A STUDY OF SOME OF THE PROPERTIES OF PYROGEN

A thesis presented by

WILLIAM ANDERSON

for the degree of

Doctor of Philosophy

of

The University of Glasgow

August 1955

CONTENTS

INTRODUCTION

Several authors[1,2,3] have reviewed the early literature dealing with bacterial pyrogen (herein called pyrogen), and have listed the observations of various workers concerning the pyrexia which had long been known to accompany certain diseases. The cause of fevers following intravenous injections has been traced, particular reference being made to the work of Wechselmann[4] and Hort and Penfold[5]. The work of Jona[6] initiated systematic study, chemical and physiological, of the nature of pyrogen, and the researches of Seibert[7,12] which were confirmed by Rademaker[8], finally identified the cause of the fevers which had been associated with the intravenous injection of a great variety of therapeutic materials. Suitable methods were then elaborated by which pyrogen could be eliminated, or prevented from gaining access to the solutions.[9]

Although the therapeutic use of vaccines continued throughout many years, little attempt was made to isolate the therapeutically active material from the complex mixture of substances present in a vaccine, until the work of Co Tui and his colleagues in 1944[10].

Since the older literature has been reviewed on several occasions, it is with a review of the recent literature that the first part of this thesis will be concerned. An account will be given of relevant chemical work which has resulted in the availability of preparations of considerable purity for physiological experiments and clinical investigation. The suspected mode of action of pyrogen will be discussed, and reference will be made to the therapeutic application of pyrogen in a variety of disorders.

The second part of this thesis will consist of a report of experimental work by the author, and it may conveniently be divided into the following sections:

Section 1

Novel methods of preparing purified pyrogen from the supernatant liquid of an inorganic medium culture of Proteus vulgaris.

Section 2

Investigation of a new index of pyrogenic activity, utilising the shift to the left occurring among rabbit neutrophils after the injection of pyrogen, and its comparison with other indices of pyrogenic activity.

Section 3

General applicability of the new index of activity using pyrogen from various sources:

a) Pseudomonas polysaccharide

b) Lipopolysaccharide from Salmonella abortus equi

c) Pyrogen from unknown sources as found in:

i) a commercial sample of Normal Saline, which was found to be pyrogenic

ii) pyrogenic distilled water

Section 4

Further similarities in the biological action of pyrogen from different bacterial sources:

a) The induction of tolerance to pyrogen from 3 bacterial sources and the demonstration of the absence of concurrent precipitin formation

b) The demonstration of cross tolerance in rabbits to pyrogen from 3 different bacterial sources

Section 5

The detection of pyrogen in therapeutic materials which induce hypothermia in the rabbit:

a) Injection of Calcium Gluconate, B.P.

b) ACTH

P A R T 1

A REVIEW OF RECENT LITERATURE DEALING WITH PYROGEN

The Chemistry of Pyrogen

The systematic work which followed the early empirical observations on the nature of pyrogen led to the recognition of its polysaccharide nature; and this provided the impetus which prompted succeeding efforts to isolate preparations of considerable purity.

It will be shown that the activities of the various workers can be channelled into two investigational approaches. Firstly, we have the isolation of pyrogenic material from complex media in which vigorous bacterial growth occurs; or from bacterial bodies inwhich some consider there is a greater abundance of pyrogen; and secondly, isolation from a simple inorganic medium culture of the organism after bacterial filtration. Both of these methods present difficulties which have made the selection of one or other, to a large extent a matter of choice by the various workers.

In the first method, contaminating material occurs in maximal concentration and more elaborate purification is required; in the second, contaminants are minimal and therefore purification need not be so drastic, but it has the disadvantage that very large volumes of liquid have to be handled.

It has not yet been shown whether any structural differences exist between the pyrogens obtained by the by the different methods, but the influence of preparatory method is probably reflected in the apparently conflicting results obtained by different workers. It is well known, for example, that the type of medium used can influence the type of toxin production by toxin producing organisms.[11]

The first serious attempt at isolation of purified pyrogenic material was made by Co Tui et al.[10] Using E. typhosa, they selected alcohol and acetone prepipitation as their method of purification and deproteinisation was effected by phenol.[13] Subsequent investigation showed that the material obtained gave the reactions of a polysaccharide and contained one glucosamine unit for evry 5-6 hexose units.

For the rabbit it was found that 0.06γ/kg satisfied their definition of the Minimum Pyrogenic Dose (MPD)

In the same year, the preparation of pyrogenic material from Triple Vaccine, Ps. pyocyaneus, and P. vulgaris was described[14]. Again, acetone precipitation dialysis, and deproteinisation formed the basis of the method. Analysis of the product confirmed the report of Co Tui et al.[10] that pyrogenic material was largely polysaccharide in nature.

Dare[15] has recently modified this method and has produced pyrogenic material (also of doubtful purity) which he has investigated in humans, as well as rabbits, finding that the minimal effective dose in rabbits was 0.02γ/kg, and the ED_{50} in man was 0.092γ/kg.[16]

Beck and Fisher[17] have investigated the pyrogenic properties of a lipopolysaccharide prepared from Serratia marcescens by Shear et al.[18] who first showed that this material possessed tumour-haemorrhagic properties in small doses(0.1γ). Significant rises in temperature could be obtained with a dose of 0.005γ/kg in rabbits and the lethal dose was about 4000 times the MPD.

In the preparation of this material, in order to lighten the burden of subsequent purification Shear and his colleagues[18] used a simple inorganic medium (+glucose) for the growth of the bacteria; and they found that the yield from such a medium was not less than that from a complex medium.

They used a chloroform precipitation method and found that after shaking 1 volume of filtrate with 4 volumes of chloroform the activity resided in the chloroform-protein emulsion layer which was removed and worked up for pyrogen. Having thus achieved substantial reduction in the bulk of the liquid, the active material was dissolved in water from which it was precipitated by 5 volumes of alcohol.

67% of the material consisted of sugar residues and phospholipid was present as a firmly bound complex. The material was heat stable and had a molecular weight of about 8 millions.[19] This was the first time that the presence of phospholipid had been reported in pyrogenic material.

Ikawa et al.[21] have isolated from E.coli a tumour-necrotising agent which has been shown to be a powerful pyrogen[22] and lipopolysaccharide in nature. Isolation was from both the bacteria and the medium in which they had been grown, the medium used being simple inorganic (with glucose and asparagine). Liquid bulk was reduced by distillation, which was followed by dialysis and electrodialysis, final purification being effected by ethanol fractionation.

The activity resided in the lipopolysaccharide pr tion, and chemical investigation showed that it was possible to isolate from the active agent, sugars and phospholipid which yielded fatty acids on hydrolysis.[23]

The preparation of a pyrogen which has been the subject of extensive clinical trials is reported by Nesset et al.[24], who harvested their pyrogen from the cells of a Pseudomonas species (ATCC No 9229) grown in a complex medium. Their method consisted of the tryptic digestion, dialysis and lyophilisation of a washed cell preparation and the product had an MPD of 0.3γ/kg for rabbits. The presence was confirmed of polysaccharide and reducing sugars and tightly bound nucleic acid - an observation made by others[25] - and the study was extended to include preparations from other Gram-negative organisms[26] in which, in all cases, free lipid was found.

It is agreed [27] that Gram-negative organisms share the property of being the most copious pyrogen producers, and Westohal and his collaborators have developed a method whereby pyrogen in a pure state can be extracted from Gram-negative organisms. Westphal favours the belief that yields are greater when bacterial bodies are used as the source of pyrogen.

An acetone-dried preparation of E.coli which has been grown in a complex medium is extracted with phenol at 65°[28] and on cooling the upper aqueous layer contains the polysaccharide and nucleic acids while the lower layer contains the protein. Dialysis[29] and evaporation of the aqueous layer is followed by precipitation with 10 volumes of alcohol, and the nucleic acids are removed by alcohol fractionation at controlled pH, further purification being effected in the preparative ultracentrifuge to yield a pure polysaccharide pyrogen free from protein and nucleic acid.

Electrophoresis[30] can be usdd in place of ultracentrifugation in the final purification, but a variety of intermediate fractions is obtained resulting in poorer overall yields.

These workers claim that when the rough form of Gram-negative is used the pyrogenically active group is attached to protein instead of polysaccharide.[30]

Fractionation of an acetic acid hydrolysate yielded a lipid-free polysaccharide which possessed only one-thousandth of the activity of the original materail; and it seems from these results that the polysaccharide is inert when stripped ofthe lipid moiety - a conclusion which is not wholly upheld by the subsequent work of others.[36]

Paper chromatography was used in the identification and estimation of the component sugars[32] which comprised about 74% of the lipopolysaccharide (Shear found 67%). Of the remaining 26%, 12-13% could be accounted for by phospholipid, leaving 13% for which the data suggest lipid.

With this lipopolysaccharide pyrogen maximum rises in temperature (2-2.5°C) in rabbits are obtained with 1γ/kg; and the MPD is given as 0.002γ/kg, the lethal dose being at least 10,000 times the MPD.

Investigation of the physical-chemical properties of coli pyrogen[33] showed that it consists of spherical fundamental units having a particle weight of about 1 million, aggregation occurring in solution to give a particle weight of about 20 million.

It is essential to mention the relationship which exists between this work and that reported in connection with the preparation of Gram-negative "endotoxins". Van Heyningen[34] has reviewed this work and draws attention to the phospholipid-polysaccharide complex first described by Boivin, when it appeared to be the complete dominant O-somatic antigen. Morgan[35] later showed, however that the complete O antigen consisted of a phospholipid-polysaccharide-protein complex.

The relationship between the O antigen and the pyrogenic lipopolysaccharide of Westphal is not clear, but degraded polysaccharide and lipopolysaccharide from the O somatic antigen of Shigella dysenteriae have been prepared and studied[36] and the following order of antigenicity and pyrogenicity were found:

Degraded polysaccharide	non-toxic non-antigenic	pyrogenic ($2-5\gamma/kg$)
Undegraded polysaccharide	poorly toxic weakly antigenic	pyrogenic ($0.05\gamma/kg$)
Lipopolysaccharide	toxic Forssman antigen	very pyrogenic ($0.002\gamma/kg$)

Lipopolysaccharide is only weakly active in producing agglutinins and precipitins in the rabbit.

The only difference between the undegraded polysaccharide and the lipopolysaccharide is that the latter contains phospholipid, and pyrogenic activity has been attributed by Westphal[29] to the presence of this phospholipid combined with polysaccharide. This phospholipid is distinguished from the inactive phospholipid which can be removed from the O antigen by formamide. Davies et al.[36] feel however, that this phospholipid may not be the only factor conferring pyrogenicity since the undegraded polysaccharide from which no phospholipid could be isolated was also found to be pyrogenicin rabbits, but they suggest that the lipid portion may also be responsible for conferring on the lipopolysaccharide the property of being a Forssman antigen.

The whole antigen is also pyrogenic, more so indeed than the undegraded polysaccharide,[36] and this fact has probably been responsible for initiating the misconception that pyrogenicity is a property of antigens. It is clear that

a) the whole ahtigen is pyrogenic
b) the lipopolysaccharide pyrogen (which is more strongly pyrogenic than the antigen) is not antigenic.

The Physiology and Pharmacology of Pyrogen

A well established sequence of reactions is recognised as following the introduction of pyrogen into the blood stream of man and animals. It is suspected, however, with good reason, that this sequence is known only in outline. This sequence may be outlined:

intravenous injection of pyrogen

latent period — 30 - 90 minutes in man.[1] No temperature change
— 15 - 30 minutes in rabbits, cats, and dogs.[1,40]
Can be reduced by larger doses.[39]

rise in body temperature

leucopenia — 45 minutes after injection;lasts at least 90 minutes and is roughly proportional to dose (41)

peak temperature 2-3 hours after injection ;may be followed by a second peak in 4-6 hours after injection

leucocytosis polymorphonuclear, with a shift to the left. Lymphopenia and eosinopenia may persist[42]

<u>The latent period</u>

The period of time which elapses between injection of pyrogen and rise in body temperature has suggested that the action of pyrogen is indirect, and that some change in its nature, or some reaction in which it takes part makes a substance available which then assumes the causal role.

Interest in the latent period began with the observation that greater fever with earlier onset and longer duration could be produced in rabbits if pyrogen were incubated, prior to injection,withnormal rabbit serum[43,44] while it had earlier been shown[45] that serum cultures of bacteria yielded about 10 times the amount of pyrogen which could be obtained from an agar culture.

The febrile blood from rabbits given an injection of pyrogen 3 hours previously, displays an abbreviated latency on reinjection[39] and this shortened latency is also observed when blood is incubated with pyrogen and reinjected. It has been suggested that a fast-acting "endogenous pyrogen" is formed in the presence of blood components, and that the endogenous pyrogen is present in the circulating blood from 30 minutes to 3 hours after the pyrogen injection. It is not known whether this ehdogehous pyrogen is modified pyrogen or a blood constituent.

Plasma from pyrogen-tolerant rabbits differs in its ability to raect with pyrogen to form endogenous pyrogen.[46] One of the features of tolerance is a prolonged latency; and the injection of the incubate of pyrogen with normal plasma into refractory animals decreases this protracted latency, whereas on incubation of pyrogen with strongly refractory plasma and subsequent injection, the protracted latent period is not shortened.

The existence of "augmenting substance" which boosts the febrile activity of pyrogen, and of "inhibitors" which postpone or prevent hypothermic responses have been suggested[47] in partial explanation of these findings. It is not fully understood why the augmenting effect should be seen only in vitro, but Farr et al.[47] have suggested that a balance between the concentrations of augmentors and inhibitors is held in vivo and that this balance may be upset by repeated pyrogen injection. resulting in a depression of the formation of endogenous pyrogen.

Tolerance to Pyrogen

Tolerance to pyrogen is evidenced by decrease in height and duration of fever, but repeated pyrogen injections fail to reduce the response below a certain minimum value which varies with the frequency and size of dosage used. Purified pyrogen and pyrogenic vaccines are alike in inducing tolerance to the pyrogenic effect.[48,49]

It has been found that tolerance in rabbits will disappear in about 3 weeks,[50] a conclusion which has been amply confirmed.[52]

Beeson[51] found that it was possible to abolish induced tolerance to pyrogen in the rabbit by blockading the reticulo-endothelial system with colloidal thorium dioxide[51] and these results have been interpreted to indicate that the development of tolerance is evidence of the onset of a functional change in the reticuloendothelial system.

Involvement of the antigen-antibody reaction in classical form, in the development of tolerance becomes less likely as evidence accumulates.[1] It has been suggested, however that the pyrogenically active group is accomodated on some larger carrier-molecule,e.g. polysaccharide.[54] If this is so then it is perhaps possible that the pyrogenic group may be separated in vivo from the carrier molecule which, if it is antigenic per se could proceed to take part in an independent antigen-antibody reaction.

Perry[55] found that the circulating antibody to a pyrogenic preparation from P. vulgaris (its purity was not indicated) does not diminish the febrile response to pyrogen, and concludes that antigenicity cannot be concerned in the phenomenon of tolerance to pyrogen.

Separation of precipitinogen and thermogenic substances (from Ps. aeruginosa) has been claimed[56], while several workers have been unable to relate the levels of serum antibody and tolerance to pyrogen using a preparation which contained both antigen and pyrogen;[48,52,58] and several others have also obtained results which do not indicate the involvement of specific antibody in pyrogen tolerance.[58,60] The slight residual antigenicity demonstrable in Shear's polysaccharyde has been attributed to persistent impurity.[61,62]

It has also been shown that no relationship exists between the pyrogen-producing capacity of E.coli and its possession of capsular, flagellar, or somatic antigen[63]

Rise in Body Temperature

It has been claimed that pyrogen fever is accompanied by increase in oxygen consumption[64] but Rodney and Welcke[25] using pure pyrogen and working with various animal tissues in a Warburg apparatus showed that pyrogen did not stimulate oxygen uptake.

Increased heat production during the chill-phase following the injection of typhoid vaccine has also been reported.[66,67]

Cutaneous vasoconstriction[68,69,70] with reduction in skin temperature[70-73] has consistently been recognised as a causal factor in the febrile response to pyrogen, and this vasoconstriction in the rabbit ear has been shown to be abolished by stellate ganglionectomy.[74]

Working with curarised dogs, Wells and Rall[75,76] found that under such conditions where increased heat production due to increased activity of skeletal muscle cannot occur, pyrogen fever can be produced, and the authors conclude that the mechanism is one of considerable heat loss.

Although the hypothalamus appears to be essential for body temperature regulation[77,78] different groups of workers have been unable to assign a definite role to the hypothalamus in the mechanism of pyrogen fever.[79,80]

In an endeavour to locate the site of action of pyrogen in the central nervous system, Chambers et al.[40] studied the effect of pyrogen on dogs and cats, normal and with various neurological lesions, and concluded that for the production of pyrogen fever which is mediated by the CNS, a functional communication of the spinal cord with one or more centres of the brain was essential; and that an integrating mechanism exists in the medulla oblongata and/or spinal cord which could mediate a pyrogenic fever when connected with the cord below the cervical region.

Stuart[81] holds the view that febrile responses are indicative of central nervous action, whereas other effects which may be elicited by sub-febrile doses of pyrogen are evidence of an effect on target tissues as well as one mediated by the adrenals.

The study of the relationship between cell stimulation or injury and pyrogenic fever has acquired a vast literature. Pyrogenic material has been obtained from sterile exudates during aseptic inflammatory injury[82,83] and the separation of a thermogenic factor from those which produce leucopenia and leucocytosis has been claimed.[84] However, Grant,[85] using Menkin's pyrexin derived from exudates, suggested that this was not the complete explanation of pyrogenic fever.

It has also been claimed that substances causing fever and leucocyte changes similar to those elicited by pyrogen are to be found in normal human urine, and that their concentrationincreases in conditions involving inflammatory processes.[86,87]

Human leucocytes are damaged as a result of in vitro contact with pyrogen,[88] and Westphal et al.[87] claim that their experiments show that pyrogen has a particular affinity for the outer layer of cells, especially erythrocytes and leucocytes, and they conclude that exogenous stimulating materials (of which pyrogen is one) become attached to the cells by means of specific receptors.[89] The specificity may be for the lipid or phospholipid group. This fixation on the cell boundaries with resultant distortion and possibly irritationis postulated to stimulate the liberation of endogenous pyrogen which is then responsible in a humoral manner for initiating the chain of events which follows.

The results obtained by Bennett and Beeson[120] may be connected wity these findings. These workers found that of the extracts of several tissues injected intravenously into rabbits, only an extract of mechanically disrupted granulocytes was effective in stimulating significant rises in temperature.

As well as altering the boundary layer potential of the cells to which it is fixed, pyrogen appears to cause increased phagocytic tendency,[53] and in this connection it is interesting to note that cortisone, which can exert a strong antipyretic action, reduces the stimulating effect of killed typhoid bacilli on the phagocytic activity of the RES.[165]

Evidence of the stimulating effect of pyrogen on the leucopoietic, phagocytic, and reticular elements of the body is found in the work of Windle et al.[91] and also of Staurt[90] who found that the skin lesions in rabbits which had been used to demonstrate the Arthus phenomenon healed more quickly when pyrogen was administered. Others [92] have demonstrated the beneficial effect of pyrogen on the survival rate in thermally injured rats, and it has also been shown to expedite the successful recovery from frostbitten skin.[93]

Characteristic changes are induced in mast cells[166] as a result of exposure to pyrogen and it has been suggested that enzyme systems are involved in connective tissue permeability changes.[81]

<u>The leucocyte response to pyrogen</u>

The alteration in blood picture following the intravenous injection of pyrogen is one of the most consistent effects of pyrogen in man and animals, and several authors[42,87,94] have observed that leucocytosis can still be elicited when the dose of pyrogen is too small to disturb body temperature.

The pattern of the white blood cell response involves a general leucopenia commencing 5 minutes after injection[1] and persisting for about 90 minutes, when it gives way to a polymorphonuclear leucocytosis with a shift to the left which attains a maximum value in rabbits 3 tp 4 hours after injection.[95] The polymorphonuclear leucocytosis is maintained in the presence of a persisting and sometimes increasing lymphopenia and eosinopenia.[42] The leucocytosis abates after 24 hours but the shift to the left requires a few days to accomplish a complete retreat.[95] This sequence has been demonstrated repeatedly in man and animals.[42,81,96-106]

The temperature response is believed to be mediated by the CNS, but it is known[42,94,107,108] that the beneficial effects of pyrogen therapy may be obtained in certain cases with the use of sub-febrile doses which are accompanied by a leucocytosis; and the artificial separation of the temperature and leucocyte responses has been achieved by Westphalet al.[30,110] whose acetylated pyrogen from E.coli is more effective in stimulating the temperature response than the leucocyte response.

The changes which have been observed[110,111,112] in endocrine organs after pyrogen administration have been described by Selye[113] as typical Alarm Reaction changes, and he states that they agree with the assumption that bacterial pyrogens exert their beneficial effect through the production of an Alarm Reaction. It os well known that cortical extracts and ACTH produce white blood cell changes similar to those seen after the injection of pyrogen,[114] and it has been shown[115] thatthere is a significant increase in urinary corticosteroid-like substances after treatment of hypertensives with pyrogen.

The relationship between inflammatory processes and factors which influence the blood picture has already been noted[84,86,87] and several of these factors have been isolated from body fluids[84,86] and from leucocytes themselves,[116] while Westphal et al [30] conclude that the effect of inflammatory processes is under the influence of the pituitary-adrenal system.

The lymphopenia and eosinopenia which follow the injection of bacterial vaccines[1,65,117] and pure pyrogen[1,42] suggest stimulation of the pituitary-adrenal system, and increased production of ACTH has been found to follow the injection of coli vaccine.[118]

Although the leucocyte response in the dog to ACTH and topyrogen are superficially similar, adrenalectomy modifies the response to ACTH but not ot pyrogen; pyrogen administered to adrenalectomised dogs induces a leucocyte response but of milder intensity.[96] In adrenalectomised cats on the other hand, the typical leucocyte response to pyrogen could not be produced,[98] but was restored by the implantation of adrenal pieces.[97]

There is also histological evidence of the participation of the pituitary-adrenal system in the pyrogenic response and this includes reports of cytological changes in the pituitary, adrenals, and other endocrine organs, as well as lymphoid and myeloid organs.[91,110,111,112]

Stuart[81] has noted an increase in plasma ascorbic acid during the leucopenia of the pyrogen reaction, and its subsequent depletion during the leucocytosis. The relation of this change in ascorbic acid distribution to the effect of ACTH, which also causes leucocytosis, on ascorbic acid[167] is not clear.

The connection between the stimulation of the tissue cells by pyrogen and the role of the pituitary-adrenal system in the pyrogenic response may possibly be found in the theory of Sayers and Sayers[117] which suggests that in conditions of stress the demand of the peripheral cells for cortical hormones increases, resulting in a reduction of the blood level of cortical hormones which stimulates ACTH production by the pituitary. The ACTH produced in turn stimulates the adrenal cortex to satisfy the demands of the cells for more cortical hormones.

Therapeutic Applications of Pyrogen

It has been shown repeatedly that the response of the human subject to the injection of pyrogen closely resembles that elicited in the laboratory animal, with the exception that humans appear to be more sensitive.[10,29,121] Why this should be so is not known, but when it is remembered that pyrogen triggers off some sort of chain raection which involves endocrine organs, a mobilisation of body defences, and a combination of other physiological effects, some explanation can perhaps be seen in the reasonable surmise that this process is more highly integrated in human beings and therefore requires less initial stimulation.

The empirical use of vaccines and protein shock therapy in a variety of disorders has been known for many years and favourable results have often been reported. With the purification of pyrogen, however, it has gradually become clear that many of the beneficial effects of the use of these crude materials can be obtained using pure pyrogen preparations in microgram doses, with tye absence of unpleasant complications and side effects seen with these older materials.

A recent significant advance in pyrogen therapy has been the successful use of a Pseudomonas polysaccharide pyrogen ('Piromen', formerly 'Pyromen') in allergy, dermatology and various other conditions in sub-febrile doses.

Some clinicians have used pyrogen in sub-febrile doses, some have used febrile doses and suppressed the fever with antipyretics, and others have used it in conjunction with other forms of therapy where the combination has proved better than either alone. The rationale for the use of pyrogen in so many varied disorders lies in its vigorous nOn-specific stimulation of the body defences.

Allergy

The similarity between the leucocyte response to ACTH cortisone and pyrogen, and the fact that pyrogen appears to stimulate the pituitary-adrenal system, together with the noted beneficial effect of these hormones in allergy, suggested the use of pyrogen in allergy.

Sub-febrile dosage is favoured[42] and the intravenous, intracutaneous, and oral routes have been used.[42,94,107,122] It is important that dosage should be individualised.[125]

An increased sense of well-being and mental alertness often accompanies pyrogen therapy.[42,94,108,124,163]

Dermatology

Febrile[126] and sub-febrile[124] dosage of pyrogen have been used in the successful treatment of certain dermatological conditions. As in the treatment of certain allergic diseases, the best results seem to be obtained when pyrogen is given in conjunction with other more specific or traditional forms of treatment.[127]

Besides being as effective in some cases as ACTH or cortisone, pyrogen therapy has the advantage of not producing endocrine imbalance or withdrawal symptoms which often accompany treatment with these hormones.[128]

Good results have been obtained in the treatment of otitis externa,[127] neurodermatitis,[108] and in other various dermatological conditions.[129,130,131]

<u>Neurology</u>

Little therapeutic application has been made of the experimental results of Windle and his colleagues[132-136,119] in the healing of transected spinal cords without glial barrier formation. One report[137] concerning the treatment of cases of spinal cord injury and disease gives a modest figure of 10% beneficial results and states that dosage and duration of treatment were inadequate and that the results justified further more comprehensive trials.

Successful use has been made of coli vaccine[138] in acute anterior poliomyelitis, and of Pseudomonas polysaccharide ('Piromen') in bulbar and encephalitic poliomyelitis.[139]

Accelerated neuroregeneration of severed nerves of the corneal epithelium has also been achieved.[142]

Some success has also been achieved in the treatment of multiple sclerosis.[140,141]

The use of pyrogen in the treatment of neurosyphilis is well known[141,143,145] and offers advantages over the use of vaccines and cabinet heat in that the dose can be more accurately measured, and the side effects are fewer.

It is not known whether fever in itself effects an increased mobilisation of body defences in addition to that effected by pyrogen (as shown by sub-febrile doses) but Doan[144] has shown that physical heat produces blood cell changes similar to those seen after pyrogen injection, and Selye[115] concludes that the effects have the common denominator of mild stress which stimulates body defence.

Malignant hypertension

Encouraging results, with the reversion of the malignant phase have been obtained in the pyrogen treatment of selected cases of malignant hypertension. Febrile dosage was employed.[146,147,148]

Repair of damaged tissue

It has already been pointed out that pyrogen will stimulate considerable regrowth of damaged tissue, and this has been partly explained by the enhancement of vascularisation in the area, and stimulation of the pituitary-adrenal and reticuloendothelial systems. This, together with the experimental results concerning the inhibition of gastric motility[150] and gastric secretion[151,152] in dogs, and the ulcer inhibiting action of pyrogen in the Shay rat,[149] seems to afford some explanation of the excellent results obtained in the treatment of duodenal ulcers in man.[153,154]

The fibrinolytic action of pyrogen

Vaccines[156] and pyrogen[155] have been observed to possess a fibrinolytic action, and use has been made of this in the lysis of lung nodules in cases of resistant tuberculosis.[157] The organisms liberated after fibrinolysis are then attacked by chemotherapeutic agents.

Other uses of pyrogen in therapeutics have been the detection of foci of infection in which pain is experienced after the injection of pyrogen,[158] the treatment of acute rheumatic disease,[159] inflammatory polyneuritis,[160] diphtheria carriers,[161] and in the treatment of drug-agranulocytosis.[162]

A considerable amount of evidence has accumulated to show that it is possible to stimulate by innocuous means a protective response in the body, which is comparable in vigour to that with which the body responds to insults such as injury, infection, etc.; and the response can be elicited by pyrogen. This was the foundation of non-specific therapy which has been reviewed by Petersen,[164] and it is tempting to speculate that pyrogen or the endogenous pyrogen whose production it is believed to stimulate, is the common active factor in such a diversity of therapeutic materials mentioned by Petersen.

Where the term pyrogen has been used, especially in the chapter dealing with its therapeutic applications, it has been used to include pyrogenic preparations from a variety of bacteria and in various conditions of purity. Sub-febrile therapy with pyrogen has largely been investigated, in America, using a pyrogen which is a Pseudomonas polysaccharide ('Piromen') whose preparation is indicated by Hösset et al.[24] On the other hand, much of the German literature refers to the use of a coli vaccine ('Pyrifer') or to the use of Westphal's lipopolysaccharide pyrogen from Salmonella abortus equi or to acetylated coli lipopolysaccharide.[23-52]

P A R T 2

A REPORT OF EXPERIMENTAL WORK

SECTION 1

Novel methods of preparing purified pyrogen from the supernatant liquid of a largely inorganic medium culture of Proteus vulgaris.

It has already been pointed out that pyrogen may be obtained from the bacterial cell,[28-32] or from the cell-free medium[18-20] in which the bacteria have grown, or it can be obtained from both at once.[21,23] Difficulties attend each method; when bacterial cells are used the subsequent purification must be vigorous and elaborate; when the cell-free supernatant liquid is used a large volume is required if useful quantities of pyrogen are to be obtained.

Clearly then, the cell-free supernatant liquid from an inorganic medium culture will provide a source of pyrogen with a minimum amount of complex material requiring removal, the remaining problem being the reduction in bulk of the liquid since the concentration of pyrogen is very small despite the considerable activity of such a solution.

Shear accomplished this[18-20] using a chloroform precipitation method followed by alcohol precipitation. It has also been found that by adding a solution of benzoic acid in an organic solvent to the aqueous supernatant liquid containing the pyrogen, the pyrogen is brought down with the precipitated benzoic acid from which it is later separated by elution with a volume of water many times smaller than that from which it was obtained.[168]

The experiments reported here were performed with the object of reducing the bulk of liquid in which the pyrogen resides after bacterial filtration of an inorganic medium culture of Proteus vulgaris. Precipitation can then be effected with alcohol and/or acetone in volumes which are more easily handled.

Medium

A simple inorganic medium (+glucose and nicotinic acid) in which Proteus vulgaris will grow luxuriantly was prepared from the following materials:

ammonium phosphate $(NH_4)_2HPO_4$	4.0 G
sodium chloride NaCl	1.0 G
potassium dihydrogen phosphate KH_2PO_4	1.0 G
magnesium sulphate $MgSO_4.7H_2O$	0.7 G
ferrous sulphate $FeSO_4.7H_2O$	trace
d-glucose (dextrose)	10 G
nicotinic acid	2×10^{-5} M
water (apyrogenic) distilled	tol litre

Care has to be taken in the preparation of this medium: the glucose cannot be autoclaved with the salts otherwise a considerable amount of caramelisation will occur and maximum bacterial growth will not occur. All the salts cannot be autoclaved together or a heavy precipitate will be obtained presumably from the magnesium and phosphate in presence of ammonium.

The medium was prepared in 3 litre lots. The magnesium sulphate was autoclaved with the glucose in 275 mls of apyrogenic water, care being taken not to allow the pressure of steam in the autoclave to exceed 10 lbs per square inch, in order to prevent caramelisation of the glucose. When this solution had cooled it was added to the autoclaved solution (about 2700 mls) of the other salts and nicotinic acid which had also ben allowed to cool to about 37°C. The medium thus prepared was then inoculated with about 20 mls of a saline suspension of Proteus vulgaris which had been grown for about 24 hours on an agar slope. Incubation was then allowed to proceed for 4 days at 37°.

Proteus vulgaris was chosen as the source of pyrogen because it produces pyrogen copiously , is relatively non-pathogenic, and grows luxuriantly in a simple medium. A smooth strain was used.

Removal of the bacterial bodies

After 4 days growth the culture was clarified using a high-speed supercentrifuge (Sharples), and sterilised by filtration through Doulton filter candles (bacterial grade) to give a cell-free supernatant liquid, which was dialysed against tap water for 8 hours and restorilised by filtration.

Concentration of pyrogen

A The use of depyrogenising charcoal

It is well known that solutions may be depyrogenised by shaking with activated charcoal.[176,177] An attempt was therefore made to adsorb the pyrogen onto charcoal in order that it might later be eluted from the charcoal by a volume of water smaller than that from which it was adsorbed.

To 1 litre lots of pyrogenic supernatant were added 15 G. depyrogenising charcoal and these were shaken during the following 3 hours after which the charcoal was removed by paper filtration, the filtrate being immediately tested for pyrogenicity. It was found that the use of 15 G charcoal per litre of pyrogen solution successfully removed the pyrogen.

Adsorption was carried out at pH 5 (the reaction of the growth after incubation) and at pH 7 (adjustment made with $\frac{N}{10}$ NaOH), and no advantage was found in adjusting the solution to pH 7 before adsorption.

Several attempts were then made to elute the pyrogen from the charcoal with apyrogenic water and with buffer solutions at pH values ranging from 5 to 9 but in no case

case could pyrogen be detected in any of the eluates obtained. It was therefore decided to abandon this method for it seemed impossible to elute any of the pyrogen from the charcoal onto which it had been adsorbed.

B The use of Seitz filter pads

Several authors have reported that asbestos filter pads will remove pyrogen from solution as filtration proceeds;[3,178] and it has been shown that the adsorbed pyrogen may be elüted from these pads by an alkaline eluting fluid, maximum elution taking place at pH 9-12, but it was reported that the eluted pyrogen rapidly decomposed in contact with the eluate.

An attempt was therefore made to recover the pyrogen before decomposition took place.

A highly pyrogenic solution was produced, clarified, dialysed and sterilised as already described. This pyrogen solution was then sucked through Seitz filter pads (Ford's sterimats, 3.6 cm diameter) and the filtrate was found to be pyrogen free (for details of the method of pyrogen testing see Section 2). It was found that these pads would depyrogenise at least 300 mls of the pyrogen solution.

An alkaline eluting fluid of pH 9 was used to elute the pyrogen from the pads since at this pH a high degree of elution occurs, and although even greater elution occurs at higher pH values, the rate of decomposition of the eluted pyrogen is considerably accelerated.

The eluting fluid used consisted of a solution of sodium phosphate ($Na_2HPO_4.12H_2O$), 30 G in 500 mls which had a pH about 9.2. Solution of NaOH at pH 9 was found to be unsatisfactory and much of the pyrogen could not be recovered, even when the eluates were adjusted immediately to pH 5 or to pH 7. Rises in temperature were of the order of 0.3 - 0.4^o, as compared with rises of about 1.5^o when equivalent volumes of the original pyrogen solution were injected. Using sodium phosphate solution as the eluting fluid, however, it appeared that at least 90% of the activity could be recovered in more concentrated form.

The eluting fluid was left in contact with the Seitz pad through which the pyrogen solution had been passed immediately beforehand, and after about 10 - 15 minutes gentle suction was applied in order to draw the liquid through at the smallest practicable speed. About 100 mls of eluting fluid were passed through each filter pad and the eluate (pH 8.6 - 9.0) was adjusted immedistely to the pH (5) of the original pyrogen solution obtained from the bacterial growth by means of $\frac{N}{2}$ HCl, the final volume of solution of eluted pyrogen being about 140 mls.

A group of 6 filter units were used at one time and typical volumes were:

volume of original pyrogen solution passed through filters	1650 mls
volume of eluting fluid	500 mls
volume of eluate after adjustment to pH 5	700 mls

It is seen that this operation has accomplished a two-fold concentration of the pyrogen solution: the pyrogenic activity of the adjusted eluate was in most cases double that of the original solution (volume for volume).

To the eluate (pH 5) 5 volumes of alcohol were added and the precipitaet was allowed to settle in the refrigerator overnight (crystals of sodium acetate were added to complete precipitation). On centrifugation the cloudiness resolved into another more viscous liquid phase together with a small amount of solid material. If, instead of centrifuging on the following morning, the precipitate were left for several days large crystals formed which appeared on testing to be sodium phosphate. It was found, however, that these crystals on removal and solution in apyrogenic water displayed pyrogenic activity. This can only be explained by assuming that it was difficult to free them from small amounts of pyrogen precipitated at the same time.

The viscous liquid and the solid material precipitatde were taken up in apyrogenic water and dialysed for 4 - 5 days first against tap water (1 day) and then against distilled water for 3 - 4 days at about 2 - 4°C.

The contents of the dialysis sac were then added to 10 volumes of acetone (+ 0.4% glacial acetic acid) and the precipitate which was allowed to settle overnight in the refrigerator was collected and dried in vac over P_2O_5.

The MPD of this material was found to be 0.5γ/kg (this gave a mean rise in temperature in rabbits of 0.63°) and spectrophotometric examination showed the absence of nucleic acid; acid hydrolysis liberated reducing sugar.

It appears that it is better to elute the pyrogen by sucking the eluting fluid through the asbestos pad rather than soaking the pad whole or broken in the eluting fluid as has been done by others.[3] It also appears to be essential to adjust the eluate immediately to a slightly acid reaction. In this manner tne pyrogenic activity can be preserved.

C Reduction of liquid bulk by freeze-drying

It is known that the dialysed supernatant liquid from an inorganic medium culture of Proteus vulgaris can be freeze-dried without loss of potency[3] and this seemed to offer a suitable method whereby the liquid bulk could suitably be reduced before precipitating with alcohol or acetone.

The apparatus available for freeze-drying had a maximum capacity of 600 mls.. The pyrogen solution was prepared as before from a growth of Proteus vulgaris and 600 mls were divided into 60 x 10ml freeze-drying ampoules and spin-freeze-dried overnight. Only the primary drying process was carried out because this removes about 98% of the water.

In a typical experiment the following working conditions prevailed in the various parts of the apparatus:-

refrigerator tempertaure	-45°C
pressure in drying chamber	0.1 mm Hg.
temperature in drying chamber	20°C rising overnight due heat of pump to 30 °C
time required for removal of water	24 hours

Freez-drying provides an exceedingly gentle method of reducing the bulk of the liquid under aseptic conditions.

The gummy contents of the tubes after drying were redissolved in water, about 30 mls sufficing to redissolve the product from 600 mls of original pyrogen solution. A straw-coloured solution with a characteristic odour was always obtained and increased viscosity was particularly noticeable. This solution was then dialysed for 1 day against tap water (12°C at that time) followed by 3 days dialysis against frequently changed distilled water in the refrigerator (2 - 4 °C). The solid material which came down in the sac during dialysis was found to be non-pyrogenic and was discarded

The liquid from the dialysis sacs was again freez-dried, the resulting material this time being a fawn-coloured powder which was dried in vac over sulphuric acid. About 1.3 mgm of this material was obtained from 600 mls of the original pyrogen solution (from the bacterial growth) and had the following order of activity:

dose	average maximal rise in temperature in rabbits
1γ/kg	0.92°C
1.86γ/kg	1.60°C

In an endeavour to obtain material with even greater activity this product was dissolved in apyrogenic water and reprecipitated 3 times using 10 volumes acetone (+ 0.4% glacial acetic acid to coagulate the precipitate), the precipitate being allowed to come down overnight in the refrigerator.

Precipitates were bulked from several similar operations, dried in vac over sulphuric acid, and were found to possess activity of the following order;

dose	average maximal rise in temperature in rabbits
0.2γ/kg	1.16°C 0.95°C

The MPD (that dose giving a mean temperature rise in rabbits of 0.6°C) was found to be 0.04γ/kg.

The temperature response was accompanied by a vigorous leucocyte response, involving a leucocytosis with a shift to the left, and a decrease in the relative percentage of small lymphocytes.

By way of comparison it was later found (see Sections 2 and 3 of this thesis) that lipopolysaccharide from Salmonella abortus equi[28-32] will give an average maximal rise in temperature in rabbits of 1.28°C with a dose of 0.1γ/kg and that Pseudomonas polysaccharide[24] ('Piromen') will, with a dose of 10γ/kg induce a rise in temperature in rabbits of 1.23°C.

Section 2

The investigation of a new index of pyrogenic activity utilising the degree of shift to the left occurring among rabbit neutrophils after the injection of pyrogen, and its comparison with other indices of pyrogenic activity.

Disadvantages which attend the measurement of pyrogenic activity by computing the average maximum rise in rectal temperature (referred to in this thesis as the "temperature response") in rabbits, have been noted by several authors.[3,41,106]

The main disadvantages are:

a) Difficulty in measuring the temperature of the rabbit: either an elaborate system of thermocouples has to be set up and maintained, or the clinical thermometer (adapted) has to be contended with (by the rabbit no less than by the experimenter).

b) The laboratory has to be maintained at a fairly even temperature, and this is not easy in the average laboratory. Humidity is even more difficult to control.

c) The accuracy of the temperature response as reported by various workers seems to vary widely with the conditions employed.

d) The temperature of the rabbit is easily upset

The persistent lack of a chemical method of assay of pyrogen, and the absence of hope for the elaboration of one in the near future, dictate the use at present of biological methods.

As the evidence accumulates , it becomes more apparent that the effect of pyrogen on the white blood cells is more direct and more closely related to its clinical efficacy than its ability to disturb the normal temperature.

