12	11	23	23	31	28	12	7	28
10	22	6	11	11	14	3	1	0	7	7	6	9	0	11	17	3	10
10	10	8	10	3	1	33	13	2	1	25	10			12	8		
26	1	16	1	23	14	28	11	16	15	4	2	11	7	1	1	26	4
3	7	2	25	18	17	9	9	8	8	7	17	7	7	10	11	13	4
14	14	16	2	19	13	19	6	0	8	7	5	1	8	6	15	2	9
18	3	14	17	5	14	6	0	1	1	6	10	1	3	13	14	11	7
9	4	5	5	16	11	13	10	6	1	10	4	9	4	6	10	28	9
14	10	12	11	5	0	3	9	2	1	18	6	25	5	9	4	6	17
15	22	21	16	3	13	18	3	7	7	7	12	13	6	2	19	0	8
12	14	10	8	1	3	31	38	7	1	3	2	1	4	11	10	1	3
13	7	2	22	13	18	2	10	12	11	4				3			
6	6	5	17	21	6	15	17	3	5	13	2	12	6	24	15	5	6
10	8	15	27	5	10	11	14	13	8	0	22	22	4	5	3	3	8
13	11	14	19	18	13	4	6	4	11	11	5	4	14	8	7	4	5
14	1	13	0	5	3	19	19	5	10	26	20	8	11	8	21	12	12
7	6	20	13	1	6	3	10	10	11								
20	8	7	20	10	16												
4	18	2	5	20	9	18	2	2	6	7				7			
15	2	14	22	9	15	7	11	14	15	19	4	16	20	7	17	18	17
17	13	3	1	6	9	7	10	15	6	18	9	20	7	5	10	3	5
15	19	4	17	5	11	2	3	29	7	7	9	11	9	4	13	8	4
3	1	15	8	22	18	16	8	13	17	14	4	1	2	7	1	13	3
2	8	24	14	5	6	1	6	20	16								

Variance = 182

Percentages of total mononuclears counted at weekly intervals.

Not followed by injection						Followed by injection of .2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
74	84	97	89	80	89	41	33	50	44	65	66	50	36	60	74	49	61
88	54	91	85	76	65	43	34	28	61	64	53	52	42	47	62	68	46
90	70	88	90	85	63	72	73	72	70	80	80	67	78	64	64	77	64
89	74	85	84	79	79	39	65	71	73	61	82			68	66		
99	84	63	80	97	66	51	65	66	58	70	73	66	68	73	74	51	79
85	87	81	97	93	94	86	88	82	82	81	89	81	83	87	85	89	71
95	92	93	86	94	90	58	68	74	87	70	79	75	83	74	88	77	84
62	83	92	93	83	89	81	73	77	79	78	84	76	71	87	87	85	81
86	76	71	80	90	85	60	63	79	73	84	69	83	79	79	64	46	82
88	89	86	85	73	82	80	70	79	77	62	70	52	68	67	72	72	63
92	97	94	93	81	88	60	76	81	81	82	86	85	83	76	92	73	82
88	89	66	85	77	80	53	36	67	72	71	75	74	71	63	83	73	73
92	85	84	95	88	92	76	83	87	86	81				78			
86	82	74	72	95	86	59	58	73	70	63	72	66	68	52	59	69	67
84	73	91	53	86	86	65	61	86	66	74	57	57	71	78	76	71	67
92	89	89	92	93	88	70	67	70	61	84	78	70	86	84	80	68	79
92	80	89	77	73	73	56	55	82	65	48	60	81	68	69	56	85	58
84	87	94	89	80	81	77	66	83	63								
94	86	82	96	85	61												
87	94	82	83	93	85	61	79	78	67	81				72			
91	84	92	96	87	91	64	84	86	88	92	79	88	93	81	91	92	90
*67	90	79	77	73	72	67	66	65	69	59	66	63	81	70	67	71	69
*91	91	82	91	81	87	79	76	47	81	79	81	62	82	70	86	81	70
*75	78	90	88	58	61	60	65	63	56	89	77	73	75	80	77	86	76
*83	87	97	89	86	83	74	70	61	58								

Mean of all the above = 76

Mean of values for first six weeks = 81

* Rabbits not previously injected.

Calculation of variance among all values of percentages of total mononuclears.

Deviations from the mean																	
2	8	21	13	4	13	35	43	26	32	11	10	26	40	16	2	27	15
12	22	15	9	0	11	33	42	48	15	12	13	24	34	29	14	8	30
14	6	12	14	9	13	4	3	4	6	4	4	9	2	12	12	1	12
13	2	9	8	3	3	37	11	5	3	15	6			8	10		
23	8	13	4	21	10	25	11	10	18	6	3	10	8	3	2	25	3
9	11	5	21	17	18	10	12	6	6	5	13	5	7	11	9	13	5
19	16	17	10	18	14	18	8	2	11	6	3	1	7	2	12	1	8
14	7	16	17	7	13	5	3	1	3	2	8	0	5	11	11	9	5
10	0	5	4	14	9	16	13	3	3	8	7	7	3	3	12	30	6
12	13	10	9	3	6	4	6	3	1	14	6	24	8	9	4	4	13
16	21	18	17	5	12	16	0	5	5	6	10	9	7	0	16	3	6
12	13	10	9	1	4	23	40	9	4	5	1	2	5	13	7	3	3
16	9	8	19	12	16	0	7	11	10	5				2			
10	6	2	4	19	10	17	18	3	6	13	4	10	8	24	17	7	9
8	3	15	23	10	10	11	15	10	10	2	19	19	5	2	0	5	9
16	13	13	16	17	12	6	9	6	15	8	2	6	10	8	4	8	3
16	4	13	1	3	3	20	21	6	11	28	16	5	8	7	20	9	18
8	11	18	13	4	5	1	10	7	13								
18	10	6	20	9	15												
11	18	6	7	17	9	15	3	2	9	5				4			
15	8	16	20	11	15	8	8	10	12	16	3	12	17	5	15	16	14
9	14	3	1	3	4	9	10	11	7	17	10	13	5	6	9	5	7
15	15	6	15	5	11	3	0	29	5	3	5	14	6	6	10	5	6
1	2	14	12	18	15	16	11	13	20	13	1	3	1	4	1	10	0
7	11	21	13	10	7	2	6	15	18								

Variance = 168

Comparison of the variances in the small lymphocyte and total mononuclear percentages.

$$F = \frac{\text{small lymphocyte variance}}{\text{total mononuclear variance}} = \frac{182}{168} = 1.08$$

$$n_1 = n_2 = 405$$

Therefore F = > 20%

Therefore the variances of the two percentages do not differ significantly.

Examination of the variance of repeated counts of the same smear.

Random smear (a)

Small lymphocytes			Total mononuclears		
Percentage	d	d^2	Percentage	d	d^2
73	4	16	78	3	9
70	1	1	78	3	9
66	3	9	73	2	4
66	3	9	73	2	4
71	2	4	76	1	1
68	1	1	73	2	4

$\sigma^2 = 8$ $\sigma^2 = 6.2$

$$F = \frac{8}{6.2} = 1.29$$

$n_1 = n_2 = 5$

Therefore $F = > 20\%$

Random smear (b)

Small lymphocytes			Total mononuclears		
Percentage	d	d^2	Percentage	d	d^2
63	1	1	65	2	4
65	1	1	68	1	1
63	1	1	66	1	1
64	0	0	67	0	0
65	1	1	67	0	0
64	0	0	66	1	1

$\sigma^2 = .8$ $\sigma^2 = 1.4$

$$F = \frac{1.4}{.8} = 1.75$$

$n_1 = n_2 = 5$

Therefore $F = > 20\%$

Therefore the variances of the percentages of small lymphocytes and total mononuclears in repeated counts of a smear seem not significantly different.

Comparison of the variances in repeated readings of the same smear with the variances in repeated smears from the same rabbit.

Rabbit A

Different smears:-

Small lymphocytes		
Percentage	d	d^2
69	0	0
73	4	16
87	18	324
80	11	121
50	19	361
54	15	225

$\sigma^2 = 209.4$

Variance in six readings of one smear from same rabbit = 8 (see previous page)

Therefore $F = \frac{209.4}{8} = 26.2$, and $n_1 = n_2 = 5$, = .1%

Rabbit B

Different smears:-

Small lymphocytes		
Percentage	d	d^2
87	3	9
91	7	49
76	8	64
89	5	25
77	7	49
83	1	1

$\sigma^2 = 39$

Variance in six readings of one smear from same rabbit = .8(see previous page)

Therefore $F = \frac{39}{.8} = 49$, and $n_1 = n_2 = 5$, = <.1%

Therefore the variance in repeated readings of the same smear is significantly less then the variance due to weekly fluctuation of the percentage of small lymphocytes.

Comparison of percentage of small lymphocytes in rabbits not previously injected with percentage in rabbits which had been injected with previous standard.

Not injected			Injected		
Percentage	d	d^2	Percentage	d	d^2
69	7	49	83	4	16
84	8	64	71	8	64
69	7	49	72	7	49
82	6	36	76	3	9
			76	3	9
			84	5	25
			85	6	36
			77	2	4
			77	2	4
			79	0	0
			87	8	64
			77	2	4
			85	6	36
			73	6	36
			73	6	36
			87	8	64
			75	4	16
			81	2	4
			80	1	1
			82	3	9
			85	6	36
Mean 76			79		
Variance		66			26

t = .75. Degrees of freedom = 23. Probability=40-50%

Therefore both samples may be said to be from the same population.

Investigation of the effect on the local white cell picture of repeated puncture of the ear vein for the withdrawal of blood, no pyrogen being injected. (Tabulated as percentages of small lymphocytes, total mononuclears, neutrophils and total granulocytes for each rabbit.)

	Time of withdrawal in hours			
	0	2	2½	3
sl	33	44	17	37
tm	39	50	25	39
n	59	45	71	55
tg	62	50	76	61
sl	79	64	56	65
tm	81	66	61	69
n	19	32	35	25
tg	20	34	40	31
sl	69	66	64	50
tm	74	70	68	55
n	23	26	29	41
tg	26	30	33	46
sl	80	67	72	67
tm	85	71	77	71
n	14	24	19	23
tg	15	28	22	28
sl	65	76	65	50
tm	70	80	71	55
n	27	19	25	44
tg	29	20	30	45
Mean	65	63	55	54

Comparison of first and last columns gives a value of t = 1.1, equivalent to probability of about 30% of there being this difference by chance. Therefore repeated puncturing may affect the local white cell picture.

Preliminary work showing the effect of pyrogen (.2 ml./kg.) on the white cell picture(Tabulated as percentages of small lymphocytes, total mononuclears, neutrophils and total granulocytes for each rabbit.)

	Before inj.	Hours after injection 1	1½	2	2½	3	4	4¼	4½	24
sl	71	60		31		15	34			68
tm	75	62		34		15	36			72
n	23	36		66		84	65			29
tg	24	38		67		84	65			29
sl	63	70		17		3	36			59
tm	67	72		19		3	38			65
n	32	25		81		96	61			41
tg	34	27		82		96	61			42
sl	46	57		55		21	20			85
tm	57	69		63		23	21			87
n	41	29		36		78	79			12
tg	42	31		37		78	79			12
sl	88	54		48		10			16	35
tm	90	58		54		13			17	45
n	10	40		44		86			82	52
tg	10	43		46		86			82	55
sl	47	53		32		25			79	33
tm	61	57		39		29			79	57
n	38	40		62		70			20	41
tg	40	42		62		71			20	42
sl	85	62		64		28			85	85
tm	88	66		64		28			85	88
n	12	32		34		71			15	11
tg	13	33		36		72			15	11
sl	81	75		61		18			18	26
tm	86	81		61		22			26	38
n	13	18		38		77			73	61
tg	14	19		39		78			73	62
sl	79	67		41		31			32	69
tm	83	71		51		31			37	77
n	17	28		49		68			62	23
tg	18	28		49		68			62	23
sl	73		51		46	70			70	
tm	77		52		46	70			70	
n	22		46		54	29			30	
tg	23		48		54	29			30	

	Before inj.	Hours after injection 1	1½	2	2½	3	4	4¼	4½	24
sl	74		47		12	17			13	
tm	76		49		12	18			16	
n	23		50		87	81			83	
tg	23		51		88	82			83	
sl	72		71		87	29			14	
tm	80		71		87	29			16	
n	19		27		13	71			82	
tg	20		28		13	71			84	
sl	83		28		23	13			41	
tm	83		31		23	15			41	
n	15		67		76	85			58	
tg	16		69		76	85			58	
sl	86		75		51	36			60	
tm	89		78		53	38			62	
n	10		22		46	61			36	
tg	12		23		46	61			37	
sl	42		33		15	11			22	
tm	46		34		17	12			22	
n	53		66		82	87			77	
tg	54		66		83	87			77	
sl	83			38	32	14		30		
tm	86			38	32	15		31		
n	13			60	66	84		68		
tg	13			61	67	85		69		
sl	78			16	20	9		7		
tm	85			16	20	10		9		
n	14			84	80	90		92		
tg	15			84	80	90		92		
sl	80			77	58	24		15		
tm	82			78	58	24		16		
n	17			21	41	76		84		
tg	18			22	41	76		84		
sl	66			30	6	58		11		
tm	67			30	7	59		14		
n	32			70	92	41		85		
tg	33			70	92	41		86		
sl	47			32	20	51		22		
tm	50			34	22	51		23		
n	50			64	78	49		77		
tg	50			66	79	49		78		

	Before inj.	Hours after injection 1	1½	2	2½	3	4	4¼	4½	24
sl	75			23	16	17		5		
tm	75			23	16	19		7		
n	23			76	83	81		91		
tg	24			76	83	82		92		
sl	89				14	18				20
tm	94				15	19				21
n	6				84	80				78
tg	6				85	80				79
sl	61				11	5				17
tm	64				12	6				19
n	33				87	93				80
tg	36				87	93				81
sl	44				3	6				12
tm	50				11	15				20
n	50				88	84				80
tg	51				89	84				81
sl	76				29	9				9
tm	79				32	15				11
n	19				65	82				86
tg	20				67	85				88
sl	74				14	3				5
tm	77				18	6				7
n	22				80	93				91
tg	23				81	94				93
sl	83				27	34				4
tm	83				28	35				5
n	16				72	65				95
tg	17				72	65				96

Rises in temperature shown by 19 rabbits to four injections each at three dose levels.

* Rabbit No.	Response to 0.2 ml. per kilogram			
1	1.13	1.24	1.18	1.23
2	1.26	1.45	2.28	0.85
3	1.30	1.53	1.63	1.21
4	0.96	0.95	1.13	0.99
5	1.40	1.76	1.40	1.13
6	1.32	1.34	1.49	1.17
7	1.27	1.24	1.27	1.52
8	0.81	1.36	1.21	1.16
9	1.03	0.94	1.68	1.76
10	1.91	2.14	1.60	1.51
11	0.27	0.78	0.26	0.11
12	1.24	0.33	1.45	1.31
13	2.10	1.87	1.48	1.44
14	0.47	0.13	0.28	1.64
15	1.08	0.40	0.81	1.24
16	0.58	0.93	0.44	0.64
17	1.13	0.95	1.41	1.51
18	1.53	1.21	1.72	1.50
19	1.24	0.69	0.88	1.15

Mean of 76 rises = 1.18°

* These numbers do not represent the same rabbits as numbers 1 - 19 in appendices five and four.