Perhaps none of the pharmacological effects of pyrogen could be said to be caused by pyrogen and by no other substance. Indeed its use in medicine is due almost entirely to its non-specific stimulating action. But the fact that other substances can also produce one or other of the effects of pyrogen in the normal animal does not preclude the use of that effect as the basis of an assay, provided that is, that the experiments are so planned that,a) during the experiment the effect is being caused only by pyrogen and is quantitative, and b) that there is a successful statistical analysis of the results. This must be so if biological assay is to be acceptable at all.

The physiological responses to pyrogen which have been deemed suitable for quantitative assessment in a simple and useful way are the temperature and the white blood cell responses. The former has been investigated using several approaches (average maximal rise in temperature, use of various instruments for measuring temper ature, and a fever index taking into account the height and duration of fever) in several species of animal. The latter (white blood cell responses) have been examined somewhat less extensively; total white counts measuring the leucopenia[41] and leucocytosis[179,169] in rabbits, differential counts, particularly small lymphocyte percentages[3] in rabbits, and total and differential counts in the dog.[106]

The leucocytosis which follows the injection of pyrogen is due to the addition to the circulation of young neutrophils, and the leucocytosis is therefore accompanied by a shift to the left. An extensive search of the literature has revealed no report of the measurement of the degree of shift to the left which occurs in response to pyrogen injection.

This section is the report of an investigation by the author into the possibility of measuring the degree of shift to the left which occurs among rabbit neutrophils 3 - 4 hours after the injection of pyrogen and of using it as an index of pyrogenic activity.

While investigating quantitatively this effect of pyrogen, temperature responses were measured concurrently. Differential counts were also performed on the blood smears, in order to calculate for comparative purposes, the values for the small lymphocyte percentage fall, an index of activity previously suggested.[3]

Method

The standard pyrogen:

A standard pyrogen is required for an investigation of this type, and in this case it was prepared from a smooth strain of Proteus vulgaris known to produce pyrogen copiously. Pyrogen preparations from this organism have been given to rabbits over prolonged periods without having any permanent effect on them.

The standard pyrogen consisted of a freeze-dried preparation of the supernatant liquid, dialysed and sterilised, from an inorganic (+glucose and nicotinic acid) medium culture of the organism.

The pyrogen preparation described in Section 1 was not used because enough could not be prepared in one batch to provide material for all the experiments we had in mind and it was thought advisable to use one batch of standard for all the experiments.

The preparation and inoculation of the medium, and clarification, sterilisation (by candle filtration), dialysis and re-sterilisation of the supernatant were carried out as described in Section 1. The sterile supernatant liquid containing the pyrogen was then distributed aseptically in 2.5 ml quantities into freeze-drying tubes and spin-freeze-dried. Both primary and secondary drying was carried out.

The conditions under which this batch was dried were:

primary drying:

refrigerator temperature: -47°C

pressure in drying chamber: 0.1 mmHg.

temperature in drying chamber: 18°C rising overnight to 27°C.

time of drying: 20 hours

secondary drying:

pressure in drying chamber: 0.005 - 0.01 mmHg.

The ampoules were distributed on three "headers" and when the ampoules of one header are being sealed it is cut off from the other two by means of an air-tight tap. The prĕssure in each header as the ampoules were sealed was 0.003 mmHg.

The seal of each ampoule was tested with a high-frequency glow-discharge tester. Other details are given in the handbook of the maufacturers of the machine (Edwards and Co.).

There are several reasons why this standard pyrogen was chosen for this investigation:-

a) it is simple to prepare and is stable over useful periods of time

b) it is reproducible

c) it may be prepared from one culture which helps to ensure even distribution of the active material which is dried directly from a sterile solution

d) the simplicity of the preparatory method avoids any degradation of the active material.

e) the absence of complex molecules from the medium diminishes the possibility of production of pyrogenic artefacts, i.e. the attachment of the pyrogenically active grouping to other"carrier" molecules.

Absolute purity of the standard cannot be claimed, but it is difficulttosee how the concentration of residual impurity in a preparation harvested thus from a largely inorganic medium, freed from bacteria bodies, could exceed to any serious extent the residual impurity in preparations derived from whole bacteria. It will be pointed out later why it is concluded that whatever persistent impurities remain do not interfere with the results.

The choice of the standard was also influenced by the fact that the effect of pyrogen on the relative percentage of small lymphocytes was investigated originally,[3] using a similar standard, hence the extent of comparison of the results possible would be increased by the use of this standard.

The experimental animals:

30 previously unused rabbits of both sexes were employed in the investigation and were divided into 6 groups of 5.

Before commencing the experiments the rabbits were thoroughly conditioned to the procedure to be adopted in the tests, for it has been observed that rabbits which have been so conditioned are more cooperative during their first two or three tests than rabbits unaccustomed to the test routine. Unaccustomed rabbits are easily upset and occasionally take fright during the first few periods in the test boxes, giving unexpected and unsatisfactory results.

The rabbits are conditioned beforehand to the procedure by being made to sit lightly harnessed in the test boxes for 4 hours at a time with rectal thermocouples inserted. It was found that this approximately constant degree of stress to which they were subjected did not deflect the blood count or upset the temperature of the rabbits beyond normal limits. Besides, this method where each rabbit is accomodated in a specially designed box, provides maximum comfort for the animals and combines with that essential, minimum opportunity for unnecessary and undesirable movement.

Dosage:

As a result of preliminary experiments to determine the potency of the standard pyrogen preparation, four dose-levels were chosen:

table 1

Dose-levels of standard pyrogen used in the investigation of the polynuclear count as the basis of an index of pyrogenic activity

	Dose		Log Dose
A	0.02	ml/kg	$\bar{2}.3010$
B	0.06324	ml/kg	$\bar{2}.8010$
C	0.1125	ml/kg	$\bar{1}.0510$
D	0.2	ml/kg	$\bar{1}.3010$

It will be seen that log B is equidistant from log A and log D and that log C is equidistant from log B and log D. D was chosen because it elicited temperature and white blood cell responses equivalent to about 75% of the maximal responses which in the writer's experience can be elicited with this pyrogen preparation under these conditions of experiment.

The contents of each ampoule of freez-dried materia (from 2.5 mls original supernatant) were dissolved in saline (apyrogenic) immediately prior to injection, the dilution being such that each dose of original supernatant (0.2 ml/kg etc.) was contained in 2 mls of solution, so that no matter what the dose of pyrogen, each animal received the same volume of solution on each occasion (2 mls/kg). Giving the same volume on each injection cancels any possible effect of administration of different volumes. The injections were warmed to about 37°C before administration.

All the rabbits received one injection per week over 12 weeks, each animal receiving each of the 4 dose-levels on 3 occasions.

The test:

Temperatures were recorded essentially by the method originally developed by Wylie and Todd.[27] The design of the test boxes and thermocouples have been described Wylie.[2] The same apparatus was used with only minor alterations and refinements (e.g. increase in the number of thermocouples).

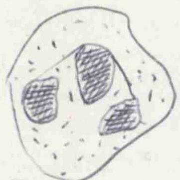
The rabbits were not fed on the day prior to the test, i.e. about 36 hours elapsed between the last meal and the test, and any food remaining in the feeding dishes was removed on the day prior to the test. This appears to decrease the amount of defaecation occurring during the test, and as rectal temperature is recorded, it helps to decrease the inaccuracy caused by partial or complete expulsion of the thermocouple during defaecation.

15 rabbits (3 groups of 5) were used in a test, this being the largest number that one experimenter can handle successfully at one time. They were placed in the test boxes and the thermocouples were inserted by 8.15 a.m. At 9.30a.m. by which time the rabbits were settled down the pre-injection temperatures were recorded, and the pre-injection blood smears made. The injections were usually completed by 10.30 a.m. and temperatures were recorded continuously (10 minute intervals) until about 2 p.m. when the post-injection smears were made.

The index of activity:

Polynuclear counts were performed and the grouping suggested by Cooke and Ponder[174] was adopted. This modification of Arneth's count (The Polynuclear Count) leads to a rapid and sensitive assessment of the overall lobar picture of the nuclei of the neutrophils. These authors suggest that lobes are joined by chromatin filaments only, so that two chromatin filaments would join 3 lobes for example; bands of nuclear material thicker than chromatin filaments are for the purposes of the count, not deemed to connect lobes, but to be part of the lobe formed by the two larger masses of nuclear material.

Thus:

3 lobes 

1 lobe 

100 neutrophils are counted and classified into 1-lobed, 2-lobed, and so on, and the totals for each class are counted; thence by multiplying the number of cells in each class by the number of lobes in a cell of that class, the total number of lobes in 100 neutrophils is found, and division of this number by the number of cells counted (100) will give the average number of lobes per neutrophil. Cooke and Ponder called this average the "Weighted Mean".

The response to the injection of pyrogen is calculated by measuring:

i the average number of lobes per neutrophil before injection of pyrogen = a

ii the average number of lobes per neutrophil 3 - 4 hours after injection of pyrogen = b

Then $\frac{a - b}{a} \times 100$ = percentage fall in the average number of lobes per neutrophil, which measures the degree of shift to the left.

Preliminary experiments showed that the maximal left-handed deflection of the average number of lobes per netrophil could be expected to occur between 3 and 4 hours after the injection of pyrogen. Then after a period of instability, the count commenced an upward return to normal and attained a steady pre-injection level about 4 - 6 days later. This episode is illustrated in fig.1 (page 52).

The end-point in the experiment is therefore the value obtained for the average number of lobes per neutrophil (degree of shift to the left) from a blood smear (from each rabbit) taken about 3½ hours after injection.

When a double-peaked temperature response occurred care was taken to wait until after the second peak had passed before removing the sample of blood, otherwise the smear was liable to display rather a scarcity of white cells (about 200 or less in one smear)-which made counting tedious and difficult- as opposed to 60 - 80 per strip of smear in one taken after the final defervescence had set in. When a biphasic

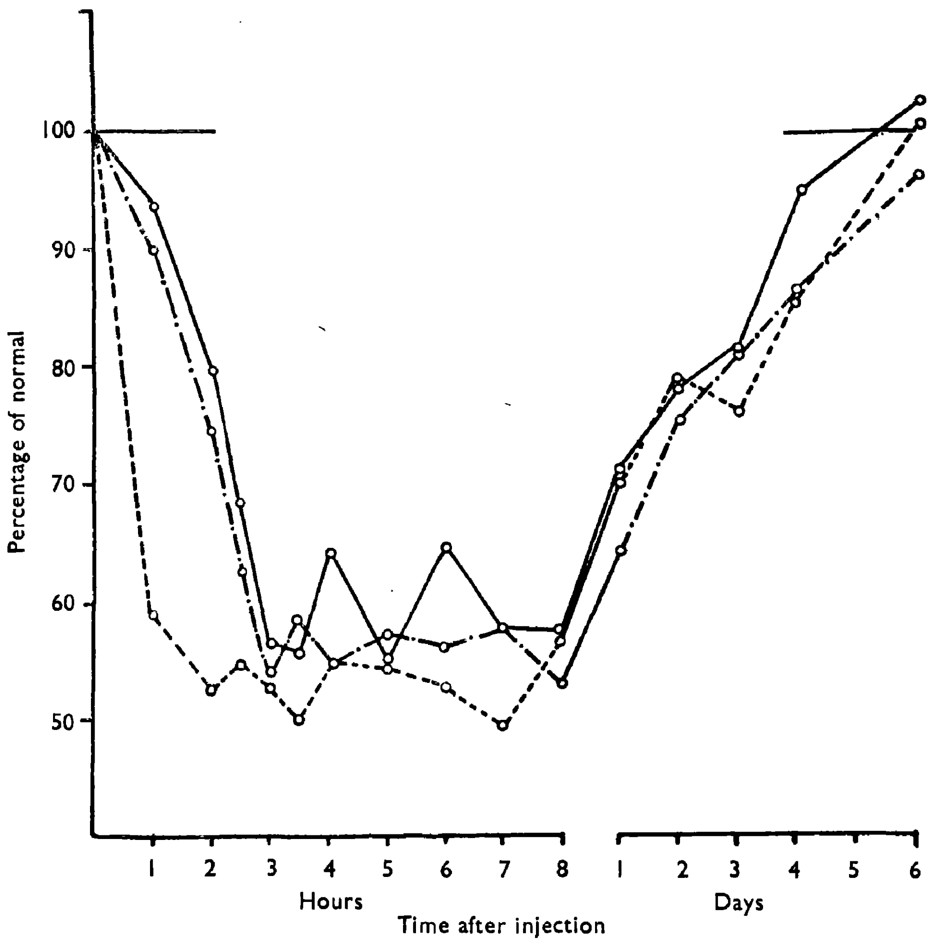


FIG. 1. Graph showing depression in average number of lobes per neutrophil after injection of pyrogen (0·2 ml./kg.) and the course of return to normality.[95]

O——O Rabbit number 1
O–·–O ,, ,, 4
O - - O ,, ,, 5

temperature response did occur, the second peak usually appeared about 3 hours after injection.

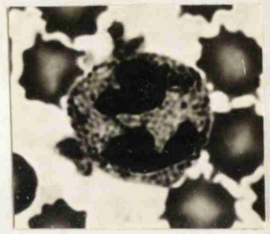
The blood smears:

Blood smears were made on alcohol-cleaned microscope slides from a drop of blood obtained from the marginal ear vein and spread evenly, care being taken to prevent the blood reaching the edges ofthe slide. The post-injection smears were taken from the ear other than that into which the injection had been given.

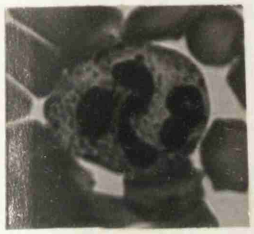
Fixing with methyl alcohol was carried out as soon as the smeared blood had dried, and the methyl alcohol was allowed to remain on the slide for at least 3 minutes, and in most cases it was there for about 10 minutes, over-fixing being almost impossible. This allowed 15 smears to be taken within about 15 - 20 minutes. The remaining methyl alcohol was shaken off and the slides covered with a 1 in 10 aqueous dilution of Giemsa's stain (prepared by the British Drug Houses Ltd), and staining was allowed to proceed for about 30 - 45 minutes. On completion of the staining process, the excess stain was washed off by flooding the slide several times with tap water, and the smears were allowed to dry.

The smears were examined using an oil immersion lens and the lobar configuration of the majority of the neutrophils could be elucidated with a magnification of 600. Amagnification of 1000 was occasionally needed.

1 lobe
X 1000

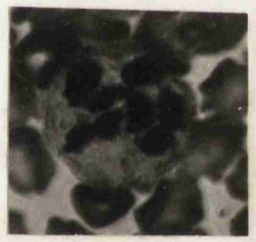


2 lobes
X 1000



3 lobes
X 1000

4 lobes
X 600



5 lobes
X 1000

Figure 2

Showing the types of neutrophil enumerated, classified according to their lobar configuration

Giemsa's Stain

Photographs of examples of the different types of neutrophil counted appear in figure 2 on page 54. Neutrophils with more than 5 lobes were not encountered.

Polynuclear and differential counts were performed on each smear. In the performance of the polynuclear count the 100 neutrophils counted were found round the edges and at the end of the smear, where many of the neutrophils accumulate during spreading. In a good thin smear about 100 neutrophils are encountered in one journey round the edges and tail-end of the smear. No special distribution of the different types of neutrophil was detected, i.e. it was not possible to say for instance that 4-lobed or 5-lobed neutrophils could more readily be found in one part of the smear and 1-lobed and 2-lobed neutrophils in another.

In the performance of the small lymphocyte count however, care must be taken to allow for the distribution of lymphocytes and granulocytes; lymphocytes predominate at the beginning and in the centre of the smear; granulocytes predominate at the edges and at the tail-end. Dawson[3] counted a strip at the beginning, a strip at the tail-end, and if by that time 300 cells had not been counted and differentiated, another strip in the centre of the smear. This leads to high figures for the small lymphocytes for the reason that there are more cells in a strip near the beginning of the smear where small lymphocytes tend to predominate, than near the end of the smear where granulocytes tend to predominate. Hence counting strips symmetrically on the smear irrespective of the number counted in the strips at the different positions on the smear, in the manner recommended by Dawson, leads to the counting of most of the 300 cells (the usualnumber for differential counts) in positions on the smear where small lymphocytes predominate.

The effect of this modification can be seen in the difference between the figure given by Dawson[3] (79%) for the normal percentage of small lymphocytes, and that found in the present series of experiments (60%) - a difference of nearly 20%.

The literature contains few references to the normal percentage of small lymphocytes in the rabbit, and the figures which are given suffer from considerable variation which seems to depend on the method of counting employed.

Andrewes[182] gave 50 - 70% as the normal value; Wintrobe[183] quotes Scarborough as giving 41.8% and recommends the coverslip method. MacGregor et al.[109] in an excellent review of the techique of the differential count (unfortunately their examples were from human blood but that does not affect the principles) conclude that the coverslip method of preparation of the smears yields results for the percentage of small lymphocytes which may be up to 15% lower than cross-sectional counting of smears made on a microscope slide.

The important consideration in an investigation such as this, however, is that the cells should be counted and the smears prepared in the same manner every time. Then, provided a reasonably small error is obtained in the normal count (in the present case the standard deviation was found to be 9.6) consistent results should be obtained.

The small lymphocyte percentage fall[3] was calculated from the smears taken during the investigation and the results have been compared with those of Dawson[3] (see later)

Normal values:

These were worked out prior to the start of the investigation and are recorded in detail on pages 135-139 and are summarised in table 2 on page 58.

It was found that some animals gave consistently lower values for the average number of lobes per neutrophil than others, e.g. the group of 5 Beverens gave a mean normal value of 2.13, whereas the mean for the group of 5 Dutch rabbits was 2.59. The means for the other 4 groups lay between the value for the 2 groups cited.

This difference did not appear to affect the magnitude of response to a given dose of pyrogen.

Table 2

Normal Values

		literature value[170]
average number of lobes per neutrophil	2.30	2.50
coefficient of variation	10%	14.4%
percentage of small lymphocytes	60	see page 56
coefficient of variation	16%	

Kennedy[170] has reported normal values for the "Weighted Mean" (average number of lobes per neutrophil) for a mixed group of rabbits and the mean value(2.50) is computed from a range of normals (one for each rabbit) which includes values ranging from 1.99 to 2.96 which are apparently accepted as normal.

The error observed in counting one smear several times was shown to be less than that involved in counting smears prepared from one rabbit at different times (weekly intervals) and both of these errors are less than the difference between counts performed on smears prepared before and after injection of the smallest dose-level of pyrogen used in the investigation. (details on pages 141, 142.)

Total white blood cell counts:

Total counts were not performed at each test because of the time-consuming technique involved, especially when 15 animals were used at once, and when the information provided by these counts was not required in the present investigation.

Several were made, however, and the results obtained are recorded in table 3

Table 3 (95)

INCREASES IN TOTAL NUMBERS OF LEUCOCYTES IN RABBITS, 3 TO 4 HOURS AFTER INJECTION OF VARIOUS DOSE-LEVELS OF PYROGEN. TEMPERATURE RESPONSES ARE ALSO GIVEN

Dose, ml./kg.	Increase in white blood cells/cu. mm.	Rise in rectal temperature, °C.
0·2	14,400	1·22
	14,800	1·57
	6,600	1·30
0·1125	7,200	1·17
	6,400	1·49
	13,200	1·10
0·06324	3,000	0·77
	8,000	1·18
	13,000	0·81
0·02	1,400	0·34
	—	0·95
	800	0·45

Post-injection counts were made between 3 and 4 hours after injection. The results of the total counts, in conjunction with the polynuclear counts made at the same time show that there is, after injection of pyrogen, an an addition to the circulation of young neutrophils - mostly 1-lobed and some 2-lobed; there also appeared to be a decrease in 3-,4-,5-lobed neutrophils and it is tempting to speculate that this may indicate an affinity of pyrogen for these older neutrophils which as a result are damaged and removed from the circulation by the time defervescence of the pyrogenic reaction has set in.

Results:

The results for each dose-level are the means of 90 readings (30 rabbits, with 3 replications at each dose-level) and the results are reported in detail on pages 143-149,165-171,184-190

The statistical analysis are also given in full and have allowed the following conclusions to be drawn:-

1. The results are normally distributed, the test being made by the use of χ^2. In one instance(the results for the temperature response at the smallest dose-level) distribution was not quite normal but the deviation from normality was not serious and certainly preclude the application of the usual statistical methods to these results.

2. The mean responses for each index of activity being investigated, together with the coefficients of

Table 4 (95)

TEMPERATURE RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN

Dose, ml./kg.	Average maximal rise in rectal temperature	
	Mean °C.	Coefficient of variation per cent.
0·2	1·38	21
0·1125	1·25	21
0·06324	0·95	30
0·02	0·61	36

Table 5 (95)

LEUCOCYTE RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN PERCENTAGE FALL IN AVERAGE NUMBER OF LOBES PER NEUTROPHIL

Dose, ml./kg.	Percentage fall in average number of lobes per neutrophil	
	Mean per cent.	Coefficient of variation, per cent.
0·2	35·6	22
0·1125	29·8	28
0·06324	25·2	33
0·02	16·8	48

Table 6 (95)

RESPONSE IN THE RABBIT TO VARIOUS DOSE-LEVELS OF PYROGEN SMALL LYMPHOCYTE PERCENTAGE FALL (6)

Dose, ml./kg.	Small lymphocyte percentage fall	
	Mean	Coefficient of variation, per cent.
0·2	56·5	27
0·1125	49·8	39
0·06324	44·8	41
0·02	27·0	62

variation are given in tables 4,5,6, on page 61.

The coefficients of variation allow comparison of the variabilities of groups of results measured in different units (e.g. temperature response and white blood cell response).

It is seen that the coefficients of variation indicate increasing variability of response in the following order: temperature response, percentage fall in the average number of lobes per neutrophil, and the small lymphocyte percentage fall.

It is interesting to note that the mean temperature response (o.61 C^o) is virtually the same figure (0.6C^o) as that adopted by the British Pharmacopoeia as the threshold value for a pyrogenic response (i.e. an average rise in temperature greater than this is taken to indicate pyrogenicity). The corresponding value for the degree of shift to the left obtained in the present experiments is $16.8 \pm 1.7 (p = 0.05)$. It will be seen later that using lipopolysaccharide from Salmonella abortus equi in a dose of 0.005γ/kg an average maximal rise in temperature of 0.61 C^o was obtained and this corresponded to a degree of shift to the left of $14.7 \pm 2.8 (p = 0.05)$.

4. Analysis of variance was conducted on each group of results, the total variance being divided into

between-rabbits, between-dose, rabbit-dose interaction, and a residual, and from the calculations based on this analysis (pages 152,174,193) we see that rabbit-dose interaction does not exist significantly. This indicates that there was no dose-level, of the four used, to which the rabbits displayed special sensitivity or lack of it. Between-rabbit variance was shown to exist significantly, showing that much of the error of the test can be explained by the natural differences between individual rabbits.

The significance of difference of the means for the various dose-levels may be tested using the information supplied by the analysis of variance. This allows more accurate testing of the significance of difference than a straight t-test on the means using the variance calculated for each dose-level, for the reason that much of the error has been properly apportioned and subtracted from the residual (error) before applying the test. The means for the four dose-levels for each of the three indices of activity have been shown to differ significantly.

5. Correlation coefficients were calculated for each response and log(100 x dose), and in each case exceeded that given for the 5% level of probability, allowing us

to regard it as being at least 19 to 1 that there is some degree of linear relationship between the three different responses and log dose over the range of values indicated. (see pages 155,177,196)

Correlation coefficients were also calculated to assess the extent of relationship between the temperature response and each of the white blood cell responses (degree of shift to the left, and small lymphocyte percentage fall) and in each case the correlation coefficient was found to be significantly greater than zero. It is emphasised that the correlation holds only over the ranges of values specified.

6. Partial correlation coefficients (the correlation coefficient referred to in 5. above have been termed "total correlation coefficients") were also calculated in order to eliminate the effect of log dose, that is to determine whether the observed correlation (total correlation) was independent of the effect of log dose .

The significance of the partial correlation coefficients indicate that the observed correlation is independent of the effect of log dose.
(see pages 183, 202, 204)

It should be borne in mind that the existence of a signifiant degree of corrleation, although providing in a single figure a convenient measure of the extent of relationship between the two variates, does not necessarily indicate a causal relationship between the two variates correlated, that is correlation between the temperature response to pyrogen and the leucocyte response to pyrogen does not suggest that it is the effect of pyrogen on the leucocytes which causes the subsequent upset in temperature. It does so happen in this case, however, that physiological evidence is coming along to indicate that some reaction between pyrogen and the leucocytes is concerned in the stimulation of the temperature response.

Correlation of a significant degree between the temperature and the leucocyte responses does strengthen the argument that both the temperature effect and the leucocyte effect are caused by the same active substance, and this evidence is specially valuable at the present time when even the purity of the most pure pyrogen preparation available is still a matter for some speculation.

In further support of this argument, it will be shown later (Section 3) that using pyrogen from other bacterial sources, prepared by widely differing methods and hence containing different residual impurities, the same degree of shift to the left is obtained for a temperature response of a given magnitude.

7. Having shown that correlation exists between the different responses and log dose we can take the matter further and construct equations for the regression lines. The regression lines are given in figures3,4,and 5 on page 67. Linearity of regression was tested by analysis of variance, and in the case of the tempertaure response only was there a slight deviation from linearity. (pages 158,180,199)

8. The residual variancesabout the regression lines were calculated for each index of activity, and thence the standard deviation of this scatter. The ratios,

$$\frac{\text{standarddeviation of the scatter about the regression}}{\text{difference between the maximum and minimum mean responses}}$$

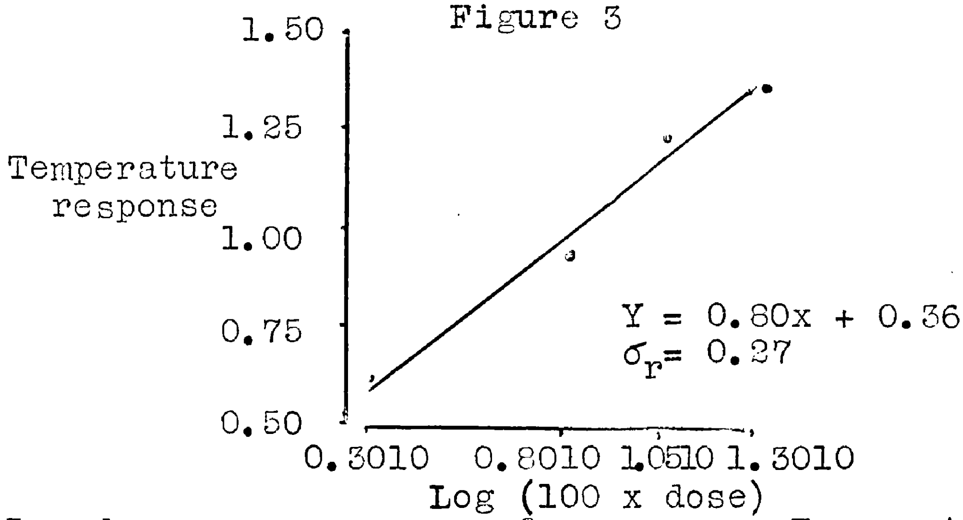
and

$$\frac{\text{standard deviation of the scatter about the regression}}{\text{mean of the four mean responses}}$$

were calculated for each index of activity. The first ratio is designated ratio A and the second, ratio B.

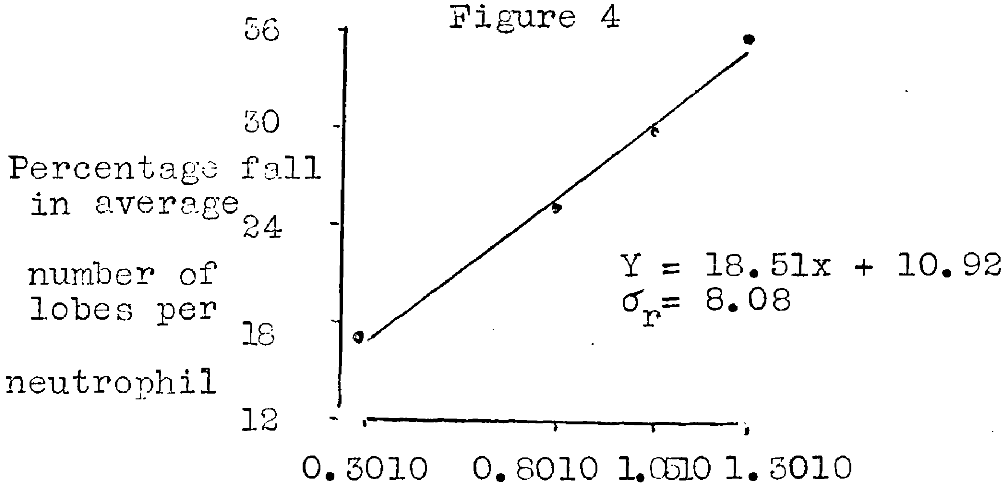
It will be seen that ratio A gives a relationship between the scatter about the response as a whole and the complete range of the response, and that ratio B gives a similar relationship with the "height" of response.

Figure 3



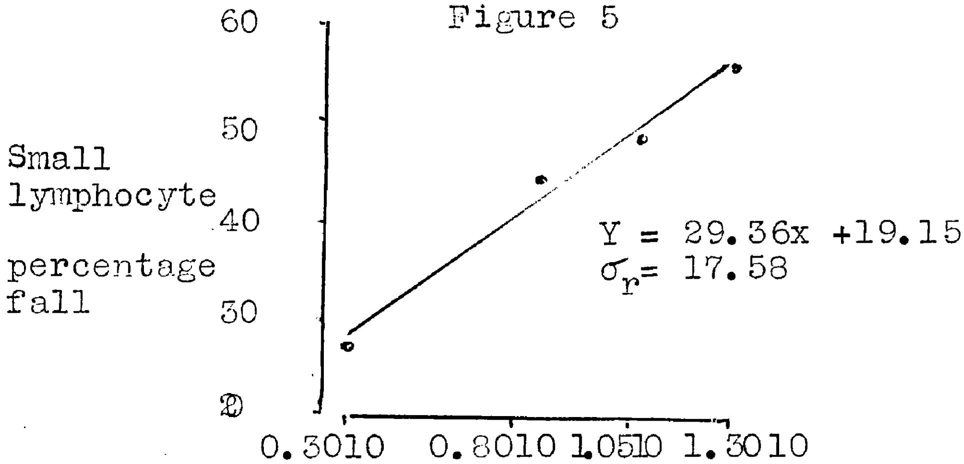
Log dose response curve for pyrogen. Temperature response

Figure 4



Log dose response curve for pyrogen. Degree of shift to the left

Figure 5



Log dose response curve for pyrogen. Small lymphocyte percentage fall

These ratios confirmed the impression given by the comparison of the coefficients of variation that the order of accuracy for the 3 indices of pyrogenic activity is, temperature response, percentage fall in the average number of lobes per neutrophil, and the small lymphocyte percentage fall. It is seen from table 7 that there is very little to choose between the temperature response and the degree of shift to the left as far as accuracy (i.e. lack of variability) is concerned.

Table 7

Comparison of ratios A and B for the three indices of pyrogenic activity investigated

	Temperature response	Percentage fall in the average number of lobes per neutrophil	Small lymphocyte percentage fall
Ratio A	0.35	0.43	0.60
Ratio B	0.26	0.30	0.40

It will be seen that, the smaller the value for these ratios the smaller the degree of variability.

Double-peaked Temperature Responses:

Occasionally, after partial defervescence at about 2 hours from the time of injection of pyrogen, the temperature recommenced to rise with the result that a biphasic temperature response occurred, the second peak usually making its appearance about 3 hours after injection.

Calculation showed that the frequency with which these biphasic temperature responses occurred tended to be higher with higher dose-levels. Table 8 shows the frequency of occurrence

Table 8

Frequency of occurrence of biphasic temperature responses at various dose-levels of pyrogen

Dose (ml/kg)	Percentage of all temperature responses which were biphasic
0.2	28
0.1125	40
0.06324	36
0.02	9

The slightly smaller frequency at the highest dose-level is difficult ot explain satisfactorily, but it was observed that at this level of response the peak temperature tended to flatten out at the summit for some time before starting to fall, giving the impression that the extra vigour of this dose-level tended to inhibit partial defervescence after the first peak, so obscuring a second peak.

Time required by the rabbit to reach maximum tempemture following injection of pyrogen:

The mean times(in minutes) taken to reach peak temperatures were calculated for each dose-level in order to determine whether there was any difference in this respect between the responses for the different dose-levels used. Results are summarised in table 9 and given in detail on pages 160-164

Tab le 9

Times required to reach peal temperatures after the injection of various dose-levels of pyrogen

Dose (ml/kg)	Time taken to reach peak temperature (minutes)
0.2	119
0.1125	122
0.06324	111
0.02	111

It is seen that there is a slight tendency for the peak temperatures stimulated by the lower dose-levels to be attained more quickly that those stimulated by the higher dose-levels. These results will bbe compared with similar measurements made during experiments with pyrogen preparations from other bacterial surces (Section 3)

Higher dose-levels of pyrogen:

Several experiments were performed using a still higher dose-level (o.3557ml/kg) whose logarothm ($\bar{1}.5510$) is as mich above dose-level D as C is below D (see tablel)

Inspection of the results (page 205) will show that the observed responses are smaller than indicated by the regression lines, showing that, in the case of all three indices of activity the pyrogenic response begins to flatten out at this level.

Conclusions:

1. Comparison of the temperature and a leucocyte method (which measures the degree of shift to the left occurring among rabbit neutrophils) of assay of pyrogen has been carried out in rabbits, and it has been found that there is little to distinguish between the two methods as far as accuracy is concerned, the temperature method showing slightly less variability.

2. The method using the small lymphocyte percentage fall as suggested by Dawson[3] was found to possess less accuracy than either of the other two methods. The responses were smaller than those reported by Dawson[3] due to the fact that the technique used in the small lymphocyte count gave lower values for the small lymphocyte percentage. This does not alter the fact that there is still a straight line relationship between the small lymphocyte percentage fall and lgo dose.

3. Correlation has been shown to exist between the

temperature response and each of the white blood cell responses, and the importance of this finding has been discussed.

4. The method of assay using the degree of shift to the left provides an alternative metdod which is easier to per form, requiring less preparation and less elaborate apparatus.

5. The small lymphocyte percentage fall was considered to possess too great variability to warrant its further use.

Section 3

The general applicability of the new index of pyrogenic activity using pyrogen from different bacterial sources and prepared by different methods.

Having found that the measurement of the degree of shift to the left occurring among rabbit neutrophils is a suitable index of pyrogenic activity, it was considered desirable to check this index using pyrogenpreparations from other bacterial sources, in order to eliminate the possibility that the quantitative effect observed with Proteus pyrogen was peculiar to that pyrogen preparation.

Two purified pyrogen preparations were chosen: a) Pseudomonas polysaccharide ('Piromen') which is prepared along the lines indicated by Nesset et al.[24] to whose work reference has already been made; it is supplied in vials containing 50 polysaccharide per ml. in normal sodium racemic-lactate solution, with merthiolate 1 in 10,000 as preservative; b) lipopolysaccharide from Salmonella abortus equi prepared by the general method indicated by Westphal et al.[28-32] and supplied in 1 mgm quantities in sealed ampoules.

These preparations were chosen because there is considerable difference in pyrogenic activity per unit of weight (0.005γ/kg Salmonella lipopolysaccharide elicits the same temperature response as 1γ/kg Pseudomonas polysaccharide) indicating differences in purity; and because the methods of preparation differ considerably. Both preparations have been used in medicine

Method:

Preliminary experiments were performed with these materials and the results of these indicated the choice of the following dose-levels:-

Pseudomonas polysaccharide: 10γ/kg; 5γ/kg; 2.5γ/kg

Salmonella lipopolysaccharide: 0.1γ/kg; 0.01γ /kg; 0.005γ/kg

It was decided to reduce the number of experiments compared with the investigation using Proteus pyrogen, and so"dilute-out"the accuracy of both the temperature and the leucocyte responses to the same extent in order to determine which index of activity retained the greater degree of reliability, because it was suspected that the reason why the variabilities of the two responses (temperature and degree of shift to the left) were so close in the experiments with Proteus pyrogen was that such a large number of experiments were performed that only gross differences would have shown up.

Accordingly, 15 rabbits in groups of five were used in the experiments with ach preparation, each rabbit receibing each dose-level on one occasion. The experimental details relating to feeding and conditioning of the animals, the conduct of the test, the measurement of rectal temperatures , and the preparation of the blood smears were the same as those described in the previous section.

It was confirmed during the preliminary experiments that the maximal change in the average number of lobes per neutrophil could, as with Proteus pyrogen, be expected to occur between three and four hours after injection.

Temperature response and the percentage fall in the average number of lobes per neutrophil (called the leucocyte response) were measured; the small lymphocyte percentage fall was not.

The results appear in full on pages 207-209, 228-230. The mean responses with the coefficients of variation are given in tables 10 and 11 on page 76.

It is seen that the leucocyte response (percentage fall in the average number of lobes per neutrophil) is attended by less variability than the temperature response in every instance.

Table 10

Temperature and leucocyte responses to Pseudomonas polysaccharide

Dose γ/kg	average maximal rise in rectal temperature C°		percentage fall in the average number of lobes per neutrophil	
	mean	V	mean	V
10	1.23	28	31.5	19
5	1.08	32	26.0	16
2.5	0.91	40	21.5	25

V = coefficient of variation per cent

Table 11

Temperature and leucocyte responses to Salmonella lipopolysaccharide

Dose γ/kg	average maximal rise in rectal temperature C°		percentage fall in the average number of lobes per neutrophil	
	mean	V	mean	V
0.1	1.28	32	32.1	18
0.01	0.77	34	19.6	23
0.005	0.61	51	14.7	34

The results were analysed in a similar manner, with some exceptions due to the different experimental design, to the results in Section 2. Normality of distribution was assumed since there is no satisfactory criterion for this number of results.

1. Analyses of variances were conducted andthe variances

were divided into between-rabbits, between-doses, and a residual (error). Between - rabbit variance was again shown to be significant, and since the between-doses variance was significant, the t-test was applied to the means for the various dose-levels, using the residual as the error.

This revealed that in two cases out of three, a two-fold increase in dose could not be distinguished using the temperature response as an index of pyrogenic activity, while in the third case, significance of difference was just established. In the case of the index which utilises the degree of shift to the left, however, two-fold increases in dose could be distinguished in every instance ($p \leqslant 0.01$).
(see pages 211,213;232,234)

Thus it is seen that, having "diluted-out" the accuracy of each index equally (as seen in the investigation reported in Section 2), that measuring the degree of shift to the left remains the more reliable.

2. Correlation coefficients were calculated between each of the two responses and log dose and were shown to be significant. Thence regression equations were worked out and analysis of variance used to test the linearity of regression. Significant departure from

linearity was not observed in any instance.

Total and partial correlation coefficients were calculated for the temperature and leucocyte responses for each preparation, and it was found that the responses were correlated. The partial correlation coefficient between temperature and leucocyte responses for Pseudomonas polysaccharide was not significantly greater than zero, however, indicating that the total correlation which was shown to exist for the two responses, was to some extent dependent on the dosesused. (pages 222,223;243,244)

4. Ratios A and B which were devised and explained in Section 2 as a means offurther comparing the relative variabilities of the different responses were calculated for both preparations, and these supported the conclusion already made that the index utilising the degree of shift to the left is the more reliable. (pages 216,217;237,238)

These results are summarised in table 12 on page79.

The temperature responses were plotted against the leucocyte responses for the experiments with the pyrogen preparations from Proteus, Pseudomonas, and Salmonella (figure 6, page 80) and the suggestion of a uniform relationship between the temper ature and leucocyte responses is seen to be very strong.

Table 12

Comparison of ratios A and B

		Temperature response	Leucocyte response
RatioA	Pseudomonas polysaccharide	1.06	0.51
	Salmonella lipopolysaccharide	0.49	0.29
RatioB	Pseudomonas polysaccharide	0.32	0.20
	Salmonella lipopolysaccharide	0.37	0.23

Timesto reach peak temperatures were calculated for both pyrogen preparations and are given in table 13

Table 13

Times (minutes) required to reach peak temperatures

	Pseudomonas polysaccharide			Salmonella lipopolysaccharide		
Dose γ/kg:	10	5	2.5	0.1	0.01	0.005
Times taken to reach peaks (means)	104	113	116	118	116	86

It is seen that with Salmonella lipopolysaccharide (as with Proteus pyrogen) there is a tendency for the pyrexia to attain a maximum value more quickly with the smallest dose-level.

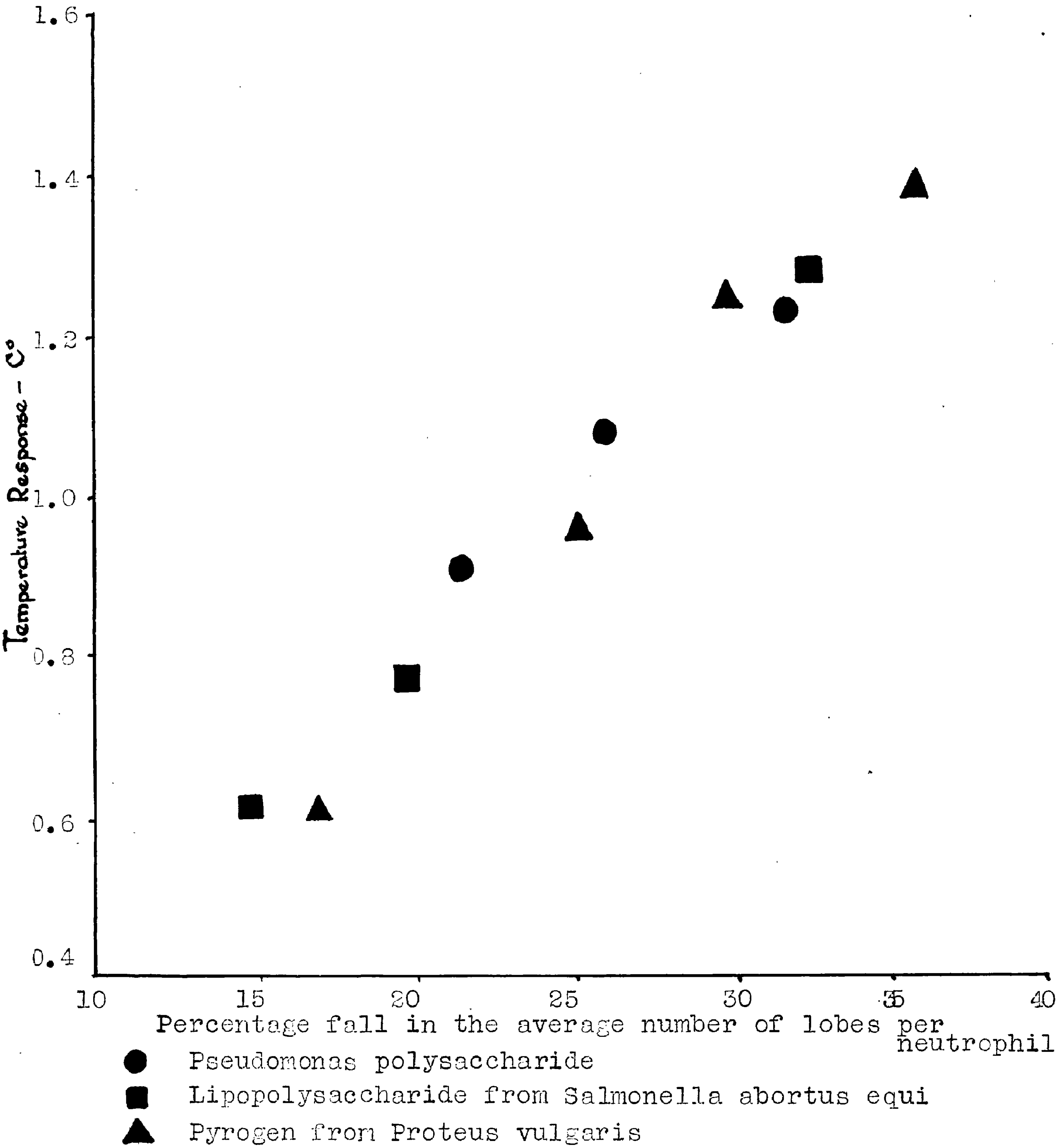


Figure 6
Relationship between the temperature and leucocyte responses for different pyrogen preparations

Having shown that the degree of shift to the left is a suitable index of pyrogenic activity for pyrogenpreparations from different bacterial sarces, it was considered of further interest to examine its utility with pyrogen from unhnown scurces as found in

i a commercial sample of Normal Saline which was found to be pyrogenic

ii pyrogenic distilled water

1 Pyrogenic Saline

A sample labelled Injection of Sodium Chloride, B.P. was received for pyrogen testing. The test was carried out as required by the B.P. under Water for Injection (which for the test may contain 0.9% pyrogen free sodium chloride) with the exception that 5 rabbits were used and blood smears were taken immediately prior to, and about $3\frac{1}{2}$ hours after, injection.

The following results were obtained:

average maximal rise in rectal temperature	1.59C° (standard deviation = 0.2)
percentage fall in the average number of lobes per neutrophil	31.2 (standard deviation = 2.8)

The sample was reported as pyrogenic.

<u>ii Pyrogenic distilled water</u>

Water was distilled as for the preparation of apyrogenic water (i.e. into pyrogen-free glass containers), but instead of sterilising the distillate immediately, it was allowed to remain in the laboratory for 48 hours, loosely-stoppered. Pyrogen-free sodium chloride (0.9%) was added and the test for pyrogen carried out as described in the British Pharmacopoeia, with the exceptios that 5 rabbits were used and blood smears were taken immediately prior to, and about 3½ hours after, injection.

The following results were obtained:

average maximal rice in rectal temperature	1.24 C°	(standard deviation = 0.17°)
percentage fall in the average number of lobes per neutrophil	28.7	(standard deviation = 2.4%)

The water was judged pyrogenic.

It willbe seen that both of these results could have been obtained without use of the temperature index, merely by the preparation and counting of two blood smears before and after injection.

The utility of the new index of pyrogenic activity based on the polynuclear count has been demonstrated with pyrogen from a variety of bacterial sources.

The investigations using the purified pyrogens from Pseudomonas and Salmonella have revealed that this index is not only more convenient to use but is attended by less variability than the temperature index.

One of the most remarkable features of these experiments is the close relationship between the temperature response and the leucocyte response, which gives strong indication that both effects are caused by one active substance. This relationship has been borne out using pyrogen preparations of widely differing purity and potency, and because of the different methods of preparation employed, containing different residual impurities.

It remains to be established whether, as Westphal suggests, the basis of this physiological activity is to be found in the phospholipid group.

Section 4

A. Tolerance to pyrogen and the absence of precipitin formation.

B. The production of cross-tolerance to pyrogen from different bacterial sources.

A At the conclusion of the experiments which were performed in the investigation of the Polynuclear count as the basis of an index of pyrogenic activity it was suspected that 8 of the 30 rabbits were beginning to show diminished temperature responses to the highest dose-level of pyrogen used. Further experiments were peformed at weekly intervals with these animals, and it was found that they were showing diminished temperature responses compared with their first responses to that dose-level.

The standard pyrogen was checked for deterioration, and in rabbits which had not been used for 3 months the expected temperature response was obtained.

Advantage was taken of this apparently incipient tolerance in the 8 rabbits were tested for precipitins using a ring test. in order to determine whether precipitins appeared at the same time as signs of tolerance.

Method

4 - 5 mls of blood were taken from the marginal ear veins of the rabbits on the day after the last injection of

pyrogen, and again 7 days later. The blood was allowed to clot and the serum to separate in the refrigerator. Serum was also obtained from a rabbit which had not had any pyrogen for 10 weeks.

Thoroughly clean (acid washed) precipitin tubes (5mm x 40mm) were used and about 0.1 - 0.2 ml undiluted serum placed in the bottom of the tubes. This was carefully overlaid with about the same amount of pyrogen solution so that two layers were formed, allowing any precipitin to be observed as a white interfacial ring.

Various dilutions of pyrogen were used:

1 reconstituted freez-dried standard

2 this diluted 1 in 2, 1 in 10, 1 in 100

Controls of normal rabbit serum and saline were run.

The tubes were read after 15 minutes, 30 minutes, 1 hour, 2 hours , 3 hours, and 16 hours and in no case could any ring of precipitin be observed.

It was concluded from these experiments that precipitin formation does not accompany the inception of tolerance to pyrogen from Proteus vulgaris.

B It has already been shown that pyrogen from Proteus vulgaris, a Pseudomonas species, and Salmonella abortus equi possess closely similar prop erties

Group A received 8 daily injections of Pseudomonas
polysaccharide - 5γ/kg.

Group B received 8 daily injections of lipopolysaccharide
from S. abortus equi - 0.04γ/kg.

Group C received 8 daily injections of the standard
pyrogen from P. vulgaris descrÅbed in Section 2
(0.02 ml /kg). (This was a different batch and
appeared to be more potent).

The daily temperâture responses are shown garphically in fig. 7. Polynuclear counts were not performed because up to five or six days are required for the count to return to normal.

On the 9th day about 6 mls of blood were removed from each rabbit, and the serum used for precipitin tests on the following day. The ring test employed has already been described. In this test the pyrogen solutions used as "antigen" were employed in the following dilutions:

Pseudomonas polysaccharide: 50γ/ml and 5γ/ml
Lipopolysaccharide from S. abortus equi: 50γ/ml and 0.05γ/ml
Pyrogen from P. vulgaris: reconstituted freeze-dried standard
and dilutions 1 in 10 and 1 in 100
of this.

The test was conducted as previously indicated.

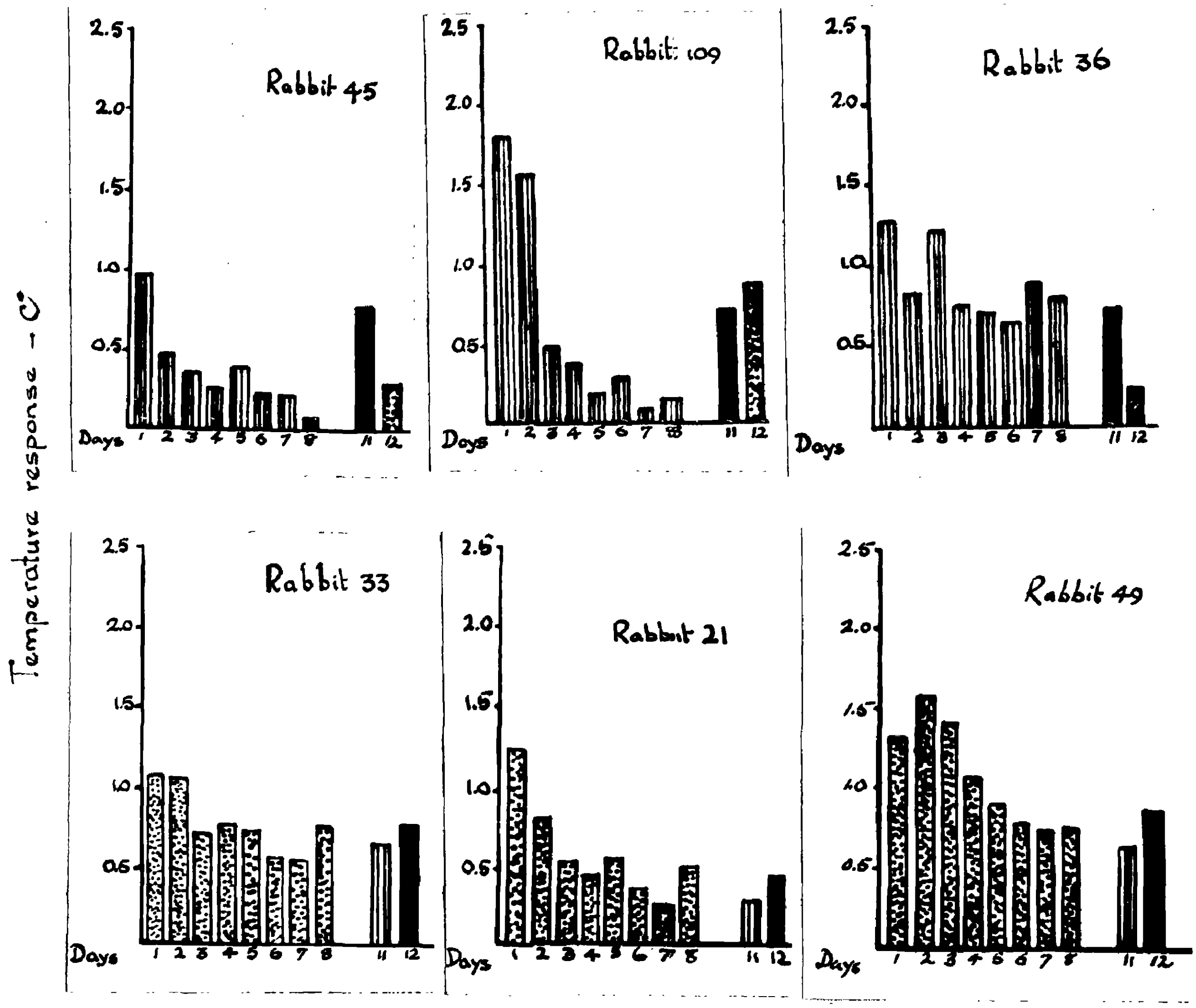


Figure 7
Individual temperature responses to different pyrogen preparations

Temperature responses to Pseudomonas polysaccharide 5γ/kg

Temperature responses to Salmonella lipopolysaccharide 0.04γ/kg

Temperature responses to Proteus pyrogen 0.02 ml/kg

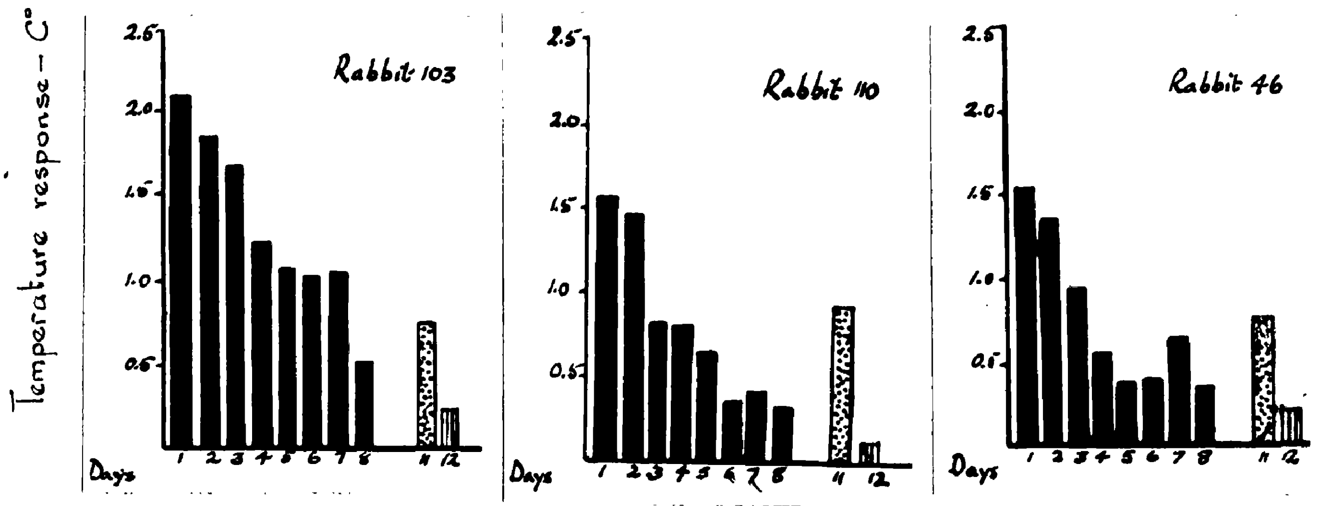


Figure 7
Individual temperature responses to different pyrogen preparations

Temperature responses to Pseudomonas polysaccharide 5γ/kg

Temperature responses to Salmonella lipopolysaccharide 0.4γ/kg

Temperature responses to Proteus pyrogen 0.02 ml/kg

On the 11th and 12th days each rabbit received an injection of the pyrogen preparations which it had not already received, for the purpose of testing for the presence of cross-tolerance.

The induction of tolerance was followed by noting the daily diminution in the temperature response, and calculation (page 257) showed that the mean responses for the 8th daily injection were significantly less than the mean responses for the same groups for the 1st injection.

The establishment of cross-tolerance is indicated by the responses for injections 9 and 10 (i.e. on the 11th and 12th days respectively) being significantly less than the responses for the 1st injections.

A larger dose of lipopolysaccharide from S. abortus equi was given to rabbits of Group C on day 11 (0.4γ/kg) because the dose of Proteus pyrogen to which these rabbits were rendered tolerant was much more powerful than 0.04γ/kg of Salmonella lipopolysaccharide which was given to Group B. 0.4γ/kg Salmonella lipopolysaccharide was approximately equivalent in strength to the dose of Proteus pyrogen to which Group C had been rendered tolerant. It is seen that this dose of Salmonella lipopolysaccharide elicits a pyrogenic response which is significantly less than that elicited by Proteus pyrogen on the first day.

The doses of Pseudomonas polysaccyaride and Salmonella lipopolysaccharide are roughly equivalent, and that of Proteus pyrogen is stronger than the other two.

Group A rabbits (rendered tolerant to Pseudomonas polysaccharide) gave a greater response to Proteus pyrogen than Group C gave on the 8th day (Group C had been rendered tolerant to the stronger dose of Proteus pyrogen). Also Pseudomonas polysaccharide given to Group B (rendered tolerant to Salmonella lipopolysaccharide) elicited a greater response than when given to Group C (rendered tolerant to Proteus pyrogen) indicating that the stronger dose of Proteus pyrogen had probably induced a greater degree of tolerance than the weaker dose of Pseudomonas polysaccharide.

By comparing the means for day 8 with those for days 11 and 12 (page259) it is seen that the degrees of cross-tolerance are approximately equivalent with the exception that Group C appeared to be more tolerant to Pseudomonas polysaccharide than to Proteus pyrogen (mean for day 12 is significantly less than that for day 8) which may in part be accounted for by the difference in strength of pyrogenic dose to which Group C were rendered tolerant.

The larger dose (0.4γ/kg) of lipopolysaccharide from S. abortus equi given on day 11 to Group C gave a greater response than Pseudomonas polysaccharide to the same group on day 12. 0.04γ/kg is roughly equivalent to the dose of Pseudomonas polysaccharide, hence it is seen that increase in dose of pyrogen from another baterial source is, to some extent, tending to overcome tolerance although in this instance the dose is still not large enough to completely overcome the tolerance which had been established. The possibility is not completely excluded that cross-tolerance is not complete and the results depend to some extent on the dosage employed.

As in the previous experiments the precipitin tests were negative in all cases, showing that the production of tolerance is independent of precipitin production.

The possibility of production of cross-tolerance is further evidence to support the contention that different pyrogen preparations (i.e. from different bacterial sorces) possess a common basis for physiological activity.

Section 5

The detection of pyrogen in therapeutic materials which induce hypothermia in the rabbit.

A-Injection of Calcium Gluconate

The present position with regard to the testing for the presence of pyrogen in substances which cause hypothermia in the rabbit is unstaisfactory and this is evidenced by the absence of a limiting test in the B.P. for pyrogen in Injection of Calcium Gluconate.

Intravenous injection of calcium gluconate causes hypothermia in the rabbit, but no data could be found in the literature to indicate the duration of this hypothermia after a given dose of calcium gluconate intravenously, or whether it would interfere with the pyrogenic response if given with pyrogen.

This problem was investigated not only with the object of finding out what happened when calcium gluconate was given intravenously with pyrogen but also to examine the possibility of employing the new index of activity (which measures the degree of shift to the left) as the basis of a pyrogen test for Injection of Calcium Gluconate.

Only one report could be found in the literature concerning the effect of intravenous injection of a calcium salt on the polynuclear count in rabbits, where the author injected 5 mls of 2½% calcium chloride and gave the result for 1 rabbit. Although he claims that there was a slight regenerative shift in the count after injection, the value of this shift is so low, that the results for a group of rabbits could possibly have been open to different interpretation.[173]

Several experiments were performed in order to elucidate certain points before commencing the investigation.

It is well known that Seitz filtration of a pyrogenic solution will effectively remove the pyrogen, and this was applied in the preparation of pyrogen-free solutions of calcium gluconate. It was ascertained that the process of Seitz filtration did not reduce the content of calcium gluconate in the solutions. This was done in the following manner:

i assayed the calcium gluconate to be used in the experiments, by the method given in the British Pharmacopoeia.

ii prepared a 10%soltuion (as for the injection) and divided this into two portions. One portion was filtered through a Seitz filter (Carlson 6cm "Special" EK Sterilising) and the other was not. From each of these two portions were taken two accurately measured 5 ml quantities (= 0.5 G. calcium gluconate) which were assayed for calcium gluconate in duplicate.

It was also ascertained that Seitz filtration effectively removed added pyrogen from Injection of Calcium Gluconate. If Seitz filtration were carried out after the addition of pyrogen to Injection of Calcium Gluconate, no pyrogenic reaction was obtained. Rabbits receiving the injection immediately after the addition of pyrogen , developed pyrexia and a shift to the left.

It is obviously desirable to use as much as possible of the substance being tested for the presence of pyrogen inorder that the smallest quantities of pyrogen which may be present are detected. But in the case where the material to be tested exerts a pharmacological action on the test animal, and especially in this case where the action includes the production of hypothermia, a compromise has to be made otherwise the object of the test may be defeated..

It was considered desirable to test Injection of Calcim m Gluconate (10%solution) without previous dilution for two reasons: a - dilution creates an avenue for the introduction of pyrogen; b - any pyrogen already present may be diluted to a subliminal value.

Several experimets were performed in which the rabbits received 5 mls/kg. of Injection of Calcium Gluconate and subsequent falls in temperature ranging from 0.66 - 3.67°C were recorded. Falls in temperature of over 1 centigrade degree were usually accompanied by signs of distress in the animals, while those rabbits which experienced falls of 3 centigrade degrees were in a state of semi-collapse during a 6-hour period. Breathing was laboured and they salivated and urinated freely. As their temperatures rose to normal the signs of distress gradually disappeared.

Unexpected results were obtained when pyrogen was added to as large a dose of calcium gluconate as this, and it was considered that 5 mls/kg of Injection of Calcium Gluconate upset the rabbits so much that it was too big for the purpose of testing the injection for pyrogen.

Subsequent experiments were performed using a dose of 2 mls/kg which was found to produce a much less severe reaction and to cause a hypothermia which was replaced by preinjection temperature within about one-and-a-half hours. This dose was chosen for reasons other than the convenience of the duration of effect.

The maximum dose recommended by the British Pharmacopoeia for Injection of Calcium Gluconate is 20 mls (intravenously or intramuscularly), and assuming the average adult to weigh 70 kg, this provides one ml for every 3.5 kg of body weight. Thus in the present experiments the rabbits are receivingabout seven times the amount of injection on a basis of body weight, and therefore about seven times the amount of pyrogen which may be présent also on a basis of body weight. But the human subject appears to be more sensitive to pyrogen than the rabbit, hence if pyrogen is not detected by the rabbits, it may be assumed that the injection is for practical purposes pyrogen-free..

The experiments were planned so that each of 22 rabbits would receive:

i - 2 mls /kg of Injection of Calcium Gluconate(Seitz-filtered to remove any adventitious pyrogen) to which had been added a dose of Proteus pyrogen sufficient to raise the rectal temperature of the rabbits about 1C.°

ii - the same dose of pyrogen alone

iii - 2 mls/kg of Injection of Calcium Gluconate (Seitz-filtered)

all on separate occasions.

Comparison of the results for each series of injections then allows observation of the effect of Injection of Calcium Gluconate on the rise in temperature and the extent of shift to the left produced by a dose of pyrogen which has given a measured rise in temperature (in the same rabbits) and leucocyte response.

Information is of-course also yielded concerning the effect of pyrogen on the hypothermic effect of Injection of Calcium Gluconate given intravenously.

The results have been summarised in tabular form (table 14) and are given in detail in appendix III on pages 245-252.

Table 14

Results of experiments to determine the effect of the presence of calcium gluconate on the pyrogen reaction in the rabbit

	G	G+P	P
Fall in temperature(C^{o})	0.66	0.42	-
Time to reach minimum temperature(minutes)	20	12	-
Duration of hypothermia (time to return to pre-injection temperature) (minutes)	80	34	-
Rise in temperature(C^{o})	-	1.18	1.21
Time to reach peak temperature(minutes)	-	112	117
Percentage fall in the average number of lobes per neutrophil	1.9	23.0	21.8

G: injection of calcium gluconate 2 mls/kg
G+P: same but containing pyrogen 0.02ml/kg
P: pyrogen 0.02ml/kg

Rectal temperatures were measured as already described, and the pre-injection temperatures were calculated as the mean of temperatures recorded at 10 minute intervals during the hour immedistely prior to injection.

It was found that within a few minutes after injection of the calcium gluconate the temperature of the animals began to fall, reaching a minimum value about 20 minutes after injection, although this was extended in some instances to as much as 45 minutes.

The return to pre-injection level was usually complete in about an hour-and-a-half and seldom exceeded two hours. Severe hypothermia was uncommon and in the one or two instances of its occurrence, both the temperature and the physical condition of the rabbit were steadily approaching pre-injection level by 3 hours after injection.

The effect of pyrogen given with calcium gluconate was to decrease the hypothermia due to the latter, accelerate the return of temperature to pre-injection level, and to stimulate a typical pyrogenic response within a time which was not significantly different from that in which pyrogen alone produced pyrexia and a shift to the left. Neither the degree of shift to the left nor the temperature rise which occurred when calcium gluconate was given with pyrogen was significantly different from the corresponding results obtained with pyrogen alone; and the times takento reach peak temperatures were not significantly different. (calculations on page252)

From these results the following conclusions may be drawn:

i The presence of pyrogen in Injection of Calcium Gluconate can be detected by measuring the rise in rectal temperature occurring in rabbits: the hypothermic effect of calcium gluconate is modified by pyrogen which stimulates a pyrogenic response of undimininshed intensity

ii The peak temperature of a pyrogenic response occurs in the same time when calcium gluconate is given with the pyrogen. The duration of a pyrogen test need not be prolonged beyond 3 hours in order to allow the effects of any pyrogen present(temperature or leucocyte response) to become apparent.

iii The degree of shift to the left occurring when pyrogen is administered with calcium gluconate is of the same magnitude as that occurring when pyrogen is given alone

In conclusion it may be said that for the purpose of a pyrogen limit test for Injection of Calcium Gluconate a dose of 2 mls/kg would be a suitable quantity to test. The period of the test need not be prolonged beyond 3 hours and either the rise in temperature or the degree of shift to the left occurring among the rabbit neutrophils may be used as an index of pyrogenic activity.

In these experiments temperature responses were measured as described in Section 2; the degree of shift to the left was also computed as described in Section 2.

B - ACTH

A problem similar to that of Injection of Calcium Gluconate but probably more complex is presented by ACTH. ACTH is a good example of a substance which might be expected to be heavily contaminated by pyrogen unless great care is taken during manufacture to exclude bacterid contamination at all stages in the process.

ACTH causes hypothermia in the rabbit when injected intravenously; it also brings about a considerable alteration in the blood picture, the change resembling that produced by pyrogen.

There is evidence that the hypothermic (and antipyretic) effect of ACTH is not due to contaminating posterior pituitary hormone.[171]

Douglas and Paton[171] concluded from experiments with ACTH and pyrogen that ACTH will delay and reduce the pyrexial response if given simultaneously with pyrogen; given after pyrogen, ACTH will exert an antipyretic effect.

The observed delay in the pyrogenic response prompted these authors to suggest that in testing ACTH for pyrogen the test should be prolonged to 5 hours in order that peak temperatures due to pyrogen might not be missed.

With a different object in view other workers[181] investigated the effect of ACTH on the febrile response of dogs to pyrogen in a series of well-planned experiments with adequate controls using a cross-over technique. They concluded that ACTH did not affect the febrile response of dogs to pyrogen, inasmuch as it failed to reduce the maximum increment in temperature for the mean 6-hour increment following pyrogen administration.

The experiments reported here were performed with the object of clarifying the position with regard to the testing of ACTH for pyrogen.

The degree of shift to the left occurring(and the temperature response) was measured in order to determine whether the leucocyte effects of ACTH and pyrogen were in any way additive.

Two groups of rabbits (15 in each group; 4 rabbits common to each group) were used. The experiments were planned so that the rabbits of each group would at separate times receive: ACTH alone; ACTH together with pyrogen; and pyrogen alone.

Group A received a dose of pyrogen (0.06 ml/kg) sufficient ot give a near maximal response (1.7 - 2.0 C^{o}); group B received a dose of pyrogen (0.02 ml/kg) sufficient to raise the temperature of the rabbits 1 - 1.5 C^{o}.

The pyrogèn used was freeze-dried standard from Proteus vulgaris (see Section 2) but being a different batch had greater potency.

The technique used in measuring temperature and in preparing blood smears was similar to that already described with the exception that the test was extended to 6 - 8 hours (from time of injection) and the pre-injection temperatures were calculated as the mean of 10 readings in the hour-and-a-half preceding injection.

The ACTH used (Organon) had a stated potency of 2.32 units permgm.

The volume of all injections was 2 mls/kg irrespective of the dose of ACTH and/or pyrogen; solutions and dilutions were made with saline.

ACTH was given in a dose of 1 unit per kg body weight to both groups. The considerations governing the choice of dose to be used in the test are similar to those discussed under Injection of Calcium Gluconate. In the experience of the writer 1 unit/kg of this ACTH is as great a quantity as can be given to a small group of rabbits without upsetting so many of them as to make the result useless and another test necessary. Doses of 2 and 3 units/kg cause severe hypothermia (falls in temperature of 2.5 - 3.5 C^{o}) lasting for 8 hours and longer and the rabbits show signs of distress more than half of them being in a semi-collapsed condition, limb movement being almost totally absent. Doses which have this effect upon the test animals are manifestly too large for the pyrpose of the test, since rabbits in this condition could hardly be expected to yield accurate results.

Douglas and Paton[171] gave one group of rabbits 3 units/kg and inspection of their results will show that several of the falls in temperature after this dose were approaching 3 centigrade degrees, and they state that considerably larger falls sometimes occurred.

The results of the present series of experiments are recorded in detail in appendix V on page 260 and the following conclusions are drawn :

Groups A and B showed similar hypothermic responses to ACTH (both groups received 1 unit/kg). There was considerable between rabbit variation in the responses and the standard deviation was more than half the mean response in both cases. The absence of a rise in temperature after the hypothermia confirmed the assurance we received that the sample was non-pyrogenic. It was also fouhd that the percentage fall in the average number of lobes per neutrophil (degree of shift to the left) for ACTH was similar for both groups (21.9;22.0)

By comparing the results for the ACTH+pyrogen series of injections with those where only ACTH was given, it is seen that when the higher dose of pyrogen was given with ACTH the hypothermia was decreased by two-thirds to three-quarters as compared with that obtained when ACTH was given alone, and when the lower dose of pyrogen was given with ACTH the hypothermia was reduced by more than half. (see table 15)

Table 15

Effect of pyrogen on the hypothermia produced by intravenous ACTH in rabbits

		mean fall in temperature C°	t	p	result
GroupA	ACTH 1unit/kg	1.73	3.766	<0.001	significantly different
	ACTH 1unit/kg +pyrogen(0.06 ml/kg)	0.49			
GroupB	ACTH 1unit/kg	1.91	3.770	<0.001	significantly different
	ACTH 1unit/kg +pyrogen(0.02 ml/kg)	0.78			

When pyrogen was given with ACTH a shift to the left occurred, but to no greater degree than when pyrogen was given alone. However, the degrees of shift to the left for ACTH given with both doses of pyrogen (separately) were greater than for ACTH given alone, showing that wiyhin this range pyrogen will augment the shift to the left produced by ACTH.

The rises in temperature elicited by the larger dose of pyrogen and this together with ACTH were not significantly different (table 16).

Table 16

Effect of ACTH on the pyrogenic response in rabbits

	rise in temperature (mean) C°	t	p	result
GroupA ACTH 1unit/kg +pyrogen(0.06 ml/kg)	1.66	0.197	0.9-0.8	significance of difference not established
pyrogen(0.06 ml/kg)	1.70			

There is however a slight tendency to a lower temperature when ACTH is given with pyrogen, and it is possible using only two or three rabbits (see 171), to get a result showing that ACTH decreases the pyrogenic response at this level.

With the weaker dose of pyrogen however, it was found that ACTH did significantly lower the peak temperature. (table 17)

Table 17

Effect of ACTH on the pyrogenic response in rabbits

		rise in temperature (mean) C°	t	p	result
GroupB	ACTH 1unit/kg +pyrogen(0.02 ml/kg)	0.88	3.795	<0.001	significantly different
	pyrogen(0.02 ml/kg)	1.54			

Thus it appears that ACTH will modify the pyrexia of smaller doses of pyrogen to some extent; and this emphasises the importance of using as much as is practicable of the substance to be tested for pyrogen.

The degrees of shift to the left for ACTH+pyrogen and for pyrogen alone (both doses) were not significantly different.

It was found that for doth groups ACTH delays the time required for pyrogen to exert its maximum pyretic effect, and it appears to do so to the same extent with both dose-levels of pyrogen. (table 18)

Table 18

Effect of ACTH on the time required by the rabbit to reach peak temperature after pyrogen injection

		Time to reach peak temperature (minutes)	t	p	result
GroupA	ACTH 1unit/kg +pyrogen(0.06 ml/kg)	179	6.822	<0.001	significantly different
	pyrogen(0.06 ml/kg)	100			
GroupB	ACTH 1unit/kg +pyrogen(0.02 ml/kg)	193	3.217	0.01-0.001	significantly different
	pyrogen(0.02 ml/kg)	121			
GroupA	ACTH 1unit/kg +pyrogen(0.06 ml/kg)	179	1.330	0.2 - 0.1	significance of difference not established
GroupB	ACTH 1unit/kg +pyrogen(0.02 ml/kg)	193			

Although the peak temperature of a pyrogenic reaction occurs about 2 hours after injection, a pyrogen test is usually prolonged to 3 hours after injection in order that late peaks may be observed; and this is important when only 3 rabbits are used in the test(as in the B.P. test). When ACTH is given with pyrogen, the maximum pyrogenic response tends to be delayed to about 3 hours, hence it would be advisable to prolong any test of ACTH for pyrogen to at least 4, and preferably 5, hours after injection.

A test of this nature for detecting the pesence of pyrogen in ACTH is not wholly satisfactory, since small amounts of pyrogen will probably be missed due to the depressing effect of ACTH on the pyrogenic response. There is no reason, however , why a test of this nature should not detect gross pyrogen contamination of samples of ACTH.

It is recommended that 1 unit/kg ACTH is the largest useful dose to be used in a pyrogen test. If a dose larger than this is used and only 5 rabbits are used in the test, it is possible that 60% of the rabbits will give either no response to any pyrogen present, or will be so seriously upset that any result obtained will not be reliable.

Beattie and Hartfall[175] reported typical "moderately severe" pyrogenic reactions after administartion of ACTH to their patients. 20-25 mgm in one litre of saline was taken as the standard dose and was administered by slow intravenous infusion over a period of 24 hours in most cases.

25 mgm for, say, 70 kg body weight is equivalent to 0.36 mgm/kg. In the case of the material used in the experiments reported here, every mgm ACTH represented 2.32 units, and each rabbit received 1 unit/kg hence for a 2.5kg rabbit (the average weight of the populatin) 2.5 units were administered at each test which is just over 1 mgm, giving a dose of 0.4 mgm ACTH per kg body weight.

Thus it is seen that 1 unit/kg is a useful and practicable quantity to test for pyrogen, although there is, in this case, little margin for the increased sensitivity of humans to pyrogen as compared with rabbits. As well as being useful, this quantity appears to be the maximum possible, if a reliable estimate of pyrogen content is to be obtained using a test of this nature.

It is not possible to use the Polynuclear count as the basis of a pyrogen test for ACTH, since although pyrogen increases the shift to the left attributable to ACTH, different doses of pyrogen when injected with the same dose of ACTH, elicit the same degree of shift to the left, whereas injected alone, they elicit significantly different degrees of shift to the left. (see table19)

Table 19

Showing the degrees of shift to the left elicited by ACTH, ACTH + pyrogen, and pyrogen

	Percentage fall in average number of lobes per neutrophil	
	Group A	Group B
ACTH 1 unit/kg	22.0	21.9
ACTH lunit /kg + pyrogen(0.06ml/kg)	35.0	-
ACTH lunit/kg + pyrogen(0.02ml/kg)	-	34.7
Pyrogen (0.06ml/kg)	38.4	-
Pyrogen (0.02ml/kg)	-	32.4

Discussion

The concept of the polysaccharide nature of pyrogen is well established and the most potent preparations so far obtained have been shown to incorporate a phospholipid group in the structure and have therefore been called lipopolysaccharides. Although the sugar residues appear to vary from one preparation to another, it is not yet known whether the structure of the phospholipid to which pyrogenic activity has been attributed, is constant for the different preparations.

The difficulty which this substance has caused in the past has to some extent been explained by the demonstration of its tremendous potency and by its ubiquity.

The physiological examination of this powerful bacterial metabolite has been discussed in detail, and although much clear-cut evidence is awaited, there is a large amount of experimental data which has shown that the production of some other substance ("endogenous pyrogen") is stimulated by the injection of pyrogen, and that it is in all probability this endogenous pyrogen which is responsible for the general stimulation and the chain of events which follow. The action of pyrogen is therefore seen to be indirect. The site of production of endogenous pyrogen is unknown.

The temperature response is believed to be mediated by CNS stimulation and the leucocyte response mainly by the pituitary-adrenal system. There also appear to be profound effects on other endocrine and haemopoietic organs and on the reticuloendothelial system.

The clinical efficiency of pyrogen has its foundation in this non-specific stimulatory action, and this has been most profitably employed in a variety of ailments when used as an adjunct to other more specific measures where these exist.

The difficulties which may be experienced in the preparation of pyrogen have been discussed and it is seen that the extensive purification required when complex media and/or bacterial bodies are used as source can be avoided when a simple largely inorganic medium is used and the bacteria removed before extraction of the pyrogen.

Two simple methods were elaborated and are reported in part two (Section 1) of this thesis. The first consisted in adsorbing the pyrogen onto asbestos pads, subsequently eluting it with an alkaline fluid, and immediately neutralising the eluate, before precipitating with alcohol. This method was suggested before, but decomposition was found to be too rapid to allow the recovery of the pyrogenic activity. The success of the present method appears to be related to the immediate neutralisation of the eluate (or its adjustment to pH 5) and probably to the method of elution employed. It appears that sucking the eluting fluid through the pads after 10 - 15 minutes soaking is quite satisfactory. The material produced had a minimum pyrogenic dose of 0.5γ/kg.

In the second method suggested the liquid bulk was reduced by drying from the frozen state under reduced pressure. This conveniently concentrates the pyrogen solution and the writer feels that if large scale freeze-drying apparatus could be obtained this method could be used to produce pyrogen in quantity. Purification of the freeze-dried material was carried out by dialysis, repetition of the freeze-drying process and finally acetone precipitation. The material obtained had an MPD of 0.04γ/kg. for rabbits

The other sections of this thesis are devoted to an examination and comparison of certain biological properties of pyrogen.

There is no more reason in using the temperature response as an index of pyrogenic activity than in calling the substance pyrogen. It was called pyrogen because it was first known to elevate body temperature, and for a long time this was the only well known physiological reaction of the substance, hence it was by this index that pyrogenic activity was, and as yet mostly is, measured. But a large volume of literature has gradually been built up which points unmistdakably to an equally important effect which pyrogen has on white blood cells. At first it was vaguely imagined that this was almost cetainly a secondary effect of some sort, and this may still prove to be so, but it dose seem at least that it is no more indirect than the pyrexial effect; and it is probably moer nearly related to the means whereby it exerts its beneficial therapeutic action. In fact, it has been shown experimentally that pyrogen has an affinity for the white blood cells, that it damages them, and also that on disruption in vitro certain white blood cells will liberate material which will upon injection elevate the body temperature of experimental animals. This does not prove that this is what happens on injection of pyrogen or even that it is thereffect of endogenous pyrogen produced in the body, or again that what is liberated from damaged cells is endogenous pyrogen which is responsible for the thermogenic stimulus. But it does at least preclude the assumption that temperature rise is a direct effect of pyrogen injection.

Similarly the leucocyte effect which has been used as an index of pyrogenic activity (degree of shift to the left) may not wholly be a direct effect oy pyrogen, because the appearance of youhg neutrophils which cases this shift to the left may possibly be brought about by a stimulation of haemopoietic organs directly by pyrogen or endogenous pyrogen, or it may be brought about as a result of pituitary-adrenal stimulation, or even as a result of of the depletion of the leucocytes which initially occurs or by a combination of these.

In view of the fact that the temperature response has been shown to be less reliable than the leucocyte response (Sections 2 and 3 of this thesis) and that the temperature response has been shown to be unnecessary in order that the therapeutic effects may be obtained (indeed rise in temperature is regarded by some as an unwanted side effect), as well as being cumbersome to measure accurately from the point of view of assay, it is suggested that the leucocyte method described replace it.

Other workers have advocated leucocyte methods of potency estimation, and these have included total leucocyte counts[41,106,172] in dogs and rabbits, and differential counts in rabbits.[3]

There is now an overwhelming amount of evidence that the various well known manifestations of the pyrogenic reaction are caused by pyrogen and not by any contaminating material present in the different preparations which have been studied by the different workers. The demonstration in this report of the existence of correlation between the temperature and the leucocyte responses over the ranges defined, and using pyrogen preparations from different bacterial sources and prepared by totally different methods adds greatly to this conclusion. It would be unlikely that the different types and quantities of possible contaminating impurities present in such a variety of different pyrogen preparations would elicit both the temperature and leucocyte responses having such a consistent relationship as has been shown to exist (Sections 2 and 3 of this thesis).