Rabbit No.	Response to .06324 ml. per kilogram			
1	0.89	0.31	0.90	0.76
2	0.86	0.32	1.05	0.26
3	1.09	0.88	1.44	0.97
4	1.29	0.44	0.73	0.86
5	1.47	0.93	1.47	1.20
6	1.21	1.29	1.24	0.73
7	1.13	1.16	1.15	0.61
8	1.07	0.87	1.31	0.74
9	1.35	1.39	1.01	1.13
10	1.75	2.16	1.67	2.21
11	0.67	1.65	1.38	0.21
12	1.38	1.37	0.99	1.26
13	1.88	1.11	1.96	1.36
14	1.11	1.09	0.64	1.32
15	1.42	1.37	1.19	1.17
16	1.06	0.97	1.80	1.00
17	1.48	1.82	1.82	1.36
18	1.20	1.61	1.50	0.86
19	1.06	1.25	1.44	1.41

Mean of 76 rises = 1.18°

Rabbit No.	Response to .02 ml. per kilogram			
1	0.70	1.43	0.80	0.54
2	0.43	0.81	0.97	0.30
3	0.81	1.06	1.41	0.93
4	0.55	0.56	0.74	-0.21
5	1.23	1.36	1.55	0.12
6	1.16	0.71	1.41	0.19
7	0.73	0.59	0.34	1.16
8	0.75	0.12	0.05	1.06
9	0.97	0.75	0.17	1.14
10	1.68	1.80	1.26	2.44
11	0.70	1.31	1.73	1.00
12	1.21	0.09	0.67	0.86
13	1.20	0.41	1.21	1.48
14	0.50	0.38	1.50	2.08
15	1.06	1.46	0.95	0.79
16	0.50	0.60	0.70	0.03
17	1.47	1.53	1.69	1.01
18	1.02	0.75	0.87	0.96
19	1.25	1.01	1.08	0.90

Mean of 76 rises = 0.93

Calculations of variances in temperature rise at the three dose levels.

Deviations at .2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
.05	.06	0	.05	.29	.87	.28	.42	.23	.50	.13	.39
.08	.27	1.10	.33	.32	.86	.13	.92	.50	.12	.04	.63
.12	.35	.45	.03	.09	.30	.26	.21	.12	.13	.48	0
.22	.23	.05	.19	.11	.74	.45	.32	.38	.37	.19	1.14
.22	.58	.22	.05	.29	.25	.29	.02	.30	.43	.62	.81
.14	.16	.31	.01	.03	.11	.06	.45	.23	.22	.48	.74
.09	.06	.09	.34	.05	.02	.03	.57	.20	.34	.59	.23
.37	.18	.03	.02	.11	.31	.13	.44	.18	.81	.88	.13
.50	.24	.15	.58	.17	.21	.17	.05	.04	.18	.76	.21
.73	.96	.42	.33	.57	.98	.49	1.03	.75	.87	.33	1.51
.91	.40	.92	1.07	.51	.47	.20	.97	.23	.38	.80	.07
.06	.85	.27	.13	.20	.19	.19	.08	.28	.84	.26	.07
.92	.69	.30	.26	.70	.07	.78	.18	.27	.52	.28	.55
.71	1.05	.90	.46	.07	.09	,54	.14	.43	.55	.57	1.15
.10	.78	.37	.06	.24	.19	.01	.01	.13	.53	.02	.14
.60	.25	.74	.54	.12	.21	.62	.18	.43	.33	.23	.90
.05	.23	.23	.33	.30	.64	.64	.18	.54	.60	.76	.08
.35	.03	.54	.32	.02	.43	.32	.32	.09	.18	.06	.03
.06	.49	.30	.03	.12	.07	.26	.23	.32	.08	.15	.03

Variance .2157 .1699 .2540

Check by χ^2 method for normality of distribution of temperature responses.

1. Dose = .2 ml./kg.

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o-f_t)^2}{f_t}$
0.00 - 0.80	13	16	3	.5625
0.81 - 1.00	10	11	1	.0909
1.01 - 1.20	10	12	2	.3333
1.21 - 1.40	19	13	6	.2769
1.41 - 1.60	13	10	3	.9000
1.61 - 2.30	11	13	2	.3077

χ^2 = 2.4713
Degrees of freedom = 5
Probability = 70 - 80 %

2. Dose = .06324 ml./kg.

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o-f_t)^2}{f_t}$
0.00 - 0.80	12	14	2	.2857
0.81 - 1.00	12	12	0	.0000
1.01 - 1.20	17	13	4	1.2308
1.21 - 1.40	16	14	2	.2857
1.41 - 1.60	8	11	3	.8182
1.61 - 2.30	11	11	0	.0000

χ^2 = 2.6204
Degrees of freedom= 5
Probability = 70 - 80 %

3. Dose = .02 ml./kg.

Range of temperature rises of group	Observed frequency	Theoretical frequency	$f_o - f_t$	$\frac{(f_o-f_t)^2}{f_t}$
-.21 - 0.40	11	10	1	.1000
0.41 - 0.60	9	9	0	.0000
0.61 - 0.80	12	11	1	.0909
0.80 - 1.00	11	11	0	.0000
1.01 - 1.20	11	9	2	.4444
1.21 - 2.50	22	24	2	.1667

$\chi^2 = .8020$
Degrees of freedom = 5
Probability = 95 - 98 %

Therefore at each dose level the responses are normally distributed.

Analysis of variance of temperature rises into between rabbit and within rabbit variance.

Dose = .2 ml./kg.

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between rabbit	8.8506	24	.3688
Within rabbit	6.1664	75	.0822
Total	15.0170	99	

$F = \frac{.3688}{.0822} \doteq 4.49 \begin{matrix} n_1 = 24 \\ n_2 = 75 \end{matrix} = <0.1\%$

Therefore between rabbit variance is significantly greater than within rabbit variance.

Dose = .06324 ml./kg.

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between rabbit	6.57237	24	.273849
Within rabbit	4.63805	75	.061841
Total	11.21042	99	

$F = \frac{.273949}{.061841} \doteq 4.43 \begin{matrix} n_1 = 24 \\ n_2 = 75 \end{matrix} = <0.1\%$

Therefore between rabbit variance is significantly greater than within rabbit variance.

Dose = .02 ml./kg.

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between rabbit	5.73605	24	.23900
Within rabbit	7.31805	75	.09759
Total	13.05500	99	

$F = \frac{.23900}{.09759} \doteq 2.449 \begin{matrix} n_1 = 24 \\ n_2 = 75 \end{matrix} \doteq .1\%$

Therefore between rabbit variance is significantly greater than within rabbit variance.

Time taken (minutes) after injection to reach maximum temperature. "+" indicates that higher temperatures might have been reached had the experiment been continued longer. Their accuracy however would have been lessened by the disturbance associated with the removal of blood.

.2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
100	80	90	110	110	130	100	100	110	100	140	170+
110	170	130	160	150	150	130	190+	160	150	100	70
90	100	100	100	130	140	140	150	100	110	140	170+
90	90	100	90	90	120	150	160	100	110	100	80
110	180+	150	140	140	140	90	150	170	160	80	50
80	140	100	90	100	110	140	170	90	70	100	160+
60	70	180+	170	80	120	110	80	130	150+	160+	130
140	110	100	140	100	90	140	110	130	90	70	150+
80	180+	90	100	80	110	90	160	130	90	150+	120
80	70	100	70	80	120	130	170+	80	90	140+	110
70	140	40	70	130	100	170	130	70	90	140+	140+
120	150	90	100	80	120	110	110	80	110	70	130
80	190+	90	100	100	110	100	110	110	140	160	150+
60	170	110	100	100	100	100	110	70	150	70	100
80	130	120	100	100	140	100	110	100	180	100	140
130	160	140	130	80	150	100	130	130	160	140	120
90	150	80	70	80	70	60	120	90	130	140	150+
80	160+	100	120	100	160+	130	120	110	170	130	160+
100	100	100	110	90	110	80	100	100	150	150	160+

Mean 110 120 120 (to nearest ten)

Leucocyte Counts

Dose = .2 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall
1	sl	35	17	18	27	5	22	49	9	40	38	12	26
	tm	41	17	24	33	5	28	50	9	41	44	12	32
2	sl	36	7	29	29	5	24	24	13	11	60	19	41
	tm	43	8	35	34	8	26	28	13	15	61	20	41
3	sl	69	9	60	71	10	61	72	7	65	65	24	41
	tm	72	9	63	73	14	59	72	9	63	70	24	36
4	sl	39	7	32	70	11	59	71	23	48	59	25	34
	tm	39	8	31	71	12	59	73	23	50	65	27	38
5	sl	44	2	42	56	9	47	57	13	44	61	4	57
	tm	51	3	48	66	12	56	58	13	45	65	5	60
6	sl	81	40	41	80	23	57	81	21	60	80	38	42
	tm	86	47	39	82	24	58	88	22	66	82	38	44
7	sl	53	10	43	66	10	56	72	10	62	80	13	67
	tm	58	12	46	68	10	58	74	11	63	87	14	73
8	sl	78	12	66	72	11	61	73	13	60	73	20	53
	tm	81	17	64	73	11	62	77	16	61	79	21	58
9	sl	59	9	50	62	16	46	78	19	59	71	43	28
	tm	60	10	50	63	16	47	79	20	59	73	44	29
10	sl	63	16	47	74	18	56	71	18	53	75	10	65
	tm	70	19	51	79	20	59	77	21	56	80	12	68
11	sl	54	9	45	75	8	67	79	19	60	79	19	60
	tm	60	10	50	76	8	68	81	20	61	81	19	62
12	sl	41	34	7	34	5	29	65	24	41	71	22	49
	tm	53	40	13	36	6	30	67	26	41	72	22	50
13	sl	74	51	23	82	33	49	84	37	47	83	23	60
	tm	76	53	23	83	35	48	87	37	50	86	25	61
14	sl	57	5	52	55	10	45	69	7	62	67	10	57
	tm	59	5	54	58	10	48	73	7	66	70	11	59
15	sl	58	13	45	85	24	61	64	14	50	61	23	38
	tm	61	13	48	86	26	60	66	15	51	65	24	41
16	sl	66	5	61	68	5	63	61	16	45	68	7	61
	tm	67	5	62	70	6	64	61	17	44	70	8	62
17	sl	53	9	44	77	22	55	62	11	51	53	15	38
	tm	56	11	44	82	24	58	65	12	53	56	16	40
18	sl	62	11	51	82	7	75	61	15	46	75	28	47
	tm	66	12	54	83	7	76	63	17	46	77	29	48

sl = small lymphocytes tm = total mononuclears

Rabbit No.	Cells	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall
19	sl	74	25	49	74	27	47	83	15	41	54	17	37
	tm	79	28	51	78	30	48	67	15	42	61	21	40
20	sl	83	14	69	86	31	55	87	27	60	79	22	57
	tm	84	15	69	86	32	54	88	27	61	84	22	62
21	sl	62	6	56	57	1	56	66	10	56	65	10	55
	tm	66	8	58	65	2	63	69	10	59	67	13	54
22	sl	75	7	68	43	31	12	79	10	69	74	29	45
	tm	76	7	69	47	32	15	81	10	71	79	30	49
23	sl	64	14	50	59	24	35	55	19	36	56	16	40
	tm	65	14	51	63	24	39	56	19	37	60	18	42
24	sl	66	12	54	52	13	39	56	5	51	71	8	63
	tm	70	14	56	61	15	46	58	7	51	74	11	63

Dose = .06324 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall
1	sl	63	32	31	63	42	21	45	13	32	33	25	8
	tm	65	34	31	66	42	24	50	13	37	36	26	10
2	sl	61	16	45	49	39	10	49	36	13	41	35	6
	tm	64	16	48	53	41	12	52	36	16	42	36	6
3	sl	79	26	53	78	79	-1	63	25	38	72	36	36
	tm	80	28	52	80	80	0	67	26	41	78	39	39
4	sl	57	47	10	82	53	29						
	tm	61	47	14	82	54	28						
5	sl	68	8	60	70	32	38	61	26	35	65	25	40
	tm	70	9	61	73	34	39	66	27	39	68	27	41
6	sl	79	54	25	89	62	27	79	44	35	79	24	55
	tm	81	56	25	89	67	22	81	44	37	83	27	56
7	sl	65	11	54	77	17	60	73	27	46	80	44	36
	tm	70	11	59	79	17	62	75	29	46	83	48	35
8	sl	78	16	62	82	26	56	73	26	47	69	28	41
	tm	78	16	62	84	26	58	76	27	49	71	30	41
9	sl	82	38	44	68	20	48	81	41	40	76	45	31
	tm	84	38	46	69	20	49	83	41	42	79	45	34
10	sl	54	21	33	66	17	49	47	26	21	67	66	1
	tm	62	27	35	70	18	52	52	28	24	68	67	1
11	sl	79	17	62	84	24	60	85	22	63	78	56	22
	tm	82	17	65	86	27	59	85	23	62	83	58	25
12	sl	69	23	46	74	38	36	73	38	35	68	50	18
	tm	71	26	45	75	40	35	74	39	35	71	51	20
13	sl	76	35	41									
	tm	81	35	46									
14	sl	59	28	31	70	23	47	60	14	46	66	23	43
	tm	63	28	35	72	24	48	66	14	52	68	24	44
15	sl	72	24	48	50	21	29	50	25	25	68	27	41
	tm	74	25	49	57	21	36	57	25	32	71	29	42
16	sl	83	17	66	77	18	59	68	15	53	86	11	75
	tm	84	17	67	78	18	60	70	15	55	86	12	74
17	sl	46	13	33	52	20	32	80	9	71	61	14	47
	tm	48	13	35	60	20	40	81	9	72	68	14	54
19	sl	79	34	45									
	tm	81	35	46									
20	sl	91	26	65	76	34	42	88	8	80	92	18	74
	tm	92	27	65	79	34	45	88	10	78	93	18	75
21	sl	54	12	42	63	16	47	52	21	31	79	19	60
	tm	59	14	45	66	17	49	63	21	42	81	24	57
22	sl	79	25	54	81	46	35	61	17	44	81	31	50
	tm	79	25	54	81	46	35	62	18	44	82	32	50
23	sl	86	33	53	76	44	32	71	11	60	74	26	48
	tm	89	33	56	77	44	33	73	12	61	75	27	48

Dose = .02 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall	percentage before injection	percentage after injection	fall
1	sl	59	39	20	69	24	45	45	20	25	58	50	8
	tm	60	40	20	74	26	48	49	22	27	61	50	11
2	sl	44	39	5	65	18	47	44	16	28	60	41	19
	tm	47	41	6	68	22	46	46	18	28	62	42	20
3	sl	61	37	24	55	30	25	75	38	37	62	40	22
	tm	64	39	25	64	32	32	77	42	35	64	44	20
4	sl	60	24	36	64	43	21						
	tm	68	27	41	66	44	22						
5	sl	71	7	64	46	13	33	76	10	66	71	61	10
	tm	73	12	61	51	15	36	79	11	68	74	62	12
6	sl	82	52	30	85	42	43	68	15	53	83	55	28
	tm	87	53	34	89	42	47	71	15	56	85	59	26
7	sl	66	33	33	87	50	37	74	11	63	81	45	36
	tm	74	34	40	88	53	35	77	12	65	84	50	34
8	sl	85	17	68	86	47	39	79	67	12	83	37	46
	tm	87	20	67	87	50	37	81	69	12	85	39	46
9	sl	78	57	21	62	72	-10	44	50	-6	81	43	38
	tm	79	59	20	64	72	-8	46	51	-5	82	44	38
10	sl	63	14	49	68	53	15	66	48	18	55	32	23
	tm	67	16	51	72	53	19	72	53	19	63	32	31
11	sl	74	63	11	91	64	27	72	63	9	80	43	37
	tm	76	64	12	92	66	26	73	67	6	82	44	38
12	sl	61	46	15	82	66	16	71	61	10	69	52	17
	tm	63	47	16	83	68	15	73	64	9	73	53	20
13	sl	75	43	32									
	tm	78	45	33									
14	sl	48	38	10	57	37	20	67	25	42	66	21	45
	tm	52	41	11	59	42	17	69	27	42	67	22	45
15	sl	77	43	34	75	42	33	64	16	48	69	46	23
	tm	78	43	35	76	45	31	67	18	49	71	46	25
16	sl	80	25	55	79	28	51	77	14	63	68	21	47
	tm	84	25	59	80	28	52	79	15	64	68	32	46
17	sl	64	27	37	51	34	17	84	12	72	55	41	14
	tm	69	30	39	56	35	21	85	13	72	58	41	17
19	sl	65	25	40									
	tm	72	27	45									
20	sl	79	13	66	89	44	45	90	32	58	89	60	29
	tm	81	13	68	91	47	44	92	32	60	90	61	29
21	sl	67	44	23	62	38	24	69	16	53	67	46	21
	tm	70	44	26	67	40	27	71	16	55	69	49	20
22	sl	68	42	26	85	67	18	80	51	29	68	53	15
	tm	70	42	28	86	68	18	81	52	29	70	55	15
23	sl	79	24	55	73	64	9	85	59	26	75	56	19
	tm	80	25	55	77	66	11	86	59	27	76	57	19

Dose = .2 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise
1	n	56	82	26	63	94	31	47	90	43	53	87	34
	tg	59	83	24	67	96	29	49	91	42	57	87	30
2	n	53	88	35	57	84	27	69	85	16	38	76	38
	tg	56	91	35	66	92	26	72	86	14	39	81	42
3	n	25	90	65	25	82	57	26	89	63	26	75	49
	tg	27	90	63	28	86	58	28	91	63	29	76	47
4	n	60	91	31	27	87	60	23	77	54	36	70	34
	tg	61	92	31	28	87	59	28	77	49	38	73	35
5	n	46	96	50	34	88	54	41	86	45	33	93	60
	tg	48	97	49	35	88	53	41	87	46	35	93	58
6	n	12	51	39	17	73	56	71	73	2	16	61	45
	tg	13	54	41	18	76	58	74	77	3	18	61	43
7	n	40	86	46	29	87	58	21	86	65	11	86	75
	tg	42	87	45	32	89	57	25	88	63	13	87	74
8	n	18	81	63	26	87	61	23	83	60	19	80	61
	tg	18	83	65	27	87	60	24	85	61	22	80	58
9	n	37	89	52	33	83	50	19	77	58	25	53	28
	tg	40	90	50	37	83	46	21	80	59	28	56	28
10	n	27	80	53	19	79	60	22	75	53	18	87	69
	tg	29	80	51	20	80	60	23	79	56	20	88	68
11	n	36	90	54	23	91	68	17	79	62	16	79	63
	tg	40	91	51	26	92	66	19	80	61	19	81	62
12	n	46	59	13	62	93	31	32	73	41	26	77	51
	tg	47	60	13	65	93	28	34	73	39	27	78	51
13	n	22	45	23	16	63	47	13	62	49	13	75	62
	tg	25	47	22	17	65	48	14	63	49	15	77	62
14	n	38	95	57	43	88	45	26	92	66	29	88	59
	tg	41	95	54	43	90	47	27	93	66	30	89	59
15	n	35	94	59	14	70	56	31	84	53	29	74	45
	tg	39	95	56	14	73	59	34	84	50	34	77	43
16	n	31	93	62	27	94	67	37	83	46	28	91	63
	tg	32	94	62	30	95	65	39	83	44	30	91	61
17	n	40	90	50	17	75	58	31	85	54	41	80	39
	tg	46	91	45	18	78	60	34	87	53	44	84	40
18	n	33	88	55	17	93	76	36	82	46	21	70	49
	tg	33	88	55	18	93	75	37	83	46	22	70	48

tg = total granulocytes n = neutrophils

Rabbit No.	Cells	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise
19	n	20	72	52	20	68	48	31	83	52	37	78	41
	tg	22	72	50	23	70	47	33	85	52	38	79	41
20	n	13	85	72	12	67	55	12	71	59	13	78	65
	tg	16	86	70	14	68	54	13	73	60	15	78	63
21	n	30	90	60	31	95	64	28	88	60	32	82	50
	tg	33	92	59	34	97	63	30	90	60	33	87	54
22	n	22	93	71	50	67	17	19	89	70	17	69	52
	tg	24	93	69	54	68	14	1[illegible]	90	71	21	70	49
23	n	35	85	50	36	75	39	42	79	35	38	78	40
	tg	35	85	50	38	76	38	43	80	37	41	82	41
24	n	30	86	56	34	84	50	42	92	50	26	88	62
	tg	30	86	56	39	85	46	42	93	51	26	88	62

Dose = .06324 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise
1	n	33	64	31	31	56	25	49	87	38	64	73	9
	tg	36	66	30	34	57	23	50	87	37	64	74	10
2	n	33	82	49	42	57	15	43	63	20	55	63	8
	tg	35	84	49	47	60	13	48	64	16	58	65	7
3	n	19	70	51	17	17	0	31	68	37	19	59	40
	tg	19	72	53	19	19	0	33	75	42	22	61	39
4	n	35	51	16	17	45	28						
	tg	38	52	14	17	46	29						
5	n	30	90	60	26	65	39	34	73	39	29	72	43
	tg	30	91	61	27	67	40	34	73	39	32	74	42
6	n	18	43	25	9	29	20	17	55	38	14	69	55
	tg	19	45	26	9	34	25	19	55	36	17	73	56
7	n	28	87	59	18	83	65	22	70	48	16	49	33
	tg	30	89	59	20	83	63	24	72	48	17	52	35
8	n	22	84	62	15	73	58	22	73	51	27	69	42
	tg	22	84	62	16	74	58	24	74	50	29	70	41
9	n	15	60	45	28	77	49	16	59	43	20	54	34
	tg	17	61	44	31	79	48	17	59	42	21	54	33
10	n	32	69	37	26	80	54	47	70	23	28	32	4
	tg	37	72	35	29	81	52	48	71	23	31	33	2
11	n	16	81	65	13	72	59	12	73	61	15	41	26
	tg	19	83	64	14	74	60	14	76	62	18	43	25
12	n	27	73	46	22	59	37	23	59	36	26	44	18
	tg	30	74	44	24	60	36	27	62	35	30	49	19
13	n	19	62	43									
	tg	19	64	45									
14	n	33	70	37	26	74	48	30	84	54	30	69	39
	tg	36	72	36	29	76	47	33	86	53	31	76	45
15	n	24	74	50	37	77	40	40	73	33	28	68	40
	tg	25	75	50	42	78	36	43	75	32	29	71	42
16	n	16	82	66	22	82	60	27	82	55	13	86	73
	tg	16	82	66	23	83	60	29	84	55	13	88	75
17	n	47	85	38	35	76	41	19	89	70	29	83	54
	tg	51	87	36	40	79	39	20	91	71	32	85	53
19	n	19	64	45									
	tg	19	64	45									
20	n	8	71	63	18	64	46	11	87	76	7	79	72
	tg	8	73	65	21	66	45	11	89	78	7	81	74
21	n	38	83	45	31	81	50	35	77	42	19	73	54
	tg	41	86	45	33	83	50	38	79	41	19	76	57
22	n	20	72	52	17	50	33	30	81	51	17	65	48
	tg	21	75	54	18	54	36	38	81	43	17	67	50
23	n	9	67	58	21	56	35	26	87	61	22	71	49
	tg	11	67	56	22	56	34	27	88	61	25	72	47

Dose = .02 ml./kg.

Rabbit No.	Cells	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise	percentage before injection	percentage after injection	rise
1	n	39	57	18	24	74	50	46	76	30	37	46	9
	tg	41	60	19	27	74	47	50	78	28	40	49	9
2	n	48	57	9	30	75	45	50	76	26	35	55	20
	tg	52	59	7	32	78	46	55	82	27	38	58	20
3	n	35	59	24	31	63	32	22	55	33	27	48	21
	tg	37	61	24	36	67	31	23	58	35	34	57	23
4	n	25	72	47	32	54	22						
	tg	32	73	41	34	55	21						
5	n	28	86	58	47	80	33	19	87	68	25	37	12
	tg	28	89	61	50	85	35	22	89	67	26	38	12
6	n	12	47	35	9	55	46	25	83	58	14	38	24
	tg	14	48	34	12	58	46	29	85	56	15	41	26
7	n	24	66	42	12	45	33	22	88	66	13	47	34
	tg	27	67	40	12	46	34	23	89	66	15	51	36
8	n	13	78	65	13	50	37	18	30	12	15	59	44
	tg	14	80	66	13	51	38	19	30	11	15	61	46
9	n	19	40	21	34	27	-7	50	47	-3	17	52	35
	tg	22	41	19	36	28	-8	55	48	-7	18	56	38
10	n	30	80	50	28	44	16	23	38	15	34	68	34
	tg	34	83	49	29	47	18	28	47	19	37	69	32
11	n	19	35	16	7	30	23	20	25	5	16	53	37
	tg	23	36	13	7	33	26	26	32	6	17	56	39
12	n	37	51	14	14	28	14	25	33	8	25	44	19
	tg	37	53	16	17	33	16	28	36	8	28	46	18
13	n	19	53	34									
	tg	22	54	32									
14	n	43	58	15	38	52	14	28	71	43	30	75	45
	tg	49	61	12	41	57	16	31	73	42	32	78	46
15	n	21	55	34	21	52	31	29	79	50	26	51	25
	tg	21	56	35	23	55	32	33	82	49	30	54	24
16	n	16	74	58	19	71	52	21	83	62	32	78	46
	tg	17	75	58	19	72	53	22	84	62	32	78	46
17	n	30	67	37	42	60	18	14	82	68	39	57	18
	tg	32	70	38	44	65	21	15	87	72	41	59	18
19	n	27	71	44									
	tg	28	73	45									
20	n	15	83	68	8	46	38	8	66	58	9	38	29
	tg	18	87	69	9	53	44	9	68	59	10	39	29
21	n	27	56	29	28	56	28	27	83	56	30	47	17
	tg	30	56	26	33	59	26	28	84	56	31	51	20
22	n	28	55	27	12	30	18	17	45	28	29	44	15
	tg	29	57	28	14	31	17	19	47	28	31	45	14
23	n	18	74	56	18	32	14	14	40	26	24	41	17
	tg	18	75	57	23	33	10	14	40	26	24	43	19

Check on the normality of small lymphocyte falls, by χ^2 test.

1. Dose = .2 ml./kg.

Range of falls of group	Observed frequency	Theoretical frequency	$(f_o - f_t)$	$\frac{(f_o - f_t)^2}{f_t}$
7 - 40.5	16	21	5	1.1905
40.5 - 50.5	21	21	0	0
50.5 - 60.5	23	19	4	0.8421
60.5 - 69	16	10	6	3.6000

$\chi^2 = 5.6326$
Probability = 10-20%

2. Dose = .06324 ml./kg.

Range of falls of group	Observed frequency	Theoretical frequency	$(f_o - f_t)$	$\frac{(f_o - f_t)^2}{f_t}$
-1 - 30.5	14	19	5	1.3158
30.5 - 40.5	20	16	4	1
40.5 - 50.5	20	17	3	0.5294
50.5 - 60.5	13	13	0	0
60.5 - 80	9	10	1	0.1000

$\chi^2 = 2.9452$
Probability = 50-70%

3. Dose = .02 ml./kg.

Range of falls of group	Observed frequency	Theoretical frequency	$(f_o - f_t)$	$\frac{(f_o - f_t)^2}{f_t}$
-10 - 10.5	10	9	1	0.1111
10.5 - 20.5	14	12	2	0.3333
20.5 - 30.5	18	16	2	0.2500
30.5 - 40.5	11	16	5	1.5625
40.5 - 50.5	10	12	2	0.3333
50.5 - 72	13	10	3	0.9000

$\chi^2 = 3.4902$
Probability = 50-70%

Therefore the falls in small lymphocytes are normally distributed.

Small lymphocyte percentage falls shown by 19 rabbits to four injections each at three dose levels.

Responses to												
.2 ml./kg.				.06324 ml./kg.				.02 ml./kg.				
51	81	82	68	49	33	71	24	34	65	56	14	
81	83	46	68	74	20	27	15	11	72	64	32	
87	86	90	63	67	-1	60	50	39	45	49	35	
95	84	77	93	88	54	57	62	90	72	87	14	
51	71	74	53	32	30	44	70	37	51	78	34	
81	85	86	84	83	78	63	45	50	43	85	44	
85	85	82	73	79	68	64	59	80	45	15	55	
85	74	76	40	54	71	49	41	27	-16	-14	47	
75	76	75	87	61	74	45	1	78	22	27	42	
83	89	76	76	78	71	74	28	15	30	13	46	
17	85	63	69	67	49	48	26	25	20	14	25	
91	82	90	85	53	67	77	65	21	35	63	68	
78	72	78	62	67	58	50	60	44	44	75	33	
92	93	74	90	75	77	78	87	69	65	82	69	
83	71	82	72	72	62	89	77	58	33	86	25	
83	64	70	72	71	55	91	80	84	51	64	33	
90	98	85	85	78	75	60	76	34	39	77	31	
91	28	87	61	68	43	72	62	38	21	36	22	
78	60	65	71	62	42	85	65	70	12	31	25	

Mean small lymphocyte percentage falls in response to -

.2 ml./kg = 76
.06324 ml./kg = 59
.02 ml./kg = 44 (to nearest unit)

Calculation of variances in small lymphocyte percentage falls at the three dose levels.