In Section 2 where the original investigation of the suitability of the polynuclear count as a basis of an index of pyrogenic activity is reported, the conclusion was drawn from the data that as far as the variability of the different responses is concerned, there is little to choose between the temperature response as an index and that which measures the degree of shift to the left (based on the polynuclear count), but thet temperature appears to be slightly less variable.

In the application of the new index to pyrogen from other bacterial sources (Section3), however a smaller number of experiments was performed in an endeavour to determine which index retained most accuracy (i.e. the accuracy was "diluted-out" equally in both cases). This was done because it was felt that with such a large number (360) experiments as was performed in the original investigation (Section 2) both indices of activity were likely to show similar accuracy, whereas with a smaller number of experiments as was performed in Section 3 , inherent inaccuracy would be more likely to show up. This was found to be the case and it was shown that the index of activity based on the polynuclear count (percentage fall inthe average number of lobes perneutrophil measuring the degree of shift to the left) was the more accurate (i.e. less variable).

Two new ratios were devised (ratio A and ratio B) in order to check the result given by the comparison of the coefficients of variation and this they were found to do. Ratio compares in a way which is independent of the units of measurement, the scatter about hte regression with the range of values composing the regression; and ratio B compares similarly the same scatter with the"height"of the response (the overall mean of all the responses). Hence the variability of the responses has been assessed in a manner which considers not only the range but also the degree ("height") of the responses..

It has also been shown that the new index of activity is suitable for detecting pyrogen contamination in water and saline, where the pyrogen is derived from a mixture of unknown bacterial sources.

Signs of incipient tolerance in some rabbits which had had slightly more than 12 weekly injections of Proteus pyrogen were detected, and further evidence was supplied to dissociate this phenomenon from thet of precipitin production.

The biological similarity of pyrogen from different sources was further demonstrated by the production of a degree of cross-tolerance to 5 different pyrogen preparations. The results of these experiments (along with those on the polynuclear count using pyrogen from different sources) suggest that in spite of the reported variation in the polysaccharide composition of the various preparations there is the basis of common pharmacological action. This basis has been indicated by Westphal to reside in the common phospholipid moiety, although proof is still awaited that the structure of this part of the molecule is constant from one pyrogen preparation to another, and in the light of other evidence already quoted,[36] that it is to be found in all pure pyrogen preparations.

An attempt was made (Section 5) to use the new index of activity in the solution of the problem of testing for pyrogen, certain therapeutic materials which produce hypothermia in the rabbit. It was shown that computation of the degree of shift to the left (percentage fall in the average number of lobes per neutrophil) would give a reliable indication of the pyrogen content of Injection of Calcium Gluconate, and also that the use of the temperature response was still a possible choice for the detection of pyrogen in this injection, the maximum rise in tempertaure being unaffected by the transient initial hypothermia produced by the calcium gluconate.

In the case of ACTH which may also be given intravenously, the leucocyte response to the ACTH itself confuses any leucocyte response due to added pyrogen. It was found, however, that a modified test involving the measurement of the rise in temperature which succeeds the initial hypothermia, when pyrogen is present in the injection would serve as a control for gross pyrogen contamination. This problem with ACTH presents difficulties which have already been discussed (Section 5) and which reduce the pyrogen testing of ACTH to the state of an inaccurate limit test. If the dose of ACTH given in the test is increased in order to detect small quantities of pyrogen, the hypothermic effect (and possibly other effects) on the rabbits is so severe as to vitiate the test. If the dose of ACTH is decreased in order to avoid this there is a chance that enough pyrogen to cause discomfort to a patient will escape detection. It was found that 1 unit per kg of the ACTH supplied by Organon Ltd (2.32 units /mgm) was a suitable compromise for the test.

Summary

1 A survey of the recent literature dealing with pyrogen has been made.

2 Two methods are described whereby potent preparations of pyrogen may be obtained from an inorganic medium culture of Proteus vulgaris.

3 A new index of pyrogenic activity has been devised and it is based on the degree of shift to the left occurring among rabbit neutrophils after the injection of pyrogen.

4 This index of activity has been used successfully with pyrogen from various bacterial sources and of different potencies, and has been shown to be more reliable than the temperature response.

5 The phenomenon of cross-tolerance has been demonstrated using pyrogen from 3 Gram-negative organisms and it has been shown that tolerance to the pyrogenic effect can be established in the rabbit without concurrent precipitin formation.

6 The problem of testing for the presence of pyrogen in substances which exert a hypothermic effect on rabbits has been studied and several recommendations have been made.

References

1 Bennett and Beeson, Medicine, 1950, 29:365.

2 Wylie, Thesis, University of Glasgow, 1949.

3 Dawson, Thesis, University of Glasgow, 1954.

4 Wechselmann, Munch.med.Wschr., 1911,58:1510.

5 Hort and Penfold, J.Hyg.Camb., 1912,12:361.
Brit.Med.J., 1911,2:1[illegible].

6 Jona, J.Hyg.Camb.,1916,15:169

7 Seibert, Amer.J.Physiol., 1923,67:90.
Ibid., 1925,71:621
Seibert and Mendel,Ibid., 1923,67:105

8 Rademaker, Ann.Surg., 1930,92:195.

9 Symposium on Pyrogens, J.Pharm.Pharmacol.,1954,6:304-316.

10 Co Tui, Hope, Schrift, and Powers, J.Lab.Clin.Med., 1944,29:58.

11 Olitzki, Bendersky, and Koch, J.Immunol.,1943,46:71.

12 Bourn and Seibert, Amer.J.Physiol.,1925,71:652.

13 Palmer and Gerlough, Science,1940,95:155

14 Robinson and Flusser, J.Biol.Chem.,1944,153:529

15 Dare, J.Pharm.Pharmacol.,1953,5:528.

16 Dare and Mogey, J.PharmPharmacol.,1954,6:325

17 Beck and Fisher, Cancer Res., 1946,6:410

18 Shear and Turnen, J.Nat.Cancer Inst.,1943,4:81

19 Hartwell, Shear, and Adams, J.Nat.cancer Inst., 1943,4:107

20 Kahler, Shear, and Hartwell, J.Nat.Cancer Inst., 1943,4:123.

21 Ikawa,Koepfli,Mudd, and Niemann, J.Nat.Cancer Inst., 1952,13:157.

22 Farr, Clark, Proffitt, and Campbell, Amer.J.Physiol., 1954,177:269.

23 Ikawa, Koepfli, Mudd, and Niemann,
J.Amer.Chem.Soc.,1952,74:5219.
Ibid.,1953,75:1035.
Ibid.,1953,75:3439.

24 Nesset,McLallen,Anthony, and Ginger,J.Amer.Pharm.Ass. (Sci.Ed.) 1950,39:456.

25 Rodney and Welcke, J.Bact.,1945,50:129.

26 Ginger,Nesset,Reigel, and Fitzsimmons, J.Amer.Pharm.Ass.(Sci.Ed.) 1951,40:421.

27 Wylie and Todd, Quart.J.Pharmacol.,1948,21:240.

28 Westphal, Luderitz, and Bister, Z.Naturf.,1952,7b:148.

29 Westphal, Luderitz, Eichenberger, and Keiderling, Z.Naturf.,1952,7b:536.

30 Westphal, Luderitz, and Keiderling, Zbl.Bakt.,1952,158:152.

31 Communication from A.G.Wander Forschungsinstitut,Berne.

32 Luderitz and Westphal, Z.Naturf.,1952,7b:540.

33 Schramm, Westphal, and Luderitz, Z.Naturf.,1952,7b:594.

34 van Heyningen, Bacterial Toxins, Blackwell Scientific Publications, Oxford,1950,pp.86-102.

35 Morgan, Biochem.J.,1937,31:2003.
and Partridge, Ibid.,1940,34:169.
Ibid.,1941,35:1140.
Davies,Morgan, and Mosiman,Ibid.,1954,56:572.

36 Davies, Morgan, and Record, Biochem.J.,1955,60:290.

37 Davies, Biochem.J.,1955,59:696.

38 Altschule, Parkhurst, and Promisol, Arch. intern. Med., 1950, 86:505.

39 Grant and Whalen, Amer. J. Physiol., 1953, 173:47.

40 Chambers, Koenig, Koenig, and Windle, Amer. J. Physiol., 1949, 159:209.

41 Chapman, Quart. J. Pharm. Pharmacol., 1942, 15:361.

42 Randolph and Rollins, Ann. Allergy, 1950, 8:626.

43 Farr and LeQuire, Proc. Soc. Biol. N. Y, 1950, 75:661.

44 LeQuire, J. Infect. Dis., 1951, 88:194.

45 Probey and Pitman, J. Bact., 1945, 50:397.

46 Grant, Amer. J. Physiol., 1953, 173:246.

47 Farr, Clark, Profitt, and Campbell, Amer. J. Physiol., 1954, 177:269.

48 Favorite and Morgan, J. Clin. Invest., 1942, 21:589.
J. Lab. Clin. Med., 1946, 31:672.

49 Bennett, J. Exp. Med., 1948, 88:267.

50 McClosky, Price, VanWinkle, Welch, and Calvery, J. Amer. Pharm. Ass. (Sci. Ed.) 1943, 32:69.

51 Beeson, J. Exp. Med., 1947, 86:29 and 39.

52 Tennent and Ott, J. Amer. Pharm. Ass. (Sci. Ed.) 1953, 42:614.

53 Fritze, Doering, Menecke, and Schoen, Schweiz. med. Wschr., 1953, 83:783.
Fritze, Verhandlung der Deutschen Gesellschaft fur innere Medizin 60 Kongress 1952.
Bergmann, Buschmann, Doering, Fritze, and Wendte, Klin. Wschr., 1954, 32:500.

54 Westphal and Luderitz, Angewandte Chemie, 1954, 66:407.

55 Perry, J. Pharm. Pharmacol., 1954, 6:340.

56 Welch, Calvery, McClosky, and Price, J. Amer. Pharm. Ass. (Sci. Ed.) 1943, 32:65.

57 Proffitt, Farr, and Clark, Memorandum Report 53-19 related to Project NM007 081.12 Naval Med. Res. Inst. Bethesda, Md.

58 Morgan, J.Immunol.,59:129.

59 Zahl,Hutner,and Cooper, Proc.Soc.Exp.Biol.N.Y., 1943,54:48.

60 Zahl and Hutner, Amer.J.Hyg.,1944,39:189.

61 Shwartzman, CancerRes.,1944,4:191.

62 Shear, J.Nat.Cancer Inst.,1943-44,4:461.

63 Mondolfo and Hounie, ElDia Medico,Buenos Aires, 1947,19:1724 (cit.referencel)

64 Delafield and Smith, Brit.J.Exp.Path., 1936,17:379.

65 Long, Endocrinology,1949,45:135.

66 Barr,Cecil,and Dubois, Arch.intern.Med.,1922,29:608.

67 Dubois, Lane Medical Lectures. The mechanism of heat loss and temperature regulation, 1937, Stanford University,University Press

68 Altschule,Freedberg, and McManus,J.Clin.Invest.,1945, 24:878.

69 Fremont-Smith,Morrison,and Makepeace,J.Clin.Invest., 1929,7:489.

70 Perera,Arch.intern.Med.,1941,68:241.

71 Ranson, Arch.intern.Med.,1938,61:285.

72 Pinkston, Amer.J.Physiol.,1934,110:448.
Ibid.,1935,111:539.

73 Friedlander,Silbert,and Bierman, Amer.J.Med.Sci., 1940,199:657.

74 Douglas, J.Physiol.,1954,126 :319.

75 Wells and Rall,Proc.Soc.Exp.Biol.N.Y.,1948,68:421.

76 Wells and Rall,Proc.Soc.Exp.Biol.N.Y.,1949,70:169.

77 Isenschmid and Schmitzler,Arch.exper.PathPharmak. 1914,76:202.

78 Keller and Hare, Proc.Soc.Exp.Biol.N.Y.,1932,29:1069.

79 Ranson,Clark,and Magoun,J.Lab.Clin.Med.,1939,25:160.

80 Haertig and Masserman, J.Neurophysiol.,1940,3:293.

81 Stuart,Amer.J.Physiol.,1952,171:No. 3 December.

82 Menkin,Science,1944,100:337.

83 Bennett, J.Exp.Med.,1948,88:279.

84 Menkin, Dynamics of Inflammation,MacMillan Co., New York, 1940.
Newer Concepts of Inflammation, C.C.Thomas, Springfield,Illinois,1950.
Int.Arch.Allergy,1953,4:131.

85 Grant,Amer.J.Physiol.,1949,159:511.
Ann.Rev.Physiol.,1951,13:75.

86 Abderhalden,Experientia,1948,4:114.

87 Westphal,Luderitz,Kickhofen,Eichenberger,and Keiderling, Symposium on the Mechanism of Inflammation, Montreal,September,1953.

88 Kerby,Proc.Soc.Exp.Biol.N.Y., 1952,79:255.
Ibid., 1952,81:129.
Ibid., 1952,81:389.

89 Westphal and Kickhofen,Z.Rheumaforsch.,1953,12:321.

90 Stuart,Fed.Proc.,1951,10:133.

91 Windle,Chambers,Ricker,Ginger,and Koenig,Amer.J.med.Sci., 1950,219:422.

92 Greene,Stuart,and Joralemon,Proc.Soc.Exp.Biol.N.Y., 1953,82:39.

93 Wexler and Tryczynski,J.Lab.Clin.Med.,1955,45:296.

94 Zindler, Ann.Allergy,1951,9:494.

95 Anderson and Todd,J.Pharm.Pharmacol.,1954,6:962.

96 Soylemezoglu and Wells, Proc.Soc.Exp.Biol.N.Y., 1951,77:43

97 Mitchell and Stuart, Amer.J.Physiol.,1951,167:810

98 Mitchell, Amer.J.Physiol.,1952,171:No.3 December

99 Kibler and McBride, J.Infect.Dis.,1917,21:13.

100 Cowie and Calha n, Arch.intern.Med.,1919,23:69.

101 Gow,Quart.J.Med.,1919,13:82.

102 CoTui,and Schrift, Proc.Soc.Exp.Bol.,N.Y.,1942,49:320

103 Smith, Amer.J.Hyg.,1939,29:15

104 Robertson and Yu, J.Hyg.Camb.,1938,38:299.

105 Oloitzki,Avi-ery, and Bendersky, J.Immunol.,1941,41:361

106 Young and Rice, J.Lab.Clin.Med.,1944,29:735.

107 Wittich, Ann.Allergy,1951,9:502.

108 Guerrieri, Clin.Med.,1952,59:29.

109 MacGregor,Richards, and Loh, J.Path.Bact.,1940,51:337

110 Keiderling,Wohler, and Westphal, Arch.exper.Path. Pharmak.,1953,217:293.

111 Windle,Wilcox.Rhines, and Clemente, Fed.Proc.,1950 9:137.

112 Windle, Anat.Rec.,1950,106:260.

113 Selye, The Physiology and Pathology of Exposure to Stress, Acta Inc.,Montreal,1950.

114 Randolph and Rollins, Proc.1st Clin.ACTH Conf., Churchill, London,1950(J.R.Mote,Ed.).

115 Corcoran and Page, J.Lab.Clin.Med.,1948.33:1326.

116 Weisberger, Heinle, and Hannah, Proc.Soc.Exp.Biol.N.Y., 1949,70:749.

117 Sayers and Sayers, Endocrinology,1947,40:265.

118 Golkel and Steindl, Arztl.Wschr.,1951,5/I:444.
(cit. reference 30).

119 Clemente,Chambers,Greene,Mitchell,and Windle,
Anat.Rec.,1951,109:280.

120 Bennett and Beeson, J.Exp.Med.,1953,98:477 and 493.

121 Dare and Mogey, J.Pharm.Pharmacol.,1954,6:325.

122 Khight, Ann.Allergy,1954,12:174.

123 Fenton, Ann.Allergy,1953,11:363.

124 Howles, J.Louis.State Med.Soc.,1954,106:54.

125 Randolph,Medical Clinics of North America,1954 ,
vol. 38, No. 2, W.B.Saunders,Co.

126 Kierland and Kulwin, Arch.Derm.Syph.N.Y.,1950,62:571.

127 Welsh, The Eye,Ear, Nose and Throat Monthly,1953,
32:585.

128 Lever, New England J.Med.,1951,245:359.

129 McCorriston, Canad.med.Ass.J.,1953,68:137.

130 Cobin, Clin.Med.,1954,61:No. 2,February.

131 Sigel, Connecticut State Med.J., 1953,17:912.

132 Windle and Chambers, J.Comp.Neurol.,1950,93:241.

133 Windle, Clemente, and Chambers, J.Comp.Neurol.,
1952,96:359.

134 Scott and Clemente, Amer.J.Physiol.,1951,167:825.

135 Windle,Clemente,Scott, and Chambers,
Arch.Neurol.Psychiat.,1952,67:553.

136 Scott and Clemente, Arch.Neurol.Psychiat.,1952,67:830

137 Friedlander and Bailey, Neurology,1953,3:684.

138 Gattner and Koeppe, Die Therap.Gegenwart., 1949, 88:204.

139 Schopp,Fellhauer,and Eigel, Med.Bull.St.Louis.Univ., 1952,4:6.

140 Chesrow,Piszczek,Ganer, and Kaplitz, Geriatrics,1953, 8:504.

141 Hoff, Deut.med.Wschr.,1937,63:340.

142 Bammer and Martini, Pflug.Arch.,1953,257:308.

143 Lonsen and Liebert, Illinois med.J.,1949,96:186.

144 Doan, Radiology, 1938,30:382.

145 Epstein and Allen, Arch.Derm.Syph.,N.Y.,1951,63:419.

146 Taylor,Corcoran,Fertig, and Page, J.Lab.Clin.Med., 1948,33:1469.

147 Page and Taylor, Modern Concepts of Cardiovascular Disease, 1949,18:51.

148 Page and Corcoran, Arterial Hypertension: Its Diagnosis and Treatment. The Year Book Publishers,Inc.,Chicago, 1949.

149 McGinty,Wilson, and Rodney, Proc.Soc.Exp.Biol.N.Y., 1949,70:334

150 Necheles,Dommers,Weiner,Olson, and Rychel., Amer.J.Physiol.,1942,137:22.

151 Walker,Olson, and Necheles, Amer.J.Physiol., 1950,163:758.

152 Blickenstaff and Grossman, Amer.J.Physiol.,1950, 160:567.

153 Olson and Necheles, Amer.J.dig.Dis,1953,20:372.

154 Hochrein and Schleicher, Deut.med.Wschr.,1951,76:936.

155 Eichenberger and Schmidhauser-Kopp, Helv.physiol.acta, 1954,12:69.

156 Meneghini, Archivo"E.Maragliano"di Patol.e Clin., 1949,4:771.

157 Favez,Agnet, and Bossy, Helv.med.acta,1954,21:464.

158 Schellong and Soestmeyer, Deut.med.Wschr.,1951, 76:761.

159 Smengler, Klin.Wschr., 1949,27:627.

160 Rosenhagen, Klin.Wschr.,1946-47,24-25:233.

161 Saarelma, Ann.Med.Intern.Fenniae, 1946,35:156.

162 Wercker, Deut.med.Wschr.,1950,75:320.

163 Fitch and Washburne, New England J.Med.,1951,245:961.

164 Petersen, Protein Shock and Non-Specific Therapy, MacMillan Co.,New York, 1922.

165 Biozzi,Benacerraf, and Halpern, Brit.J.Exp.Path., 1955,36:226.

166 Stuart and Windle, Amer.J.Physiol.,1950,163:753.

167 Cortisone and ACTH, Butterworth & Co. Ltd., London, 1953, (W.S.C.Coepman,Ed.)

168 Harper, Unpublished results.

169 Dorche and Castaing, Ann.pharm.franc.,1950,8:353.

170 Kennedy, Quart.J.exp.Physiol.,1931-32,21:253.

171 Douglas and Paton, Lancet,1952,1:342.

172 Windle, Trans.N.Y.Acad.Sci.,1952,14:159.

173 Climenko, Quart.J.exp.Physiol.,1930,20:369.

174 Cooke and Ponder, The Polynuclear Count, Griffin & Co., London, 1927.

175 Beattie and Hartfall, Brit.Med.J.,1955,June25,p.1494.

176 Lees and Levvy, Brit.Med.J., 1940,1:430.

177 Brindle and Rigby, Quart.J.Pharm.Pharmacol.,1946,19:302

178 CoTui and Wright, Ann.Surg.,1942,116:412.

179 Bandelin, J.Amer.Pharm.Ass.(Sci.Ed.),1945,34:48.

180 Fevold,Personal Communication.

181 Rall and Wells, Proc.Soc.Exp.Biol.N.Y., 1952,80:380.

182 Andrewes, Lancet,1910,2:8.

183 Wintrobe,Clinical Hematology, Kimpton,London,1947.

Acknowledgements

The author thanks Professor J.P.Todd, of the School of Pharmacy, The Royal Technical College, Glasgow, who supervised the work and provided much helpful criticism and stimulating advice.

Thanks are also due to the following: Mr J.C.Eaton, M.A., for advice and discussion about the statistical analysis of the results; Miss A.C.Inglis, B.Sc., F.P.S., who performed some of the small lymphocyte counts; Dr Edkins of Organon, Ltd., who supplied the ACTH used; Dr Fevold of Travenol Labs., Inc., Illinois, who supplied the Pseudomonas polysaccharide; Prof. Dr Westphal of the Wander Forschungsinstitut, who supplied the Salmonella lipopolysaccharide; the Council of the Pharmaceutical Society of Great Britain, who provided an educational grant for the maintenance of the author during the period when this work was being carried out.

A P P E N D I X I

Normal values for the 'Average number of lobes per neutrophil'. (x)

Rabbit No.	x	x^2	Rabbit No.	x	x^2	Rabbit No.	x	x^2
	2.53	6.4009		2.13	4.5369		2.04	4.1616
	2.29	5.2441		2.08	4.3264		2.15	4.6225
	2.34	5.4756		1.93	3.7249		2.36	5.5696
1.	2.65	7.0225	8.	2.20	4.8400	15.	2.37	5.6169
	2.24	5.0176		2.18	4.7524		1.77	3.1329
	2.38	5.6644		1.82	3.3124		1.93	3.7249
mean	2.41			2.06			2.10	
	2.01	4.0401		2.46	6.0516		2.05	4.2025
	2.11	4.4521		2.54	6.4516		2.01	4.0401
	2.11	4.4521		2.33	5.4289		2.14	4.5796
2.	2.09	4.3681	9.	2.24	5.0176	16.	2.58	6.6564
	2.29	5.2441		2.33	5.4289		1.96	3.8416
	2.37	5.6169		2.15	4.6225		1.91	3.6481
mean	2.16			2.34			2.11	
	2.19	4.7961		2.27	5.1529		2.33	5.4289
	2.24	5.0176		2.16	4.6656		2.41	5.8081
	2.05	4.2025		2.04	4.1616		2.51	6.3001
3.	2.07	4.2849	10.	2.29	5.2441	17.	2.51	6.3001
	2.05	4.2025		2.20	4.8400		2.56	6.5536
	1.99	3.9601		2.21	4.8841		2.79	7.7841
mean	2.10			2.20			2.52	
	2.16	4.6656		2.11	4.4521		2.07	4.2849
	1.90	3.6100		2.14	4.5796		2.21	4.8841
	2.30	5.2900		1.93	3.7249		1.91	3.6481
4.	2.43	5.9049		2.02	4.0804	319.	19.6	3.8416
	2.30	5.2900	11.	2.11	4.4521		1.89	3.5721
	2.33	5.4289		1.88	3.5344		1.85	3.4225
mean	2.24			2.03			1.98	
	2.44	5.9536		2.26	5.1076		2.56	6.5536
	2.29	5.2441		2.03	4.1209		2.32	5.3824
	2.49	6.2001		1.97	3.8809		2.46	6.0516
5.	2.26	5.1076		2.02	4.0804	101.	2.78	7.7284
	2.25	5.0625	12.	2.17	4.7089		2.80	7.8400
	2.42	5.8564		2.16	4.6655		2.79	7.7841
mean	2.36			2.10			2.62	
	2.56	6.5536		2.11	4.4521		2.77	7.6729
	2.32	5.3824		2.09	4.3681		2.58	6.6564
	2.30	5.2900		2.23	4.9729		2.65	7.0225
6.	2.38	5.6644		2.16	4.6656	102.	2.48	6.1504
	2.25	5.0625	13.	2.16	4.6656		274	7.5076
	2.37	5.6169		2.02	4.0804		2.50	6.2500
mean	2.36			2.13			2.62	
	2 42	5.8564		2.21	4.8841		2.76	7.6716
	2.44	5.9536		2.10	4.4100		2.48	6.1504
	2.09	4.3681		1.94	3.7636		2.55	6.5025
7.	2.19	4.7961	14.	2.24	5.0176	103.	2.76	7.6176
	2.23	4.9729		2.41	5.8081		2.63	6.9169
	2.03	4.1209		2.20	4.8400		2.26	5.1076
mean	2.23			2.18			2.57	

continued.

Rabbit No.	x	x^2	Rabbit No.	x	x^2	Rabbit No.	x	x^2
	2.80	7.8400		2.48	6.1504		2.17	4.7089
	2.47	6.1009		2,45	6.0025		2.21	4.8841
	2.63	6.9169		2.60	6.7600		2.18	4.7524
104.	2.22	4.9284	108.	2.53	6.4009	400.	2.00	4.0000
	2.50	6.2500		2.24	5.0176		2.20	4.8400
	2.59	6.7081		2.24	5.0176		2.12	4.4944
mean	2.54			2.42			2.15	
	2.55	6.5025		2.54	6.4516		2.24	5.0176
	2.76	7.6176		2.29	5.2441		2.20	4.8400
	2.34	5.4756		2.44	5.9536		2.23	4.9729
105.	2.70	7.2900	109.	2.36	5.5696	402.	2.08	4.3264
	2.63	6.9169		2.30	5.2900		2.27	5.1529
	2.47	6.1009		2.73	7.4529		2.22	4.9284
mean	2.58			2.44			2.21	
	2.80	7.8400		2 34	5.4756		2.50	6.2500
	2.39	5.7121		2.37	5.6169		2.26	5.1076
	2.47	6.1009		2 37	5.6169		2.46	6.0516
107.	2.23	4.9729	110.	2.38	5.6644	112.	2.42	5.8564
	2.45	6.0025		2.47	6.1009		2.20	4.8400
	2.40	5.7600		2.21	4.8841		2 53	6.4009
mean	2.46			2.36			2.40	

Total number of rabbits = 30
Number values per rabbit = 6
Therefore total number of observations = 180. (= n)

$\sum x = 413.75$ therefore mean $\bar{x} = \frac{413.75}{180} = \underline{2.30}$

$\sum(x^2) = 960.8297$ and $\frac{(\sum x)^2}{n} = 951.0503$

therefore variance $\sigma^2 = \frac{\sum(x^2) - \frac{(\sum x)^2}{n}}{n - 1}$

$$= \frac{960.8297 - 951.0503}{179} = \underline{0.0546}$$

and hence standard deviation $\sigma = \underline{0.23}$
and coefficient of variation $V = \underline{10}$

continued.

The six groups comprised the following rabbits. Values are given for the average number of lobes per neutrophil. Each value given is the mean of six readings.

Group I (Beveren)

Rabbit No.	average number of lobes per neutrophil
2	2.16
4	2.24
11	2.03
13	2.13
16	2.11
	mean =2.13

Group II (Dutch)

Rabbit No.	average number of lobes per neutrophil
101	2.62
102	2.62
103	2.57
104	2.54
105	2.58
	mean=2.59

Group III (Havana)

1	2.41
5	2.36
6	2.36
7	2.23
9	2.34
	mean =2.34

Group IV (Havana)

3	2.10
10	2.20
12	2.10
15	2.10
17	2.52
	mean =2.20

Group V (Chinchilla)

8	2.06
14	2.18
108	2.42
109	2.44
112	2.40
	mean=2.30

Group VI (Mixed)

110	2.36
319	1.98
400	2.15
402	2.21
107	2.46
	mean=2.23

<u>Normal values for the percentage of small lymphocytes.</u>

Rabbit No.	x	x^2	Rabbit No.	x	x^2	Rabbit No.	x	x^2
	64.3	4134.49		68.4	4678.56		55.0	3025.00
	68.1	4637.61		63.9	4083.21		54.0	2916.00
	56.6	3203.56		66.4	4408.96		53.2	2830.24
1	60.4	3648.16	103	75.2	5655.04	112	52.7	2777.29
	71.1	5055.21		67.7	4583.29		47.2	2227.84
	60.1	3612.01		59.4	3528.36		39.9	1592.01
	63.9	4083.21		56.4	3180.96		39.4	1552.36
	62.0	3844.00		72.6	5270.76		62.5	3906.25
	71.4	5097.96		71.4	5097.96		61.4	3769.96
5	59.0	3481.00	104	52.5	2756.25	2	67.1	4502.41
	70.6	4984.36		81.8	6691.24		58.5	3422.25
	62.6	3918.76		65.4	4277.16		66.5	4422.25
	62.5	3906.25		47.3	2237.29		73.0	5329.00
	66.8	4462.24		70.5	4970.25		76.5	5852.25
	67.9	4610.41		62.0	3844.00		81.2	6593.44
6	67.5	4556.25	105	55.2	3047.04	4	65.4	4277.16
	57.1	3260.41		53.0	2809.00		42.7	5285.29
	78.5	6162.25		71.1	5055.21		64.7	4186.09
	62.1	3856.41		57.9	3352.41		56.8	3226.24
	60.9	3708.81		65.4	4277.16		63.2	3994.24
	76.5	5852.25		50.9	2590.81		72.6	5270.76
	67.1	4502.41	8	41.0	1681.00	11	67.4	4542.76
7	72.8	5299.84		49.2	2420.64		72.4	5241.76
	74.1	5490.81		31.2	973.44		59.8	3576.04
	59.8	3576.04		42.8	1831.84		68.5	4692.25
	50.4	2540.16		58.9	3469.21		59.0	3481.00
	63.8	4070.44		51.4	2641.96		69.9	4886.01
9	73.1	5343.61		47.1	2218.41	13	62.0	3844.00
	41.5	1722.25	14	59.8	3576.04		52.7	2777.29
	59.6	3552.16		54.3	2948.49		60.6	3672.36
	52.2	2724.84		58.2	3387.24		62.5	3906.25
	48.5	2352.25		47.5	2256.25		61.9	3831.61
	57.6	3317.76		57.8	3340.84		72.4	5241.76
101	61.9	3831.61		38.7	1497.69	16	54.3	2948.49
	56.3	3169.69	108	57.2	3271.84		45.4	2061.16
	50.2	2520.04		58.8	3457.44		62.4	3893.76
	58.4	3410.56		54.0	2916.00		45.0	2025.00
	67.9	4610.41		63.0	3969.00		49.5	2450.25
	73.9	5461.21		66.5	4422.25		63.2	3994.24
102	64.4	4147.36	109	51.1	2611.21	110	50.4	2540.16
	56.7	3214.89		53.7	2883.69		59.0	3481.00
	59.7	3564.09		62.8	3943.84		55.7	3102.49

continued.

Rabbit No.	x	x^2	Rabbit No.	x	x^2	Rabbit No.	x	x^2
	44.7	1998.09		67.8	4596.84		70.2	4928.04
	45.3	2052.09		75.0	5625.00		58.8	3457.44
	65.5	4290.25		74.3	5520.49		58.1	3375.61
319	56.1	3147.21	107	69.9	4886.01	12	53.9	2905.21
	58.6	3433.96		38.6	1489.96		69.8	4872.04
	47.1	2218.41		66.6	4435.56		56.4	3180.96
	58.5	3422.25		60.8	3696.64		68.6	4705.96
	64.0	4096.00		67.5	4556.25		60.8	3696.64
	66.9	4475.61		68.9	4747.21		73.2	5358.24
400	51.9	2693.61	3	66.4	4408.96	15	69.0	4761.00
	42.8	1831.84		53.7	2883.69		59.6	3552.16
	59.2	3504.64		62.7	3931.29		64.4	4147.36
	53.0	2809.00		73.3	5372.89		61.6	3794.56
	46.4	2152.96		68.5	4692.25		66.6	4435.56
	61.8	3819.24		72.5	5256.25		68.0	4624.00
402	46.8	2190.24	10	34.7	1204.09	17	64.7	4186.09
	46.1	2125.21		51.5	2652.25		62.3	3881.29
	42.9	1840.41		54.2	2937.64		62.9	3956.41

$\Sigma x = 10850.1$ therefore mean $= \dfrac{10850.1}{180} = 60.3$

$\Sigma(x^2) = 670542.11$ and $\dfrac{(\Sigma x)^2}{n} = 654025.94$

therefore variance $\sigma^2 = \dfrac{670542.11 - 654025.94}{179}$

$= \underline{92.27}$

and hence standard deviation $\sigma = \underline{9.6}$
and coefficient of variation $V = \underline{16}$

Percentage fall in the average number of lobes per neutrophil occurring in "blank" experiments when only apyrogenic saline (2mls/kg) was injected.

Rabbit No.	x	x^2
1	0	0
5	2.8	7.84
6	0	0
7	0	0
9	0	0
101	7.4	54.76
102	4.1	16.81
103	0	0
104	1.8	3.24
105	3.3	10.89
8	5.4	29.16
14	0	0
108	5.4	29.16
109	1.4	1.96
112	4.0	16.00
2	0	0
4	2.8	7.84
11	0	0
13	0	0
16	1.0	1.00
110	0	0
319	0	0
400	0	0
402	4.3	18.49
107	1.9	3.61
3	0	0
10	6.1	37.21
12	6.1	37.21
15	10.8	116.64
17	4.7	22.09

$\Sigma x = 73.3$ therefore mean $= 2.4$

$\Sigma(x^2) = 413.91$ and $\frac{(\Sigma x)^2}{n} = 179.10$

therefore variance $= \frac{413.91 - 179.10}{29}$

$= \underline{8.10}$

and standard deviation $= \underline{2.8}$

Comparison of the variance observed in counting one smear several times and that observed in counting smears taken from one rabbit at weekly intervals

Results of counts of several smears taken at weekly intervals:

x	x^2
2.30	5.2900
2.05	4.2025
1.99	3.9601
2.15	4.6225
2.14	4.5796
1.99	3.9601
2.07	4.2849
2.14	4.5796
2.01	4.0401
1.93	3.7249
2.03	4.1209
2.07	4.2849

$\sum x = 24.87 \qquad \frac{(\sum x)^2}{n} = 51.5431$

$\sum(x^2) = 51.6501$

therefore variance $= \frac{51.6501 - 51.5431}{11}$

$\underline{0.0097}$

Results of one smear counted several times:

2.20	4.8400
2.18	4.7524
2.16	4.6656
2.22	4.9284
2.24	5.0176
2.28	5.1984
2.26	5.1076
2.20	4.8400
2.19	4.7961
2.20	4.8400
2.22	4.9284
2.21	4.8841

$\sum x = 26.56 \qquad \frac{(\sum x)^2}{n} = 58.7861$

$\sum(x^2) = 58.7986$

therefore variance $= \frac{58.7986 - 58.7861}{11}$

$= \underline{0.0011}$

$F = \frac{0.0097}{0.0011} = \underline{8.8}$ which is greater than the five percent value for the variance ratio ($n_1 = 11$; $n_2 = 11$) hence the variance observed in counting one smear several times is less than that observed in counting smears taken from one rabbit at weekly intervals.

continued.

The investigation reported on the previous page was repeated with smears from a different rabbit.

Results of counts of several smears taken at weekly intervals:

x	x^2
2.33	5.4289
2.06	4.2436
2.18	4.7524
2.02	4.0804
2.07	4.2849
1.95	3.8025
2.13	4.5369
2.13	4.5369
2.03	4.1209
2.00	4.0000
2.05	4.2025
2.03	4.1209

$$\sum x = 24.98 \qquad \frac{(\sum x)^2}{n} = 52.0000$$

$$\sum(x^2) = 52.1108$$

$$\text{therefore variance} = \frac{52.1108 - 52.0000}{11} = \underline{0.0101}$$

Results of one smear counted several times:

2.48	6.1504
2.38	5.6644
2.44	5.9536
2.46	6.0516
2.44	5.9536
2.40	5.7600
2.45	6.0025
2.39	5.7121
2.44	5.9536
2.47	6.1009
2.42	5.8564
2.48	6.1504

$$\sum x = 29.25 \qquad \frac{(\sum x)^2}{n} = 71.2969$$

$$\sum(x^2) = 71.3095$$

$$\text{therefore variance} = \frac{71.3095 - 71.2969}{11} = \underline{0.0002}$$

$F = \frac{0.0101}{0.0002} = \underline{50.5}$ which is greater than the five percent value for the variance ratio ($n_1 = 11; n_2 = 11$) hence the variance observed in counting one smear several times is less than that observed in counting smears taken from one rabbit at weekly intervals.

Temperature responses of 30 rabbits to 4 dose-levels of pyrogen from *Proteus vulgaris*.

Rabbit No.	0.2ml/kg.			0.1125ml/kg.		
1	1.62	1.37	1.05	1.15	1.15	1.39
5	1.45	1.45	1.07	1.01	1.03	1.05
6	1.39	1.53	1.34	0.80	1.20	1.14
7	1.63	1.40	1.23	1.13	1.52	1.37
9	1.60	1.49	1.43	1.54	1.16	1.35
101	1.50	0.80	1.39	1.08	1.17	1.19
102	2.50	1.16	1.61	1.16	1.17	1.32
103	1.57	1.35	1.41	0.92	1.10	1.51
104	1.35	1.53	1.47	0.88	1.17	1.44
105	1.38	0.86	1.30	0.84	0.98	1.11
8	1.20	1.24	1.26	0.83	1.00	1.15
14	1.46	1.47	1.49	1.22	1.12	1.07
108	1.74	1.48	1.22	1.17	1.17	1.54
109	1.93	1.70	1.57	1.64	1.49	1.49
112	1.78	1.97	1.50	1.66	1.10	1.47
2	1.07	1.08	1.47	1.70	1.44	1.45
4	1.25	1.50	1.26	1.35	1.48	1.39
11	0.98	0.93	1.07	1.75	0.81	1.43
13	1.21	1.52	1.01	1.22	1.40	0.93
16	1.13	0.94	1.35	1.45	1.20	1.42
110	1.57	1.20	1.13	0.87	1.48	1.14
319	1.61	2.31	1.75	1.68	2.12	1.46
400	1.32	0.77	1.46	0.96	1.23	0.73
402	1.55	1.41	1.62	1.13	1.73	0.91
107	1.51	1.30	1.45	1.32	1.72	0.99
3	1.29	0.89	0.96	1.27	1.10	0.96
10	1.77	1.72	1.17	1.28	1.14	1.76
12	1.56	1.37	1.09	1.18	1.35	1.08
15	1.28	1.23	1.47	1.32	1.06	1.03
17	1.44	1.11	1.10	1.39	1.12	1.04

continued.

Rabbit No.	0.06324ml/kg.			0.02ml/kg.		
1	0.76	0.99	0.95	0.73	0.64	0.76
5	1.11	0.77	0.93	0.69	0.75	0.61
6	1.12	0.96	0.89	0.44	0.69	0.75
7	0.78	0.94	0.98	0.72	0.69	0.64
9	1.14	1.08	1.23	0.56	0.53	0.69
101	1.22	0.66	0.63	0.69	0.49	0
102	0.59	0.76	0.87	0.57	0.31	0.50
103	0.87	1.01	0.34	0.52	0.74	0.46
104	0.71	0.73	0.59	0.48	0.77	0.33
105	0.32	0.62	0.59	0.46	0.78	0.42
8	0.68	0.66	0.62	0.43	0.05	0.27
14	0.49	0.87	1.05	0.73	0.51	0.86
108	1.36	1.37	0.77	0.53	0.72	0.34
109	1.13	1.51	1.18	1.15	1.18	0.95
112	1.35	1.37	0.81	0.99	0.44	0.45
2	0.79	1.00	1.42	0.82	0.69	0.76
4	0.97	1.07	1.33	0.64	0.58	0.51
11	0.93	1.10	1.47	0.72	0.47	0.77
13	1.49	0.99	1.29	0.78	0.58	0.48
16	0.81	1.14	1.33	0.70	0.67	0.97
110	0.94	0.76	0.55	0.40	0.41	0.68
319	1.01	0.69	1.04	0.90	0.26	1.13
400	0.48	0.55	0.73	0.31	0.33	0.31
402	0.93	0.81	0.86	0.39	0.57	0.38
107	0.71	1.13	0.57	0.64	0.40	0.74
3	1.16	1.22	0.78	0.44	0.40	0.68
10	0.98	1.47	1.00	0.73	0.38	0.80
12	1.32	0.94	1.01	0.93	0.54	0.69
15	1.29	0.32	1.10	0.79	0.42	0.56
17	1.14	1.30	0.97	0.78	0.78	0.59

Calculations of the means, variances, standard deviations, and coefficients of variation of the temperature responses(x) to the 4 dose-levels of pyrogen from Proteus vulgaris.