Deviations at .2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
25	5	6	8	10	26	12	35	10	21	12	30
5	7	30	8	15	39	32	44	33	28	20	12
11	10	14	13	8	60	1	9	5	1	5	9
19	8	1	17	29	5	2	3	46	28	43	30
25	5	2	23	27	29	15	11	7	7	34	10
5	9	10	8	24	19	4	14	6	1	41	0
9	9	6	3	20	9	5	0	36	1	29	11
9	2	0	36	5	12	10	18	17	60	58	3
1	0	1	11	2	15	14	58	34	22	17	2
7	13	0	0	19	12	15	31	29	14	31	2
59	9	13	7	8	10	11	33	19	24	30	19
15	6	14	9	6	8	18	6	23	9	19	24
2	4	2	14	8	1	9	1	0	0	31	11
16	17	2	14	16	18	19	28	25	21	38	25
7	5	6	4	13	3	30	18	14	11	42	19
7	12	6	4	12	4	32	21	40	7	20	11
14	22	9	9	19	16	1	17	10	5	33	13
15	48	11	15	9	16	13	3	6	23	8	22
2	16	11	5	3	17	26	6	26	32	13	19
Variance		217			404				581		

Check by χ^2 method for normality of distribution of small lymphocyte responses.

1. Dose = .2 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$f_o - f_t$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
17 - 60	9	9	0	0	0.0000
61 - 70	9	16	7	49	3.0625
71 - 80	20	16	4	16	1.0000
81 - 98	38	25	13	169	6.7600

χ^2 = 10.8225
Degrees of freedom = 3
Probability = 1 - 2 %

2. Dose = .06324 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$f_o - f_t$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
-1 - 40	11	14	3	9	0.6429
41 - 50	12	12	0	0	0.0000
51 - 60	10	13	3	9	0.6923
61 - 70	16	14	2	4	0.2857
71 - 91	27	18	9	81	4.5000

χ^2 = 6.1209
Degrees of freedom = 4
Probability = 10 - 20 %

3. Dose = .02 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$f_o - f_t$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
-16 - 20	11	12	1	1	0.0833
21 - 30	11	10	1	1	0.1000
31 - 40	16	12	4	16	1.3333
41 - 50	11	10	1	1	0.1000
51 - 70	14	20	6	36	1.8000
71 - 90	13	8	5	25	3.1250

χ^2 = 6.5416
Degrees of freedom = 5
Probability = 20 - 30%

Therefore distributions are not so far from normal that standard statistical calculations are not applicable.

Analysis of variance of small lymphocyte percentage fall into between rabbit and within rabbit variance.

Dose = .2 ml./kg.

Let the small lymphocyte percentage fall = x

Number of rabbits = m = 19.

Number of values per rabbit = n = 4.

1. $\Sigma(x^2) = 454920$
2. Total for each rabbit squared, squares added and the sum divided by the number of values for each rabbit
   $= 444663$
3. $\frac{(\Sigma x)^2}{76} = 438672$

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Components
Between rabbit	2-3=5991	m-1 = 18	333	$n\sigma_A^2 + \sigma_B^2$
Within rabbit	1-2=10257	mn-m = 57	180	σ_B^2
Total	1-3=16248	mn-1 = 75		

Therefore $n\sigma_A^2$ eexists.

To see if it exists significantly -

$$F = \frac{333}{180} \quad \begin{matrix} n_1 = 18 \\ n_2 = 57 \end{matrix} = 1.85 = 5\%$$

Therefore it may be said that the fluctuation in the percentage fall of small lymphocytes within a rabbit is as great as the fluctuation between rabbits.

Dose = .06324 ml./kg.

1. $\Sigma(x^2) = 296847$
2. Total for each rabbit squared, squares added and the sum divided by the number of values for each rabbit
   $= 278469$
3. $\frac{(\Sigma x)^2}{76} = 266566$

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Components
Between rabbit	11903	18	661	$n\sigma_A^2 + \sigma_B^2$
Within rabbit	18378	57	322	σ_B^2
Total	30281	75		

To see if $n\sigma_A^2$ exists significantly -

$$F = \frac{661}{322} \quad \begin{matrix} n_1 = 18 \\ n_2 = 57 \end{matrix} = 2.05 = 5\%$$

Therefore it may be said that the fluctuation in the percentage fall of small lymphocytes within a rabbit is as great as the fluctuation between rabbits.

Dose = .02 ml./kg.

1. $\Sigma(x^2) = 189955$

2. Total for each rabbit squared, squares added and the sum divided by the number of values for each rabbit

$= 162101$

3. $\frac{(\Sigma x)^2}{76} = 146345$

Source of variance	Sums of squares	Degrees of freedom	Mean squares	Components
Between rabbits	15756	18	875	$n\sigma_A^2 + \sigma_B^2$
Within rabbits	27854	57	489	σ_B^2
Total	43610	75		

To see if $n\sigma_A^2$ exists significantly -

$$F = \frac{875}{489} \quad \begin{matrix} n_1 = 18 \\ n_2 = 57 \end{matrix} \quad 1.79 = 5\%$$

Therefore it may be said that the fluctuation in the percentage fall of small lymphocytes within a rabbit is as great as the fluctuation between rabbits.

Comparison of the small lymphocyte percentage falls in rabbits not previously injected with those of rabbits which had been injected with previous standard. Dose = .2 ml./kg.

Not injected			Injected		
Percentage fall	d	d^2	Percentage fall	d	d^2
90	15	225	71	5	25
67	8	64	70	6	36
69	6	36	82	6	36
			87	11	121
			62	14	196
			84	8	64
			81	5	25
			69	7	49
			78	2	4
			81	5	25
			59	17	289
			87	11	121
			73	3	9
			87	11	121
			77	1	1
			72	4	16
Mean 75			76		
Variance		163			76

t ≙ .13. Degrees of Freedom = 17. Probability=90%

Therefore both samples may be said to be from the same population.

Dose = .06324 ml./kg.

Not injected			Injected		
Percentage fall	d	d^2	Percentage fall	d	d^2
72	6	36	44	14	196
61	5	25	34	24	576
64	2	4	44	14	196
			65	7	49
			44	14	196
			67	9	81
			68	10	100
			54	4	16
			45	13	169
			63	5	25
			48	10	100
			66	8	64
			59	1	1
			79	21	441
			75	17	289
			74	16	256
Mean 66			58		
Variance		33			184

t ≐ 1.6. Degrees of freedom = 17. Probability=20%

Result inconclusive.

Dose = .02 ml./kg.

Not injected			Injected		
Percentage fall	d	d^2	Percentage fall	d	d^2
45	9	81	42	3	9
29	7	49	45	0	0
35	1	1	42	3	9
			66	21	441
			50	5	25
			56	11	121
			49	4	16
			11	34	1156
			42	3	9
			26	19	361
			21	24	576
			47	2	4
			49	4	16
			71	26	676
			51	6	36
			58	13	169
Mean 36			45		
Variance		66			242

t ±1.5. Degrees of freedom = 17. Probability=20%

Result inconclusive.

Comparison of standard deviations in the temperature rises and small lymphocyte percentage falls.

	Dose (ml./kg.) .2	.06324	.02
Mean temperature response	1.18	1.18	0.93
Standard deviation	0.46	0.41	0.50
Coefficient of variation	39%	35%	54%
Mean small lymphocyte percentage fall	76	59	44
Standard deviation	15	20	24
Coefficient of variation	20%	34%	55%

Total mononuclear percentage falls shown by 19 rabbits in response to four injections each at three dose levels.

Responses to												
.2 ml./kg.				.06324 ml./kg.				.02 ml./kg.				
59	85	82	73	48	36	74	28	33	65	55	18	
81	76	54	67	75	23	27	14	13	68	61	32	
88	81	88	66	65	0	61	50	39	50	45	31	
94	82	78	92	87	53	59	60	84	71	86	14	
45	71	75	54	31	25	46	67	39	53	79	31	
80	85	85	84	84	78	61	42	54	40	84	40	
79	85	80	73	79	69	64	58	77	43	15	54	
83	75	75	40	55	71	51	43	25	-13	-11	46	
73	75	73	85	56	74	46	1	76	26	26	49	
83	89	75	77	79	69	73	30	16	28	8	46	
25	83	61	69	63	47	47	28	25	18	12	27	
91	83	90	84	56	67	79	65	21	29	61	67	
79	70	77	63	66	63	56	59	45	41	73	35	
93	91	72	89	80	77	79	86	70	65	81	68	
80	71	82	71	73	67	89	79	57	38	85	29	
82	63	70	74	71	57	89	81	84	48	65	32	
88	97	86	81	76	74	67	70	37	40	77	29	
91	32	88	62	68	43	71	61	40	21	36	21	
78	62	66	70	63	43	84	64	69	14	31	25	

Mean total mononuclear response to -

.2 ml./kg. = 76
.06324 ml./kg. = 59
.02 ml./kg. = 44 (to nearest unit)

Calculation of variances in total mononuclear percentage falls at the three dose levels.

Deviations at .2 ml./kg.				Deviations at .06324 ml./kg.				Deviations at .02 ml./kg.			
17	9	6	3	11	23	15	31	11	21	11	26
5	0	22	9	16	36	32	45	31	24	17	12
12	5	12	10	6	59	2	9	5	6	1	13
18	6	2	16	28	6	0	1	40	27	42	30
31	5	1	22	28	34	13	8	5	9	35	13
4	9	9	8	25	19	2	17	10	4	40	4
3	9	4	3	20	10	5	1	33	1	29	10
7	1	1	36	4	12	8	16	19	57	55	2
3	1	3	9	3	15	13	58	32	18	18	5
7	13	1	1	20	10	14	29	28	16	36	2
51	7	15	7	4	12	12	31	19	26	32	17
15	7	14	8	3	8	20	6	23	15	17	23
3	6	1	13	7	4	3	0	1	3	29	9
17	15	4	13	21	18	20	27	26	21	37	24
4	5	6	5	14	8	30	20	13	6	41	15
6	13	6	2	12	2	30	22	40	4	21	12
12	21	10	5	17	15	8	11	7	4	33	15
15	44	12	14	9	16	12	2	4	23	8	23
2	14	10	6	4	16	25	5	25	30	13	19
Variance		184				394				551	

Calculation of variances in total mononuclear percentage falls (means per animal) at the three dose levels.

Deviations at .2 ml./kg.	Deviations at .06324 ml./kg.	Deviations at .02 ml./kg.
1	13	1
6	25	0
5	16	3
11	5	20
15	18	7
8	6	11
3	8	3
8	5	32
1	16	0
5	3	19
16	14	24
11	7	1
4	1	5
10	21	27
0	17	8
4	15	13
12	12	2
8	1	14
7	4	9
Standard deviations 9	13	15
Variances 74	175	212

Check by χ^2 method for normality of distribution of total mononuclear responses.

1. Dose = .2 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$(f_o - f_t)$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
25 - 70	19	17	2	4	0.2353
71 - 80	24	19	5	25	1.3158
81 - 97	33	24	9	81	3.3750

χ^2 = 4.9261
Degrees of freedom = 2
Probability = 5 - 10%

2. Dose = .06324 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$f_o - f_t$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
0 - 40	11	14	3	9	0.6429
41 - 50	10	12	2	4	0.3333
51 - 60	11	13	2	4	0.3077
61 - 70	19	14	5	25	1.7857
71 - 89	25	17	8	64	3.7647

χ^2 = 6.8343
Degrees of freedom = 4
Probability = 10 - 20%

3. Dose = .02 ml./kg.

Range of responses of group	observed frequency	theoretical frequency	$f_o - f_t$	$(f_o - f_t)^2$	$\frac{(f_o - f_t)^2}{f_t}$
-13 - 20	11	12	1	1	0.0833
21 - 30	13	10	3	9	0.9000
31 - 40	16	12	4	16	1.3333
41 - 60	14	22	8	64	2.9091
61 - 70	10	9	1	1	0.1111
71 - 86	12	7	5	25	3.5714

χ^2 = 8.9082
Degrees of freedom = 5
Probability = 10 - 20%

Therefore the distributions are not so far from normal that standard statistical calculations are not applicable.

Mean responses per animal.

Temperature response at .2	.06324	.02 ml./kg.	Small lymphocyte response at .2	.06324	.02 ml./kg.	Mononuclear response at .2	.06324	.02 ml./kg.
1.20	0.72	0.87	71	44	42	75	47	43
1.46	0.62	0.63	70	34	45	70	35	44
1.42	1.10	1.05	82	44	42	81	44	41
1.01	0.83	0.41	87	65	66	87	65	64
1.42	1.27	1.07	62	44	50	61	42	51
1.33	1.12	0.87	84	67	56	84	66	55
1.33	1.01	0.71	81	68	49	79	68	47
1.14	1.00	0.50	69	54	11	68	55	12
1.35	1.22	0.76	78	45	42	77	44	44
1.79	1.95	1.80	81	63	26	81	63	25
0.36	0.98	1.19	59	48	21	60	46	20
1.08	1.25	0.71	87	66	47	87	67	45
1.72	1.58	1.08	73	59	49	72	61	49
0.63	1.04	1.12	87	79	71	86	81	71
0.88	1.29	1.07	77	75	51	76	77	52
0.65	1.21	0.46	72	74	58	72	75	57
1.25	1.62	1.43	90	72	45	88	72	46
1.49	1.29	0.90	67	61	29	68	61	30
0.99	1.29	1.06	69	64	35	69	64	35

Calculation of variances in mean temperature rises at the three dose levels.

Deviations at .2 ml./kg.	Deviations at .06324 ml./kg.	Deviations at .02 ml./kg.
.02	.46	.06
.28	.56	.30
.24	.08	.12
.17	.35	.52
.24	.09	.14
.15	.06	.06
.15	.17	.22
.04	.18	.43
.17	.04	.17
.61	.77	.87
.82	.20	.26
.10	.07	.22
.54	.40	.15
.55	.14	.19
.30	.11	.14
.53	.03	.47
.07	.44	.50
.31	.11	.03
.19	.11	.13
Standard deviations	.37	.31	.34
Variances	.1364	.0987	.1169

Calculation of variances in mean small lymphocyte percentage falls at the three dose levels.

Deviations at .2 ml./kg.	.06324 ml./kg.	.02 ml./kg.
5	15	2
6	25	1
6	15	2
11	6	22
14	15	6
8	8	12
5	9	5
7	5	33
2	14	2
5	4	18
17	11	23
11	7	3
3	0	5
11	20	27
1	16	7
4	15	14
14	13	1
9	2	15
7	5	9
Standard deviations	9	13	15
Variances	81	165	220

Comparison of standard deviations in the temperature rises and small lymphocyte percentage falls (mean responses).

	Dose (ml./kg.) .2	.06324	.02
Mean temperature response	1.18	1.18	0.93
Standard deviation	.37	.31	.34
Coefficient of variation	31%	26%	37%
Mean small lymphocyte percentage fall	76	59	44
Standard deviation	9	13	15
Coefficient of variation	12%	22%	34%

Comparison of the variances in the small lymphocyte and total mononuclear percentage falls.

1. Dose level .2 ml./kg.

$$F = \frac{217}{184} = 1.18 = >20\%$$

2. Dose level .06324 ml./kg.

$$F = \frac{404}{394} = 1.03 = >20\%$$

3. Dose level .02 ml./kg.

$$F = \frac{581}{551} = 1.05 = >20\%$$

Therefore there appears to be no significant difference between the variances of the two responses at any one dose level.

Calculation of the chances of false positive results, i.e. chance of the normal fluctuation in small lymphocytes being as great as the fluctuation due to any of the three doses of pyrogen administered.