0.2 ml/kg.

x	x^2	x	x^2
1.62	2.6244	1.07	1.1449
1.45	2.1025	1.25	1.5625
1.39	1.9321	0.98	0.9604
1.63	2.6569	1.21	1.4641
1.60	2.5600	1.13	1.2769
1.37	1.8769	1.08	1.1664
1.45	2.1025	1.50	2.2500
1.53	2.3409	0.93	0.8649
1.40	1.9600	1.52	2.3104
1.49	2.2201	0.94	0.8836
1.05	1.1025	1.47	2.1609
1.07	1.1449	1.26	1.5876
1.34	1.7956	1.07	1.1449
1.23	1.5129	1.01	1.0201
1.48	2.1904	1.35	1.8225
1.50	2.2500	1.57	2.4649
2.50	6.2500	1.61	2.5921
1.57	2.4649	1.32	1.7424
1.35	1.8225	1.55	2.4025
1.38	1.9044	1.51	2.2801
0.80	0.6400	1.20	1.4400
1.16	1.3456	2.21	4.8841
1.35	1.8225	0.77	0.5929
1.53	2.3409	1.41	1.9881
0.86	0.7396	1.30	1.6900
1.39	1.9321	1.13	1.2769
1.61	2.5921	1.75	3.0625
1.41	1.9881	1.46	2.1316
1.47	2.1609	1.62	2.6244
1.30	1.6900	1.45	2.1025
1.20	1.4400	1.29	1.6641
1.46	2.1316	1.77	3.1329
1.74	3.0276	1.56	2.4336
1.93	3.7249	1.28	1.6384
1.78	3.1684	1.44	2.0736
1.24	1.5376	0.89	0.7921
1.47	2.1609	1.72	2.9584
1.48	2.1904	1.37	1.8769
1.70	2.8900	1.23	1.5129
1.97	3.8809	1.11	1.2321
1.26	1.5876	0.96	0.9216
1.49	2.2201	1.17	1.3689
1.22	1.4884	1.09	1.1881
1.57	2.4649	1.47	2.1609
1.30	1.6900	1.10	1.2100

continued.

x	x^2
1.15	1.3225
1.01	1.0201
0.80	0.6400
1.13	1.2769
1.54	2.3716
1.15	1.3225
1.03	1.0609
1.20	1.4400
1.52	2.3104
1.16	1.3456
1.34	1.7956
1.05	1.1025
1.14	1.2996
1.37	1.8769
1.35	1.8225
1.08	1.1664
1.16	1.3456
0.92	0.8464
0.88	0.7744
0.84	0.7056
1.17	1.3689
1.17	1.3689
1.10	1.2100
1.17	1.3689
0.98	0.9604
1.19	1.4161
1.32	1.7424
1.51	2.2801
1.44	2.0736
1.11	1.2321
0.83	0.6889
1.22	1.4884
1.17	1.3689
1.64	2.6896
1.66	2.7556
1.00	1.0000
1.12	1.2544
1.17	1.3689
1.49	2.2201
1.10	1.2100
1.15	1.3225
1.07	1.1449
1.54	2.3716
1.49	2.2201
1.47	2.1609

<u>0.1125 ml/kg.</u>

x	x^2
1.70	2.8900
1.35	1.8225
1.75	3.0625
1.22	1.4884
1.45	2.1025
1.44	2.0736
1.48	2.1904
0.81	0.6561
1.40	1.9600
1.20	1.4400
1.45	2.1025
1.39	1.9321
1.43	2.0449
0.93	0.8649
1.42	2.0164
0.87	0.7569
1.68	2.8224
0.96	0.9216
1.13	1.2769
1.32	1.7424
1.48	2.1904
2.12	4.4944
1.23	1.5129
1.73	2.9929
1.72	2.9584
1.14	1.2996
1.46	2.1316
0.73	0.5329
0.91	0.8281
0.99	0.9801
1.27	1.6129
1.28	1.6384
1.18	1.3924
1.32	1.7424
1.39	1.9321
1.10	1.2100
1.14	1.2996
1.35	1.8225
1.06	1.1236
1.12	1.2544
0.96	0.9216
1.76	3.0976
1.08	1.1664
1.03	1.0609
1.04	1.0816

continued.

<u>0.06324ml/kg.</u>

x	x^2	x	x^2
0.76	0.5776	0.79	0.6241
1.11	1.2321	0.97	0.9409
1.12	1.2544	0.93	0.8649
0.78	0.6084	1.49	2.2201
1.14	1.2996	0.81	0.6561
0.99	0.9801	1.00	1.0000
0.77	0.5929	1.07	1.1449
0.96	0.9216	1.10	1.2100
0.94	0.8836	0.99	0.9801
1.08	1.1664	1.14	1.2996
0.95	0.9025	1.42	2.0164
0.93	0.8649	1.33	1.7689
0.89	0.7921	1.47	2.1609
0.98	0.9604	1.29	1.6641
1.23	1.5129	1.33	1.7689
1.22	1.4884	0.94	0.8836
0.59	0.3481	1.01	1.0201
0.87	0.7569	0.48	0.2304
0.71	0.5041	0.93	0.8649
0.32	0.1024	0.71	0.5041
0.66	0.4356	0.76	0.5776
0.76	0.5776	0.69	0.4761
1.01	1.0201	0.55	0.3025
0.73	0.5329	0.81	0.6561
0.62	0.3844	1.13	1.2769
0.63	0.3969	0.55	0.3025
0.87	0.7569	1.04	1.0816
0.34	0.1156	0.73	0.5329
0.59	0.3481	0.86	0.7396
0.68	0.4624	0.57	0.3249
0.49	0.2401	1.16	1.3456
1.36	1.8496	0.98	0.9604
1.13	1.2769	1.32	1.7424
1.35	1.8225	1.29	1.6641
0.66	0.4356	1.14	1.2996
0.87	0.7569	1.22	1.4884
1.37	1.8769	1.47	2.1609
1.51	2.2801	0.94	0.8836
1.37	1.8769	0.32	0.1024
0.62	0.3844	1.30	1.6900
1.05	1.1025	0.78	0.6084
0.77	0.5929	1.00	1.0000
1.18	1.3924	1.01	1.0201
0.81	0.6561	1.10	1.2100
0.59	0.3481	0.97	0.9409

continued.

0.02 ml/kg.

x	x^2
0.73	0.5329
0.69	0.4761
0.44	0.1936
0.72	0.5184
0.56	0.3136
0.64	0.4096
0.75	0.5625
0.69	0.4761
0.69	0.4761
0.53	0.2809
0.76	0.5776
0.61	0.3721
0.75	0.5625
0.64	0.4096
0.69	0.4761
0.69	0.4761
0.57	0.3249
0.52	0.2704
0.48	0.2304
0.46	0.2116
0.49	0.2401
0.31	0.0961
0.74	0.5476
0.77	0.5929
0.78	0.6084
0.00	0.0000
0.50	0.2500
0.46	0.2116
0.33	0.1089
0.42	0.1764
0.48	0.2304
0.73	0.5329
0.53	0.2809
1.15	1.3225
0.99	0.9801
0.05	0.0025
0.51	0.2601
0.72	0.5184
1.18	1.3924
0.44	0.1936
0.27	0.0729
0.86	0.7396
0.34	0.1156
0.95	0.9025
0.45	0.2025

x	x^2
0.82	0.6724
0.64	0.4096
0.72	0.5184
0.78	0.6084
0.70	0.4900
0.69	0.4761
0.58	0.3364
0.47	0.2209
0.58	0.3364
0.67	0.4489
0.76	0.5776
0.51	0.2601
0.77	0.5929
0.48	0.2304
0.97	0.9409
0.40	0.1600
0.90	0.8100
0.31	0.0961
0.39	0.1521
0.64	0.4096
0.41	0.1681
0.26	0.0676
0.33	0.1089
0.57	0.3249
0.40	0.1600
0.68	0.4624
1.13	1.2769
0.31	0.0961
0.38	0.1444
0.74	0.5476
0.44	0.1936
0.73	0.5329
0.93	0.8649
0.79	0.6241
0.78	0.6084
0.40	0.1600
0.38	0.1444
0.54	0.2916
0.42	0.1764
0.78	0.6084
0.68	0.4624
0.80	0.6400
0.69	0.4761
0.56	0.3136
0.59	0.3481

continued.

0.2ml/kg.

$\Sigma x = 124.17$ therefore mean $(\bar{x}) = \frac{124.17}{90} = 1.38^{o}$

$\Sigma(x^2) = 178.7291$ and $\frac{(\Sigma x)^2}{n} = 171.3132$

therefore variance, $\sigma^2 = \frac{\Sigma(x^2) - \frac{(\Sigma x)^2}{n}}{n-1}$

$= \frac{178.7291 - 171.3132}{89} = 0.0833$

and hence standard deviation, $\sigma = 0.29^{o}$
and coefficient of variation, $V = (\frac{100\ \sigma}{\bar{x}}) = 21.$

0.1125ml/kg.

$\Sigma x = 112.07$ therefore mean $= \frac{112.07}{90} = 1.25^{o}$

$\Sigma(x^2) = 145.5769$ and $\frac{(\Sigma x)^2}{n} = 139.5521$

therefore variance, $\sigma^2 = \frac{145.5769 - 139.5521}{89} = 0.0677$

and hence standard deviation, $\sigma = 0.26^{o}$
and coefficient of variation, $V = 21$

0.06324ml/kg.

$\Sigma x = 85.25$ therefore mean $= \frac{85.25}{90} = 0.95^{o}$

$\Sigma(x^2) = 87.8823$ and $\frac{(\Sigma x)^2}{n} = 80.7507$

therefore variance, $\sigma^2 = \frac{87.8823 - 80.7507}{89} = 0.0801$

and hence standard deviation, $\sigma = 0.28^{o}$
and coefficient of variation, $V = 30.$

0.02ml/kg.

$\Sigma x = 54.56$ therefore mean $= \frac{54.56}{90} = 0.61^{o}$

$\Sigma(x^2) = 37.2790$ and $\frac{(\Sigma x)^2}{n} = 33.0755$

therefore variance, $\sigma^2 = \frac{37.2790 - 33.0755}{89} = 0.0472$

and hence standard deviation, $\sigma = 0.22^{o}$
and coefficient of variation, $V = 36.$

A check on the normality of distribution of the temperature responses, using the χ^2 test.

0.2ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0.00-1.00	8	8	0	0
1.01-1.20	15	16	1	0.1
1.21-1.40	24	23	1	0
1.41-1.60	28	22	6	1.6
1.61-1.80	11	14	3	0.6
1.81-2.50	4	7	3	1.3

$\chi^2 = \sum\frac{(O-E)^2}{E} = 3.6$; n = 5; hence p = 0.5-0.7

Conclusion: These results are normally distributed

0.1125ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0.00-0.90	7	8	1	0.1
0.91-1.10	20	17	3	0.5
1.11-1.30	27	27	0	0
1.31-1.50	23	23	0	0
1.51-1.70	8	11	3	0.3
1.71-2.20	5	4	1	0.3

$\chi^2 = \sum\frac{(O-E)^2}{E} = 1.7$; n = 5; hence p = 0.8-0.9

Conclusion: These results are normally distributed

0.06324ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0.00-0.50	5	5	0	0
0.51-0.70	13	12	1	0.1
0.71-0.90	20	22	2	0.2
0.91-1.10	25	25	0	0
1.11-1.30	15	17	2	0.2
1.31-1.51	12	8	4	2.0

$\chi^2 = 2.5$; n = 5; hence p = 0.7 - 0.8

Conclusion: These results are normally distributed

continued.

<u>0.02ml/kg.</u>

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0.00-0.35	9	11	2	0.4
0.36-0.50	21	17	4	0.9
0.51-0.65	19	24	5	1.0
0.66-0.80	31	21	10	4.8
0.81-0.95	5	12	7	4.1
0.96-1.18	5	5	0	0

$\chi^2 = 11.2$; $n = 5$; hence $p = 0.02 - 0.05$

Conclusion: These results show a slight deviation from normality, but are not so far removed from it as to preclude the use of the usual statistical procedures

Analysis of variance of the temperature response
Incomplete three factor analysis : two factors with replication.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 6.8218	$n_1 - 1 =$ 29	0.2352	$n_2n_3\sigma_1^2+n_3\sigma_{12}^2+\sigma_o^2$
between doses	3 - 4 = 31.8758	$n_2 - 1 =$ 3	10.6253	$n_1n_3\sigma_2^2+n_3\sigma_{12}^2+\sigma_o^2$
rabbit x dose interaction	5.4829	$(n_1-1)(n_2-1) =$ 87	0.0630	$n_3\sigma_{12}^2+\sigma_o^2$
residual	5 - 1 = 12.4712	$(n_1n_2)(n_3-1) =$ 240	0.0520	σ_o^2
total	5 - 4 = 56.6517	$n_1n_2n_3-1=$ 359		

n_1 = no. of rabbits = 30

n_2 = no. of dose-levels = 4

n_3 = no. of replications = 3

σ_1^2= variance due to difference between rabbits
σ_2^2= variance due to difference between dose-levels
σ_{12}^2= variance of rabbit x dose interaction
σ_o^2= residual error

The sums of squares for the rabbit x dose interaction is obtained by subtracting the total sums of squares for the the other three compnnents from the total sum of squares.

1 the total of the three responses for each rabbit for each dose-level is squared and the squares for the 4 dose-levels summed, and this sum divided by 3 (since there are 3 responses per dose-level epr rabbit) = 436.9961

2 the 12 responses for each rabbit(3 at each of 4 dose-levels) are summed, the sums are squared, the squares are summed, and this total is divided by 12(the number of individuals per total being squared). = 399.6374

continued.

3 similarly with the 90 responses for each dose-level
= 424.6914

4 the grand total for the 360 responses is found, squared and divided by the grand total of individuals (360)
= 392.8156

5 every individual squared and the squares summed
= 449.4673

Existence of interaction:

$F = \frac{0.0630}{0.0520} = 1.21$ which is not greater than the value given by the tables for the variance ratio at the five per cent level ($n_1 = 87; n_2 = 240$) hence there is no rabbit-dose interaction, hence the between rabbit and between dose variances can be assessed against the residual which gives greater precision since there are more degrees of freedom associated with the residual than with the interaction.

Between rabbit variance:

$F = \frac{0.2352}{0.0520} = 4.52$ which is greater than the five per cent level ($n_1=29; n_2=240$) hence between rabbit variance exists significantly

Between dose variance:

$F = \frac{10.6253}{0.0520} = 204.33$ hence between dose variance exists

continued.

Significance of difference of the means for the 4 dose-levels:

Using the error variance (residual) we can test the significance of difference thus:

variance of a mean of 90 observations = $\frac{0.0520}{90}$, and the variance of the difference between two means is twice this

hence S.D. = $\sqrt{(0.0520 \times \frac{2}{90})}$ = 0.034

hence to be significantly different at the

5 per cent level	a difference of	1.97 x 0.034=	0.07	is required
1 percent level	" " "	2.60 x 0.034=	0.09	" "
0.1 percent level	" "	3.37 x 0.034=	0.12	" "

and from the following table

dose	means	difference
0.2ml/kg.	1.38	
0.1125 "	1.25	0.13
0.06324"	0.95	0.30
0.02 "	0.61	0.34

it will be seen that the means for the 4 dose-levels differ significantly ($p = < 0.001$)

Calculation of the correlation coefficient for the temperature response and log(100 x dose)

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$

where x = temperature response, and y = log(100 x dose)

$$\sum(x-\bar{x})^2 = \sum(x^2) - \frac{(\sum x)^2}{n} = 56.6517$$

$$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum y)^2}{n} = 49.21875$$

$$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 39.32536$$

Therefore $r = \frac{39.32536}{\sqrt{(56.6517 \times 49.21875)}}$ = 0.74473; n=358; p=<0.001

Therefore the correlation coefficient is significantly greater than zero.

Computation of the above figures:

$\sum(x^2)$		$\frac{(\sum x)^2}{n}$	
	178.7291		124.17
	145.5769		112.07
	87.8823		85.25
	37.2790		54.56
	449.4673		376.05

$$\frac{(376.05)^2}{360} = 392.8156$$

449.4673 - 392.8156 = 56.6517

$$\sum(y^2) = 90(1.69260100+1.10460100+0.64160100+0.09060100)$$
$$= 317.64636000$$

$$\sum y = 90(1.3010+1.0510+0.8010+0.3010) = 310.8600$$

hence $\frac{(\sum y)^2}{n} = \frac{(310.8600)^2}{360} = 268.42761$

and 317.64636) 268.42761 = 49.21875

$$\sum(xy) - \frac{\sum x \sum y}{n} = 364.04456 - \frac{376.05 \times 310.86}{360} = 39.32536$$

The equation for the regression line - temperature response and log(100 x dose)

The regression of y upon x is given by

$$Y = \bar{y} + b(x-\bar{x})$$

where Y is the predicted value of the temperature response for a given value for log(100 x dose)

$\bar{y}$ = mean of all the temperature responses (i.e. 360) = 1.045
$\bar{x}$ = mean of all the corresponding values for log(100 x dose) = 0.8635

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{\sum(xy) - \frac{\sum x \sum y}{n}}{\sum(x^2) - \frac{(\sum x)^2}{n}} = \frac{39.32536}{49.21875} = 0.79899$$

(see calculation of the correlation coefficient for the computation of these figures)

hence $Y = 1.045 + 0.79899(x-0.8635)$
therefore $Y = 0.79899x + 0.355$

log(100 x dose)

1.3010	$Y = 1.045+0.79899(1.3010-0.8635) = 1.39$
1.0510	$Y = 1.045+0.79899(1.0510-0.8635) = 1.19$
0.8010	$Y = 1.045+0.79899(0.8010-0.8635) = 1.00$
0.3010	$Y = 1.045+0.79899(0.3010-0.8635) = 0.60$

Using these values for Y the regression line has been drawn (fig. 3)

The residual variance about the regression line, σ_r^2

from this we calculate the standard deviation of the scatter about the regression line which is given by

$$\sigma_r = \sqrt{(1 - r^2)}\sqrt{\frac{\Sigma(y-\bar{y})^2}{n-2}}$$

$$= \sqrt{1 - 0.74473^2}\sqrt{\frac{56.6517}{358}}$$

$$= \underline{0.27}$$

hence

$$\text{ratio A} = \frac{0.27}{1.38 - 0.61} = \underline{0.35}$$

$$\text{ratio B} = \frac{0.27}{1.045} = \underline{0.26}$$

Analysis of variance to test linearity of relationship between temperature response and log(100 x dose)

We calculate:

1 Sum of squares of the temperature responses = 449.4673

2 Sum of temperature responses for each dose-level, square these sums and add these squares and divide by 90 (90 responses per dose-level) = 424.6914

3 Grand total for the 360 temperature responses, square this total and divide by 360 = 392.8156

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	31.8758 (2-3)	3	10.6253
within doses	24.7759 (1-2)	356	0.0696
total	56.6517 (1-3)	359	

It can be seen by inspection that the between dose mean square is significant, and the between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\Sigma(y-\bar{y})(x-\bar{x}))^2}{\Sigma(x-\bar{x})^2} = \frac{39.32536^2}{49.21875} = 31.42063$$

x = log(100 x dose)
y = corresponding temperature response

and which is now subtracted from the between dose sum of squares: 31.8758 - 31.42063 = 0.45517.

These values ate entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	31.42063	1	31.42063
deviation from regression	0.45517	2	0.22759
within doses (residual)	24.7759	356	0.0696
Total	56.6517	359	

The general significance of the regression line can be tested by comparing the regression mean square with the residual, thus $F = \frac{31.42063}{0.0696} = 451.4453$ which is very highly significant; and the significance of the deviation from linearity by comparing the deviation mean square with the residual, thus $F = \frac{0.22759}{0.0696} = 3.2699$.

The five per cent value for F $(n_1 = 2; n_2 = 356) = 2.99$ hence there is a very slight departure from linearity.

<u>Time required to reach maximum temperature after the injection of various dose-levels of pyrogen from *P. vulgaris*.</u>

<u>0.2ml/kg.</u>

x	x^2	x	x^2	x	x^2
110	12100	180	32400	90	8100
130	16900	160	25600	90	8100
100	10000	140	19600	200	40000
100	10000	160	25600	110	12100
100	10000	120	14400	80	6400
110	12100	130	16900	190	36100
110	12100	120	14400	130	16900
90	8100	90	8100	100	10000
80	6400	90	8100	110	12100
70	4900	100	10000	100	10000
110	12100	140	19600	130	16900
160	25600	150	22500	140	19600
90	8100	130	16900	120	14400
130	16900	70	4900	140	19600
110	12100	100	10000	120	14400
90	8100	120	14400	150	22500
60	3600	160	25600	150	22500
60	3600	180	32400	80	6400
110	12100	110	12100	150	22500
90	8100	180	32400	110	12100
110	12100	100	10000	140	19600
110	12100	70	4900	150	22500
120	14400	90	8100	120	14400
120	14400	120	14400	150	22500
110	12100	90	8100	150	22500
100	10000	180	32400	180	32400
60	3600	160	25600	70	4900
60	3600	150	22500	130	16900
80	6400	150	22500	120	14400
80	6400	140	19600	130	16900

$\sum x = 10670$ therefore mean $(\bar{x}) = \frac{x}{n} = \frac{10670}{90} = 119$ minutes

$\frac{(\sum x)^2}{n} = 1264987.8$ and $\sum(x^2) = 1349700$

hence variance, $\sigma^2 = \dfrac{\sum(x^2) - \frac{(\sum x)^2}{n}}{n-1} = 951.8$

and standard deviation, $\sigma = 31$

continued.

0.1125ml/kg.

x	x^2	x	x^2	x	x^2
130	16900	120	14400	90	8100
120	14400	110	12100	90	8100
130	16900	140	19600	190	36100
110	12100	110	12100	100	10000
110	12100	150	22500	90	8100
140	19600	80	6400	90	8100
130	16900	70	4900	200	40000
130	16900	190	36100	70	4900
130	16900	90	8100	90	8100
140	19600	60	3600	80	6400
120	14400	120	14400	170	28900
120	14400	100	10000	190	36100
90	8100	100	10000	90	8100
80	6400	130	16900	80	6400
90	8100	120	14400	100	10000
190	36100	140	19600	110	12100
210	44100	130	16900	90	8100
210	44100	120	14400	90	8100
80	6400	120	14400	130	16900
200	40000	160	25600	70	4900
160	25600	170	28900	200	40000
80	6400	160	25600	100	10000
120	14400	140	19600	100	10000
140	19600	110	12100	200	40000
80	6400	110	12100	170	28900
120	14400	110	12100	110	12100
110	12100	90	8100	100	10000
110	12100	110	12100	140	19600
120	14400	120	14400	120	14400
90	8100	120	14400	150	22500

$\Sigma x = 10990$ therefore mean $= \frac{10990}{90} = 122$ minutes

$\frac{(\Sigma x)^2}{n} = 1342001.1$ and $\Sigma(x^2) = 1458700$

hence variance, $\sigma^2 = 1311.2$

and standard deviation $\sigma = 36$

continued.

0.06324ml/kg.

x	x^2	x	x^2	x	x^2
80	6400	110	12100	80	6400
110	12100	140	19600	140	19600
120	14400	100	10000	100	10000
70	4900	100	10000	110	12100
70	4900	100	10000	100	10000
110	12100	110	12100	100	10000
120	14400	160	25600	130	16900
80	6400	80	6400	100	10000
120	14400	80	6400	120	14400
130	16900	130	16900	120	14400
90	8100	150	22500	140	19600
120	14400	90	8100	170	28900
70	4900	80	6400	80	6400
100	10000	110	12100	170	28900
100	10000	130	16900	150	22500
90	8100	90	8100	160	25600
90	8100	90	8100	130	16900
100	10000	90	8100	160	25600
100	10000	90	8100	150	22500
80	6400	100	10000	120	14400
110	12100	170	28900	120	14400
100	10000	120	14400	110	12100
70	4900	170	28900	130	16900
120	14400	80	6400	120	14400
70	4900	160	25600	110	12100
90	8100	110	12100	110	12100
100	10000	180	32400	110	12100
90	8100	70	4900	120	14400
130	16900	80	6400	100	10000
110	12100	90	8100	100	10000

$\Sigma x = 9960$ therefore mean $= \frac{9960}{90} = 111$ minutes

$\frac{(\Sigma x)^2}{n} = 1102240.0$ and $\Sigma(x^2) = 1167600$

hence variance $\sigma^2 = 734.4$

and standard deviation $\sigma = 27$

continued.

<u>0.02ml/kg.</u>

x	x^2	x	x^2	x	x^2
130	16900	90	8100	80	6400
130	16900	70	4900	80	6400
70	4900	70	4900	140	19600
120	14400	70	4900	120	14400
140	19600	150	22500	70	4900
90	8100	100	10000	120	14400
130	16900	80	6400	120	14400
130	16900	80	6400	140	19600
90	8100	130	16900	120	14400
80	6400	60	3600	110	12100
150	22500	180	32400	130	16900
90	8100	80	6400	100	10000
160	25600	100	10000	130	16900
50	2500	160	25600	110	12100
130	16900	150	22500	120	14400
120	14400	80	6400	0	0
120	14400	90	8100	120	14400
70	4900	160	25600	150	22500
130	16900	190	36100	150	22500
90	8100	150	22500	130	16900
80	6400	140	19600	100	10000
80	6400	160	25600	80	6400
80	6400	100	10000	90	8100
70	4900	140	19600	70	4900
80	6400	100	10000	110	12100
90	8100	150	22500	130	16900
80	6400	140	19600	90	8100
130	16900	140	19600	70	4900
80	6400	110	12100	140	19600
110	12100	160	25600	120	14400

$\Sigma x = 9920$ therefore mean $= \frac{9920}{89} = 111$ minutes
n = 89 here because there is one zero result which should strictly be recorded as infinite time to raech maximum temperature, hence it is not included in the calculation

$\frac{(\Sigma x)^2}{n} = 1105689.9$ and $\Sigma(x^2) = 1190800$
hence variance, $\sigma^2 = 967.2$ and standard deviation, $\sigma = 31$

To test the significance of the difference between the mean times taken to reach maximum temperatures for different dose-levels of pyrogen from P. Vulgaris.

$$t = \frac{\bar{x} - \bar{x}'}{\sigma} \sqrt{\frac{n \times n'}{n + n'}}$$

$$\sigma = \sqrt{\Sigma(x^2) - \frac{(\Sigma x)^2}{n} + \Sigma(x'^2) - \frac{(\Sigma x')^2}{n'} / (n + n' - 2)}$$

0.2 ml/kg : $\bar{x}$ = 119 minutes; n = n' = 90
0.1125 " : $\bar{x}'$ = 122 minutes

$$\sigma = \sqrt{\frac{84712 + 116699}{178}} \quad \sqrt{\frac{n \times n'}{n + n'}} = 6.7; \; \bar{x} - \bar{x}' = 3$$

therefore t = 0.845; p = 0.3 - 0.4; therefore the mean times do not differ significantly

---

0.1125 ml/kg: $\bar{x}$ = 122 minutes; n = n' = 90
0.06324 " : $\bar{x}'$ = 111 minutes

$$\sigma = \sqrt{\frac{116699+65860}{178}} \quad \sqrt{\frac{n \times n'}{n + n'}} = 6.7; \; \bar{x} - \bar{x}' = 11$$

therefore t = 2.310; p = 0.05 - 0.02; therefore the mean times differ significantly

---

0.06324ml/kg: $\bar{x}$ = 111 minutes therefore $\bar{x} - \bar{x}' = 0$
0.02 " : $\bar{x}'$ = 111minutes

Leucocyte responses (degree of shift to the left) of 30 rabbits to 4 dose-levels of pyrogen from Proteus vulgaris.

Rabbit No.	0.2ml/kg.			0.1125ml/kg.		
1	37.6	42.1	29.6	26.1	21.8	37.5
5	36.7	47.0	34.3	16.4	23.9	39.7
6	41.1	34.5	25.9	20.0	22.0	33.8
7	36.2	43.9	27.5	11.2	26.3	29.2
9	43.6	36.3	28.9	22.7	27.1	28.9
101	37.7	20.6	40.9	17.1	28.0	32.8
102	28.7	23.1	43.0	17.5	37.6	36.0
103	30.7	29.9	23.0	9.6	33.9	36.0
104	37.3	25.0	38.1	23.2	35.3	35.7
105	20.6	[illegible]	26.3	22.3	31 .2	35.3
8	31.9	32.8	33.3	19.2	27.8	31.6
14	40.4	34.6	31.4	13.9	28.3	34.9
108	37.5	36.8	32.5	25.5	33.8	33.8
109	42.2	35.3	26.2	31.1	33.3	34.5
112	37.2	27.0	38.6	31.9	31.3	35.5
2	27.5	42.1	40.8	37.0	31.8	32.4
4	28.2	36.1	34.0	35.9	40.1	39.8
11	21.4	35.5	44.3	38.5	33.6	33.3
13	25.0	40.7	32.0	37.5	37.9	34.3
16	32.1	38.9	33.6	27.4	31.1	29.5
110	56.5	37.3	44.5	42.8	35.3	42.6
319	44.3	44.3	44.8	44.0	38.0	38.8
400	36.9	39.9	43.9	31.2	36.8	44.7
402	40.5	43.5	40.3	33.6	30.1	38.6
107	30.1	38.7	46.2	26.2	27.9	47.9
3	43.9	41.6	22.0	24.1	30.1	17.3
10	39.0	43.0	24.3	12.2	25.0	25.1
12	38.9	32.1	49.8	27.4	33.0	24.9
15	38.7	37.6	24.0	2.1	31.6	19.2
17	42.9	37.2	17.6	21.8	36.1	17.6

continued.

Rabbit No.	0.06324ml/kg.			0.02ml/kg.		
1	21.4	23.2	26.4	16.1	4.4	8.1
5	33.0	25.6	26.9	22.2	13.7	7.0
6	31.3	31.4	31.0	9.9	9.5	8.7
7	9.5	23.6	28.8	22.1	10.9	7.4
9	29.7	18.4	15.0	16.8	6.4	18.5
101	25.7	12.6	15.0	12.1	10.1	10.8
102	30.0	37.7	4.4	25.7	16.9	9.8
103	30.3	35.3	23.4	27.8	15.7	9.1
104	25.7	32.6	22.3	30.4	22.2	11.1
105	30.1	38.0	15.3	19.6	14.1	9.1
8	18.7	32.4	32.5	24.4	37.4	17.8
14	28.8	28.8	30.8	38.1	23.7	12.3
108	30.3	34.0	37.5	22.3	21.4	25.1
109	29.4	22.0	36.5	9.3	18.9	10.7
112	25.7	40.8	34.4	35.0	23.7	15.6
2	16.1	32.2	35.1	15.0	17.7	15.0
4	26.7	19.1	29.3	14.1	23.1	10.4
11	31.6	20.5	29.2	18.7	25.9	15.9
13	24.2	24.9	35.0	14.9	28.7	4.7
16	27.7	22.7	27.1	6.1	21.5	2.6
110	19.1	28.5	22.3	32.9	23.7	29.2
319	30.8	0	19.7	30.0	25.0	23.2
400	28.2	32.5	16.5	10.2	24.7	14.9
402	25.5	0	23.6	14.5	11.9	18.7
107	12.1	17.1	26.9	15.1	19.5	30.2
3	17.1	32.7	18.5	24.9	8.8	13.5
10	15.0	28.3	1.1	21.8	16.5	13.0
12	16.9	31.2	30.0	16.6	20.0	6.2
15	24.6	29.4	20.4	4.2	13.9	14.0
17	28.1	33.3	22.3	0	11.5	8.6

<u>Calculations of the means, variances, standard deviations, and coefficients of variation of the leucocyte responses (x) (degree of shift to the left) to the 4 dose-levels of pyrogen from Proteus vulgaris.</u>

<u>0.2ml/kg.</u>

x	x^2	x	x^2
37.6	1413.76	27.5	756.25
36.7	1346.89	28.2	795.24
41.1	1689.21	21.4	457.96
36.2	1310.44	25.0	625.00
43.6	1900.96	32.1	1030.41
42.1	1772.41	42.1	1772.41
47.0	2209.00	36.1	1303.21
34.5	1190.25	35.5	1260.25
43.9	1927.21	40.7	1656.49
36.3	1317.69	38.9	1513.21
29.6	876.16	40.8	1664.64
34.3	1176.49	34.0	1156.00
25.9	670.81	44.3	1962.49
27.5	756.25	38.0	1444.00
28.9	835.21	33.6	1128.96
37.7	1421.29	56.5	3192.25
28.7	823.69	44.3	1962.49
30.7	942.49	36.9	1361.61
37.3	1391.29	40.5	1640.25
20.6	424.36	30.1	906.01
20.6	424.36	37.3	1391.29
23.1	533.61	44.3	1962.49
29.9	894.01	39.9	1592.01
25.0	625.00	43.5	1892.25
28.7	823.69	38.7	1497.69
40.9	1672.81	44.5	1980.25
43.0	1849.00	44.8	2007.04
28.0	784.00	43.9	1927.21
36.1	1303.21	40.3	1624.09
26.3	691.69	46.2	2134.44
31.9	1017.61	43.9	1927.21
40.4	1632.16	39.0	1521.00
37.5	1406.25	38.9	1513.21
42.2	1780.84	42.9	1840.41
37.2	1383.84	38.7	1497.69
32.8	1075.84	41.6	1730.56
34.6	1197.16	43.0	1849.00
36.8	1354.24	32.1	1030.41
35.3	1246.09	37.6	1413.76
27.0	729.00	37.2	1383.84
33.3	1108.89	22.0	484.00
31.4	985.96	24.3	590.49
32.5	1056.25	49.8	2480.04
26.2	686.44	24.8	615.04
38.6	1489.96	17.6	309.76

continued.

<u>0.1125ml/kg.</u>

x	x^2	x	x^2
26.1	681.21	37.0	1369.00
16.4	268.96	35.9	1288.81
20.0	400.00	38.5	1482.25
11.2	125.44	37.5	1406.25
22.7	515.29	27.4	750.76
21.8	475.24	31.8	1011.24
23.9	571.21	40.1	1608.01
22.0	484.00	33.6	1128.96
26.3	691.69	37.9	1436.41
27.1	734.41	31.1	967.21
37.5	1406.25	32.4	1049.76
39.7	1576.09	39.8	1584.04
33.8	1142.44	33.3	1108.89
29.2	852.64	34.3	1176.49
28.9	835.21	29.5	870.25
17.1	292.41	42.8	1831.84
17.5	306.25	44.0	1936.00
9.6	92.16	31.2	973.44
23.2	538.24	33.6	1128.96
22.3	497.29	28.2	795.24
28.0	784.00	35.3	1246.09
37.6	1413.76	38.0	1444.00
33.8	1142.44	36.8	1354.24
35.3	1246.09	30.1	906.01
31.2	973.44	27.9	778.41
32.8	1075.84	42.6	1814.76
36.0	1296.00	38.8	1505.44
36.0	1296.00	44.7	1998.09
35.7	1274.49	38.6	1489.96
35.3	1246.09	42.9	1840.41
19.2	368.64	24.1	580.81
13.9	193.21	12.2	148.84
25.5	650.25	27.4	750.76
31.1	967.21	2.1	4.41
31.9	1017.61	21.8	475.24
27.2	739.84	30.1	906.01
28.3	800.89	25.0	625.00
33.8	1142.44	33.0	1089.00
33.3	1108.89	31.6	998.56
31.3	979.69	36.1	1303.21
31.6	998.56	17.3	299.29
34.9	1218.01	25.1	630.01
33.8	1142.44	24.9	620.01
34.5	1190.25	18.2	331.24
35.5	1260.25	17.6	309.76

continued.

<u>0.06324ml/kg.</u>

x	x^2
21.4	457.96
33.0	1089.00
31.3	979.69
9.5	90.25
29.7	882.09
23.2	538.24
25.6	655.36
31.4	985,96
23.6	556.96
18.4	338.56
26.4	696.96
26.9	723.61
31.0	961.00
28.8	829.44
15.0	225.00
25.7	660.49
30.0	900.00
30.3	918.09
25.7	660.49
30.1	906.01
12.6	158.76
37.7	1421.29
35.3	1246.09
32.6	1062.76
38.0	1444.00
15.0	225.00
4.4	19.36
23.4	547.56
22.3	497.29
15.3	234.09
18.7	349.69
28.8	829.44
30.3	918.09
29.4	864.36
25.7	660.49
32.4	1049.76
28.8	829.44
34.0	1156.00
22.0	484.00
40.8	1664.64
32.5	1056.25
30.8	948.64
37.5	1406.25
36.5	1332.25
34.4	1183.36

x	x^2
16.1	259.21
26.7	712.89
31.6	998.56
24.2	585.64
27.7	767.29
32.2	1036.84
19.1	364.81
20.5	420.25
24.9	620.01
22.7	515.29
35.1	1232.01
29.3	858.49
29.2	852.64
35.0	1225.00
27.1	734.41
19.1	364.81
30.8	948.64
28.2	795.24
25.5	650.25
12.1	146.41
28.5	812.25
0	0
32.5	1056.25
0	0
17.1	292.41
22.3	497.29
19.7	388.09
16.5	272.25
23.6	556.96
26.9	723.61
17.1	292.41
15.0	225.00
16.9	285.61
24.6	605.16
28.1	789.61
32.7	1069.29
28.3	800.89
31.2	973.44
29.4	864.36
33.3	1108.89
18.5	342.25
1.1	1.21
30.0	900.00
20.4	416.16
22.3	497.29

continued.

<u>0.02ml/kg.</u>

x	x^2	x	x^2
16.1	259.21	15.0	225.00
22.2	492.84	14.1	198.81
9.9	98.01	18.7	349.69
22.1	488.41	14.9	222.01
16.8	282.24	6.1	37.21
4.4	19.36	17.7	313.29
13.7	187.69	23.1	533.61
9.5	90.25	25.9	670.81
10.9	118.81	28.7	823.69
6.4	40.96	21.5	462.25
8.1	65.61	15.0	225.00
7.0	49.00	10.4	108.16
8.7	75.69	15.9	252.81
7.4	54.76	4.7	22.09
18.5	342.25	2.6	6.76
12.1	146.41	32.9	1082.41
25.7	660.49	30.0	900.00
27.8	772.84	10.2	104.04
30.4	924.16	14.5	210.25
19.6	384.16	15.1	228.01
10.1	102.01	23.7	561.69
16.9	285.61	25.0	625.00
15.7	246.49	24.7	610.09
22.2	492.84	11.9	141.61
14.1	198.81	19.5	380.25
10.8	116.64	29.2	852.64
9.8	96.04	23.2	538.24
9.1	82.81	14.9	222.01
11.1	123.21	18.7	349.69
9.1	82.81	30.2	912.04
24.4	595.36	24.9	620.01
38.1	1451.61	21.8	475.24
22.3	497.29	16.6	275.56
9.3	86.49	0	0
35.0	1225.00	4.2	17.64
37.4	1398.76	8.8	77.44
23.7	561.69	16.5	272.25
21.4	457.96	20.0	400.00
18.9	357.21	13.9	193.21
23.7	561.69	11.5	132.25
17.8	316.84	13.3	176.89
12.3	151.29	13.0	169.00
25.1	630.01	6.2	38.44
10.7	114.49	14.0	196.00
15.6	243.36	8.6	73.96

continued.

<u>0.2ml/kg.</u>

$\Sigma x = 3202.8$ therefore mean $(\bar{x}) = \frac{3202.8}{90} = 35.6$

$\Sigma(x^2) = 118932.08$ and $\frac{(\Sigma x)^2}{n} = 113976.98$

therefore variance, $\sigma^2 = \frac{118932.08 - 113976.98}{89} = 55.68$

and hence standard deviation, $\sigma = 7.5$
and coefficient of variation, $V = 21$

(see page 149 for formulae used in calculating these statistics)

<u>0.1125ml/kg.</u>

$\Sigma x = 2685.9$ therefore mean $(\bar{x}) = \frac{2685.9}{90} = 29.8$

$\Sigma(x^2) = 86366.13$ and $\frac{(\Sigma x)^2}{n} = 80156.21$

therefore variance, $\sigma^2 = \frac{86366.13 - 80156.21}{89} = 69.77$

and hence standard deviation, $\sigma = 8.4$
and coefficient of variation, $V = 28$

<u>0.06324ml/kg.</u>

$\Sigma x = 2269.3$ therefore mean $(\bar{x}) = \frac{2269.3}{90} = 25.2$

$\Sigma(x^2) = 63503.39$ and $\frac{(\Sigma x)^2}{n} = 57219.14$

therefore variance $\sigma^2 = \frac{63503.39 - 57219.14}{89} = 70.61$

and hence standard deviation, $\sigma = 8.4$
and coefficient of variation, $V = 33$

<u>0.02 ml/kg.</u>

$\Sigma x = 1513.2$ therefore mean $(\bar{x}) = \frac{1513.2}{90} = 16.8$

$\Sigma(x^2) = 31316.52$ and $\frac{(\Sigma x)^2}{n} = 25441.94$

therefore variance, $\sigma^2 = \frac{31316.52 - 25441.94}{89} = 66.01$

and hence standard deviation, $\sigma = 8.1$
and coefficient of variation, $V = 48$

A check on the normality of distribution of the leucocyte response (degree of shift to the left) using the χ^2 test.