Fall due to pyrogen -

(a) .2 ml./kg. = 76% = fall of 55 in 72
(b) .06324 ml./kg. = 59% = fall of 42 in 72
(c) .02 ml./kg. = 44% = fall of 32 in 72

Mean of 406 results for normal percentage of small lymphocytes = 72
Standard deviation = 13

Chance of normal counts deviating to this extent -

(a) $\frac{55}{13}$ = greater than tabulated values. Therefore the probability of a normal count deviating to the extent "really" due to .2 ml./kg. is very remote.

(b) $\frac{42}{13}$ = 3.231 ≅ 49.93%. Therefore percentage outwith this is 0.07%. Therefore 0.07% of normal counts may deviate to the extent due to .06324 ml./kg.

(c) $\frac{32}{13}$ = 2.462 ≅ 49.31%. Therefore percentage outwith this is 0.69%. Therefore 0.69% of normal counts may deviate to the extent due to .02 ml./kg.

Comparison of the small lymphocyte percentage falls due to the three dose levels.

Dose (ml./kg.)	Percentage fall in small lymphocytes	Variances
.2	76	217
.06324	59	404
.02	44	581

Comparison of .2 and.06324 groups

t ≐ 2. Degrees of freedom = 150. Probability = 2 - 5%

Comparison of .06324 and .02 groups

t ≐ 1.4. Degrees of freedom = 150. Probability = 1-2%

Therefore the three doses produce significantly different responses. Therefore the small lymphocyte percentage fall is quantitative for a higher dose than the temperature response. This calculation also shows that the dose/response curve deviates significantly from being parallel to the abscissa.

Comparison of the temperature rises due to the three dose levels.

Dose (ml./kg.)	Temperature rises	Variances
.2	1.18	.2157
.06324	1.18	.1699
.02	0.93	.2540

Comparison of .2 and .06324 groups

No difference in response

Comparison of .06324 and .02 groups

t = 3.6. Degrees of freedom = 150. Probability = very remote.

Therefore only the two lower levels produce responses significantly different.

Investigation of the correlation between temperature and small lymphocyte responses, considering mean responses per animal.

Dose = .2 ml./kg.

Dev. from $\bar{X} = x$	x^2	Dev. from $\bar{Y} = y$	y^2	xy
+.02	.0004	-5	25	-0.1
+.28	.0784	-6	36	-1.68
+.24	.0576	+6	36	+1.44
-.17	.0289	+11	121	-1.87
+.24	.0576	-14	196	-3.36
+.15	.0225	+8	64	+1.20
+.15	.0225	+5	25	+0.75
-.04	.0016	-7	49	+0.28
+.17	.0289	+2	4	+0.34
+.61	.3721	+5	25	+3.05
-.82	.6724	-17	289	+13.94
-.10	.0100	+11	121	-1.10
+.54	.2916	-3	9	-1.62
-.55	.3025	+11	121	-6.05
-.30	.0900	+1	1	-.30
-.53	.2809	-4	16	+2.12
+.07	.0049	+14	196	+0.98
+.31	.0961	-9	81	-2.79
-.19	.0361	-7	49	+1.33

$$r = \frac{+6.56}{\sqrt{2.4500 \times 1464}}$$

$$\pm +0.1$$

$$n = 17$$

Therefore there is no correlation.

Investigation of the correlation between temperature and small lymphocyte responses, considering mean responses per animal.

Dose = .06324 ml./kg.

Dev. from $\overline{X} = x$	x^2	Dev. from $\overline{Y} = y$	y^2	xy
-.46	.2116	-15	225	+6.90
-.56	.3136	-25	625	+14.00
-.08	.0064	-15	225	+1.20
-.35	.1225	+6	36	-2.10
+.09	.0081	-15	225	-1.35
-.06	.0036	+8	64	-0.48
-.17	.0289	+9	81	-1.53
-.18	.0324	-5	25	+0.90
+.04	.0016	-14	196	-0.56
+.77	.5929	+4	16	+3.08
-.20	.0400	-11	121	+2.20
+.07	.0049	+7	49	+0.49
+.40	.1600	0	0	0.00
-.14	.0196	+20	400	-2.80
+.11	.0121	+16	256	+1.76
+.03	.0009	+15	225	+0.45
+.44	.1936	+13	169	+5.72
+.11	.0121	+2	4	+0.22
+.11	.0121	+5	25	+0.55

$$r = \frac{+28.65}{\sqrt{1.7769 \times 2967}}$$

$$\doteq +.39$$

$$n = 17$$

Therefore there is no correlation.

Investigation of the correlation between temperature and small lymphocyte responses, considering mean responses per animal.

Dose = .02 ml./kg.

Dev. from $\bar{X} = x$	x^2	Dev. from $\bar{Y} = y$	y^2	xy
-.06	.0036	-2	4	+0.12
-.30	.0900	+1	1	-0.30
+.12	.0144	-2	4	-0.24
-.52	.2704	+22	484	-11.44
+.14	.0196	+6	36	+0.84
-.06	.0036	+12	144	-0.72
-.22	.0484	+5	25	-1.10
-.43	.1849	-33	1089	+14.19
-.17	.0289	-2	4	+0.34
+.87	.7569	-18	324	-15.66
+.26	.0676	-23	529	-5.98
-.22	.0484	+3	9	-0.66
+.15	.0225	+5	25	+0.75
+.19	.0361	+27	729	+5.13
+.14	.0196	+7	49	+0.98
-.47	.2209	+14	196	-6.58
+.50	.2500	+1	1	+0.50
-.03	.0009	-15	225	+0.45
+.13	.0169	-9	81	-1.17

$$r = \frac{-20.55}{\sqrt{2.1036 \times 3959}}$$

$$\triangleq -.23$$

$$n = 17$$

Therefore there is no correlation.

Investigation of the correlation between temperature and small lymphocyte responses, considering individual results.

Dose = .2 ml./kg.

Deviations from $\bar{X}$ = x				Deviations from $\bar{Y}$ = y				xy			
-.05	+.06	0	+.05	-25	+5	+6	-8	+1.25	+0.30	0.00	-0.40
+.08	+.27	+1.10	-.33	+5	+7	-30	-8	+0.40	+1.89	-33.00	+2.64
+.12	+.35	+.45	+.03	+11	+10	+14	-13	+1.32	+3.50	+6.50	-0.39
-.22	-.23	-.05	-.19	+19	+8	+1	+17	-4.18	-1.84	-0.05	-3.23
+.22	+.58	+.22	-.05	-25	-5	-2	-23	-5.50	-2.90	-0.44	+1.15
+.14	+.16	+.31	-.01	+5	+9	+10	+8	+0.70	+1.44	+3.10	-0.08
+.09	+.06	+.09	+.34	+9	+9	+6	-3	+0.81	+0.54	+0.54	-1.02
-.37	+.18	+.03	-.02	+9	-2	0	-36	-3.33	-0.36	0.00	+0.72
-.15	-.24	+.50	+.58	-1	0	-1	+11	+0.15	0.00	-0.50	+6.38
+.73	+.96	+.42	+.33	+7	+13	0	0	+5.11	+12.48	0.00	0.00
-.91	-.40	-.92	-1.07	-59	+9	-13	-7	+53.69	-3.60	+11.96	+7.49
+.06	-.85	+.27	+.13	+15	+6	+14	+9	+0.90	-5.10	+3.78	+1.17
+.92	+.69	+.30	+.26	+2	-4	+2	-14	+1.84	-2.76	+0.60	-3.64
-.71	-1.05	-.90	+.46	+16	+17	-2	+14	-11.36	-17.85	+1.80	+6.44
-.10	-.78	-.37	+.06	+7	-5	+6	-4	-0.70	+3.90	-2.22	-0.24
-.60	-.25	-.74	-.54	+7	-12	-6	-4	-4.20	+3.00	+4.44	+2.16
-.05	-.23	+.23	+.33	+14	+22	+9	+9	-0.70	-5.06	+2.07	+2.97
+.35	+.03	+.54	+.32	+15	-48	+11	-15	+5.25	-1.44	+5.94	-4.80
+.06	-.49	-.30	-.03	+2	-16	-11	-5	+0.12	+7.84	+3.30	+0.15

$$r = \frac{+60.64}{\sqrt{16.1798 \times 16284}}$$

$$\doteq +.12$$

$$n = 74$$

Therefore there is no correlation.

Dose = .06324 ml./kg.

Deviations from $\bar{X}$ = x				Deviations from $\bar{Y}$ = y				xy			
-.29	-.87	-.28	-.42	-10	-26	+12	-35	+2.90	+22.62	-3.36	+14.70
-.32	-.86	-.13	-.92	+15	-39	-32	-44	-4.80	+33.54	+4.16	+40.48
-.09	-.30	+.26	-.21	+8	-60	+1	-9	-0.72	+18.00	+0.26	+1.89
+.11	-.74	-.45	-.32	+29	-5	-2	+3	+ 3.19	+3.70	+0.90	-0.96
+.29	-.25	+.29	+.02	-27	-29	-15	+11	-7.83	+7.25	-4.35	+0.22
+.03	+.11	+.06	-.45	+24	+19	+4	-14	+0.72	+2.09	+0.24	+6.30
-.05	-.02	-.03	-.57	+20	+9	+5	0	-1.00	-0.18	-0.15	0.00
-.11	-.31	+.13	-.44	-5	+12	-10	-18	+0.55	-3.72	-1.30	+7.92
+.17	+.21	-.17	-.05	+2	+15	-14	-58	+0.34	+3.15	+2.38	+2.90
+.57	+.98	+.49	+1.03	+19	+12	+15	-31	+10.83	+11.76	+7.35	-31.93
-.51	+.47	+.20	-.97	+8	-10	-11	-33	-4.08	-4.70	-2.20	+32.01
+.20	+.19	-.19	+.08	-6	+8	+18	+6	-1.20	+1.52	-3.42	+0.48
+.70	-.07	+.78	+.18	+8	-1	-9	+1	+5.60	+0.07	-7.02	+0.18
-.07	-.09	-.54	+.14	+16	+18	+19	+28	-1.12	-1.62	-10.26	+3.92
+.24	+.19	+.01	-.01	+13	+3	+30	+18	+3.12	+0.57	+0.30	-0.18
-.12	-.21	+.62	-.18	+12	-4	+32	+21	-1.44	+0.84	+19.84	-3.78
+.30	+.64	+.64	+.18	+19	+16	+1	+17	+0.57	+10.24	+0.64	+3.06
+.02	+.43	+.32	-.32	+9	-16	+13	+3	+0.18	-6.88	+4.16	-0.96
-.12	+.07	+.26	+.23	+3	-17	+26	+6	-0.36	-1.19	+6.76	+1.38

$$r = \frac{+195.07}{\sqrt{12.7449 \times 30285}}$$

$$\doteq +.31$$

$$n = 74$$

Therefore there is correlation.

Dose = .02 ml./kg.

Deviations from $\bar{X}$ = x				Deviations from $\bar{Y}$ = y				xy			
-.23	+.50	-.13	-.39	-10	+21	+12	-30	+2.30	+10.50	-1.56	+11.70
-.50	-.12	+.04	-.63	-33	+28	+20	-12	+16.50	-3.36	+0.80	+7.56
-.12	+.13	+.48	0	-5	+1	+5	-9	+0.60	+0.13	+2.40	0.00
-.38	-.37	-.19	-1.14	+46	+28	+43	-30	-17.48	-10.36	-8.17	+34.20
+.30	+.43	+.62	-.81	-7	+7	+34	-10	-2.10	+3.01	+21.08	+8.10
+.23	-.22	+.48	-.74	+6	-1	+41	0	+1.38	+0.22	+19.68	0.00
-.20	-.34	-.59	+.23	+36	+1	-29	+11	-7.20	-0.34	+17.11	+2.53
-.18	-.81	-.88	+.13	-17	-60	-58	+3	+3.06	+48.60	+51.04	+0.39
+.04	-.18	-.76	+.21	+34	-22	-17	-2	+1.36	+3.96	+12.92	-0.42
+.75	+.87	+.33	+1.51	-29	-14	-31	+2	-21.75	-12.18	-10.23	+3.02
-.23	+.38	+.80	+.07	-19	-24	-30	-19	+4.37	-9.12	-24.00	-1.33
+.28	-.84	-.26	-.07	-23	-9	+19	+24	-6.44	+7.56	-4.94	-1.68
+.27	-.52	+.28	+.55	0	0	+31	-11	0.00	0.00	+8.68	-6.05
-.43	-.55	+.57	+1.15	+25	+21	+38	+25	-10.75	-11.55	+21.66	+28.75
+.13	+.53	+.02	-.14	+14	-11	+42	-19	+1.82	-5.83	+0.84	+2.66
-.43	-.33	-.23	-.90	+40	+7	+20	-11	-17.20	-2.31	-4.60	+9.90
+.54	+.60	+.76	+.08	-10	-5	+33	-13	-5.40	-3.00	+25.08	-1.04
+.09	-.18	-.06	+.03	-6	-23	-8	-22	-0.54	+4.14	+0.48	-0.66
+.32	+.08	+.15	-.03	+26	-32	-13	-19	+8.32	-2.56	-1.95	+0.57

$$r = \frac{+192.88}{\sqrt{19.0537 \times 43611}}$$

$$\triangleq +.21$$

$$n = 74$$

Therefore there may be slight correlation.

Rises in temperature expressed as percentages of the normal temperature.

.2 ml./kg.				.06324 ml./kg.				.02 ml./kg.			
2.91	3.20	3.03	3.15	2.28	0.79	2.30	1.96	1.81	3.71	2.05	1.39
3.23	3.75	6.00	2.18	2.22	0.82	2.70	0.67	1.10	2.09	2.50	0.77
3.37	4.04	4.26	3.14	2.87	2.30	3.82	2.58	2.12	2.77	3.71	2.48
2.46	2.47	2.96	2.53	3.31	1.12	1.90	2.23	1.42	1.44	1.92	-0.54
3.63	4.64	3.66	2.95	3.82	2.40	3.81	3.12	3.21	3.54	4.03	0.31
3.42	3.48	3.88	3.09	3.16	3.36	3.25	1.92	3.01	1.81	3.67	0.48
3.27	3.24	3.26	3.92	2.93	2.96	3.00	1.59	1.88	1.51	0.87	3.00
2.11	3.52	3.13	3.00	2.77	2.24	3.44	1.95	1.94	0.31	0.13	2.74
2.64	2.38	4.30	4.51	3.45	3.55	2.59	2.91	2.49	1.92	0.44	2.94
4.97	5.69	4.14	3.88	4.55	5.72	4.36	5.82	4.44	4.75	3.33	6.46
0.68	2.03	0.70	0.30	1.74	4.37	3.68	0.55	1.82	3.45	4.62	2.60
3.22	0.85	3.80	3.41	3.61	3.56	2.57	3.30	3.17	0.23	1.77	2.27
5.40	4.89	3.82	3.71	4.87	2.86	5.12	3.52	3.09	1.04	3.14	3.77
1.20	0.33	0.72	4.36	2.82	2.77	1.63	3.38	1.27	0.97	3.74	5.46
2.85	1.04	2.09	3.22	3.70	3.57	3.07	3.04	2.76	3.88	2.48	2.06
1.50	2.44	1.13	1.65	2.69	2.49	4.68	2.55	1.28	1.55	1.81	0.08
2.93	2.44	3.66	3.86	3.75	4.62	4.67	3.49	3.73	3.93	4.43	2.65
3.95	3.14	4.48	3.87	3.07	4.14	3.83	2.20	2.61	1.92	2.27	2.48
3.27	1.79	2.32	3.01	2.82	3.31	3.75	3.70	3.28	2.66	2.82	2.36

Mean percentage rises -

3.07	3.05	2.41

Standard deviations -

1.21	1.08	1.32

Coefficient of variation -

39.4	35.4	54.8

Investigation of correlation between percentage of small lymphocytes before injection and subsequent fall. Dose = .2 ml./kg.

Deviations from mean percentage before injection = x				Deviations from mean fall = y				xy			
-29	-37	-15	-26	-31	-27	-9	-23	+899	+999	+135	+598
-28	-35	-40	-4	-20	-25	-38	-8	+560	+875	+1520	+32
+5	+7	+8	+1	+11	+12	+16	-8	+55	+84	+128	-8
-20	-3	-8	-7	-7	+8	-2	-5	+140	-24	+16	+35
+17	+17	+16	+16	-8	+11	+8	-7	-136	+187	+128	-112
-11	+2	+8	+16	-6	+7	+13	+18	+66	+14	+104	+288
+14	+8	+9	+9	+17	+12	+11	+4	+238	+96	+99	+36
-5	-2	+14	+7	+1	-3	+10	-21	-5	+6	+140	-147
+11	-1	+10	+7	+16	-2	+7	+4	+176	+2	+70	+28
-10	+11	+15	+15	-4	+18	+11	+11	+40	+198	+165	+165
-23	-30	+1	+7	-42	-20	-8	0	+966	+600	-8	0
-7	-9	+5	+3	+3	-4	+13	+8	-21	+36	+65	+24
-3	-6	+21	0	-11	-4	+12	+1	+33	+24	+252	0
+4	+2	+4	-3	+12	+12	+14	-4	+48	+24	+56	+12
-11	-11	+13	-2	-11	-5	+6	+2	+121	+55	+78	-4
+15	+19	+22	+23	+8	+20	+6	+11	+120	+380	+132	+253
+1	-2	-7	+2	+6	+7	+7	+ 7	+6	-14	-49	+14
+10	+11	-21	+15	-4	+19	-37	+20	-40	+209	+777	+300
-8	0	-5	-9	-9	+1	-14	-13	+72	0	+70	+117

$$r = \frac{+12598}{\sqrt{15873 \times 15315}}$$

$$\doteq +.81$$

$$n = 74$$

Therefore there is a high degree of correlation.

Dose = .06324 ml./kg.

Deviations from mean percentage before injection = x				Deviations from mean fall = y				xy			
-7	-7	-25	-37	-11	-21	-10	-34	+77	+147	+250	+1258
-9	-21	-21	-29	+3	-32	-29	-36	-27	+672	+609	+1044
+9	+8	- 7	+2	+11	-43	-4	-6	+99	-344	+28	-12
-2	0	-9	-5	+18	-4	-7	-2	-36	0	+63	+10
+9	+19	+9	+9	-17	-15	-7	+13	-153	-285	-63	+117
-5	+7	+3	+10	+12	+18	+4	-6	-60	+126	+12	-60
+8	+12	+3	-1	+20	+14	+5	-1	+160	+168	+15	+1
+12	-2	+11	+6	+2	+6	-2	-11	+24	-12	-22	-66
-16	-4	-23	-3	-9	+7	-21	-41	+144	-28	+483	+123
+9	+14	+15	+8	+20	+18	+21	-20	+180	+252	+315	-160
-1	+4	+3	-2	+4	-6	-7	-24	-4	-24	-21	+48
-11	0	-10	-4	-11	+5	+4	+1	+121	0	-40	-4
+2	-20	-20	-2	+6	-13	-17	-1	+12	+260	+340	+2
+13	+7	-2	+16	+24	+17	+11	+33	+312	+119	-22	+528
-24	-18	+10	-9	-9	-10	+29	+5	+216	+180	+290	-45
+21	+6	+18	+22	+23	0	+38	+32	+483	0	+684	+704
-16	-7	-18	+9	0	+5	-11	+18	0	-35	+198	+162
+9	+11	-9	+11	+12	-7	+2	+8	+108	-77	-18	+88
+16	+6	+1	+4	+11	-10	+18	+6	+176	-60	+18	+24

$$r = \frac{+9772}{\sqrt{12232 \times 22025}}$$

$$\triangleq +.6$$

$$n = 74$$

Therefore there is a high degree of correlation.

Dose = .02 ml./kg.

Deviations from mean percentage before injection = x				Deviations from mean fall = y				xy			
-11	-1	-25	-12	-11	+14	-6	-23	+121	-14	+150	+276
-26	-10	-5	-26	-26	-12	+16	-3	+676	+120	-80	+78
-9	-15	+5	-8	-7	-6	+6	-9	+63	+90	+30	+72
+1	+1	-24	+6	+33	-21	+2	+35	+33	-21	-48	+210
+12	+13	+15	-2	-1	-3	+12	+22	-12	-39	+180	-44
-4	+17	+4	+11	+2	+6	+32	+5	-8	+102	+128	+55
+15	+16	+13	+9	+37	+8	+15	-19	+555	+128	+195	-171
+8	-8	-26	+11	-10	-41	-37	+7	-80	+328	+962	+77
-7	-2	-4	-15	+18	-16	-13	-8	-126	+32	+52	+120
+4	+21	+2	+10	-20	-4	-22	+6	-80	-84	-44	+60
-9	+12	+1	-1	-16	-15	-21	-14	+144	-180	-21	+14
-22	-13	-3	-4	-21	-11	+11	+14	+462	+143	-33	-56
+7	+5	-1	-6	+3	+2	-8	+17	+21	+10	+8	-102
+10	+9	-2	+7	+24	+20	+16	+32	+240	+180	-32	+224
-6	-19	+14	-15	+6	-14	+41	-17	-36	+266	+574	+255
+9	+19	+20	+19	+35	+14	+27	-2	+315	+266	+540	-38
-3	-8	-1	-3	-8	-7	+22	-10	+24	+56	-22	+30
-2	+15	+10	-2	-5	-13	-2	-16	+10	-195	-20	+32
+9	+3	+15	+5	+24	-22	-5	-12	+216	-66	-75	-60

$$r = \frac{+7136}{\sqrt{10899 \times 24977}}$$

$$\triangleq +.43$$

$$n = 74$$

Therefore there is a high degree of correlation

Investigation of correlation between percentage of small lymphocytes before injection and subsequent fall, considering mean responses per animal.

Dose = .2 ml./kg.

Percentage before injection	Deviations from mean percentage before injection = x	Fall	Deviations from mean fall = y	xy
37	-27	27	-22	+594
37	-27	26	-23	+621
69	+5	57	+8	+40
55	-9	48	-1	+9
81	+17	50	+1	+17
68	+4	57	+8	+32
74	+10	60	+11	+110
68	+4	46	-3	-12
71	+7	55	+6	+42
72	+8	58	+9	+72
53	-11	32	-17	+187
62	-2	54	+5	-10
67	+3	49	0	0
66	+2	58	+9	+18
61	-3	47	-2	+6
84	+20	60	+11	+220
63	-1	56	+7	-7
68	+4	49	0	0
59	-5	40	-9	+45

$$r = \frac{+1984}{\sqrt{2687 \times 2040}}$$

$$\doteq +.85$$

$$n = 17$$

Therefore there is a high degree of correlation

Dose = .06324 ml./kg.

Percentage before injection	Deviations from mean percentage before injection = x	Fall	Deviations from mean fall = y	xy
51	-19	23	-19	+361
50	-20	19	-23	+460
73	+3	32	-10	-30
66	-4	43	+1	-4
82	+12	36	-6	-72
74	+4	49	+7	+28
76	+6	52	+10	+60
77	+7	41	-1	-7
59	-11	26	-16	+176
82	+12	52	+10	+120
71	+1	34	-8	-8
64	-6	42	0	0
60	-10	36	-6	+60
79	+9	63	+21	+189
60	-10	46	+4	-40
87	+17	65	+23	+391
62	-8	45	+3	-24
76	+6	46	+4	+24
77	+7	48	+6	+42

$$r = \frac{+1726}{\sqrt{2052 \times 2680}}$$

$$\pm +.74$$

$$n = 17$$

Therefore there is a high degree of correlation

Dose = .02 ml./kg.

Percentage before injection	Deviations from mean percentage before injection = x	Fall	Deviations from mean fall = y	xy
58	-12	25	-6	+72
53	-17	25	-6	+102
63	-7	27	-4	+28
66	-4	43	+12	-48
80	+10	39	+8	+80
77	+7	42	+11	+77
83	+13	41	+10	+130
66	-4	11	-20	+80
63	-7	26	-5	+35
79	+9	21	-10	-90
71	+1	15	-16	-16
60	-10	29	-2	+20
71	+1	35	+4	+4
76	+6	54	+23	+138
64	-6	35	+4	-24
87	+17	50	+19	+323
66	-4	30	-1	+4
75	+5	22	-9	-45
78	+8	27	-4	-32

$$r = \frac{+828}{\sqrt{1530 \times 2322}}$$
$$\triangleq +.44$$
$$n = 17$$

Therefore there is slight correlation

Investigation of correlation between percentage of small lymphocytes before injection and subsequent percentage fall, considering mean responses per animal.

Dose = .2 ml./kg.

Deviations from mean percentage before injection = x	Deviations from mean percentage fall = y	xy
-27	-5	+135
-27	-6	+162
+5	+6	+30
-9	+11	- 99
+17	-14	-238
+4	+8	+32
+10	+5	+50
+4	-7	-28
+7	+2	+14
+8	+5	+40
-11	-17	+187
-2	+11	-22
+3	-3	-9
+2	+11	+22
-3	+1	-3
+20	-4	-80
-1	+14	-14
+4	-9	-36
-5	-7	+35

$$r = \frac{+178}{\sqrt{2687 \times 1464}}$$

$$= +.1$$

$$n = 17$$

Therefore there is no correlation

Dose = .06324 ml./kg.

Deviations from mean percentage before injection = x	Deviations from mean percentage fall = y	xy
-19	-15	+285
-20	-25	+500
+3	-15	-45
-4	+6	-24
+12	-15	-180
+4	+8	+32
+6	+9	+54
+7	-5	-35
-11	-14	+154
+12	+4	+48
+1	-11	-11
-6	+7	-42
-10	0	0
+9	+20	+180
-10	+16	-160
+17	+15	+255
-8	+13	-104
+6	+2	+12
+7	+5	+35

$$r = \frac{+954}{\sqrt{6088284}}$$
$$= .39$$
$$n = 17$$

Therefore there is very slight correlation.

Dose = .02 ml./kg.

Deviations from mean percentage before injection = x	Deviations from mean percentage fall = y	xy
-12	-2	+24
-17	+1	-17
-7	-2	+14
-4	+22	-88
+10	+6	+60
+7	+12	+84
+13	+5	+65
-4	-33	+132
-7	-2	+14
+9	-18	-162
+1	-23	-23
-10	+3	-30
+1	+5	+5
+6	+27	+162
-6	+7	-42
+17	+14	+238
-4	+1	-4
+5	-15	-75
+8	-9	-72

$$r = \frac{+285}{\sqrt{1530 \times 3959}}$$

$$= +.1$$

$$n = 17$$

Therefore there is no correlation

Comparison of the percentages of small lymphocytes over the six weeks when no pyrogen was injected with the percentages over the twelve weeks when pyrogen was injected weekly.

These comparisons are made only on the rabbits completing the course.

t-tests on the following pairs of results to see if the groups are significantly different -

1. All rabbits in first six weeks compared with all rabbits during the injection weeks.
   t = 7.04. Degrees of freedom = 340. Probability = <.1%
2. Rabbits which had been injected with previous standard and rested for about six weeks before this series of experiments compared with the same rabbits during the injection weeks.
   t = 7.1. Degrees of freedom = 286. Probability < .1%
3. Group of new rabbits compared with the same rabbits during the injection weeks.
   t = 1.46. Degrees of freedom = 52. Probability =10-20%
4. Old group over the first six weeks compared with new group over same period.
   t = 1.57. Degrees of freedom = 112. Probability =10-20%
5. Old group over the injection weeks compared with new group over the same period.
   t = 0.5. Degrees of freedom = 226. Probability = 60%

Therefore the weekly injection of pyrogen appears to affect the normal percentage of small lymphocytes as measured by differential count.

To examine the validity of the United States Pharmacopoeia's exclusion from use in pyrogen tests of rabbits whose normal temperature is > 39.8° or whose temperature on the test morning is < 38.9° and > 39.8°.

Investigation of the responses of these groups.

1. Dose = .2 ml./kg.

Mean rise of group whose normal temperature is < 38.9 = 1.23
Number in group = 59
Variance = .2061
Mean rise of group whose normal temperature is
38.9 - 39.8 = 1.08
Number in group = 16
Variance = .2050
Only one rabbit on one occasion had a normal temperature of > 39.8° (39.97°) therefore this group could not be calculated.

t = 1.15. Degrees of freedom = 73. Therefore probability =20-30% .
This result is inconclusive.

2. Dose = .06324 ml./kg.

Mean rise of low group = 1.21
Number in group = 53
Variance =.1703
Mean rise of middle group = 1.10
Number in group = 23
Variance =.1679
No rabbit had a normal temperature in the high group.

t = 1.1. Degrees of freedom = 74. Therefore probability =20-30% .
This result is inconclusive.

3. Dose = .02 ml./kg.

Mean rise of low group = 1.05
Number in group = 52
Variance =.2246
Mean rise of middle group = .63
Number in group = 23
Variance =.2005
Only one rabbit had a normal temperature in the high group.
t ≥ 3.8. Degrees of freedom = 73. Probability = < .001
Therefore the responses of the groups are significantly different.

To examine the validity of the exclusion, in a proposed pharmacopoeial test, of rabbits whose normal temperature is <38.3° when restrained.

1. Dose = .2 ml./kg.

Mean rise of group of normal temp. < 38.2° = 1.26
Number in group = 14
Variance = .3809
Mean rise of group of normal temp. > 38.2° = 1.18
Number in group = 61
Variance = .1716
t ≐ .47. Degrees of freedom = 73. Probability = 50-60%
Therefore restrained rabbits of temperature < 38.2° need not be excluded.

2. Dose = .06324 ml./kg.

Mean rise of group of normal temp. < 38.2° = 1.26
Number in group = 16
Variance = .2496
Mean rise of group of normal temp. > 38.2° = 1.16
Number in group = 60
Variance = .1509
t = .77. Degrees of freedom = 74. Probability = 40 - 50%
Therefore restrained rabbits of temperature < 38.2° need not be excluded.

3. Dose = .02 ml./kg.

Mean rise of group of normal temp. < 38.2° = 1.34
Number in group = 19
Variance = .2093
Mean rise of group of normal temp. > 38.2° = 0.78
Number in group = 56
Variance = .1856
t = 4.67. Degrees of freedom = 73. Probability = < .001

Therefore at this dose level the responses of the two groups are significantly different.