0.2ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
0 -25.5	10	9	1	1.1
25.6-30.5	14	14	0	0
30.6-35.5	15	22	7	2.2
35.6-40.5	26	21	5	1.2
40.6-45.5	21	15	6	2.4
45.6-57.5	4	9	5	2.8

$\chi^2 = \sum\frac{(O-E)^2}{E} = 9.7$; n = 5; hence p = 0.05 - 0.1

Conclusion: These results are normally distributed

0.1125ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
o -20.5	13	12	1	0.1
20.6-25.5	12	15	3	0.6
25.6-30.5	15	20	5	1.3
30.6-35.5	28	23	5	1.1
35.6-40.5	18	13	5	1.9
40.6-45.5	4	6	2	0.7

$\chi^2 = 5.7$; n = 5; hence p = 0.3-0.5

Conclusion: these results are normally distributed

0.06324ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
0 -15.5	11	11	0	0
15.6-20.5	13	15	2	0.3
20.6-25.5	14	21	7	2.3
25.6-30.5	27	20	7	2.5
30.6-35.5	20	14	6	2.6
35.6-41.5	5	8	3	1.1

$\chi^2 = 8.8$; n = 5; hence p = 0.1 - 0.2

Conclusion: These results are normally distributed

continued.

<u>0.02ml/kg.</u>

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0 - 5.5	5	7	2	0.6
5.6-10.5	19	12	7	4.1
10.6-15.5	20	20	0	0
15.6-20.5	17	22	5	1.1
20.6-25.5	17	16	1	0.1
25.6-38.5	12	13	1	0.1

$\chi^2 = 6.0$; $n = 5$; hence $p = 0.3 - 0.5$

Conclusion: these results are normally distributed.

<u>Analysis of variance of the leucocyte response which measures the degree of shift to the left. Incomplete three factor analysis : two factors with replication.</u>

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 3485.87	$n_1 - 1 =$ 29	120.20	
between doses	3 - 4 = 16982.84	$n_2 - 1 =$ 3	5660.95	AS ON PAGE 152
rabbit x dose interaction	5766.54	$(n_1-1)(n_2-1)=$ 87	66.28	
residual	5 - 1 = 14071.45	$n_1n_2(n_3-1)=$ 240	58.63	
total	5 - 4 = 40306.70	$n_1n_2n_3-1 =$ 359		

$n_1, n_2, n_3, \sigma_1^2, \sigma_2^2, \sigma_{12}^2$, and σ_0^2 have the same meaning as the same symbols on page , and the sum of squares for the rabbit x dose interaction is calculated in the same manner as shown on page 152.

1 = 286046.67
2 = 263297.29
3 = 276794.26
4 = 259311.42
5 = 300118.12

1, 2, 3, 4, 5, were calculated as for the temperature response using the results obtained for this index of activity.

continued.

Existence of interaction:

$F = \frac{66.28}{58.63} = 1.13$ which is not greater than the value given by the tables for the variance ration at the five per cent level ($n_1 = 87$; $n_2 = 240$), hence there is no rabbit-dose interaction, hence the between rabbit and between dose variances can be assessed against the residual which gives greater precision since there are more degrees of freedom associated with the residual than with the interaction.

Between rabbit variance:

$F = \frac{120.20}{58.63} = 2.05$ which is greater than the five percent level ($n_1 = 29$; $n_2 = 240$) hence between rabbit variance exists significantly.

Between dose variance:

$F = \frac{5660.95}{58.63} = 96.55$ hence between dose variance exists

continued.

Significance of difference of the means for the 4 dose-levels :

Using the error variance (residual) we can test the significance of difference thus:

variance of a mean of 90 observations = $\frac{58.63}{90}$ and the variance of the difference between two means is twice this

hence S.D. = $\sqrt{(58.63 \times \frac{2}{90})} = 1.14$

hence to be significantly different at the

5 percent level a difference of 1.97 x 1.14=2.25 is required
1 percent level " " " 2.60 x 1.14=2.96 " "
0.1percent level" " " 3.37 x 1.14=3.85 " "

and from the following table

dose	means	difference
0.2ml/kg.	35.6	
		5.8
0.1125 "	29.8	
		4.6
0.06324"	25.2	
		8.4
0.02 "	16.8	

it will be seen that the means forthe 4 dose-levels differ significantly ($p = < 0.001$)

Calculation of the correlation coefficient for the leucocyte response (degree of shift to the left) and log(100 x dose)

Correlation coefficient $r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{(\Sigma(x-\bar{x})^2 \Sigma(y-\bar{y})^2)}}$

where x = leucocyte response (degree of shift to the left)

and y = log(100 x dpse)

$$\Sigma(x-\bar{x})^2 = \Sigma(x^2) - \frac{(\Sigma x)^2}{n} = 40306.70$$

$$\Sigma(y-\bar{y})^2 = \Sigma(y^2) - \frac{(\Sigma y)^2}{n} = 49.21875$$

$$\Sigma(x-\bar{x})(y-\bar{y}) = \Sigma(xy) - \frac{\Sigma x \Sigma y}{n} = 910.8210$$

Therefore $r = \frac{910.8210}{\sqrt{(49.21875 \times 40306.70)}} = \underline{0.6467}; n=358; p=<0.001$

Therefore the correlation coefficient is significantly greater than zero

Computation of the above figures:

$\Sigma(x^2)$		$\frac{(\Sigma x)^2}{n}$	
	118932.08		3203.8
	86366.13		2685.9
	63503.39		2269.3
	31316.52		1513.2
	300118.12		9671.2

$$\frac{(9671.2)^2}{360} = 259811.42$$

$$300118.12 - 259811.42 = \underline{40306.70}$$

$\Sigma(y^2)$ and $\frac{(\Sigma y)^2}{n}$ are calculated on page 155.

$$\Sigma(xy) - \frac{\Sigma x \Sigma y}{n} = 9261.90220 - \frac{9671.2 \times 310.8600}{360}$$

$$= \underline{910.8210}$$

The equation for the regression line - leucocyte response (degree of shift to the left) and log(100 x dose)

As in the case of the temperature response, the components of the equation $Y = \bar{y} + b(x-\bar{x})$ are calculated. Here, Y is the predicted value of the leucocyte response (degree of shift to the left) for a given value for log(100 x dose)

$\bar{y}$ = mean of all the leucocyte responses (360) = 26.9
$\bar{x}$ = mean of all the corresponding values for log(100 x dose) = 0.8635

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{\sum(xy) - \frac{\sum x \sum y}{n}}{\sum(x^2) - \frac{(\sum x)^2}{n}} = \frac{910.8210}{49.21875} = \underline{18.50557}$$

(see calculation of the correlation coefficient for the computation of these figures)

hence $Y = 26.9 + 18.50557(x - 0.8635)$
therefore $\underline{Y = 18.50557x + 10.920}$

log(100 x dose)

1.3010	$Y = 26.9+18.50557(1.3010-0.8635) = 35.0$
1.0510	$Y = 26.9+18.50557(1.0510-0.8635) = 30.4$
0.8010	$Y = 26.9+18.50557(0.8010-0.8635) = 25.7$
0.3010	$Y = 26.9+18.50557(0.3010-0.8635) = 16.5$

Using these values forY the regression line has been drawn (fig. 4)

<u>The residual variance about the regression line, σ_r^2</u>

from this we calculate the standard deviation of the scatter about the regression line which is given by

$$\sigma_r = \sqrt{(1 - r^2)}\ \sqrt{\frac{\Sigma(y-\bar{y})^2}{n-2}}$$

$$= \sqrt{(1 - 0.6467^2)}\ \sqrt{\frac{40306.70}{358}}$$

$$= \underline{8.08}$$

hence

$$\text{ratio A} = \frac{8.08}{35.6 - 16.8} = \underline{0.43}$$

$$\text{ratio B} = \frac{8.08}{26.9} = \underline{0.30}$$

Analysis of variance to test linearity of relationship between leucocyte response (degree of shift to the left) and log(100 x dose)

In the manner shown on page[158] we calculate as for the temperature response the following values

1 300118.12
2 276794.26
3 259811.42

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	16982.84 (2-3)	3	5660.95
within doses	23323.86 (1-2)	356	65.52
total	40306.70 (1-3)	359	

It can be seen by inspection that the between dose mean square is significant, and the between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\Sigma(y-\bar{y})(x-\bar{x}))^2}{\Sigma(x-\bar{x})^2} = \frac{910.3210^2}{49.21875} = 16855.26134$$

x = lgo(100 x dose)
y = corresponding response (degree of shift to the left)

and which is now subtracted from the between dose sum of squares: 16982.84 - 16855.26 = 127.58.

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	16855.26	1	16855.26
deviation from regression	127.58	2	63.79
within dpses (residual)	23323.86	356	65.52
total	40306.70	359	

The general significance of the regression line can be tested by comparing the regression mean square with the residual, thus

$$F = \frac{16855.26}{65.52} = 257.25$$

which is very highly significant; and the significance of the deviation from linearity by comparing the deviation mean square with the residual, thus

$$F = \frac{63.79}{65.52}$$

which is less than 1 and hence not significant, hence the data can be represented by a straight line.

Calculation of the correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left)

$$\text{Correlation coefficient } r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$$

where x = temperature response
and y = leucocyte response (degree of shift to the left)

$$\sum(x-\bar{x})^2 = \sum(x^2) - \frac{(\sum x)^2}{n} = 56.6517$$

$$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum y)^2}{n} = 40306.70$$

$$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 822.127$$

Therefore $r = \frac{822.127}{\sqrt{(56.6517 \times 40306.70)}} = \underline{0.5441}; n=358; p=<0.001$

Therefore the correlation coefficient is significantly greater than zero

$\sum(x-\bar{x})^2$ and $\sum(y-\bar{y})^2$ are calculated on pages 155 and 177.

$$\sum(xy) - \frac{\sum x \sum y}{n} = 10924.501 - \frac{376.05 \times 9671.2}{360}$$

$$= \underline{822.127}$$

Calculation of the partial correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left)

The partial correlation coefficient measures the extent of relationship between the two variables when other factors (in this case dose) are kept constant.

Let 1 represent the leucocyte response (degree of shift to the left)

2 represent the temperature response

3 represent lgo(100 x dose)

then r_{12} means the total correlation between the leuccoyte response and the temperature response(= 0.5441)

r_{13} means the total correlation between the leucocyte response and log(100 x dose) (=0.6467)

r_{23} means the total correlation between the temperature response and log(100 x dose) (= 0.7447)

The partial correlation coefficient

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1-r_{13}^2)(1-r_{23}^2)}}$$

$$= \frac{0.5441 - 0.6467 \times 0.7447}{\sqrt{(1-0.6467^2)(1-0.7447^2)}}$$

$$= \underline{0.1228}$$

Significance of the partial correlation coefficient:
This may be tested by calculating t,

$$t = \frac{r\sqrt{N-3}}{\sqrt{1-r^2}} = \frac{0.1228\sqrt{357}}{\sqrt{1 - -.1228^2}} = \underline{2.338}; n=357; p=0.02-0.05$$

Therefore the partial correlation coefficient is significantly greater than zero.

Small lymphocyte responses (small lymphocyte percentage fall) of 30 rabbits to 4 dose-levels of pyrogen from Proteus vulgaris.

Rabbit No.	0.2ml/kg.			0.1125ml/kg		
1	66.4	59.2	21.9	53.6	50.4	53.4
5	52.9	52.9	49.9	49.0	32.7	60.6
6	62.2	42.7	39.8	3.8	60.7	54.7
7	41.8	54.9	13.0	0	33.6	45.2
9	53.1	49.0	57.7	7.5	24.1	48.1
101	38.3	50.6	75.6	48.3	67.6	64.8
102	42.9	56.3	60.7	21.7	55.6	66.8
103	55.8	72.2	72.0	46.9	54.9	60.9
104	80.7	64.3	56.8	54.9	53.3	66.8
105	58.4	59.6	75.7	50.5	58.6	80.2
8	29.9	57.1	64.0	25.5	64.0	73.6
14	42.3	49.3	40.6	44.1	53.7	62.8
108	27.9	62.5	43.4	35.5	54.2	71.1
109	65.7	74.4	57.0	49.0	82.3	54.9
112	45.6	57.8	56.5	37.6	53.3	67.1
2	59.2	35.3	77.8	65.8	68.6	37.0
4	38.8	53.2	32.7	54.3	55.5	13.7
11	52.3	52,5	61.3	59.6	61.1	57.5
13	61.6	68.3	63.4	36.8	62.4	48.2
16	60.9	65.5	83.1	36.6	38.0	80.9
110	64.8	72.8	65.8	50.9	83.1	72.4
319	76.1	74.9	87.3	57.9	59.5	78.0
400	61.6	64.3	45.7	52.6	49.0	70.8
402	46.8	82.9	67.6	51.1	40.6	78.9
107	49.5	63.8	72.3	74.8	66.4	75.3
3	71.9	43.2	32.4	49.3	63.2	45.1
10	60.3	73.2	69.4	3.1	45.3	26.2
12	54.0	72.5	33.1	28.8	42.4	0
15	41.6	59.4	20.7	20.4	42.7	25.0
17	50.1	57.2	19.3	52.5	31.0	16.6

continued.'

Rabbit No.	0.06324ml/kg			0.02ml/kg		
1	34.5	32.9	52.4	23.7	25.1	6.7
5	34.2	40.2	56.8	31.8	32.4	25.8
6	60.7	29.7	56.9	39.3	54.4	24.0
7	68.7	36.2	43.1	48.7	0.3	17.5
9	48.0	32.9	35.3	29.7	9.0	17.3
101	75.6	44.5	38.7	42.2	12.1	18.4
102	23.0	24.8	25,3	12.1	27.8	13.8
103	51.6	37,5	27.7	22.6	0	34.9
104	47.0	65.1	48.0	50.2	31.2	20.0
105	66.1	50.9	39.3	35.8	49.3	0
8	29.4	59.3	67.0	0	22.6	37.6
14	26.7	28.7	52.4	28.3	21.3	0
108	50.0	73.3	46.4	41.8	27.5	18.3
109	75.3	64.1	64.5	62.5	49.1	11.0
112	63.7	61.6	56.5	58.7	39.3	34.8
2	30.6	42.6	69.7	44.0	36.8	18.0
4	42.7	50.6	61.5	39.0	19.8	0
11	18.6	59.7	67.2	40.0	51.0	14.9
13	41.1	71.8	61.6	30.0	24.3	14.2
16	40.6	39.1	67.9	1.6	33.3	48.3
110	47.3	46.3	19.6	32.6	25.8	70.1
319	75.6	0	58.3	0	40.2	55.1
400	53.6	53,4	47.5	14.8	31.0	34.5
402	21.3	25.3	7.6	1.0	22.1	45.0
107	41.2	48.1	27.8	21.7	44.7	53.5
3	49.9	65.1	13.4	49.5	27.2	35.5
10	34.0	63.2	10.9	13.9	36.4	35.4
12	13.9	72.6	31.3	1.0	71.7	22.9
15	12.7	68.6	26.2	0	0	43.2
17	10.0	58.8	27.8	25.6	22.9	13.2

Calculation of the means, variances, standard deviations, and coefficients of variation of the small lymphocyte percentage fall (x) for 4 dose-levels of pyrogen from *Proteus vulgaris*.

0.2ml/kg.

x	x^2	x	x^2
66.4	4408.96	59.2	3504.64
52.9	2798.41	38.8	1505.44
62.2	3868.84	52.3	2735.29
41.8	1747.24	61.6	3794.56
53.1	2819.61	60.9	3708.81
59.2	3504.64	85.3	7276.09
52.9	2798.41	53.2	2830.24
42.7	1823.29	52.5	2756.25
54.9	3014.01	68.3	4664.89
49.0	2401.00	65.5	4290.25
21.9	479.61	77.8	6052.84
49.9	2490.01	32.7	1069.29
39.8	1584.04	61.3	3757.69
13.0	169.00	63.4	4019.56
57.7	3329.29	83.1	6905.61
38.3	1466.89	64.8	4199.04
42.9	1840.41	76.1	5791.21
55.8	3113.64	61.6	3794.56
80.7	6512.44	46.8	2190.24
58.4	3410.56	49.5	2450.25
50.6	2560.36	72.8	5299.84
56.3	3169.69	74.9	5610.01
72.2	5212.84	64.3	4134.49
64.3	4134.49	82.9	6872.41
59.6	3552.16	63.8	4070.44
75.6	5715.36	65.8	4329.64
60.7	3684.49	87.3	7621.29
72.0	5184.00	45.7	2088.49
56.8	3226.24	67.6	4569.76
75.7	5730.49	72.3	5227.29
29.9	894.01	71.9	5169.61
42.3	1789.29	60.3	3636.09
27.9	778.41	54.0	2916.00
65.7	4316.49	41.6	1730.56
45.6	2079.36	50.1	2510.01
57.1	3260.41	43.2	1866.24
49.3	2430.49	73.2	5358.24
62.5	3906.25	72.5	5256.25
74.4	5535.36	59.4	3528.36
64.0	4096.00	57.2	3271.84
40.6	1648.36	32.4	1049.76
43.4	1883.56	69.4	4816.36
57.0	3249.00	33.1	1095.61
56.5	3192.25	20.7	428.49
57.8	3340.84	19.3	372.49

continued.

0.1125ml/kg.

x	x^2	x	x^2
53.6	2872.96	65.8	4329.64
49.0	2401.00	54.3	2948.49
3.8	14.44	59.6	3552.16
0	0	36.8	1354.24
7.5	56.25	26.6	707.56
50.4	2540.16	68.6	4705.96
32.7	1069.29	55.5	3080.25
60.7	3684.49	61.1	3733.21
33.6	1128.96	62.4	3893.76
24.1	580.81	38.0	1444.00
53.4	2851.56	37.0	1369.00
60.6	3672.36	13.7	187.69
64.7	4186.09	57.5	3306.25
45.2	2043.04	48.2	2323.24
48.1	2313.61	80.9	6544.81
48.3	2332.89	50.9	2590.81
21.7	470.89	57.9	3352.41
46.9	2199.61	52.6	2766.76
54.9	3014.01	51.1	2611.21
50.5	2550.25	74.8	5595.04
67.6	4569.76	83.1	6905.61
55.6	3091.36	59.5	3540.25
54.9	3014.01	49.0	2401.00
53.3	2840.89	40.6	1648.36
58.6	3433.96	66.4	4408.96
64.8	4199.04	72.4	5241.76
66.8	4462.24	78.0	6084.00
60.9	3708.81	70.8	5012.64
66.8	4462.24	78.9	6225.21
80.2	6432.04	75.3	5670.09
25.5	650.25	49.3	2430.49
44.1	1944.81	3.1	9.61
35.5	1260.25	28.8	829.44
49.0	2401.00	20.4	416.16
37.6	1413.76	52.5	2756.25
64.0	4096.00	63.2	3994.24
53.7	2883.69	45.3	2052.09
54.2	2937,64	42.4	1797.76
82.3	6773.29	42.7	1823.29
53.3	2840.89	31.0	961.00
73.6	5416.96	45.1	2034.01
62.8	3943.84	26.2	686.44
71.1	5055.21	0	0
54.0	2916.00	25.0	625.00
67.1	4502.41	16.6	275.56

continued.

<u>0.06324ml/kg.</u>

x	x^2	x	x^2
34.5	1190.25	20.6	424.36
34.2	1169.64	42.7	1823.29
60.7	3684.49	18.6	345.96
68.7	4719.69	41.1	1689.21
48.0	2304.00	40.6	1648.36
32.9	1082.41	42.6	1814.76
40.2	1616.04	50.6	2560.36
29.7	882.09	59.7	3564.09
36.2	1310.44	71.8	5155.24
32.9	1082.41	39.1	1528.81
52.4	2745.76	69.7	4858.09
56.8	3226.24	61.5	3782.25
56.9	3237.61	67.2	4515.84
43.1	1857.61	61.6	3794.56
35.3	1246.09	67.9	4610.41
75.6	5715.36	47.3	2237.29
23.0	529.00	75.6	5715.36
51.6	2662.56	53.6	2872.96
47.0	2209.00	21.3	453.69
66.1	4369.21	41.2	1697.44
44.5	1980.25	46.3	2143.69
24.8	615.04	0	0
37.5	1406.25	53.4	2851.56
65.1	4238.01	25.3	640.09
50.9	2590.81	48.1	2313.61
38.7	1497.69	19.6	384.16
25.3	640.09	58.3	3398.89
27.7	767.29	47.5	2256.25
48.0	2304.00	7.6	57.76
39.3	1544.49	27.8	772.84
29.4	864.36	49.9	2490.01
26.7	712.89	34.0	1156.00
50.0	2500.00	13.9	193.21
75.3	5670.09	12.7	161.29
63.7	4057.69	10.0	100.00
59.3	3516.49	65.1	4238.01
28.7	823.69	63.2	3994.24
73.3	5372.89	72.6	5270.76
64.1	4108.81	68.6	4705.96
61.6	3794.56	58.8	3457.44
67.0	4489.00	13.4	179 56
52.4	2745.76	10.9	118.81
46.4	2152.96	31.3	979.69
64.5	4160.25	26.2	686.44
56.5	3192.25	27.8	772.84

continued.

x	x^2
23.7	561.69
31.8	1011.24
39.3	1544.49
48.7	2371.69
29.7	882.09
25.1	630.01
32.4	1049,76
54.4	2959.36
0.3	0.09
9.0	81.00
6.7	44.89
25.8	665.64
24.0	576.00
17.5	306.25
17.3	299.29
42.2	1780.84
12.1	146.41
22.6	510.76
50.2	2520.04
35.8	1281.64
12.1	146,41
27.8	772.84
0	0
31.2	973.44
49.3	2430.49
18.4	338.56
13.8	190.44
34.9	1218.01
20.0	400.00
0	0
0	0
28.3	800.89
41.8	1747.24
62.5	3906.25
38.7	1497.69
22.6	510.76
21.3	453.69
27.5	756.25
49.1	2410.81
39.3	1544.49
37.6	1413.76
0	0
18.5	342.25
11.0	121.00
34.8	1211.04

<u>0.02ml/kg.</u>

x	x^2
44.0	1936.00
39.0	1521.00
40.0	1600.00
30.0	900.00
1.6	2.56
36.8	1354.24
19.8	392.04
51.0	2601.00
24.3	590.49
33.3	1108.89
18.0	324.00
0	0
14.9	222.01
14.2	201.64
48.3	2332.89
32.6	1062.76
0	0
14.8	219.04
1.0	1.00
21.7	470.89
25.8	665.64
40.2	1616.04
31.0	961.00
22.1	488.41
44.7	1998.09
70.1	4914.01
55.1	3036.01
34.5	1190.25
45.0	2025.00
53.5	2862.25
49.5	2450.25
13.9	193.21
1.0	1.00
0	0
25.6	655.36
27.2	739.84
36.4	1324.96
7.7	59.29
0	0
22.9	524.41
35.5	1260.25
35.4	1253.16
22.9	524.41
43.2	1866.24
13.2	174.24

continued.

0.2ml/kg.

$\sum x = 5081.7$ therefore mean $(\bar{x}) = \frac{5081.7}{90} = 56.5$

$\sum(x^2) = 308276.87$ and $\frac{(\sum x)^2}{n} = 286929.72$

therefore variance, $\sigma^2 = \frac{308276.87 - 286929.72}{89} = 239.86$

and hence standard deviation $\sigma = 15.5$
and coefficient of variation $V = 27$

(see page for formulae used in calculating these statistics)

0.1125ml/kg.

$\sum x = 4485.9$ therefore mean $(\bar{x}) = 49.8 = \frac{4485.9}{90}$

$\sum(x^2) = 257458.73$ and $\frac{(\sum x)^2}{n} = 223592.21$

therefore variance, $\sigma^2 = \frac{257458.73 - 223592.21}{89} = 380.5$

and hence standard deviation $\sigma = 19.5$
and coefficient of variation $V = 39$

0.06324ml/kg.

$\sum x = 4033.1$ therefore mean $(\bar{x}) = \frac{4033.1}{90} = 44.8$

$\sum(x^2) = 211000.95$ and $\frac{(\sum x)^2}{n} = 180732.17$

therefore variance $\sigma^2 = \frac{211000.95 - 180732.17}{89} = 340.10$

and hence standard deviation $\sigma = 18.4$
and coefficient of variation $V = 41$

0.02ml/kg.

$\sum x = 2430.8$ therefore mean $(\bar{x}) = \frac{2430.8}{90} = 27.0$

$\sum(x^2) = 90033.26$ and $\frac{(\sum x)^2}{n} = 65653.2$

therefore variance $\sigma^2 = \frac{90033.26 - 65653.21}{89} = 273.93$

and hence standard deviation $\sigma = 16.6$
and coefficient of variation $V = 62$

A check on the normality of distribution of the small lymphocyte response (small lymphocyte percentage fall) using the χ^2 test.

0.2ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
13.0-40.5	12	14	2	0.3
40.6-50.5	16	17	1	0.1
50.6-60.5	24	25	1	0
60.6-70.5	20	18	2	0.2
70.6-88.5	18	15	3	0.6

$\chi^2 = 1.2 = \frac{(O-E)^2}{E}$; $n = 4$; $p = 0.8-0.9$

Conclusion: These results are normally distributed

0.1125ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
0 - 25.5	12	9	3	1.0
25.6- 35.5	7	14	7	3.5
35.6- 45.5	11	16	5	1.6
45.6-55.5	25	18	7	2.7
55.6- 65.5	15	17	2	0.2
65.6- 83.5	20	15	5	1.7

$\chi^2 = 10.7$; $n = 5$; $p = 0.05 - 0.1$

Conclusion: These results are normally distributed

0.06324ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O-E)^2}{E}$
0 -20.5	8	9	1	0.1
20.6-30.5	14	10	4	1.6
30.6-40.5	13	19	6	1.9
40.6-50.6	19	20	1	0.1
50.6-60.5	14	18	4	0.9
60.6-76.5	22	13	9	6.2

$\chi^2 = 10.8$; $n = 5$; $p = 0.05 - 0.1$

Conclusion: These results are normally distributed

continued.

0.02ml/kg.

Range of response of groups	O observed frequency	E expected frequency	O - E	$\frac{(O - E)^2}{E}$
0 -10.5	15	14	1	0.1
10.6-20.5	16	17	1	0.1
20.6-30.5	20	21	1	0.1
30.6-40.5	21	19	2	0.2
40.6-50.5	12	12	0	0
50.6-71.5	6	7	1	0.1

$\chi^2 = 0.6; n = 5; p = 0.98 - 0.99$

Conclusion: These results are normally distributed

Analysis of variance of the small lymphocyte response (small lymphocyte percentage fall)
Incomplete three factor analysis : two factors with replication.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 21570.72	$n_1 - 1$ = 29	743.82	
between doses	3 - 4 = 42993.44	$n_2 - 1$ = 3	14331.15	AS ON PAGE 152
rabbit x dose interaction	20687.86	$(n_1-1)(n_2-1)$= 87	237.79	
residual	5 - 1 = 67603.92	$n_1n_2(n_3-1)$ = 240	281.68	
total	5 - 4 = 152855.94	$n_1n_2n_3-1$= 359		

n_1, n_2, n_3, σ_1^2, σ_2^2, σ_{12}^2, and σ_0^2 have the same meaning as the same symbols on page , and the sum of squares for the rabbit x dose interaction is calculated in the same manner as shown on page 152

1 = 799165.89
2 = 735484.59
3 = 756907.31
4 = 713913.87
5 = 866769.81

1, 2, 3, 4, 5, were calculated as for the temperature response using the results obtained for this index of activity.

continued.

Existence of interaction:

$$F = \frac{237.79}{281.68}$$ which is less than 1, hence there is no rabbit-dose interaction, hence between rabbit and between dose variances can be assessed against the residual which gives greater precision since there are more degrees of freedom associated with the residual than with the interaction.

Between rabbit variance:

$$F = \frac{743.82}{281.68} = 2.64$$ which is greater than the five per cent level ($n_1 = 29; n_2 = 240$) hence between rabbit variance exists significantly

Between dose variance:

$$F = \frac{14331.15}{281.68} = 50.88$$ hence between dose variance exists.

continued.

Significance of difference of the means for the 4 dose-levels:

Using the error variance (residual) we can test the significance of difference thus:

variance of a mean of 90 observations $= \frac{281.68}{90}$ and the variance of the difference between two means is twice this

hence S.D. $= \sqrt{(281.68 \times \frac{2}{90})} = 2.5$

hence to be significantly different at the

5 percent level a difference of 1.97 x 2.5=4.9 is required
1 percent level " " " 2.60 x 2.5=6.5 is required
0.1percent level" " " 3.37 x 2.5=8.4 is required

and from the following table

dose	means	difference
0.2ml/kg	56.5	
		6.7
0.1125"	49.8	
		5.0
0.06324"	44.8	
		17.8
0.02 "	27.0	

it will be seen that all means are significantly different the five percent level; the means for the two highest dose-levels are significantly at the 1 percent level; and the two lowest at the 0.1 percent level.

Calculation of the correlation coefficient for the small lymphocyte response (small lymphocyte percentage fall) and log(100 x dose)

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2}}$

where x = small lymphocyte percentage fall

and y = log(100 x dose)

$$\sum(x-\bar{x})^2 = \sum(x^2) - \frac{(\sum x)^2}{n} = 152855.94$$

$$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum y)^2}{n} = 49.21875$$

$$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 1444.95625$$

Therefore $r = \frac{1444.95625}{\sqrt{(152855.94 \times 49.21875)}} = \underline{0.5268}; n=358; p=<0.001$

Therefore the correlation coefficient is significantly greater than zero.

Computation of the above figures:

$\sum(x^2)$		$\frac{(\sum x)^2}{n}$				
	308276.87		5081.7			
	257458.73		4485.9			
	211000.95		4033.1	$\frac{(16031.5)^2}{360}$	=	713913.87
	90033.26		2430.8			
	866769.81		16031.5			

866769.81 - 713913.87 = 152855.94

$\sum(y^2)$ and $\frac{(\sum y)^2}{n}$ are calculated on page 155.

$$\sum(xy) - \frac{\sum x \sum y}{n} = 15288.1565 - \frac{16031.5 \times 310.8600}{360}$$

$$= \underline{1444.95625}$$

<u>The equation for the regression line - small lymphocyte response (small lymphocyte percentage fall) and log (100 x dose)</u>

As in the case of the other two responses, the components of the equation $Y = \bar{y} + b(x-\bar{x})$ are calculated. Here, Y is the predicted value for the small lymphocyte percentage fall for a given value for log(100 x dose)

$\bar{y}$ = mean of all the small lymphocyte percentage falls(360) = 44.5

$\bar{x}$ = mean of all the corresponding values for log(100 x dose) = 0.8635

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{\sum(xy) - \frac{\sum x \sum y}{n}}{\sum(x^2) - \frac{(\sum x)^2}{n}} = \frac{1444.95625}{49.21875} = \underline{29.35784}$$

(see calculation of the correlation coefficient for the computation of these figures)

hence $Y = 44.5 + 29.35784(x - 0.8635)$
therefore $\underline{Y = 29.35784x + 19.150}$

log(100 x dose)

1.3010	$Y = 44.5+29.35784(1.3010-0.8635)$ $= 57.3$
1.0510	$Y = 44.5+29.35784(1.0510-0.8635)$ $= 50.0$
0.8010	$Y = 44.5+29.35784(0.8010-0.8635)$ $= 42.7$
0.3010	$Y = 44.5+29.35784(0.3010-0.8635)$ $= 28.0$

Using these values for Y the regression line has been drawn (fig. 5)

<u>The residual variance about the regression line, σ_r^2</u>

from this we calculate the standard deviation of the scatter about the regression line which is given by

$$\sigma_r = \sqrt{(1 - r^2)}\ \sqrt{\frac{\Sigma(y-\bar{y})^2}{n-2}}$$

$$= \sqrt{(1 - 0.5268^2)}\ \sqrt{\frac{152855.94}{358}}$$

$$= \underline{17.58}$$

hence

$$\text{ratio A} = \frac{17.58}{56.5 - 27.0} = \underline{0.60}$$

$$\text{ratio B} = \frac{17.58}{44.5} = \underline{0.40}$$

<u>Analysis of variance to test linearity of relationship between small lymphocyte percentage fall and log(100 x dose)</u>

In the manner shown on page 158 we calculate as for the temperature response the following values

1 866769.81
2 756907.31
3 713913.87

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	42993.44 (2-3)	3	14331.15
within doses	109862.50 (1-2)	356	308.60
total	152855.94 (1-3)	359	

It can be seen by inspection that the between dose mean square is significant, and the between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\sum(y-\bar{y})(x-\bar{x}))^2}{\sum(x-\bar{x})^2} = \frac{(1444.95625)^2}{49.21875} = 42420.79623$$

x = log(100 x dose)
y = corresponding response(small lymphocyte percentage fall)

and which is now subtracted from the between dose sum of squares: 42993.44 - 42420.79263 = 572.64377.

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	42420.80	1	42420.80
deviation from regression	572.64	2	286.32
within doses (residual)	109862.50	356	308.60
total	152855.94	359	

The general significance of the regression line can be tested by comparing the regression mean square with the residual, thus

$$F = \frac{42420.80}{308.60} = 137.46$$

which is very highly significant; and the significance of the deviation from linearity by comparing the deviation mean square with the residual, thus

$$F = \frac{286.32}{308.60}$$

which is less than 1 and hence not significant, hence the data can be represented by a straight line.

Calculation of the correlation coefficient for the temperature response and the small lymphocyte percentage fall

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2 \sum(y-\bar{y})^2)}}$

where x = small lymphocyte percentage fall
and y = temperature response

$\sum(x-\bar{x})^2 = \sum(x^2) - \frac{(\sum x)^2}{n} = 152855.94$

$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum x)^2}{n} = 56.6517$

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} \quad 1406.434$

Therefore $r = \frac{1406.434}{\sqrt{(152855.94 \times 56.6517)}} = \underline{0.4779}; n=358; p=<0.001$

Therefore the correlation coefficient is significantly greater then zero

Computation of the above figures:

$\sum(x-\bar{x})^2$ and $\sum(y-\bar{y})^2$ are calculated on pages 196 and 155.

$\sum(xy) - \frac{\sum x \sum y}{n} = 18152.672 - \frac{16031.5 \times 376.05}{360}$

$= \underline{1406.434}$

Calculation of the partial correlation coefficient for the temperature response and the small lymphocyte percentage fall

Let 1 represent the small lymphocyte percentage fall

2 represent the temperature response

3 represent log(100 x dose)

then r_{12} means the total correlation between the temperature response and the small lymphocyte percentage fall (=0.4779)

r_{13} means the total correlationbetween the small lymphocyte percentage fall and log(100 x dose) (=0.5268)

r_{23} means the total correlation between the temperature response and log(100 x dose) (=0.7447)

The partial correlation coefficient

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1-r_{13}^2)(1-r_{23}^2)}}$$

$$= \frac{0.4779 - 0.5268 \times 0.7447}{\sqrt{(1-0.5268^2)(1-0.7447^2)}}$$

$$= \underline{0.1509}$$

Significance of the partial correlation coefficient:

$$t = \frac{r\sqrt{N - 3}}{\sqrt{1 - r^2}} = \frac{0.1509\sqrt{357}}{\sqrt{1 - 0.1509^2}} = \underline{2.885};\ p=0.01-0.001$$

Therefore the patial correlation coefficient is significantly greater than zero.

Calculation of the correlation coefficient for the small lymphocyte percentage fall and the degree of shift to the left.

Correlation coefficient $r = \dfrac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$

where x = degree of shift to the left
and y = small lymphocyte percentage fall

$$\sum(x-\bar{x})^2 = \sum(x^2) - \frac{(\sum x)^2}{n} = 40306.70$$

$$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum y)^2}{n} = 152855.94$$

$$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 45253.46$$

Therefore $r = \dfrac{45253.46}{\sqrt{(40306.70 \times 152855.94)}} = \underline{0.5765}; n=358; p=<0.001$

Therefore the correlation coefficient is significantly greater than zero.

Calculation of the above figures:

$\sum(x-\bar{x})^2$ and $\sum(y-\bar{y})^2$ are calculated on pages 177 and 196.

$$\sum(xy) - \frac{\sum x \sum y}{n} = 475930.80 - \frac{96712 \times 16031.5}{360}$$

$$= \underline{45253.46}$$

Calculation of the partial correlation coefficient for the small lymphocyte percentage fall and the degree of shift to the left.

Let 1 represent the small lymphocyte percentage fall

2 represent the degree of shift to the left

3 represent log(100 x dose)

then r_{12} means the total correlation between the small lymphocyte percentage fall and the degree of shift to the left (= 0.5765)

r_{13} means the total correlation between the small lymphocyte percentage fall and log(100 x dose) (= 0.5268)

r_{23} means the total correlation between the degree of shift to the left and log(100 x dose) (= 0.6467)

The partial correlation coefficient

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1-r_{13}^2)(1-r_{23}^2)}}$$

$$= \frac{0.5765 - 0.5268 \times 0.6467}{\sqrt{(1-0.5268^2)(1-0.6467^2)}}$$

$$= \underline{0.3637}.$$

Significance of the partial correlation coefficient:

$$t = \frac{r\sqrt{N-3}}{\sqrt{1-r^2}} = \frac{0.3637\sqrt{357}}{\sqrt{1-0.3637^2}} = \underline{7.379};\ p < 0.001$$

Therefore the partial correlation coefficient is significantly greater than zero.

Results of injection of a fifth dose-level: 0.3557 ml/kg.

Rabbit No.	Temperature response(x)	x^2	percentage fall in average number of lobes per neutrophil (x)	x^2
1	1.05	1.1025	34.5	1190.25
5	1.72	2.9584	28.6	817.96
6	1.51	2.2801	34.5	1190.25
7	1.40	1.9600	33.2	1102.24
9	1.26	1.5876	30.1	906.01
8	1.06	1.1236	43.7	1909.69
14	1.55	2.4025	40.3	1624.09
108	1.81	3.2761	37.6	1413.76
109	2.00	4.0000	38.7	1497.69
112	2.06	4.2436	41.6	1730.56
101	1.36	1.8496	39.6	1568.16
102	1.36	1.8496	37.2	1383.34
103	1.20	1.4400	33.5	1122.25
104	1.36	1.8496	35.3	1246.09
105	1.47	2.1609	33.5	1122.25

	small lymphocyte percentage fall(x)	x^2
1	48.9	2106.81
5	39.5	1560.25
6	57.3	3283.29
7	40.9	1672.81
9	43.9	1927.21
8	71.0	5041.00
14	64.3	4134.49
108	65.8	4329.64
109	71.5	5112.25
112	65.9	4342.81
101	63.6	4044.96
102	52.7	2777.29
103	47.7	2275.29
104	60.8	3696.64
105	71.6	5126.56

Calculations of the means, variances, standard deviations, and coefficients of variation of the results for the 0.3557ml/kg. dose-level.

Temperature response

$\sum x = 22.17$ therefore mean $= \frac{22.17}{15} = \underline{1.48^{\circ}}$

$\sum(x^2) = 34.0841$ and $\frac{(\sum x)^2}{n} = 32.7673$

therefore variance $= \frac{34.0841 - 32.7673}{14} = \underline{0.0941}$

and hence standard deviation = $\underline{0.31}$
and coefficient of variation = $\underline{21}$

Percentage fall in average number of lobes per neutrophil (degree of shift to the left)

$\sum x = 541.9$ therefore mean $= \frac{541.9}{15} = \underline{36.1}$

$\sum(x^2) = 19825.09$ and $\frac{(\sum x)^2}{n} = 19577.04$

therefore variance $= \frac{19825.09 - 19577.04}{14} = \underline{17.72}$

and hence standard deviation = $\underline{4.2}$
and coefficient of variation = $\underline{12}$

Small lymphocyte percentage fall

$\sum x = 862.4$ therefore mean $= \frac{862.4}{15} = \underline{57.5}$

$\sum(x^2) = 51431.30$ and $\frac{(\sum x)^2}{n} = 49582.25$

therefore variance $= \frac{51431.30 - 49582.25}{14} = \underline{132.08}$

and hence standard deviation = $\underline{11.5}$
and coefficient of variation = $\underline{20}$

APPENDIX II

Temperature and leucocyte (degree of shift to the left) responses of 15 rabbits to Pseudomonas polysaccharide at 3 dose-levels.

Temperature responses

Rabbit No.	10γ/kg.	5γ/kg.	2.5γ/kg.
21	0.86	0.37	0.39
23	1.57	0.82	0.65
30	1.04	1.12	0.62
32	1.24	0.95	0.81
35	1.04	0.72	0.74
25	1.19	1.26	1.02
36	1.74	1.46	1.51
110	1.13	1.16	0.58
33	1.25	0.82	0.84
104	0.96	1.02	1.21
85	0.89	1.18	1.43
22	1.05	1.05	0.84
113	2.04	1.35	1.52
14	1.52	1.17	0.89
107	1.00	1.19	0.56

Leucocyte responses
(degree of shift to the left)

Rabbit No.	10γ/kg.	5γ/kg.	2.5γ/kg.
21	23.0	21.8	19.4
23	26.9	26.8	25.5
30	36.0	22.2	17.7
32	29.0	22.1	13.5
35	32.7	20.5	18.2
25	29.6	28.1	22.7
36	38.0	29.2	20.1
110	44.2	27.7	34.4
33	31.9	25.7	24.3
104	24.3	25.6	16.1
85	21.8	31.5	15.4
22	32.0	25.4	23.8
113	36.6	21.2	24.8
14	35.9	34.6	25.5
107	29.9	27.5	20.8

Calculations of the means, variances, standard deviations, and coefficients of variation of the temperature responsse (x) to the 3 dose-levels of Pseudomonas polysaccharide.

10γ/kg.		5γ/kg.		2.5γ/kg.	
x	x^2	x	x^2	x	x^2
0.86	0.7396	0.37	0.1369	0.39	0.1521
1.57	2.4649	0.82	0.6724	0.65	0.4225
1.04	1.0816	1.12	1.2544	0.62	0.3844
1.24	1.5376	0.95	0.9025	0.81	0.6561
1.04	1.0816	0.72	0.5184	0.74	0.5476
1.19	1.4161	1.26	1.5876	1.02	1.0404
1.74	3.0276	1.46	2.1316	1.51	2.2801
1.13	1.2769	1.16	1.3456	0.58	0.3364
1.25	1.5625	0.82	0.6724	0.84	0.7056
0.96	0.9216	1.02	1.0404	1.21	1.4641
0.89	0.7921	1.18	1.3924	1.43	2.0449
1.05	1.1025	1.05	1.1025	0.84	0.7056
2.04	4.1616	1.85	3.4225	1.52	2.3104
1.52	2.3014	1.17	1.3689	0.89	0.7921
1.00	1.0000	1.19	1.4161	0.56	0.3136

10γ/kg.

$\Sigma x = 18.52$ therefore mean, $\bar{x} = \frac{18.52}{15} = \underline{1.23^{\circ}}$

$\Sigma(x^2) = 24.4766$ and $\frac{(\Sigma x)^2}{n} = \frac{(18.52)^2}{15} = 22.8660$

therefore variance, $\sigma^2 = \frac{\Sigma(x^2) - \frac{(\Sigma x)^2}{n}}{n-1} = \frac{24.4766-22.8660}{14}$

$= \underline{0.1150}$

and hence standard deviation, $\sigma = \underline{0.34}$
and coefficient of variation, V = 28

5γ/kg.

$\Sigma x = 16.14$ therefore mean, $\bar{x}_2 = \frac{16.14}{15} = \underline{1.08^{\circ}}$

$\Sigma(x^2) = 18.9646$ and $\frac{(\Sigma x)^2}{n} = 17.3666$

therefore variance, $\sigma^2 = \frac{18.9646 - 17.3666}{14} = \underline{0.1141}$

and hence standard deviation $\sigma = \underline{0.34}$
and coefficient of variation V = 32.

2.5γ/kg.

$\Sigma x = 13.61$ therefore mean, $\bar{x} = \frac{13.61}{15} = \underline{0.91^{\circ}}$

$\Sigma(x^2) = 14.1559$ and $\frac{(\Sigma x)^2}{n} = 12.3488$

therefore variance $\sigma^2 = \frac{14.1559 - 12.3488}{14} = \underline{0.1291}$

and hence standard deviation $\sigma = 0.36$
and coefficient of variation V = 40

<u>Calculations of the means, variances, standard deviations, and coefficients of variation of the leucocyte responses (degree of shift to the left) (x) to the 3 dose-levels Of Pseudomonas polysaccharide.</u>

10γ/kg.		5γ/kg.		2.5γ/kg.	
x	x^2	x	x^2	x	x^2
23.0	529.00	21.8	475.24	19.4	376.36
26.9	723.61	26.8	718.24	25.5	650.25
36.0	1296.00	22.2	492.84	17.7	313.29
29.0	841.00	22.1	488.41	13.5	182.25
32.7	1069.29	20.5	420.25	18.2	331.24
29.6	876.16	28.1	789.61	22.7	515.29
38.0	1444.00	29.2	852.64	20.1	404.01
44.2	1953.64	27.7	767.29	34.4	1183.36
31.9	1017.61	25.7	660.49	24.3	590.49
24.3	590.49	25.6	655.36	16.1	259.21
21.8	475.24	31.5	992.25	15.4	237.16
32.0	1024.00	25.4	645.16	23.8	566.44
36.6	1339.56	21.2	449.44	24.8	615.04
35.9	1288.81	34.6	1197.16	25.5	650.25
29.9	894.01	27.5	756.25	20.8	432.63

<u>10γ/kg.</u>

$\Sigma x = 471.8$ therefore mean, $\bar{x} = \frac{471.8}{15} = \underline{31.5}$

$\Sigma(x^2) = 15362.42$ and $\frac{(\Sigma x)^2}{n} = 14839.68$

therefore variance $\sigma = \frac{15362.42 - 14839.68}{14} = \underline{37.34}$

and hence standard deviation $\sigma = \underline{6.11}$

and coefficient of variation $V = \underline{19}$

<u>5γ/kg.</u>

$\Sigma x = 389.9$ therefore mean $\bar{x} = \frac{389.9}{15} = \underline{26.0}$

$\Sigma(x^2) = 10360.63$ and $\frac{(\Sigma x)^2}{n} = 10134.80$

therefore variance $\sigma = \frac{10360.63 - 10134.80}{14} = \underline{16.13}$

and hence standard deviation $\sigma = \underline{4.0}$

and coefficient of variation $V = \underline{16}$

<u>2.5γ/kg.</u>

$\Sigma x = 322.2$ therefore mean $\bar{x} = \frac{322.2}{15} = \underline{21.5}$

$\Sigma(x^2) = 7307.28$ and $\frac{(\Sigma x)^2}{n} = 6920.86$

therefore variance $\sigma = \frac{7307.28 - 6920.86}{14} = 27.60$

and hence standard deviation $\sigma = 5.3$

and coefficient of variation $V = \underline{24}$

Andysis of variance of the temperature response to *Pseudomonas polysaccharide*.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 3.7194	$n_1 - 1 = 14$	0.2657	$n_2\sigma_1^2 + \sigma_0^2$
between doses	3 - 4 = 0.8039	$n_2 - 1 = 2$	0.4020	$n_1\sigma_2^2 + \sigma_0^2$
residual (error)	1+4-2-3= 1.2962	$(n_1-1)(n_2-1)=$ 28	0.0463	σ_0^2
total	1 - 4 = 5.8195	$n_1 n_2 - 1 =$ 44		

n_1 = number of rabbits = 15
n_2 = number of dose-levels = 3

σ_1^2 = variance due to differences between rabbits
σ_2^2 = variance due to differences between dose-levels
σ_0^2 = residual variance (error)

1 Square every individual response and add the squares = 57.5971
2 Total for each rabbit for the three dose-levels, square each total, sum the squares, and divide this total by the number of dose-levels (3) = 55.4970
3 Total for each dose-level, square the totals, sum the squares, and divide by the number of rabbits (15) = 52.5815
4 Total for all 45 responses, square this total and divide by 45 = 51.7776

Between rabbit variance:

$$F = \frac{0.2657}{0.0463} = 5.74$$

which is greater than the five percent value for the variance ratio F (n_1=14; n_2=28) indicating that between rabbit variance exists

Between doses variance:

$$F = \frac{0.4020}{0.0463} = 8.68$$

which is also greater than the five percent value for $F(n_1=2; n_2=28)$. Note: in the F-tests n_1 and n_2 are different from the n_1 and n_2 in the table at the top of the page.

continued.

Significance of difference of the means for the temperature response to the three dose-leveld of Pseudomonas polysaccharide:

variance of a mean of 15 observations $= \frac{0.0463}{15}$

and the variance of the difference between two means is twice this, hence

$$S.D. = \sqrt{0.0463 \times \frac{2}{15}} = 0.08$$

hence to be significantly different at the

5 percent level a difference of $2.05 \times 0.08 = 0.16^{\circ}$ is required

(for n = 28, t = 2.05)

and from the following table

dose	means °C	difference
10γ/kg.	1.23	
		0.15
5 "	1.08	
		0.17
2.5 "	0.91	

it is seen thatusing this index of activity the two highest dose-levels cannot be distinguished. The difference between the response for 2.5γ/kg and 10γ/kg is 0.32°, and these two dose-levels can be distinguished $(p=<0.001)$

Analysis of variance of the leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 633.61	$n_1 - 1 = 14$	45.26	AS ON PAGE 210
between doses	3 - 4 = 748.25	$n_2 - 1 = 2$	374.13	
residual (error)	1+4-2-3= 501.38	$(n_1-1)(n_2-1) = 28$	17.91	
total	1 - 4 = 1883.24	$n_1 n_2 - 1 = 44$		

$n_1, n_2, \sigma_1^2, \sigma_2^2, \sigma_0^2$ have the same meaning as the same symbols on page 210

1 33030.33
2 31780.70
3 31895.34
4 31147.09

1, 2, 3, 4, were calculated in the same manner as the same numbers on page using, of-course the results for this index of activity.
Between rabbit variance:

$$F = \frac{45.26}{17.91} = 2.53$$

which is greater than the five percent value for the variance ratio (n_1=14;n_2=28) indicating that between rabbit variance exists significantly

Between doses variance:

$$F = \frac{374.13}{17.91} = 20.89$$

which is also greater than the five percent value for F ($n_1 = 2; n_2 = 28$) indicating that between doses variance exists significantly.

continued.

Significance of difference of the means for the leucocyte responses(degree of shift to the left) to the three dose-levels of Pseudomonas polysaccharide:

variance of a mean of 15 observations = $\frac{17.91}{15}$

and the variance of the difference between two means is twicethis, hence

$$S.D. = \sqrt{17.91 \times \frac{2}{15}} = 1.55$$

hence to be significantly different at the

5 percent level a difference of 2.05 x 1.55=3.2 is required
1 percent level a difference of 2.76 x 1.55=4.3 is required

amd from the following table

dose	means	difference
10 /kg.	31.5	
		5.5
5 "	26.0	
		4.5
2.5 "	21.5	

it is seen that using this index of activity two-fold increases in dose can be distinguished (p=<0.01)

<u>Calculation of the correlation coefficient for the temperature response to Pseudomonas polysaccharide and log dose.</u>

Correlation coefficient $r = \dfrac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$

where x = tempetature response
and y = log dose

$\sum(x-\bar{x})^2 = 5.8195$

$\sum(y-\bar{y})^2 = 2.71893340$

$\sum(x-\bar{x})(y-\bar{y}) = 1.478158$

Therefore $r = \dfrac{1.478518}{\sqrt{(5.8195 \times 2.71893340)}} = \underline{0.3716}$

n = 45; p = 0.02 - 0.01 therefore the correlation coefficient is significantly greater than zero

---

Computation of the above figures:

$\sum(x-x)^2$ is calculated on page 210

$\sum(y-y)^2 = \sum(y^2) - \dfrac{(\sum y)^2}{n} = 15(1^2+0.6990^2+0.3979^2) - \dfrac{(15 \times 2.0969)^2}{45}$

$= \underline{2.71893340}$

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \dfrac{\sum x \sum y}{n} = ((1 \times 18.52)+(.699 \times 16.14) + (.3979 \times 13.61)) - \dfrac{48.27 \times 31.4535}{45}$

$= \underline{1.478158}$

<u>Calculation of the correlation coefficient for the leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide and log dose.</u>

Correlation coefficient $r = \dfrac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$

where x = leucocyte response (degree of shift to the left)
and y = log dose

$\sum(x-\bar{x})^2 = 1883.24$

$\sum(y-\bar{y})^2 = 2.71893345$

$\sum(x-\bar{x})(y-\bar{y}) = 45.0372$

Therefore $r = \dfrac{45.0372}{\sqrt{(1883.24 \times 2.71893345)}}$ $\underline{0.6294}$

n = 45; $p = < 0.001$; therefore the correlation coefficient is significantly greater than zero

---

Computation of the above figures:

$\sum(x-\bar{x})^2$ is calculated on page 219

$\sum(y-y)^2$ is calculated on page 214

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \dfrac{\sum x \sum y}{n} =$

$((1 \times 471.8)+(.699 \times 389.9)+(.3979 \times 322.2)) - \dfrac{1183.9 \times 31.4535}{45}$

$= \underline{45.0372}$

The equation for the regression line - temperature response to Pseudomonas polysaccharide and log dose.

The components of the equation $Y = \bar{y} + b(x-\bar{x})$ are calculated. Y is the predicted value fo the temperature response for a given value for log dose.

$\bar{y}$ = mean of all the temperature responses = 1.07
$\bar{x}$ = mean of all the corresponding values for log dose = 0.6990

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{1.478158}{2.71893340} = \underline{0.5437}$$

(see calculation of the correlation coefficient for the computation of these figures)

$$\text{hence } Y = 1.07 + 0.5437(x-0.6990) = \underline{0.5437x + 0.69}$$

log dose

1.0000 $\quad Y = 1.07 + 0.5437(1 - 0.6990) = 1.23$

0.6990 $\quad Y = 1.07 + 0.5437(0.6990 - 0.6990) = 1.07$

0.3979 $\quad Y = 1.07 + 0.5437(0.3979 - 0.6990) = 0.91$

The standard deviation of the scatter about the regression line is given by

$$\sigma_r = \sqrt{1 - r^2}\sqrt{\frac{\sum(y-\bar{y})^2}{n-2}} = \sqrt{1 - 0.3716^2}\sqrt{\frac{5.8195}{43}} = \underline{0.3416}$$

$$\text{hence ratio } A = \frac{0.34}{1.23 - 0.91} = 1.06$$

$$\text{ratio } B = \frac{0.34}{1.07} = 0.32$$

The equation for the regression line - leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide and log dose.

In the equation $Y = \bar{y} + b(x-\bar{x})$, Y isthe predicted value for the leucocyte response for a given value for log dose

$\bar{y}$ = mean of all the leucocyte responses = 26.3
$\bar{x}$ = mean of all the corresponding values for log dose = 0.6990

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{45.0372}{2.71893340} = \underline{16.5643}$$

(see calculation of the correlation coefficient for the computation of these figures)

$$\text{hence } Y = 26.3 + 16.5645(x - 0.6990)$$
$$= \underline{16.5645x + 14.72}$$

log dose

1.0000 $Y = 26.3 + 16.5645(1 - 0.6990)$
$= 31.3$

0.6990 $Y = 26.3 + 16.5643(0.6990 - 0.6990)$
$= 26.3$

0.3979 $Y = 26.3 + 16.5643(0.3979 - 0.6990)$
$= 21.3$

The standard deviation of the scatter about the regression line is givem by

$$\sigma_r = \sqrt{1 - r^2}\sqrt{\frac{\sum(y-\bar{y})^2}{n-2}} = \sqrt{1 - 0.6294^2}\sqrt{\frac{1883.2}{43}} = \underline{5.14}$$

$$\text{hence ratio A} = \frac{5.14}{31.3 - 21.3} = \underline{0.51}$$

$$\text{ratio B} = \frac{5.14}{26.3} = \underline{0.20}$$

Analysis of variance to test linearity of relationship between the leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide and log dose.

In the manner shown on page 158 we calculate the following values

1 33030.33
2 31895.34
3 31147.09

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	758.25 (2-3)	2	347.13
within doses	1134.99 (1-2)	42	27.02
total	1883.24 (1-3)	44	

Significance of the between dose mean square:

$$F = \frac{347.13}{27.02} = 13.85$$ which is greater than the

five percent value for the variance ratio-F ($n_1=2; n_2=42$) and hence significant.
The between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\sum(y-\bar{y})(x-\bar{x}))^2}{\sum(x-\bar{x})^2} = \frac{45.0372^2}{2.71893310} = 746.0093$$

x = log dose
y = corresponding response (leucocyte response)

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	746.0093	1	746.0093
deviation from regression	2.2407	1	2.2407
within doses (residual)	1134.99	42	27.02
total	1883.24	44	

The sum of squares for the deviation from regression is obtained by subtracting the sum of squares attributable to the regression line from the between doses sum of squares.

The generalsignifficance of the regression line can be tested by comparing the regression mean square with the residual, thus

$$F = \frac{746.0093}{27.02} = 27.61$$ which is greaterthan

the five percent value for the variance ratio, $F(n_1=1; n_2=42)$

The significance of the devaition from linearity is tested by comparing the deviation mean square with the residual thus,

$$F = \frac{2.2407}{27.02}$$ = less than 1, hence the data

can be represented by a straight line.

Analysis of variance to test linearity of relationship between the temperature response to Pseudomonas polysaccharide and log dose.

In the manner shown on page 158 we calculate the following values

1 57.5971
2 52.5815
3 51.7776

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	0.8039 (2-3)	2	0.4020
within doses	5.0156 (1-2)	42	0.1194
total	5.8195 (1-3)	44	

Significance of between dose mean square:

$$F = \frac{0.4020}{0.1194} = 3.3668$$ which is greater than the five percent value for the variance ratio-F-($n_1=2;n_2=42$) and hence significant.

The between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\Sigma(y-\bar{y})(x-\bar{x}))^2}{\Sigma(x-\bar{x})^2} = \frac{1.478158^2}{2.71893310} = \underline{0.8036}$$

x = log dose
y = corrseponding response (temperature)

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	0.8036	1	0.8036
deviation from regression	0.0003	1	0.0003
within doses (residual)	5.0516	42	0.1194
total	5.8195	44	

The sum of squares for the deviation from regression is obtained by subtracting the sum of squares attributableto the regression line from the between doses sum of squares.

The general significance of the regression line can be tested be comparing hthe regression mean square with the residual, thus

$$F = \frac{0.8036}{0.1194} = 6.75$$ which is greater than

the five percent value for the variance ratio, $F\ (n_1=1; n_2=42)$.

The significance of the deviation from linearity is tested by comparing the deviation mean square with the residual thus,

$$F = \frac{0.0003}{0.1194} = \text{less than } 1$$, hence the data

can be represented by a straight line.

<u>Calculation of the correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide.</u>

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2 \sum(y-\bar{y})^2)}}$

where x = temperature response
and y = leucocyte response (degree of shift to the left)

$\sum(x-x)^2 = 5.8195$

$\sum(y-y)^2 = 1883.24$

$\sum(x-\bar{x})(y-\bar{y}) = 37.323$

Therefore $r = \frac{37.323}{\sqrt{(5.8195 \times 1883.24)}} = \underline{0.3656}$

n=43; p=0.02-0.05; <u>therefore r is</u> significantly greater than zero

Computation of the above figures:

$\sum(x-\bar{x})^2$ is calculated on page 210

$\sum(y-\bar{y})^2$ is calculated on page 212

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 1307.253 - \frac{48.27 \times 1193.9}{45}$

$= \underline{37.323}$

Calculation of the partial correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left) to Pseudomonas polysaccharide.

In this case the effect of log dose on the correlation is eliminated.

Let 1 representthe leucocyte response

2 represent the temperature response

3 represent log dose

then r_{12} means the total correlation coefficient between the leucocyte response and the temperature response (= 0.3565)

r_{13} means the total correlation coefficient between the leucocyte response and log dose (= 0.6294)

r_{23} means the total correlation coefficint between the temperature response and log dose(= 0.3716)

The partial correlation coefficient

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1-r_{13}^2)(1-r_{23}^2)}}$$

$$= \frac{0.3565 - 0.6294 \times 0.3716}{\sqrt{(1 - 0.6294^2)(1 - 0.3716^2)}}$$

= 0.1700 which for 42 degrees of freedom is not significantly greater than zero.

Time required to reach maximum temperature after the injection of various dose-levels of different pyrogen preparations.

I Pseudomonas polysaccharide

10γ/kg x	x^2	5γ/kg x	x^2	2.5γ/kg x	x^2
90	8100	210	44100	100	10000
90	8100	70	4900	120	14400
110	12100	220	48400	100	10000
90	8100	200	40000	90	8100
140	19600	100	10000	100	10000
80	6400	100	10000	70	4900
190	36100	80	6400	90	8100
170	28900	90	8100	90	8100
120	14400	70	4900	100	10000
90	8100	130	16900	180	32400
70	4900	80	6400	230	52900
80	6400	100	10000	90	8100
70	4900	80	6400	90	8100
90	8100	70	4900	200	40000
80	6400	100	10000	90	8100

10γ/kg

$\sum x = 1560$ therefore mean $= \frac{1560}{15} = 104$ minutes

$\sum(x^2) = 180600$ and $\frac{(\sum x)^2}{n} = 162240$

therefore variance $= \frac{180600-162240}{14} = 1311.4$

5γ/kg

$\sum x = 1700$ therefore mean $= \frac{1700}{15} = 113$ minutes

$\sum(x^2) = 231400$ and $\frac{(\sum x)^2}{n} = 192667$

therefore variance $= \frac{231400-192667}{14} = 2736.7$

2.5γ/kg

$\sum x = 1740$ therefore mean $= \frac{1740}{15} = 116$ minutes

$\sum(x^2) = 23320$ and $\frac{(\sum x)^2}{n} = 201840$

therefore variance $= \frac{23320-201840}{14} = 2240.0$

continued.

Significance of difference of the mean times taken to reach maximum temperatures.

$$t = \frac{\bar{x} - \bar{x}'}{\sigma} \sqrt{\frac{n \times n'}{n + n'}}$$

where $\sigma = \sqrt{\left(\left(\Sigma(x^2) - \frac{(\Sigma x)^2}{n} + \Sigma(x'^2) - \frac{(\Sigma x')^2}{n'}\right) / (n+n'-2)\right)}$

For 10γ/kg $\bar{x} = 104$ and $n = n' = 15$
5γ/kg $\bar{x}' = 113$

therefore $t = \frac{9}{45.2} \times 2.7 = 0.538$

therefore the means do not differ significantly

For 5γ/kg $\bar{x} = 113$ and $n = n' = 15$
2.5γ/kg $\bar{x}' = 116$

therefore $t = \frac{3}{50.0} \times 2.7 = 0.162$

therefore the means do not differ significantly

continued.

II Lipopolysaccharide from S. abortus equi

0.1γ/kg x	x^2	0.01γ/kg x	x^2	0.005γ/kg x	x^2
95	9025	95	9025	125	15625
85	7225	95	9025	55	3025
195	38025	66	4356	15	225
105	11025	105	11025	55	3025
95	9025	105	11025	55	3025
155	24025	115	13225	95	9025
115	13225	125	15625	105	11025
195	38025	105	11025	105	11025
95	9025	85	7225	85	7225
195	38025	145	21025	85	7225
95	9025	205	42025	105	11025
95	9025	205	42025	75	5625
85	7225	85	7225	105	11025
75	5625	115	13225	105	11025
95	9025	85	7225	125	15625

0.1γ/kg

$\sum x = 1775$ therefore mean $= \frac{1775}{15} = 118$ minutes

$\sum(x^2) = 236575$ and $\frac{(\sum x)^2}{n} = 210042$

therefore variance $= \frac{236575-210042}{14} = 1895.2$

0.01γ/kg

$\sum x = 1736$ therefore mean $= \frac{1736}{15} = 116$ minutes

$\sum(x^2) = 224306$ and $\frac{(\sum x)^2}{n} = 200913$

therefore variance $= \frac{224306-200913}{14} = 1670.9$

0.005γ/kg

$\sum x = 1295$ therefore mean $= \frac{1295}{15} = 86$ minutes

$\sum(x^2) = 124775$ and $\frac{(\sum x)^2}{n} = 111802$

therefore variance $= \frac{124775-111802}{14} = 926.6$

continued.

<u>Significance of difference of the mean times taken to reach maximum temperatures.</u>

For o.1γ/kg $\bar{x} = 118$
θ.01γ/kg $\bar{x}' = 116$ and $n = n' = 15$

$$\text{therefore } t = \frac{2}{42.2} \times 2.7 = 0.128$$

therefore the means do not differ significantly

For θ.01γ/kg $\bar{x} = 116$
0.005γ/kg $\bar{x}' = 86$ and $n = n' = 15$

$$\text{therefore } t = \frac{30}{36.0} \times 2.7 = 2.25 \quad (n=28;p=0.02-0.05)$$

therefore the means differ significantly

Temperature and leucocyte (degree of shift to the left) responses of 15 rabbits to lipopolysaccharide from Salmonella abortus equi.

Temperature responses

Rabbit No.	0.1γ/kg.	0.01γ/kg.	0.005γ/kg.
46	1.29	0.42	0.05
49	1.47	0.97	0.59
50	1.31	0.65	0.37
30	1.17	0.87	0.27
35	1.09	0.87	0.44
41	0.98	1.22	0.57
43	1.39	1.10	0.58
44	0.78	0.82	0.51
45	0.96	1.00	0.46
A	0.64	0.88	0.73
110	1.67	0.66	0.51
109	1.69	0.44	1.05
107	1.29	0.67	0.91
112	2.29	0.30	1.29
32	1.20	0.64	0.79

Leucocyte responses
(degree of shift to the left)

Rabbit No.			
46	36.3	26.7	14.7
49	34.1	20.7	9.6
50	35.1	15.2	11.8
30	32.1	22.6	13.0
35	18.6	22.9	6.1
41	28.3	23.2	10.2
43	33.3	16.9	10.5
44	28.1	24.8	21.4
45	22.4	14.0	13.6
A	29.8	20.5	16.1
110	34.0	20.2	25.8
109	38.9	14.6	22.1
107	36.8	22.1	18.2
112	38.4	11.9	16.3
32	35.5	17.6	12.1

<u>Calculation of the means, variances, standard deviations, and coefficients of variation of the temperature response(x) to the 3 dose-levels of lipopolysaccharide from Salmonella abortus equi.</u>

0.1γ/kg.		0.01γ/kg.		0.005γ/kg.	
x	x^2	x	x^2	x	x^2
1.29	1.6641	0.42	0.1764	0.05	0.0025
1.47	2.1609	0.97	0.9409	0.59	0.3481
1.31	1.7161	0.65	0.4225	0.37	0.1369
1.17	1.3689	0.87	0.7569	0.27	0.0729
1.09	1.1881	0.87	0.7569	0.44	0.1936
0.98	0.9604	1.22	1.4884	0.57	0.3249
1.39	1.9321	1.10	1.2100	0.58	0.3364
0.78	0.6084	0.82	0.6724	0.51	0.2601
0.96	0.9216	1.00	1.0000	0.46	0.2116
0.64	0.4096	0.88	0.7744	0.73	0.5329
1.67	2.7889	0.66	0.4356	0.51	0.2601
1.69	2.8561	0.44	0.1936	1.05	1.1025
1.29	1.6641	0.67	0.4489	0.91	0.8281
2.29	5.2441	0.30	0.0900	1.29	1.6641
1.20	1.4400	0.64	0.4096	0.79	0.6241

<u>0.1γ/kg.</u>

$\Sigma x = 19.22$ therefore mean $\bar{x} = \frac{19.22}{15} = \underline{1.28^\circ}$

$\Sigma(x^2) = 26.9234$ and $\frac{(\Sigma x)^2}{n} = 24.6272$

therefore variance $\sigma^2 = \frac{26.9234 - 24.6272}{14} = \underline{0.1640}$

and hence standard deviation $\sigma = \underline{0.41}$
and coefficient of variation $V = \underline{32}$

<u>0.01γ/kg.</u>

$\Sigma x = 11.51$ therefore mean $\bar{x} = \frac{11.51}{15} = \underline{0.77^\circ}$

$\Sigma(x^2) = 9.7765$ and $\frac{(\Sigma x)^2}{n} = 8.8320$

therefore variance $\sigma^2 = \frac{9.7765 - 8.8320}{14} = \underline{0.0675}$

and hence standard deviation $\sigma = \underline{0.26}$
and coefficient of variation $V = \underline{34}$

<u>0.005γ/kg.</u>

$\Sigma x = 9.12$ therefore mean $\bar{x} = \frac{9.22}{15} = \underline{0.61^\circ}$

$\Sigma(x^2) = 6.8988$ and $\frac{(\Sigma x)^2}{n} = 5.5450$

therefore variance $\sigma^2 = \frac{6.8988 - 5.5450}{14} = \underline{0.0967}$

and hence standard deviation $\sigma = \underline{0.31}$
and coefficient of variation $V = \underline{51}$

Calculations of the means, variances, standard deviations, and coefficients of variation of the leucocyte response (x) (degree of shift to the left) to the 3 dose-levels of lipopolysaccharide from Salmonella abortus equi.

0.1γ/kg.		0.01γ/kg.		0.005γ/kg.	
x	x^2	x	x^2	x	x^2
36.3	1317.69	26.7	712.89	14.7	216.09
34.1	1162.81	20.7	428.49	9.6	92.16
35.1	1232.01	15.2	231.04	11.8	139.24
32.1	1030.41	22.6	510.76	13.0	169.00
18.6	345.96	22.9	524.41	6.1	37.21
28.3	800.89	23.2	538.24	10.2	104.04
33.3	1108.89	16.9	285.61	10.5	110.25
28.1	789.61	24.8	615.04	21.4	457.96
22.4	501.76	14.0	196.00	13.0	169.00
29.8	888.04	20.5	420.25	16.1	259.21
34.0	1156.00	20.2	408.04	25.8	665.64
38.9	1513.21	14.6	21316	22.1	448.41
36.8	1354.24	22.1	488.41	18.2	331.24
38.4	1474.56	11.9	141.61	16.3	265.69
35.5	1260.25	17.6	309.76	12.1	146.41

0.1γ/kg.

$\Sigma x = 481.7$ therefore mean $\bar{x} = \frac{481.7}{15} = \underline{32.1}$

$\Sigma(x^2) = 15936.33$ and $\frac{(\Sigma x)^2}{n} = 15468.99$

therefore variance $\sigma^2 = \frac{15936.33 - 15468.99}{14} = \underline{33.38}$

and hence standard deviation $\sigma = \underline{5.8}$

and coefficient of variation $V = \underline{18}$

0.01γ/kg.

$\Sigma x = 293.9$ therefore mean $\bar{x} = \frac{293.9}{15} = \underline{19.6}$

$\Sigma(x^2) = 6023.71$ and $\frac{(\Sigma x)^2}{n} = 5758.48$

therefore variance $\sigma^2 = \frac{6023.71 - 575848}{14} = \underline{18.95}$

and hence standard deviation $\sigma = \underline{4.4}$

and coefficient of variation $V = \underline{23}$

0.005γ/kg.

$\Sigma x = 220.9$ therefore mean $\bar{x} = \frac{220.9}{15} = \underline{14.7}$

$\Sigma(x^2) = 3611.55$ and $\frac{(\Sigma x)^2}{n} = 3253.12$

therefore variance $\sigma^2 = \frac{3611.55 - 3253.12}{14} = \underline{25.60}$

and hence standard deviation $\sigma = 5.1$

and coefficient of variation $V = 34$

Analysis of variance of the temperature response to lipopolysaccharide from S. abortus equi.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 2.2544	$n_1 - 1 = 14$	0.1610	$n_2 o_1^2 + o_0^2$
between doses	3 - 4 = 3.7148	$n_2 - 1 = 2$	1.8574	$n_1 o_2^2 + o_0^2$
residual (error)	1+4-2-3= 2.3401	$(n_1-1)(n_2-1) = 28$	0.0836	o_0^2
total	8.3093 (1-4)	$n_1 n_2 - 1 = 44$		

$n_1, n_2, o_1^2, o_2^2, o_0^2$ have the same meaning as the same symbils on page 210

1 43.5987
2 37.5438
3 39.0042
4 35.2894

1,2,3,4, are calculated as shown on page 210

Between rabbit variance:
$$F = \frac{0.1610}{0.0836} = 1.93$$ which just fails to be significant at the five percent level of significance

Between doses variance:
$$F = \frac{1.8574}{0.0836} \; 22.22$$ which is greater than the five percent value for F ($n_1=2; n_2=28$) hence between dose variance exists significantly.

continued.

Significance of difference of the means for the temperature response to the three dose-levels of lipopolysacchairde from S. abortus equi:

variance of a mean of 15 observations = $\frac{0.0836}{15}$

and the variance of the difference between two means is twice this, hence

$$S.D. = \sqrt{0.0836 \times \frac{2}{15}} = 0.11$$

hence to be significantly different at the

5 per cent level a difference of 2.05 x 0.11 =0.23° is required.

and from the following table

dose	means	difference
0.1γ/kg	1.28	0.51
0.01 "	0.77	0.16
0.005"	0.61	

it is seen that the temperature responses for the 0.005γ/kg and 0.01γ/kg dose-levels are not significantly different (P=0.05)

Analysis of variance of the leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi.

source of variance	sums of squares	degrees of freedom	mean squares	components of variance
between rabbits	2 - 4 = 432.28	$n_1 - 1 = 14$	30.88	$n_2 o_1^2 + o_o^2$
between doses	3 - 4 = 2413.65	$n_2 - 1 = 2$	1206.83	$n_1 o_2^2 + o_o^2$
residual (error)	1+4-2-3 658.72	$(n_1-1)(n_2-1) = 28$	23.53	o_o^2
total	1 - 4 = 3504.65	$n_1 n_2 - 1 = 44$		

$n_1, n_2, o_1^2, o_2^2, o_o^2$ have the same meaning as the same symbols on page 210

1 25571.59
2 22499.23
3 24480.59
4 22066.94

1, 2, 3, 4, were calculated in the same manner as on page 210

Between rabbit variance:

$$F = \frac{30.88}{23.53} = 1.31$$ which is not significant at the five percent level.

Between doses variance:

$$F = \frac{1206.83}{23.53} = 51.29$$ which is greater than the five percent value for F ($n_1 = 2; n_2 = 28$) hence between dose variance exists significantly

continued.

Significance of difference of the means for the leucocyte responses (degree of shift to the left) to the three dose-levels of lipopolysaccharide from S. abortus equi:

variance of a mean of 15 observations = $\frac{23.53}{15}$ = 1.57

and the variance of the difference between two means is twice this, hence

$$S.D. = \sqrt{23.53 \times \frac{2}{15}} = 1.77$$

hence to be significantly different at the

5 percent level a difference of 2.05 x 1.77 = 3.63 is required
1 percent level a difference of 2.76 x 1.77 = 4.89 is required

and from the following table

dose	means	difference
0.1 γ/kg	32.1	12.5
0.01 γ/kg	19.6	4.9
0.005 γ/kg	14.7	

it is seen that the means for the 0.01 γ/kg and 0.005 γ/kg doses differ significantly (p = 0.01)

Caculation of the correlation coefficient for the temperature response to lipopolysaccharide from S. abortus equi, and log(1000 x dose).

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2\sum(y-\bar{y})^2)}}$

where x = temperature response
and y = log(1000 x dose)

$\sum(x-\bar{x})^2 = 8.3093$

$\sum(y-\bar{y})^2 = 13.91601$

$\sum(x-\bar{x})(y-\bar{y}) = 7.189830$

Therefore $r = \frac{7.189830}{\sqrt{(8.3093 \times 13.91601)}} = \underline{0.6686}$

n = 43; p = <0.001; therefore the correlation coefficient is significantly greater than zero.

Computation of the above figures:

$\sum(x-\bar{x})^2$ is calculated on page 231

$\sum(y-\bar{y})^2 = \sum(y^2) - \frac{(\sum y)^2}{n} = 15(2^2+1^2+0.6990^2) - \frac{(15 \times 3.6990)^2}{45}$

$= \underline{13.91601}$

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = \{(2 \times 19.22)+(1 \times 11.51) + (.699 \times 9.12)\} - \frac{39.85 \times 55.4850}{45}$

$= \underline{7.189830}$

Calculation of the correlation coefficient for the leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi, and log(1000 x dose).

Correlation coefficient $r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2 \sum(y-\bar{y})^2)}}$

where x = leucocyte response (degree of shift to the left)
and y = log (1000 x dose)

$\sum(x-\bar{x})^2 = 3504.65$

$\sum(y-\bar{y})^2 = 13.91601$

$\sum(x-\bar{x})(y-\bar{y}) = 183.0246$

Therefore $r = \frac{183.0246}{\sqrt{(3504.65 \times 13.91601)}} = \underline{0.8288}$

$n = 43; p = < 0.001$; therefore the correlation coefficient is significantly greater than zero

---

Computation of the above figures:

$\sum(x-x)^2$ is calculated on page 233

$\sum(y-y)^2$ is calculated on page 235

$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} =$

$((2 \times 481.7)+(1 \times 293.9)+(.699 \times 220.9)) - \frac{996.5 \times 55.4850}{45}$

$= \underline{183.02460}$

<u>The equation for the regression line - temperature response to lipopolysaccharide from S. abortus equi, and log (1000 x dose).</u>

In the equation $Y = \bar{y} + b(x-\bar{x})$, Y is the predicted value for the temperature response for a given value for log (1000 x dose)

$\bar{y}$ = mean of all the temperature responses = 0.89
$\bar{x}$ = mean of all the corresponding values for log(1000xdose) = 1.233

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{7.189830}{13.19601} = \underline{0.51666}$$

(see calculation of the correlation coefficient for the computation of these figures)

hence $Y = 0.89 + 0.51666(x - 1.233)$
$= \underline{0.51666x + 0.253}$

log (1000 x dose)

2	$Y = 0.89 + 0.51666(2 - 1.233)$ $= 1.29$
1	$Y = 0.89 + 0.51666(1 - 1.233)$ $= 0.77$
0.6990	$Y = 0.89 + 0.51666(.699 - 1.233)$ $= 0.61$

The standard deviation of the scatter about the regression line is given by

$$\sigma_r = \sqrt{1 - r^2}\sqrt{\frac{(y-\bar{y})^2}{n-2}} = \sqrt{1 - 0.6686^2}\sqrt{\frac{8.3093}{43}} = \underline{0.3272}$$

hence ratio A $= \frac{0.33}{1.28 - 0.61} = \underline{0.49}$

ratio B $= \frac{0.33}{0.89} = \underline{0.37}$

The equation for the regression line - leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi, and log (1000 x dose).

In the equation $Y = \bar{y} + b(x-\bar{x})$, Y is the predicted value for the leucocyte response for a given value for log(1000 x dose)

$\bar{y}$ = mean of all the leucocyte responses = 22.1
$\bar{x}$ = mean of all the corresponding values for log (1000 x dose) = 1.233

$$b = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sum(x-\bar{x})^2} = \frac{183.0246}{13.91601} = \underline{13.1521}$$

(see calculation of the correlation coefficient for computation of these figures)

hence

$$Y = 22.1 + 13.1521(x - 1.233)$$
$$= \underline{13.1521x + 5.88}$$

log (1000 x dose)

2 $\quad Y = 22.1 + 13.1521(2 - 1.233) = 32.2$

1 $\quad Y = 22.1 + 13.1521(1 - 1.233) = 19.1$

0.6990 $\quad Y = 22.1 + 13.1521(.699 - 1.233) = 15.1$

The standard deviation of the scatter about the regression line is given by

$$\sigma_r = \sqrt{1 - r^2}\sqrt{\frac{(y-\bar{y})^2}{n-2}} = \sqrt{1 - 0.8288^2}\sqrt{\frac{3504.65}{43}} = \underline{5.04}$$

hence ratio $A = \frac{5.04}{32.1 - 14.7} = \underline{0.29}$

ratio $B = \frac{5.04}{22.1} = \underline{0.23}$

Analysis of variance to test linearity of relationship between the temperature response to lipopolysaccharide from S. abortus equi and log (1000 x dose).

In the manner shown on page 158 we calculate the following values

1 43.5987
2 39.0042
3 35.2894

and enter in table as below:

source of variance	sums of squares	degrees of freedom	mean squares
between doses	3.7148 (2-3)	2	1.8574
within doses	4.5945 (1-2)	42	0.1094
total	8.3093 (1-3)	44	

Significance of the between dose mean square:

$$F = \frac{1.8574}{0.1094} = 16.9781$$ which is greater

than the fiev percent value for the variance ratio $(n_1=2; n_2=42)$

The between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\Sigma(y-\bar{y})(x-\bar{x}))^2}{\Sigma(x-\bar{x})^2} = \frac{7.18983^2}{13.91601} = 3.71468944$$

x = log (1000 x dose)
y = corresponding response (leucocyte)

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	3.7147	1	3.7147
deviation from regression	0.0001	1	0.0001
within doses (residual)	4.5945	42	0.1094
total	8.3093	44	

The sum of squares for the deviation from regression is obtained by subtracting the sum of squares attributable to the regression line from the between doses sum of squares.

The general significance of the regression line can be tested by comparing the regression mean square with the residual, thus

$$F = \frac{3.7147}{0.1094} = 33.95521$$ which is greater than

the five percent value forthe variance ratio ($n_1=1; n_2=42$) and hence significant.

The significance of the deviation from the regression is tested by comparing the deviation mean square with the residual, thus

$$F = \frac{0.0001}{0.1094}$$ which is less than 1 and hence

not significant, hence the data can be represented by a straight line.

Analysis of variance to test the linearity of relationship between the leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi, and log(1000 x dose)

IN the manner shown on page158we calculate the following values

1 25571.59
2 24480.59
3 22066.94

and enter in table as below

source of variance	sums of squares	degrees of freedom	mean squares
between doses	2413.65 (2-3)	2	1206.83
within doses	1091.00 (1-2)	42	25.98
total	3504.65 (1-3)	44	

Significance of the between doses mean square:

$$F = \frac{1206.83}{25.98} = 46.45$$ which is greater

than the five percent value for the variance ratio ($n_1=2;n_2=42$) and hence significant.