Equation of line joining lower and middle temperature responses plotted against log.dose.

General equation is $y = mx + c$, where m is the gradient and c is the point of intersection of line and y-axis.

$$m = \frac{\text{difference of y's}}{\text{difference of x's}} = \frac{1.18 - 0.93}{\log.0.06324 - \log.0.02} = .5$$

$c =$

$1.18 = .5 \times -1.1990 + c$

$c = 1.7795$

Or

$.93 = .5 \times -1.6990 + c$

$c = 1.7795$

Therefore equation of log.dose/temperature response line is

$$y = .5x + 1.78$$

Use of this equation to find theoretical response to high dose -
In the equation $y = .5x + 1.78$, if $x = -0.6990$, $y = 1.43$.

This is significantly different from the observed value

($t = 3.34$)

To see if a straight line fits the data from log. dose plotted against small lymphocyte percentage fall.

Data from three lots of 76 results in 19 rabbits -

Dose ml./kg.	Log. dose X	Small lymphocyte percentage fall Y
.02	-1.6990	44
.06324	-1.1990	59
.2	-0.6990	76

1. Sum of squares for every (228) value of Y = 941722
2. Values of Y for every dose level summed, squared, squares summed and divided by the number at each dose level (76) = 851583
3. Every value of Y summed, sum squared and divided by 228 = 812421

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between columns	39162	2	19581
Within columns	90139	225	401
Total	129301	227	

$$F = \frac{19581}{401} \triangleq 48.83 \quad \begin{matrix} n_1 = 2 \\ n_2 = 225 \end{matrix} = < .1\%$$

Therefore between column (i.e. between dose) variance is significant.

Divide the between column sum of squares into that due to linear regression and that due to departure from it.
Sum of squares attributable to regression line is

$$\frac{[\Sigma(Y - \bar{Y})(X - \bar{X})]^2}{\Sigma(X - \bar{X})^2} = \frac{(1219.5)^2}{37.999748} = 39137$$

Total sum of squares attributable to differences between columns = 39162

Therefore sum of squares due to deviations from regression = 25

Source of variance	Sums of squares	Degrees of freedom	Mean squares
Between columns			
Regression	39137	1	39137
Deviation from regression	25	1	25
Within columns (residual)	90139	225	401

Test general significance of the regression line by comparing regression mean square with residual -

$$F = \frac{39137}{401} \triangleq 97 \begin{matrix} n_1 = 1 \\ n_2 = 225 \end{matrix} = <.1\% = \text{highly significant.}$$

Test to see if departure from straight line is significant -

$$F = \frac{25}{401} \triangleq .06 \begin{matrix} n_1 = 1 \\ n_2 = 225 \end{matrix} = >20\%$$

Therefore data may be represented by a straight line .

Calculation of the regression equation -

Best estimate of the regression coefficient of Y on X

$$= \frac{1219.5}{37.999748} = 32 = m$$

Mean of x's $= -1.1990$

Mean of y's $= \frac{13610}{228} = 59.693$

Calculate $y = mx + c$ from this, to find c -

$59.693 = 32 \times -1.1990 + c$

Therefore $c = 98.061$

Therefore equation of the regression line is $y = 32x + 98$.

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THE ASSAY OF BACTERIAL PYROGENS

By M. Dawson and J. P. Todd

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Received July 4, 1952

The methods based on temperature response at present in use in the testing of solutions for pyrogen are useful as limit tests for fever-producing effect, but for estimation of effect involving comparison of preparations which do not differ markedly they are of little value for two reasons—the lack of a stable reference standard and the variation in the temperature responses of the rabbit. This work describes the preparation of a standard and its use in investigating variations in rabbit responses.

The Preparation of a Standard

As a source of standard we chose first *Escherichia coli* because it had been shown to produce pyrogen copiously,[1] to grow well in simple media of known chemical composition and to be relatively non-pathogenic. The pyrogenic supernatant liquid from cultures of this organism was, however, found to be unstable to even mild degrees of heat, whether the liquid was heated at the *p*H value of the growth, 4·7 to 4·9, or whether it was adjusted to *p*H 7 before attempting to concentrate by heating. The results of heating are shown in Table I.

TABLE I

Loss of pyrogenic effect from *Escherichia coli* provisional standard on heating under reduced pressure

Dilution required that response might fall within the quantitative range	Time of heating (minutes)	Temperature ° C.	Average rise in temperature in groups of 5 rabbits	
			Solution before heating	Solution after heating
0·2 per cent.	120	55	0·49	0·37
0·2 „ „	45	50	1·13	0·47
0·2 „ „	20	40	1·19	0·47
0·1 „ „ *	20	40	0·94	0·58

* Solution adjusted to *p*H7 before heating.

The pyrogenic effect also decreased on storage (Table II).

Attempts were made to store this pyrogen in the dry state by adsorbing it on asbestos pads and storing these in a desiccator. Complete adsorption of pyrogen on to a 3·6-cm. asbestos pad took place from 100 ml. of solution of pyrogen at *p*H 4·7 to 4·9, which was the normal *p*H value of a 4-days' growth of *E. coli*. Complete elution took place at *p*H 9 to 12. The dried pad retained the activity but the eluate soon decomposed. This method of storing pyrogen was soon abandoned as it was not convenient to elute the pyrogen and free the solution from asbestos fibres before each experiment. Table III compares the residual activity after storing the pyrogen on the pad and in the eluted form.

TABLE II

LOSS OF PYROGENIC EFFECT FROM *Escherichia coli* PROVISIONAL STANDARD ON STORAGE

Dilution	Period of storage days	Average rise in temperature in groups of 5 rabbits	
		Solution before storage ° C.	Solution after storage ° C.
1·0 per cent.	6	0·65	0·16
0·2 ,, ,,	7	*1·56	0·83
0·2 ,, ,,	12	*1·56	0·35
0·2 ,, ,,	9	0·67	0·14
1·0 ,, ,,	40	0·85	0·38

* Same solution.

TABLE III

COMPARISON OF THE LOSS OF PYROGEN IN THE ADSORBED AND ELUTED STATES ON STORAGE FOR 5 DAYS

Average rise in temperature in groups of 5 rabbits due to		
Pyrogen adsorbed, immediately eluted and immediately injected	Pyrogen stored on a pad for 5 days, eluted and immediately injected	Pyrogen adsorbed, immediately eluted and eluate stored for 5 days
0·77	0·80	0·31
0·95	0·75	0·49

No attempt was made to freeze-dry this preparation on account of its lack of stability.

E. coli was now discarded as a source of pyrogen and a standard prepared from *Proteus vulgaris*. The organism was grown in simple medium and separated from the liquid by continuous, high-speed centrifuge. The liquid was filtered through sterile, unglazed porcelain candles into sterile freeze-drying tubes and spin-freeze-dried. After the secondary drying under vacuum and with phosphorus pentoxide the ampoules were sealed by fusion of the glass and tested for faulty sealing by a high-frequency, glow-discharge tester. No loss of pyrogen occurred in the freeze-drying process and the material suffered no obvious storage loss during the 20 months it was used as the standard for the temperature response experiments. In carrying out these experiments the supply of this standard was exhausted, the amount prepared being limited by the capacity of the freeze-drying unit.

Freeze-drying of eluate from pads in an attempt to prepare a purer standard was not a success, as shown in Table IV. Neutralisation of the eluate before freeze-drying did not prevent loss of pyrogen.

A new standard was prepared from *P. vulgaris*. The culture was centrifuged and the supernatant liquid filtered as before. In order to obtain a purer product the filtrate was dialysed through cellophane to free it from inorganic salts. It was then re-sterilised by filtration and freeze-dried. No pyrogen was lost during drying and no obvious storage loss occurred while this standard was in use for the leucocyte response experiments.

TABLE IV

LOSS OF PYROGEN DURING FREEZE-DRYING OF ELUATE

*p*H value of solution before drying	Average rise in temperature in groups of 5 rabbits	
	Eluate before drying	Eluate dried and reconstituted
9·9	0·86	0·34
10·4	*1·33	0·69
6·7	*1·33	0·75

* Same solution.

RABBIT TEMPERATURE RESPONSE TO PYROGEN STANDARD

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg.

Method. The animals were placed in boxes adjustable for size and held lightly and comfortably in a normal sitting position and the temperatures were read by thermocouple junctions balanced against a junction in a water bath of known temperature, as described by us elsewhere.[1]

When the rabbit basic temperature was reached it was noted and the pyrogenic solution then injected, *via* the marginal ear vein, at 37° C. and diluted to a volume of 2 ml./kg. of body weight. Temperatures were read half an hour after injection and then at 10-minute intervals until they had risen to a peak and had begun to show a definite fall. The rabbits were kept awake throughout the experiment. Each of 25 rabbits was injected 4 times with each of 3 dose levels of pyrogen standard, the doses being 0·2 ml./kg., 0·06324 ml./kg. and 0·02 ml./kg., the middle dose being chosen so that its logarithm was equidistant from that of the other two.

TABLE V

TEMPERATURE INCREASES DUE TO INJECTIONS OF PYROGEN STANDARD

Dose	0·02 ml./kg.	0·06324 ml./kg.	0·2 ml./kg.
Mean of 100 responses	0·90	1·14	1·21
Standard deviation	0·36	0·34	0·39
Mean of 25 mean responses ..	0·90	1·14	1·20
Standard deviation	0·24	0·26	0·30

Results. The temperature increases due to these injections are shown in Table V along with their standard deviations. In every case this is a large fraction of the response and it is questionable whether a test showing a deviation of this magnitude can be regarded as of value except for limit tests, as used in the B.P. It is not sufficiently accurate for systematic work involving comparisons of solutions of approximately the same concentration.

Investigation of Results. Increased response or decreased variance would lessen the error. Seibert[2] showed that the response could not be increased beyond a maximum by further increase in dose and Wylie and Todd[1] found that maximum under the present conditions of experiment to be 1·3° C. For this reason causes of variance were sought in

order to lessen the error by their elimination. The possibilities considered were (*a*) variance within rabbits and variance between rabbits, (*b*) variance due to breed, sex, weight and colour.

(*a*) Analysis of variance of temperature response within and between rabbits showed the latter to be the greater. Comparison of an unknown sample of pyrogen with a standard would therefore be more accurate if carried out on the same rabbits.

(*b*) Dutch and Blue Fox rabbits were the predominating breeds in the population and no difference between the responses of the 2 groups was brought to light by t-tests which showed 30 to 40 per cent. probability of the 2 samples coming from the same population. Similar results were obtained from a comparison of the responses of bucks and does (40 to 50 per cent.) and those of dark-eared rabbits and light-eared rabbits which it was thought might radiate differently (70 to 80 per cent.). Pearson's correlation coefficient was also calculated to see if the following pairs of measurements were related, basic temperature and rise in temperature, weight and basic temperature, and weight and rise in temperature. The results were inconclusive.

Methods of measuring the response other than by simple rise in temperature were now considered. These were the use of only the maximum temperature attained as opposed to the use of the difference between this and the temperature at injection, and a measure taking into account not only the height of the rise but also the time taken to reach it. Emmens[3] says that the measure of the response after test is as useful as a comparison of the before-test and after-test states where the first is variable and the two are correlated. Applying this to pyrogen, the results were noted for the maximum temperature attained after each injection. These results (Table VI) show that the maximum temperature gives no real information and that the basic temperature must be taken into account.

300 graphs were drawn plotting rise against time until the maximum temperature was attained. From this point a perpendicular was dropped to the time axis and the area enclosed measured by planimeter. The average area and its standard deviation for each of the 3 dose levels was calculated and found to be as variable as height of rise alone and therefore of little use in a quantitative assay. The magnitude of the standard deviation in the results of all the above experiments led us to believe that temperature response in the rabbit is not an accurate method to use for the quantitative assay of pyrogen.

TABLE VI

MAXIMUM TEMPERATURES ATTAINED AFTER INJECTION OF PYROGEN STANDARD

Dose ml./kg.	Mean of 100 maxima °C.
0·02	39·44
0·06324	39·72
0·2	39·47

PRELIMINARY REPORT ON RABBIT LEUCOCYTE RESPONSE TO INJECTED PYROGEN

Pyrogen has several pharmacological properties, the main properties being an effect on the white blood cell picture,[4,5,6,7,8,9,10,11] inhibition of thermal panting in dogs,[12] ulcer inhibiting action,[13] an effect on peripheral

circulation,[14] reduction of gastric acid secretion[15] and reactions of tissues to the administration of pyrogen.[16] Of these it was decided that changes in the white cell picture as the basis of a method of assay warranted investigation. No quantitative examination seems to have been carried out on the changes in the relative numbers of the different types of white cells due to pyrogen.

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg. Some had been members of the population used in the first part of this work. Others were new, replacing those whose ear veins had become occluded due to repeated injection.

Standard pyrogen. The standard pyrogen used for these experiments was the dialysed standard previously described.

Methods. Some preliminary work on differential white cell counts was done. This established that (*a*) the error in repeated readings of the same smear was less than the difference between smears from the same rabbit on successive days and that (*b*) this in turn was less than the difference between smears before and after injection of pyrogen. The white cell count did not, of course, fluctuate as rapidly as temperature, and the departure from normal was greatest about 3 hours after injection.

In the main investigation the population of 24 was given 4 injections each, at weekly intervals, of 0·2 ml./kg. of standard. The temperature responses were measured as before and, at the same time, differential white cell counts were made from drops of blood from the marginal ear veins, the cells being stained with Leishman's stain and examined at a magnification of 600. Smears were made before injection and 3 hours after injection. In the differential counts the cells counted were classed as large lymphocytes, small lymphocytes, monocytes, eosinophils, basophils and neutrophils.

The usual number of cells counted in differential white counts is 300. Error may be introduced by the tendency of small lymphocytes to stay at the beginning of the smear or to be drawn along the centre and for granulocytes to be drawn to the end of the smear or to lie along the edges. To avoid this error strips across each end and the middle of the smear were counted and, if by then a total of 300 had not been attained, 2 intermediate strips between the centre and each end were added. This gave various totals of more than 300 for each smear. To make the results more readily comparable, all the individual cell counts were expressed as percentage of the total number counted, thus giving figures for the percentage of large lymphocytes, etc.

Results. Normally small lymphocytes predominate. After injection a fall in the percentage of small lymphocytes and a rise in the percentage of neutrophils occurred. In the other less numerous types there were no significant differences. We considered from the general appearance of the smears that there was probably an absolute as well as the measured relative increase in the neutrophils but the present work is restricted to differential counts and their use as an index of pyrogenic effect. Total counts were not carried out.

The changes in percentage of small lymphocytes were first considered.

The differences between the percentages of small lymphocytes before and after injection were extremely variable from one rabbit to another. It was considered that this was because the percentage before injection was itself a variable. To overcome this difficulty the differences were expressed as percentages of the small lymphocyte percentage before injection. These results are referred to as the percentage falls in small lymphocyte count. The mean percentage fall in small lymphocytes in the 96 responses was 75 per cent. with a standard deviation of 15. When the average for each rabbit was calculated from its 4 results and the 24 averages considered, the mean fall was still 75 per cent. with a smaller standard deviation, in this case 9.