The between dose sum of squares is now divided into that due to the linear regression and that due to departure from it, by calculation of the sum of squares attributable to the regression line which is given by

$$\frac{(\Sigma(x-\bar{x})(y-\bar{y}))^2}{\Sigma(x-x)^2} = \frac{183.0246^2}{13.91601} = 2407.15580$$

These values are entered up in the following table:

continued.

source of variance	sums of squares	degrees of freedom	mean squares
between doses:			
regression	2407.1558	1	2407.1558
deviation from regression	6.4947	1	6.4947
within doses (residual)	1091.00	42	25.98
total	3504.65	44	

The sum of squaresfor the deviatinofrom regression is obtained by subtracting the sum of squares attributable to the regression line from the between dose sum of squares.

The general significance ofthe regression line can be tested by comparing the regression mean square with the residual, thus

$$F = \frac{2407.1558}{25.98} = 92.6542$$ which is greater

than the five percent value for the varience ratio ($n_1=1;n_2=42$) and hence significant.

The significance of the deviation from linearity is tested by comparing the deviation mean square with the residual, thus

$$F = \frac{6.4947}{25.98}$$ = less than one and therefore

not significant, hence the data can be represented by a straight line.

Calculation of the correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi.

$$\text{Correlation coefficient } r = \frac{\sum(x-\bar{x})(y-\bar{y})}{\sqrt{(\sum(x-\bar{x})^2 \sum(y-\bar{y})^2)}}$$

where x = leucocyte response
and y = temperature response

$\sum(x-\bar{x})^2 = 3504.65$

$\sum(y-\bar{y})^2 = 8.3093$

$\sum(x-\bar{x})(y-\bar{y}) = 125.800$

$$\text{therefore } r = \frac{125.800}{\sqrt{(3504.65 \times 8.3093)}} = \underline{0.7372}$$

$n=43$; $p=<0.001$; therefore r is significantly greater than zero.

Computation of the above figures:

$\sum(x-\bar{x})^2$ is calculated on page 233

$\sum(y-\bar{y})^2$ is calculated on page 231

$$\sum(x-\bar{x})(y-\bar{y}) = \sum(xy) - \frac{\sum x \sum y}{n} = 1008.256 - \frac{996.5 \times 39.85}{45}$$

$$= \underline{125.800}$$

Calculation of the partial correlation coefficient for the temperature response and the leucocyte response (degree of shift to the left) to lipopolysaccharide from S. abortus equi.

In this case the effect of log(1000 x dose) on the correlation is eliminated

Let 1 represent the leucocyte response
2 represent the temperature response
3 represent log(1000 x dose)

then r_{12} means the total correlation coefficient between the leucocyte response and the temperature response (= 0.7372)

r_{13} means the total correlation coefficient between the leucocyte response and log(1000 x dose) (= 0.8288)

r_{23} means the total correlation coefficient between the temperature response and log(1000 x dose) (= 0.6686)

The partial correlation coefficient

$$r_{12.3} = \frac{r_{12} - r_{13}r_{23}}{\sqrt{(1-r_{13}^2)(1-r_{23}^2)}}$$

$$= \frac{0.7372 - 0.8288 \times 0.6868}{\sqrt{(1 - 0.8288^2)(1 - 0.6686^2)}}$$

$$= \underline{0.4400}$$

n=42; p=0.01-0.001; therefore the partial correlation coefficient is significantly greater than zero

A P P E N D I X III

Results of experiments with Injection of Calcium Gluconate and pyrogen.

Injection of Calcium Gluconate 2ml/kg (Pyrogen free)

Rabbit No.	t	t^2	f	f^2	d	d^2
103	40	1600	1.89	3.5721	180	32400
104	15	225	0.74	0.5476	70	4900
107	14	196	0.66	0.4356	69	4761
108	10	100	0.88	0.7744	60	3600
22	10	100	0.47	0.2209	76	5776
34	10	100	1.11	1.2321	86	7396
101	5	25	1.17	1.3689	125	15625
109	34	1156	0.20	0.0400	64	4096
112	13	169	0.70	0.4900	61	3721
24	13	169	0.36	0.1296	41	1681
30	29	841	0.35	0.1225	89	7921
36	2	4	0.22	0.0484	30	900
33	8	64	0.08	0.0064	28	784
14	0	0	0	0		
21	15	225	0.40	0.1600	45	2025
102	30	900	0.60	0.3600	100	10000
110	10	100	0.61	0.3721	40	1600
25	13	169	0.87	0.7569	93	8649
32	25	625	0.61	0.3721	85	7225
35	45	2025	0.69	0.4761	115	13225
113	35	1225	0.96	0.9216	115	13225
8	45	2025	0.84	0.7056	115	13225

t = time (minutes) required to reach maximum fall in temperature

f = maximum fall in temperature (C^o)

d = time (minutes) required to reach pre-injection temperature

continued.

Time to reach maximum fall in temperature

$\sum t = 421$ therefore mean = 20 (n=21)

$\sum(t^2) = 12043$ and $\frac{(\sum t)^2}{n} = 8440$

therefore variance $= \frac{12043 - 8440}{20} = 180.2$

and standard deviation = 13.4

Fall in temperature

$\sum f = 14.41$ therefore mean = 0.66 (n=22)

$\sum(f^2) = 13.1129$ and $\frac{(\sum f)^2}{n} = 9.4386$

therefore variance = 0.18
and standard deviation = 0.42

Time to reach pre-injection temperature

$\sum d = 1687$ therefore mean = 80 (n=21)

$\sum(d^2) = 162735$ and $\frac{(\sum d)^2}{n} = 135522$

therefore variance = 1361
and standard devition = 37

continued.

Injection of Calcium Gluconate 2 ml/kg containing Pyrogen (0.02ml/kg)

Rabbit No.	t'	t'^{2}	f'	f'^{2}	d'	d'^{2}	r	r^2	x	x^2
103	35	1225	0.48	0.2304	65	4225	1.17	1.3689	145	21025
104	23	529	0.52	0.2704	38	1444	1.06	1.1236	98	9604
107	12	144	0.51	0.7601	37	1369	1.13	1.2769	117	13689
108	9	81	0.45	0.2025	29	841	1.89	3.5721	79	6241
22	15	225	0.32	0.1024	50	2500	1.01	1.0201	110	12100
34	14	196	0.90	0.8100	54	2916	1.00	1.0000	134	17956
101	5	25	0.27	0.0729	20	400	1.35	1.8225	85	7225
109	10	100	0.60	0.3600	26	676	1.18	1.3924	115	13225
112	15	225	0.85	0.7225	40	1600	1.73	2.9929	90	8100
24	16	256	0.48	0.2304	36	1296	1.82	3.3124	186	34596
30	26	676	0.22	0.0484	46	2116	1.04	1.0316	126	15876
36	5	25	0.21	0.0441	10	100	1.60	2.5600	95	9025
33	7	49	0.46	0.2116	32	1024	0.91	0.8281	92	8464
14	5	25	0.28	0.0784	15	225	0.54	0.2916	155	24025
21	5	25	0.87	0.7569	40	1600	0.36	0.1296	80	6400
102	5	25	0.48	0.2304	50	2500	0.39	0.1521	160	25600
110	17	289	0.29	0.0841	27	729	0.94	0.8836	87	7569
25	5	25	0.17	0.0289	15	225	1.47	2.1609	115	13225
32	6	36	0.53	0.2809	46	2116	1.37	1.8769	116	13456
35	5	25	0.04	0.0016	5	25	1.17	1.3689	95	9025
113	8	64	0.19	0.0361	33	1089	1.99	3.9601	83	6889
8	16	256	0.12	0.0144	36	1296	0.84	0.7056	106	11236

t' = time (minutes) required to reach maximum fall in temperature

f' = maximum fall in temperature (C°)

d' = time (minutes) required to reach pre-injection temperature

r = rise in temperature (C°)

x = time (minutes) required to reach maximum temperature

continued.

Time to reach maximum fall in temperature

$\Sigma t' = 264$ therefore mean = 12

$\Sigma(t'^2) = 4526$ and $\frac{(\Sigma t')^2}{n} = 3168$

therefore variance = 64.7
and standard deviation = 8.0

Fall in temperature

$\Sigma f' = 9.24$ therefore mean = 0.42

$\Sigma(f'^2) = 5.0774$ and $\frac{(\Sigma f')^2}{n} = 3.8808$

therefore variance = 0.0570
and standard deviation = 0.24

Time to reach pre-injection temperature

$\Sigma d' = 750$ therefore mean = 34

$\Sigma(d'^2) = 30312$ and $\frac{(\Sigma d')^2}{n} = 25568$

therefore variance = 225.9
and standard deviation = 15

Rise in temperature

$\Sigma r = 25.96$ therefore mean = 1.18

$\Sigma(r^2) = 34.8808$ and $\frac{(\Sigma r)^2}{n} = 30.6328$

therefore variance = 0.20
and standard deviation = 0.45

Time to reach maximum temperature

$\Sigma x = 2469$ therefore mean = 112

$\Sigma(x^2) = 294551$ and $\frac{(\Sigma x)^2}{n} = 277089$

therefore variance = 832
and standard deviation = 29

continued.

Injection of Pyrogen 0.02ml/kg.

Rabbit No.	r'	r'²	x'	x'²
103	1.05	1.1025	98	9604
104	0.97	0.9409	124	15376
107	1.21	1.4641	112	12544
108	1.26	1.5876	90	8100
22	1.15	1.3225	149	22201
34	0.99	0.9801	100	10000
101				
109	1.14	1.2996	165	27225
112	1.19	1.4161	105	11025
24	1.34	1.7956	100	10000
30	1.20	1.4400	215	46225
36	1.31	1.7161	120	14400
33	1.21	1.4641	104	10816
14	0.96	0.9216	119	14161
21	0.99	0.9801	172	29584
102	1.57	2.4649	100	10000
110	1.50	2.2500	100	10000
25				
32	0.86	0.7396	100	10000
35	1.21	1.4641	80	6400
113	1.91	3.6481	75	5625
8				

r' = rise in temperature (C°)

x' = time (minutes) required to reach maximum temperature

Rise in temperature

$\Sigma r' = 23.02$ therefore mean = 1.21 (n=19)

$\Sigma(r'^2) = 28.9976$ and $\frac{(\Sigma r')^2}{n} = 27.8905$

therefore variance = 0.06

and standard deviation = 0.25

Time to reacp peak temperature

$\Sigma x' = 2228$ therefore mean = 117

$\Sigma(x'^2) = 283286$ and $\frac{(\Sigma x')^2}{n} = 261263$

therefore variance = 1223.6

and standard deviation = 35

continued.

Effect of Injection of Calcium Gluconate, administered alone and with pyroegn, and pyrogen, on the Polynuclear Count.

Rabbit No.	A x	A x^2	B x	B x^2	C x	C x^2
103	1.9	3.61	38.6	1489.96	16.0	256.00
104	0	0	25.1	630.01	12.5	156.25
107	1.0	1.00	16.4	268.96	30.1	906.01
108	5.8	33.64	21.1	445.21	11.6	134.56
22	0.5	0.25	32.2	1036.84	12.9	166.41
34	0	0	29.9	894.01	21.0	441.00
101	0	0	26.8	718.24		
109	3.4	11.56	37.1	1376.41	17.3	299.29
112	1.8	3.24	19.5	380.25	18.4	338.56
24	0	0	21.0	441.00	22.8	519.84
30	0	0	32.2	1036.84	26.0	676.00
36	4.0	16.00	16.7	278.89	35.2	1239.04
33	6.3	39.69	35.0	1225.00	21.9	479.61
14	0	0	20.4	416.16	17.5	306.25
21	0	0	5.2	27.04	20.4	416.16
102	0	0	23.6	556.96	36.9	1361.61
110	1.8	3.24	20.2	408.04	34.3	1176.49
25	9.4	88.36	15.6	243.36		
32	0	0	22.9	524.41	16.3	265.69
35	3.2	10.24	8.1	65.61	21.4	457.96
113	0.5	0.25	20.8	432.64	22.5	506.25
8	2.8	7.84	18.3	334.89		

A = leucocyte response (degree of shift to the left) following Injection of Calcium Gluconate

B = same but with added pyrogen(0.02 ml/kg)

C = leucocyte response (degree of shift to the left) following pyrogen alone (0.02 ml/kg.)

continued.

A Injection of Calcium Gluconate

$\sum x = 42.4$ therefore mean = 1.93

$\sum(x^2) = 218.92$ and $\frac{(\sum x)^2}{n} = 81.72$

therefore variance = 6.53
and standard deviation = 2.6

B Injection of Calcium Gluconate + pyrogen

$\sum x = 506.7$ therefore mean = 23.0

$\sum(x^2) = 13230.73$ and $\frac{(\sum x)^2}{n} = 11670.2$

therefore variance = 74.3
and standard deviation = 8.6

C Pyrogen

$\sum x = 415.0$ therefore mean = 21.8

$\sum(x^2) = 10102.98$ and, $\frac{(\sum x)^2}{n} = 9064.5$

therefore variance = 57.7
and standard deviation = 7.6

Significance of difference of the various means.

Fall in temperature

mean for Injection of Calcium Gluconate = 0.66
mean for Injection of Calcium Gluconate + pyrogen = 0.42

$$t = \frac{0.24}{0.34 \times 0.3} = \underline{2.353}; n=42; p=0.02-0.05$$

Time to reach maximum fall in temperature

mean for Injection of Calcium Gluconate = 20
mean for Injection of Calcium Gluconate + pyrogen = 12

$$t = \frac{8}{11 \times 0.3} = \underline{2.424}; p=0.02-0.05$$

Duration of upset in temperature

mean for Injection of Calcium Gluconate = 80
mean for Injection of Calcium Gluconate + pyrogen = 34

$$t = \frac{46}{27.9 \times 0.3} = \underline{5.496}; p= 0.001$$

Rise in temperature

mean for Injection of Calcium Gluconate + pyrogen = 1.18
mean for pyrogen = 1.21

These means are obviously not significantly different

Time to reach Peak temperature

mean for Injection of Calcium Gluconate + pyrogen = 112
mean for pyrogen = 117

These means are obviously not significantly different

A P P E N D I X IV

Tolerance experiments

Tolerance in 8 rabbits

1st injection of Proteus pyrogen (0.2ml/kg)

Rabbit No.	x	x^2
1	1.62	2.6244
5	1.45	2.1025
6	1.39	1.9321
7	1.63	2.6569
101	1.50	2.2500
102	2.50	6.2500
103	1.57	2.4649
104	1.35	1.8225

$\Sigma x = 13.07$ $\frac{(\Sigma x)^2}{n} = 21.1575$

$\Sigma(x^2) = 22.1033$ $\bar{x} = 1.63$

$\Sigma(x^2) - \frac{(\Sigma x)^2}{N} = 0.9458$

13th and 14th injections of Proteus pyrogen

Rabbit No.	x	x^2	x	x^2
1	0.81	0.6561	0.81	0.6561
5	0.86	0.7396	0.67	0.4489
6	0.88	0.7744	0.91	0.8281
7	0.90	0.8100	0.96	0.9216
101	0.86	0.7396	0.83	0.6889
102	1.13	1.2769	0.91	0.8281
103	0.90	0.8100	0.95	0.9025
104	1.16	1.3456	0.89	0.7921

$\Sigma x = 7.50$ $\frac{(\Sigma x)^2}{n} = 7.0313$

$\Sigma(x^2) = 7.1522$ $\bar{x} = 0.94$

$\Sigma(x^2) - \frac{(\Sigma x)^2}{n} = 0.1209$

$\Sigma x = 6.93$ $\frac{(\Sigma x)^2}{n} = 6.0031$

$\Sigma(x^2) = 6.0063$ $\bar{x} = 0.87$

$\Sigma(x^2) - \frac{(\Sigma x)^2}{n} = 0.0032$

Significance of difference of the means:

mean for 1st injection 1.63
mean for 13thinjection 0.94

$t = \frac{0.69}{0.28 \times 0.5} = \underline{4.928}$

$n=14; p= 0.001$

mean for 1st injection 1.63
mean for 14thinjection 0.87

$t = \frac{0.76}{0.26 \times 0.50} = \underline{5.846}$

$n=14; p= 0.001$

Therfore the means are significantly different

Individual temperature responses of 3 groups of rabbits to 3 different pyrogen preparations, administered at daily intervals.

I Pseudomonas polysaccharide 5γ/kg.

	Rabbit No.	days 1	2	3	4	5	6	7	8
Group A	45	0.97	0.45	0.35	0.26	0.38	0.23	0.22	0.08
	109	1.77	1.55	0.49	0.36	0.17	0.27	0.09	0.16
	36	1.28	0.84	1.22	0.77	0.70	0.65	0.89	0.81
	means	1.34							0.35

II Lipopolysaccharide from S. abortus equi 0.04γ/kg.

	Rabbit No.	1	2	3	4	5	6	7	8
Group B	33	1.05	1.03	0.69	0.76	0.69	0.57	0.56	0.75
	21	1.23	0.77	0.53	0.42	0.52	0.35	0.23	0.47
	49	1.33	1.58	1.41	1.06	0.94	0.79	0.73	0.75
	means	1.20							0.66

III Standard pyrogen from P. vulgaris 0.02ml/kg.

	Rabbit No.	1	2	3	4	5	6	7	8
Group C	103	2.09	1.85	1.70	1.23	1.09	1.02	1.05	0.51
	110	1.56	1.49	0.84	0.82	0.67	0.37	0.41	0.34
	49	1.56	1.35	0.96	0.56	0.38	0.39	0.65	0.35
	means	1.74							0.40

Temperature responses of the tolerant rabbits to pyrogen other than that which was used to induce tolerance.

	Rabbit No.	Pyrogen (day 11)	Response	Pyrogen (day 12)	Response
Group A	45	Proteus pyrogen 0.02ml/kg	0.79	Lipopolysaccharide (0.04γ/kg)	0.26
	109		0.70		0.86
	36		0.72		0.24
	means		0.74		0.45
Group B	33	Pseudomonas polysaccharide (5γ/kg.)	0.65	Proteus pyrogen (0.02ml/kg.)	0.76
	21		0.28		0.42
	49		0.65		0.87
	means		0.53		0.68
Group C	103	Lipopolysaccharide (0.4γ/kg.)	0.75	Pseudomonas polysaccharide (5γ/kg)	0.22
	110		0.93		0.13
	49		0.74		0.19
	means		0.81		0.18

Significance of difference of the mean temperature responses for the 3 groups of rabbits on different days.

Day 1

	x	x^2	
Group A	0.97	0.9409	$\Sigma(x^2) = 5.7122$ and $\frac{(\Sigma x)^2}{n} = 5.3868$
	1.77	3.1329	
	1.28	1.6384	therefore variance $= \frac{0.3254}{2} = 0.1627$
	4.02	5.7122	
Group B	1.05	1.1025	$\Sigma(x^2) = 4.3843$ and $\frac{(\Sigma x)^2}{n} = 4.3440$
	1.23	1.5129	
	1.33	1.7689	therefore variance $= \frac{0.0403}{2} = 0.0202$
	3.61	4.3843	
Group C	2.09	4.3681	$\Sigma(x^2) = 9.2353$ and $\frac{(\Sigma x)^2}{n} = 9.0480$
	1.56	2.4336	
	1.56	2.4336	therefore variance $= \frac{0.1873}{2} = 0.0937$
	5.21	9.2353	

Day 8

	x	x^2	
Group A	0.08	0.0064	$\Sigma(x^2) = 0.6881$ and $\frac{(\Sigma x)^2}{n} = 0.3675$
	0.16	0.0256	
	0.81	0.6561	therefore variance $= \frac{0.3206}{2} = 0.1603$
	1.05	0.6881	
Group B	0.75	0.5625	$\Sigma(x^2) = 1.3459$ and $\frac{(\Sigma x)^2}{n} = 1.2936$
	0.47	0.2209	
	0.75	0.5625	therefore variance $= \frac{0.0523}{2} = 0.0262$
	1.97	1.3459	
Group C	0.51	0.2601	$\Sigma(x^2) = 0.4982$ and $\frac{(\Sigma x)^2}{n} = 0.4800$
	0.34	0.1156	
	0.35	0.1225	therefore variance $= \frac{0.0182}{2} = 0.0091$
	1.20	0.4982	

continued.

Day 11

Group A

x	x^2
0.79	0.6241
0.70	0.4900
0.72	0.5184
2.21	1.6325

$\Sigma(x^2) = 1.6325$ and $\frac{(\Sigma x)^2}{n} = 1.6280$

therefoer variance = $\frac{0.0045}{2} = 0.0023$

Group B

x	x^2
0.65	0.4225
0.28	0.0784
0.65	0.4225
1.58	0.9234

$\Sigma(x^2) = 0.9234$ and $\frac{(\Sigma x)^2}{n} = 0.8321$

therefore variance $= \frac{0.0913}{2} = 0.0457$

Group C

x	x^2
0.75	0.5625
0.93	0.8649
0.74	0.5476
2.42	1.9750

$\Sigma(x^2) = 1.9750$ and $\frac{(\Sigma x)^2}{n} = 1.9521$

therefore variance = $\frac{0.0229}{2} = 0.0115$

Day 12

Group A

x	x^2
0.26	0.0676
0.86	0.7396
0.24	0.0576
1.36	0.8648

$\Sigma(x^2) = 0.8648$ and $\frac{(\Sigma x)^2}{n} = 0.6165$

therefore variance = $\frac{0.2483}{2} = 0.1242$

Group B

x	x^2
0.76	0.5776
0.42	0.1681
0.87	0.7569
2.05	1.5026

$\Sigma(x^2) = 1.5026$ and $\frac{(\Sigma x)^2}{n} = 1.4008$

therefore variance = $\frac{0.1018}{2} = 0.0509$

Group C

x	x^2
0.22	0.0484
0.13	0.0169
0.19	0.0361
0.54	0.1014

$\Sigma(x^2) = 0.1014$ and $\frac{(\Sigma x)^2}{n} = 0.0972$

therefore variance = $\frac{0.0042}{2} = 0.0021$

continued.

t was calculated for the following pairs of mean responses:

day 1 and day 8, to show whether a significantly lower response was obtained after 8 daily injections of pyrogen.

day 1 and days 11 and 12, to show whether there was any cross tolerance.

day 8 and days 11 and 12, to show whether there was any difference in the degree of cross tolerance to the different pyrogen preparations.

$$t = \frac{\bar{x} - \bar{x}'}{\sigma\sqrt{\frac{n+n'}{n \times n'}}} \quad \text{where } \sigma = \sqrt{\left(\Sigma(x^2) - \frac{(\Sigma x)^2}{n} + \Sigma(x'^2) - \frac{(\Sigma x')^2}{n'}\right)/(n+n'-2)}$$

Group A: mean for day 1 = 1.34 = $\bar{x}$, $n = n' = 3$
mean for day 8 = 0.35 = $\bar{x}'$

$$t = \frac{0.99}{0.40 \times 0.82} = 3.018; \quad p = 0.02\text{-}0.05$$

therefore the means are significantly different

Group B: mean for day 1 = 1.20
mean for day 8 = 0.66

$$t = \frac{0.54}{0.15 \times 0.82} = 4.390; \quad p = 0.01\text{-}0.02$$

therefore the means are significantly different

GroupC: mean for day 1 = 1.74
mean for day 8 = 0.40

$$t = \frac{1.34}{0.21 \times 0.82} = 7.781; \quad p = 0.01\text{-}0.001$$

therefore the means are significantly different

Hence a significantly lower response was obtained with all three groups of rabbits on the 8th daily injection

continued.

Group A: mean for day 1 = 1.34
mean for day11 = 0.74

$$t = \frac{0.60}{0.29 \times 0.82} = 2.523; \ p = 0.02 - 0.05$$

mean for day 1 = 1.34
mean for day12 = 0.45

$$t = \frac{0.89}{0.38 \times 0.82} = 2.856; \ p = 0.02 - 0.05$$

Group B: mean for day 1 = 1.20
mean for day11 = 0.53

$$t = \frac{0.67}{0.18 \times 0.82} = 4.539; \ p = 0.01 - 0.02$$

mean for day 1 = 1.20
mean for day12 = 0.68

$$t = \frac{0.52}{0.19 \times 0.82} = 3.338; \ p = 0.02 - 0.05$$

Group C: mean for day 1 = 1.74
mean for day11 = 0.81

$$t = \frac{0.93}{0.23 \times 0.82} = 4.931; \ p = 0.01 - 0.001$$

mean for day 1 = 1.74
mean for day12 = 0.18

$$t = \frac{1.56}{0.22 \times 0.82} = 8.647; \ p = < 0.001$$

continued.

Group A: mean for day 8 = 0.35
mean for day11 = 0.74

$$t = \frac{0.39}{0.29 \times 0.82} = 1.640; p = 0.1 - 0.2$$

mean for day 8 = 0.35
mean for day12 = 0.45

$$t = \frac{0.10}{0.38 \times 0.82} = 0.321; p = 0.7 - 0.8$$

Group B: mean for day 8 = 0.66
mean for day11 = 0.53

$$t = \frac{0.13}{0.19 \times 0.82} = 0.834; p = 0.4 - 0.5$$

mean for day 8 = 0.66
mean for day12 = 0.68

here t is obviously not significant

Group C: mean for day 8 = 0.40
mean for day11 = 0.81

$$t = \frac{0.41}{0.10 \times 0.82} = 5.000; p = 0.01 - 0.001$$

mean for day 8 = 0.40
mean for day12 = 0.18

$$t = \frac{0.22}{0.07 \times 0.82} = 3.833; p = 0.01 - 0.02$$

Group A: mean for day 11 = 0.74
Group C: mean for day 8 = 0.40

$$t = \frac{0.34}{0.08 \times 0.82} = 5.183; p = 0.01 - 0.001$$

Group B: mean for day 11 = 0.53
Group C: mean for day 12 = 0.18

$$t = \frac{0.35}{0.15 \times 0.82} = 2.846; p = 0.02 - 0.05$$

APPENDIX V

continued.

Injection of ACTH 1 unit/kg.

Rabbit No.	f	f^2	c	c^2
41	0.64	0.4096	21.8	475.24
43	1.03	1.0609	16.1	259.21
44	2.06	4.2436	2.1	4.41
45	3.68	13.5424	20.8	432.64
A	1.96	3.8416	15.6	243.36
42	0.53	0.2809	14.3	204.49
46	0.73	0.5329	20.7	428.49
48	1.14	1.2996	0	0
49	2.48	6.1504	29.7	882.09
50	0.64	0.4096	25.1	630.01
27	3.35	11.2225	35.8	1281.64
34	2.51	6.3001	29.6	876.16
112	1.31	1.7161	33.3	1108.89
113	2.63	6.9159	30.3	918.09
14	1.25	1.5625	34.4	1183.36

f = maximum fall in temperature (C^o)

c = degree of shift to the left (percentage fall in the average number of lobes per netrophil)

Fall in temperature

$\sum f = 25.94$ therefore mean $= \frac{25.94}{15} = \underline{1.73}$

$\sum(f^2) = 59.4896$ and $\frac{(\sum f)^2}{n} = 44.8589$

therefore variance $= \frac{59.4896 - 44.8589}{14} = \underline{1.051}$

and standard deviation = $\underline{1.02}$

Degree of shift to the left

$\sum c = 329.6$ therefore mean $= \frac{329.6}{15} = \underline{22.0}$

$\sum(c^2) = 8928.08$ and $\frac{(\sum c)^2}{n} = 7242.41$

therefore variance $= \frac{8928.08 - 7242.41}{14} = \underline{120.41}$

and standard deviation = $\underline{11.0}$

continued.

Injection of ACTH 1 unit /kg with pyrogen 0.06ml/kg

Rabbit No.	f'	f'^{2}	r	r^2	t	t^2	c'	c'^{2}
41	0.11	0.0121	2.28	5.1984	180	32400	32.6	1062.76
43	0.50	0.2500	2.11	4.4521	235	55225	31.9	1017.61
44	0.08	0.0064	2.21	4.8841	165	27225	40.1	1608.01
45	2.11	4.4521	0.59	0.3481	235	55225	44.0	1936.00
A	0.15	0.0225	1.53	2.3409	145	21025	40.2	1616.04
42	0	0	1.86	3.4596	185	34225	36.3	1317.69
46	0.06	0.0036	2.02	4.0804	125	15625	27.9	778.41
48	0.33	0.1089	1.58	2.4964	155	24025	37.3	1391.29
49	2.35	5.5225	0	0			27.9	778.41
50	0	0	2.20	4.8400	195	38025	35.5	1260.25
27	0.52	0.2704	1.20	1.4400	195	38025	42.2	1780.84
34	0.43	0.1849	1.44	2.0736	135	18225	33.3	1108.89
112	0.17	0.0289	2.08	4.3264	165	27225	35.9	1288.81
113	0	0	2.59	6.7081	195	38025	40.0	1600.00
14	0.56	0.3136	1.16	1.3456	195	38025	19.7	388.09

f' = maximum fall in temperature

r = maximum rise in temperature

t = time (minutes) required to reach maximum temperature

c' = degree of shift to the left (percentage fall in average number of lobes per neytrophil)

Fall in Temperature

$\Sigma f' = 7.37$ therefore mean $= \frac{7.37}{15} = \underline{0.49}$

$\Sigma(f'^2) = 11.1759$ and $\frac{(\Sigma f')^2}{n} = 3.6211$

therefore variance $= \frac{11.1759 - 3.6211}{14} = \underline{0.5396}$

and standard deviation $= \underline{0.73}$

continued.

Rise in temperature

$\sum r = 24.85$ therefore mean $= \frac{24.85}{15} = \underline{1.66}$

$\sum(r^2) = 47.9937$ and $\frac{(\sum r)^2}{n} = 41.1681$

therefore variance $= \frac{47.9937 - 41.1681}{14} = \underline{0.4875}$

and standard deviation $= \underline{0.70}$

Time to reach peak temperature

$\sum t = 2505$ therefore mean $= \frac{2505}{14} = \underline{179}$

$\sum(t^2) = 462525$ and $\frac{(\sum t)^2}{n} = 448216$

therefore variance $= \underline{1100.7}$

and standard deviation = 33
(n = 14 because in one instance there was no peak)

Degree of shift to the left

$\sum c' = 524.8$ therefore mean $= \frac{524.8}{15} = \underline{35.0}$

$\sum(c'^2) = 18933.10$ and $\frac{(\sum c')^2}{n} = 18361.07$

therefore variance $= \underline{40.86}$

and standarddeviation $= \underline{6.4}$

continued.

Injection of Pyrogen (0.06ml/kg)

Rabbit No.	r'	r'^2	t'	t'^2	x	x^2
41	1.46	2.1316	100	10000	47.2	2227.84
43	2.13	4.5369	100	10000	39.7	1576.09
44	1.72	2.9584	80	6400	41.0	1681.00
45	1.61	2.5921	80	6400	37.7	1421.29
A	1.42	2.0164	100	10000	38.5	1482.25
42	2.72	7.3984	145	21025	29.9	89.01
46	1.77	3.1329	75	5625	25.8	665.64
48	1.86	3.4596	75	5625	26.1	1303.21
49	1.84	3.3856	185	34225	32.3	1043.29
50	1.60	2.5600	95	9025	38.2	1459.24
27	1.38	1.9044	80	6400	40.6	1648.36
34	1.39	1.9321	100	10000	37.0	1369.00
112	1.62	2.6244	80	6400	39.9	1592.01
113	1.49	2.2201	100	10000	44.6	1989.16
14	1.43	2.0449	100	10000	47.3	2237.29

r' = maximum rise in temperature

t' = time (minutes(required to reach maximum temperature

x = degree of shift to the left (percentage fall in the average number of lobes per neutrophil)

continued.

Rise in temperature

$\sum r' = 25.44$ therefore mean $= \frac{25.44}{15} = \underline{1.70}$

$\sum (r'^2) = 44.8978$ and $\frac{(\sum r')^2}{n} = 43.1462$

therefore variance = $\underline{0.1255}$

and standard deviation = $\underline{0.35}$

Time to reach peak temperature

$\sum t' = 1495$ therefore mean $= \frac{1495}{15} = \underline{100}$

$\sum (t'^2) = 161125$ and $\frac{(\sum t')^2}{n} = 149001.7$

therefore variance = $\underline{865.9}$

and standard deviation = $\underline{29}$

Degree of shift to the left

$\sum x = 575.8$ therefore mean $= \frac{575.8}{15} = \underline{38.4}$

$\sum (x^2) = 22589.68$ and $\frac{(\sum x)^2}{n} = 22103.04$

therefore variance = $\underline{34.76}$

and standard deviation = $\underline{5.9}$

Results of experiments with ACTH and pyrogen.

Injection of ACTH 1unit/kg.

Rabbit No.	f	f^2	c	c^2
21	2.97	8.8209	26.9	723.61
8	0.78	0.6084	11.0	121.00
24	1.63	2.6569	23.7	561.69
25	1.56	2.4336	15.7	246.49
36	3.67	13.4689	34.1	1162.81
42	1.24	1.5376	15.9	252.81
34	1.07	1.1449	22. 9	524.41
27	1.24	1.5376	19.0	361.00
113	1.47	2.1609	29.7	882.09
33	0.97	0.9409	26.5	702.25
1a	3.18	10.1124	17.5	306.25
2a	1.25	1.5625	14.8	219.04
3a	3.55	12.0625	27.4	750.76
4a	2.19	4.7961	27.4	750.76
5a	1.92	3.6864	16.5	272.25

f = maximum fall in temperature (C^o)

c = degree of shift to the left (percentage fall in average number of lobes per neutrophil)

Fall in temperature

$\Sigma f = 28.69$ therefore mean $= \frac{28.69}{15} = \underline{1.91}$

$\Sigma(f^2) = 68.0705$ and $\frac{(\Sigma f)^2}{n} = 54.8744$

therefore variance = 0.9426
and standard deviation = 0.97

Degree of shift to the left

$\Sigma c = 329.0$ therefore mean = 21.9

$\Sigma(c^2) = 7837.22$ and $\frac{(\Sigma c)^2}{n} = 7216.07$

therefore variance = 44.37
and standard deviation = 6.7

continued.

<u>Injection of ACTH 1 unit /kg with pyrogen 0.02ml/kg</u>

Rabbit No.	f'	f'^2	r	r^2	t	t^2	c'	c'^2
21	0.49	0.2401	1.02	1.0404	185	34225	34.6	1197.16
8	0.85	0.7225	1.17	1.3639	185	34225	42.5	1806.25
24	0.28	0.0784	0.18	0.0324	205	42025	30.3	918.09
25	1.74	3.0276	0.58	0.3364	325	105625	36.7	1346.89
36	2.08	4.3264	0.32	0.1024	305	93025	35.2	1239.04
42	0.40	0.1600	1.51	2.2801	135	18225	34.0	1156.00
34	0.71	0.5041	0.49	0.2401	235	55225	40.0	1600.00
27	1.18	1.3924	0	0	0	0	37.0	1369.00
113	0.26	0.0676	1.22	1.4884	205	42025	37.3	1391.29
33	1.71	2.9241	0	0	0	0	37.2	1383.84
1a	0.44	0.1936	1.62	2.6244	175	30625	34.0	1156.00
2a	0.11	0.0121	1.59	2.5281	115	13225	27.6	761.76
3a	0.43	0.1849	1.46	2.1316	145	21025	32.6	1062.76
4a	0.50	0.2500	0.89	0.7921	145	21025	26.9	723.61
5a	0.58	0.3364	1.10	1.2100	145	21025	34,6	1197.16

f' = maximum fall in temperature

r = maximum rise in temperature

t = time (minutes) required to reach maxmium temperature

c' = degree of shift to the left(percentage fall in the average number of lobes perneutrophil)

continued.

Fall in temperature

$\Sigma f' = 11.76$ therefore mean = 0.78

$\Sigma(f'^2) = 14.4202$ and $\frac{(\Sigma f')^2}{n} = 9.2198$

therefore variance = 0.3715
and standard deviation = 0.61

Rise in temper ature

$\Sigma r = 13.15$ therefore mean = 0.88

$\Sigma(r^2) = 16.1753$ and $\frac{(\Sigma r)^2}{n} = 11.5282$

therefore variance = 0.3319

and standard deviation = 0.58

Time to reach peak temperature

$\Sigma t = 2505$ therefore mean $= \frac{2505}{13} = 193$

(n=13 because there are two negative results, and in the case of 'time to reach peak temperature' a negative result has no meaning or it means that an infinitely long time was required to reach a peak, and because of this difficulty they are excluded from the calcul-tion, even though the corresponding negative result for the rise in temperature is included)

$\Sigma(t^2) = 531525$ and $\frac{(\Sigma t)^2}{n} = 482694$

therefore variance = 4069.3
and standard deviation = 64

Degree of shift to the left

$\Sigma c' = 520.5$ therefore mean = 34.7

$\Sigma(c'^2) = 18308.85$ and $\frac{(\Sigma c')^2}{n} = 18061.55$

therefore variance = 17.68
and standard deviation = 4.2

continued.

Injection of Pyrogen (0.02ml/kg)

Rabbit No.	r'	r'^2	t'	t'^2	x	x^2
21	1.01	1.0201	85	7225	36.3	131769
8	1.38	1.9044	95	9025	36.0	1296.00
24	1.92	3.6864	215	46225	32.4	1049.76
25	1.30	1.6900	85	7225	31.0	961.00
36	2.37	5.6169	65	4225	40.0	1600.00
42	1.41	1.9881	100	10000	31.6	998.56
34	1.71	2.9241	150	22500	18.8	353.44
27	1.89	3.5721	210	44100	25.7	660.49
113	1.48	2.1904	80	6400	26.8	718.24
33	1.24	1.5376	100	10000	22.2	492.84
1a	1.47	2.1609	70	4900	32.7	1069.29
2a	1.64	2.6896	170	28900	37.6	1413.76
3a	1.34	1.7956	90	8100	39.4	1552.36
4a	1.75	3.0625	210	44100	36.7	1346.89
5a	1.25	1.5625	90	8100	38.8	1505.44

r' = maximum rise intemperature

t' = time (minutes) required to reach maximum tempeature

x = degree of shift to the left(percentage fall in the average number of lobes per neutrophil)

continued.

<u>Rise in temperature</u>

$\Sigma r' = 23.16$ therefore mean = <u>1.54</u>

$\Sigma(r'^2) = 37.4013$ and $\frac{(\Sigma r')^2}{n} = 35.7590$

therefore variance = <u>0.1173</u>
and standard devaiti on = <u>0.34</u>

<u>Time to reach peak temperature</u>

$\Sigma t' = 1815$ therefore mean = <u>121</u>

$\Sigma(t'^2) = 261025$ and $\frac{(\Sigma t')^2}{n} = 219615$

therefore variance = <u>2958</u>
and standard deviation = <u>54</u>

<u>Degree of shift to the left</u>

$\Sigma x = 486.0$ therefore mean = <u>32.4</u>

$\Sigma(x^2) = 16385.76$ and $\frac{(\Sigma x)^2}{n} = 15746.40$

therefore variance = <u>42.10</u>
and standard deviation = <u>6.5</u>

Significance of difference of the various means

Group A (pyrogen dose: 0.06ml/kg)

Fall in temperature

mean for ACTH	1.73
mean for ACTH + pyrogen	0.49

$$t = \frac{1.24}{0.89 \times 0.37} = \underline{3.766}; n=28; p=<0.001$$

Rise in temperature

mean for ACTH + pyrogen	1.66
mean for pyrogen	1.70

$$t = \frac{0.04}{0.55 \times 0.37} = \underline{0.1966}; p=0.8-0.9$$

Degree of shift to the left

mean for ACTH	22.0
mean for ACTH + pyrogen	35.0

$$t = \frac{13.0}{9.0 \times 0.37} = \underline{3.904}; p=<0.001$$

mean for ACTH + pyrogen	35.0
mean for pyrogen	38.4

$$t = \frac{3.4}{6.1 \times 0.37} = \underline{1.506}; p=0.1 - 0.2$$

Time to reach peak temperatures

mean for ACTH + pyrogen	179
mean for pyrogen	100

$$t = \frac{79}{31.3 \times 0.37} = \underline{6.822}; p=<0.001$$

continued.

Group B (pyrogen dose: 0.02ml/kg)

Fall in temperature

mean for ACTH 1.91
mean for ACTH + pyrogen 0.78

$$t = \frac{1.13}{0.81 \times 0.37} = 3.770; n=28; p=<0.001$$

Rise in temperature

mean for ACTH + pyrogen 0.88
mean for pyrogen 1.54

$$t = \frac{0.66}{0.47 \times 0.37} = 3.795; p=<0.001$$

Degree of shift to the left

mean for ACTH 21.9
mean for ACTH + pyrogen 34.7

$$t = \frac{12.8}{5.6 \times 0.37} = 6.178; p=<0.001$$

mean for ACTH + pyrogen 34.7
mean for pyrogen 32.4

$$t = \frac{2.3}{5.5 \times 0.37} = 1.130; p=0.2-0.3$$

Time to reach peak temperatures

mean for ACTH + pyrogen 193
mean for pyrogen 121

$$t = \frac{72}{58.9 \times 0.38} = 3.217; p=0.01 - 0.001$$

Groups A and B

Fall in temperature

mean for ACTH+pyrogen (0.06) 0.49
mean for ACTH+pyrogen (0.02) 0.78

$$t = \frac{0.29}{0.67 \times 0.37} = 1.170; p=0.2-0.3$$

Time to reach peak temperatures

mean for ACTH+pyrogen (0.06) 179
mean for ACTH+pyrogen (0.02) 193

$$t = \frac{14}{27.7 \times 0.38} = 1.330; p=0.1-0.2$$