Similar calculations were done for "total mononuclears," i.e., large and small lymphocytes and monocytes. The mean of the 96 percentage falls was 75 per cent. with a standard deviation of 14. The mean of the 24 was 75 and the standard deviation 9. The temperature results obtained at the same time as the white blood cell counts were comparable with those in Table V, the population mean being 1·18° C. and the standard deviation 0·37° C. considering mean rises, 0·45° C. considering individual rises. It was established that there was no correlation between temperature rise and white blood cell change, i.e., a rabbit sensitive to pyrogen by one response was not necessarily sensitive by the other.

Discussion

In this preliminary investigation these figures seem to indicate that small lymphocyte count is a more accurate method of assay of pyrogen than temperature measurement. The standard deviation of the temperature responses is a larger fraction of the response than in the case of white cell responses giving an assay with wider limits of error. Work is in progress on the effect on the differential count of different dose levels of pyrogen and of pyrogen from different organisms.

Summary

1. The preparation of a provisonal standard pyrogen has been described.

2. Temperature rises and white blood cell changes in the rabbit in response to this pyrogen standard have been investigated.

3. A smaller variance was found in white blood cell changes than in temperature rises.

The authors gratefully acknowledge the help of J. C. Eaton, M.A., of the Mathematics Department of Glasgow Royal Technical College for advice on the statistical analysis of the results, of W. H. Martin, B.Sc., D.R.T.C., of the Electrical Engineering Department for advice on the welding of thermocouples, of J. Wallace, B.Sc., M.D., of the Glasgow and West of Scotland Blood Transfusion Service for use of thefreeze-drying unit, of the Trustees of the McCallum Bequest for the provision of a refrigerator, and one of us (M.D.) thanks the Trustees of the Wellcome Pharmaceutical Research Fellowship, during the tenure of which this work was carried out.

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THE LEUCOCYTE RESPONSE IN THE RABBIT TO PYROGEN FROM PROTEUS VULGARIS

PART I * MONONUCLEAR AND TEMPERATURE RESPONSES

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This paper was read at the Symposium on the Assay and Detection of Pyrogens, held by the Pharmaceutical Society and the Society of Public Analysts in University College, London, in December 1953. It is in press in the Journal of Pharmacy and Pharmacology.

The Leucocyte Response in the Rabbit to Pyrogen from Proteus vulgaris. Part I. Mononuclear and Temperature Responses.

By Mary Dawson and J.P.Todd, School of Pharmacy,
The Royal Technical College, Glasgow.

Summary

Rabbit leucocyte response to a freeze-dried pyrogen from Proteus vulgaris was investigated and differential counts were carried out. The relative percentage of small lymphocytes fell, the maximum fall occurring about three hours after injection. The fall was expressed as a percentage of the initial level. At the high dose level injected, the small lymphocyte percentage fall had a coefficient of variation of 20% and the temperature rise a coefficient of 39%. At the two lower dose levels the responses were of equal variability. The small lymphocyte response remained quantitative over the three doses but the temperature response failed to distinguish between the middle and high doses.

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In the quantitative assay of pyrogens only the method based on temperature response has so far been examined in detail, and the results obtained by this method suffer from very wide variation. Attempts to find a less variable method of estimation led us to examine the reported[1-17] effects on the white blood cell picture as a means of controlling the estimation of pyrogen.

These workers injected pyrogenic preparations from various micro-organisms, such as S.typhosa, Ps.aeruginosa and E.coli into different species of animal - the guinea-pig, the rabbit and the dog - and into man. The response

generally noted was a leucopenia about an hour after injection, a temperature rise about one and a half to two hours after injection and then a leucocytosis. Some workers[2,12] reported a shift to the left in the Arneth count. Some[4,17] reported a fall in eosinophils, but various types of leucocyte have been reported to be affected in the leucopenias and leucocytoses and reports on the relative sensitivities of temperature rise and white cell count as responses to pyrogen are conflicting.

In the present work the source, preparation and method of using the pyrogen standard, and the preparation and method of counting smears were as previously described[18].

It was first established that the error involved in counting cells was less than the normal week-to-week fluctuation in count. To do this the variance in repeated counts of the same smear was compared with the variance in counts of different smears from the same animal. This was repeated on different animals until it was established that the differences in variance were significant. It was established that the error involved in counting cells and the normal fluctuation were less than the effect due to the injection of the doses of pyrogen used. It was also established that the time between injection and maximum white cell change in the differential count was about three hours.

Table I shows the fluctuation at weekly intervals of the small lymphocyte percentages of the rabbit population. The mean percentage of small lymphocytes in rabbits not previously used was not significantly different from that of rabbits used in a previous series of experiments involving the injection of pyrogen.

TABLE I

Percentage of small lymphocytes in 25 rabbits counted at weekly intervals.

Rabbit No.	Percentage of small lymphocytes					
1	66	81	96	86	78	88
2	81	46	68	83	74	56
3	82	50	78	83	83	58
4	82	62	80	82	75	73
5	98	73	56	73	95	58
6	75	79	74	97	90	89
7	86	86	88	74	91	85
8	54	69	86	89	77	86
9	81	68	67	77	88	83
10	86	82	84	83	67	72
11	87	94	93	88	75	85
12	84	86	62	80	73	75
13	85	79	74	94	85	90
14	78	66	67	55	93	78
15	82	64	87	45	77	82
16	85	83	86	91	90	85
17	86	71	85	72	67	69
18	79	78	92	85	73	78
19	92	80	79	92	82	56
20	76	90	74	77	92	81
21	87	74	86	94	81	87
* 22	55	85	75	71	66	63
* 23	87	91	76	89	77	83
* 24	69	73	87	80	50	54
* 25	74	80	96	86	77	78

Mean percentage of small lymphocytes = 79

* Rabbits not previously injected

Repeated puncturing of the ear vein at hourly intervals for the removal of blood in itself produced a slight fall in the percentage of small lymphocytes, even without the injection of pyrogen. As was stated the time after injection when the change in white cell count was greatest was about three hours after injection. Cell counts were therefore carried out only twice in each experiment, before injection and three hours after injection.

The fall in the percentage of small lymphocytes after injection was variable. It was observed in some rabbits with a high initial percentage, that the fall was actually greater than the total initial percentage of small lymphocytes for other rabbits. There was however a high degree of correlation between the initial percentage of small lymphocytes present in any animal and the subsequent fall in that animal. For this reason the fall in small lymphocytes was related to the initial percentage by expressing it as a percentage of that figure. This method of expressing the change due to pyrogen gave an answer of smaller variance than that obtained by subtracting the percentage after injection from the percentage before injection.

Although no correlation was found between temperature before injection and subsequent rise, the accuracy of expressing the rise as a percentage of initial temperature was investigated. Thus the temperature response was expressed in terms analogous to those used for small lymphocyte response but no increase in accuracy was found.

These small lymphocyte percentage falls and temperature rises measured simultaneously are recorded in Table II. No significant difference was found in the small lymphocyte responses of new and previously used members of the population.

TABLE II

Small lymphocyte percentage falls and temperature rises.

Rabbit No.	High dose				Middle dose				Low dose.			
1	*1.13	1.24	1.18	1.23	0.69	0.31	0.80	0.76	0.70	1.43	0.80	0.54
	'51	81	82	68	49	33	71	24	34	65	56	14
2	*1.26	1.45	2.28	0.85	0.66	0.32	1.05	0.26	0.43	0.81	0.97	0.30
	'81	83	46	68	74	20	27	15	11	72	64	32
3	*1.30	1.53	1.63	1.21	1.09	0.48	1.44	0.97	0.81	1.06	1.41	0.93
	'87	86	90	83	67	-1	60	50	39	45	49	35
5	*0.96	0.95	1.13	0.99	1.29	0.44	0.73	0.86	0.55	0.56	0.74	-0.21
	'95	84	77	93	88	54	57	62	90	72	87	14
6	*1.40	1.76	1.40	1.13	1.47	0.93	1.47	1.20	1.23	1.36	1.55	0.12
	'51	71	74	53	32	30	44	70	37	51	78	34
7	*1.32	1.34	1.49	1.17	1.21	1.29	1.24	0.73	1.16	0.71	1.41	0.19
	'81	85	86	84	83	78	63	45	50	43	85	44
8	*1.27	1.24	1.27	1.52	1.13	1.16	1.15	0.61	0.73	0.59	0.34	1.16
	'85	85	82	73	79	68	6	29	80	45	15	55
9	*0.81	1.36	1.21	1.16	1.07	0.87	1.31	0.747	0.75	0.12	0.05	1.06
	'85	74	76	40	54	71	49	41	27	-16	-14	47
10	*1.03	0.94	1.68	1.76	1.35	1.39	1.01	1.13	0.97	0.75	0.17	1.14
	'75	76	75	87	61	74	45	1	78	22	27	42
11	*1.91	2.14	1.60	1.91	1.75	2.16	1.67	2.21	1.68	1.80	1.26	2.44
	'83	89	76	76	78	71	74	28	15	30	13	46
12	*0.27	0.78	0.26	0.11	0.67	1.65	1.38	0.41	0.70	1.31	1.73	1 00
	'17	85	63	69	67	49	48	26	25	20	14	25
14	*1.24	0.33	1.45	1.31	1.38	1.37	0.99	1.26	1.21	0.09	0.67	0.86
	'91	82	90	85	53	67	77	65	21	35	63	68
15	*2.10	1.67	1.48	1.44	1.68	1.11	1.96	1.36	1.20	0.41	1.21	1.48
	'78	72	78	62	67	58	50	60	44	44	75	33
16	*0.47	0.13	0.28	1.64	1.11	1.09	0.64	1.32	0.50	0.38	1.50	2.08
	'92	93	74	90	75	77	78	87	69	65	82	69
17	*1.08	0.40	0.81	1.24	1.42	1.37	1.19	1.17	1.06	1.46	0.95	0.79
	'83	71	82	72	72	62	89	77	58	33	86	25
21	*0.58	0.93	0.44	0.64	1.06	0.97	1.80	1.00	0.50	0.60	0.70	0.03
	'83	64	70	72	71	55	91	80	84	51	64	33
22	*1.13	0.95	1.41	1.51	1.48	1.82	1.82	1.56	1.47	1.53	1.69	1.01
	'90	78	85	85	78	75	60	76	34	39	77	31
23	*1.53	1.21	1.74	1.50	1.60	1.41	1.5	0.66	1.02	0.75	0.87	0.96
	'91	28	87	61	68	43	72	62	38	21	36	22
24	*1.24	0.69	0.48	1.15	1.06	1.25	1.44	1.41	1.45	1.01	1.08	0.90
	'78	60	65	71	62	42	85	65	70	12	31	25

*temperature rise in C° 'percentage fall in small lymphocytes

Comparison of the coefficient of variation in the temperature and small lymphocyte responses, Table III, shows that at the high dose level the white cell method is the more accurate. At the other two dose levels the methods are of equal accuracy.

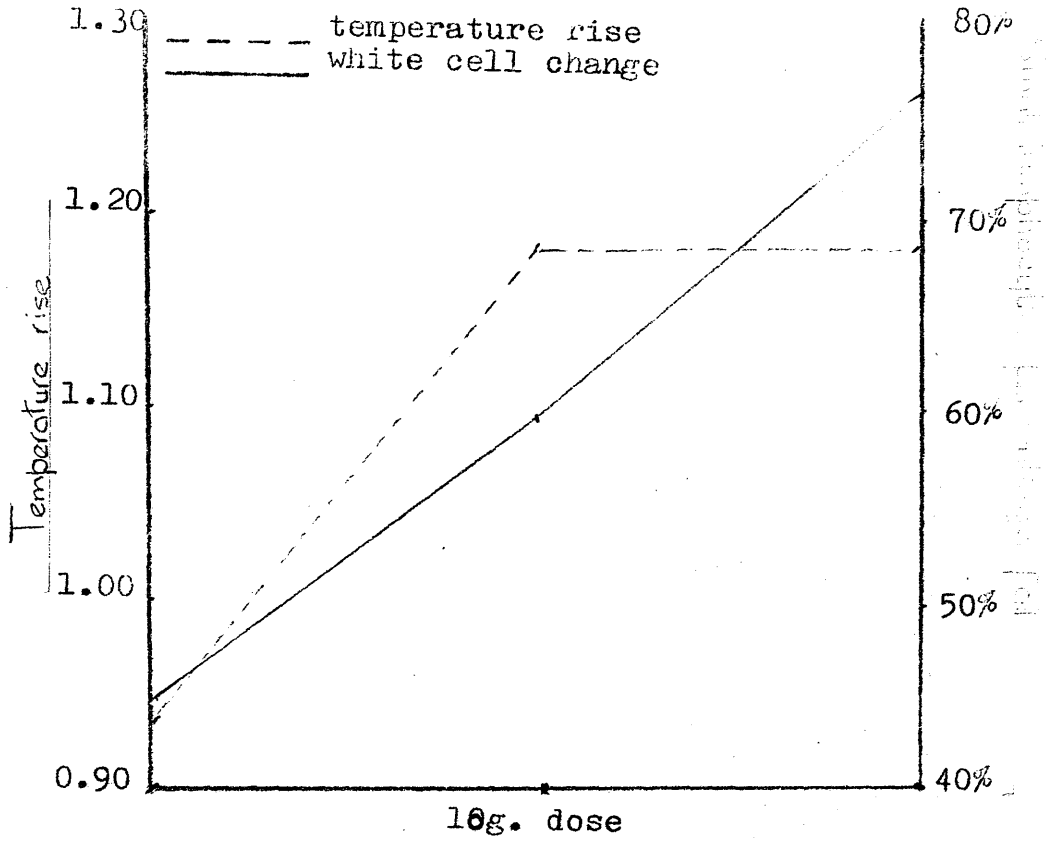
TABLE III

COMPARISON OF COEFFICIENT OF VARIATION IN TEMPERATURE AND SMALL LYMPHOCYTE RESPONSES

	High dose	Middle dose	Low dose
Mean temperature response	1.18	1.18	0.93
Standard deviation	0.46	0.41	0.50
Coefficient of variation	39%	35%	54%
Mean small lymphocyte percentage fall	76	59	44
Standard deviation	15	20	24
Coefficient of variation	20%	34%	55%

It is seen from Fig.I that the white cell method was quantitative over the dose range used whereas the temperature method failed to distinguish between the middle and high doses. Each point on the graph is the mean of the responses to 76 injections. When the responses were calculated in terms of total mononuclears, i.e. large and small lymphocytes and monocytes, the points coincided with those for small lymphocytes. It was established that the three white cell responses were significantly different and that a straight line fitted the data.

FIGURE I


temperature rise
white cell change
1.30
1.20
1.10
1.00
0.90
Temperature rise
80%
70%
60%
50%
40%
10g. dose

Comparison of figures within any one column of Table II shows that rabbits of high sensitivity and of low sensitivity are encountered in both responses. It was established by calculation of Pearson's Correlation Coefficient that, in any one rabbit, the magnitude of the response by one method was not necessarily related to the magnitude of the response by the other method.

Analysis of total variance into between-rabbit and within-rabbit variance components showed that fluctuation of leucocyte response within rabbits could be said to be as great as that between rabbits.

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