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REVIEW ARTICLE

THE PITUITARY-ADRENAL RELATIONSHIP

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INTRODUCTION

THE success which has accompanied the recent clinical use of adreno-corticotrophic hormone and cortisone has stimulated an immense amount of research on the physiology of the pituitary-adrenal relationship. Physiologists have devoted much attention to a study of the mechanism by which the adrenocorticotrophic activity of the pituitary gland is controlled, and pharmacologists have concerned themselves with the development of convenient methods for the biological standardisation of adrenocorticotrophic hormone. It is with these two aspects of the study of the adrenocorticotrophic hormone that this review is concerned.

Although some understanding of the function of the adrenal glands began in the latter half of the nineteenth century, it was not until comparatively recently that the influence of the pituitary gland on the adrenal cortex was appreciated. The existence of a pituitary adrenocorticotrophic hormone became evident in 1930 when Smith[1] showed, in rats, that removal of the pituitary gland resulted in atrophy of the adrenal cortex. Smith[1] found that the adrenal cortical atrophy, which normally followed hypophysectomy, could be prevented by daily transplants of anterior pituitary tissue. Evans[2] obtained similar results with aqueous alkaline extracts of anterior pituitary glands, and Collip, Anderson and Thomson[3] demonstrated the same effects with purified protein preparations obtained from pituitary tissue.

THE NATURE OF ADRENOCORTICOTROPHIC HORMONE

In 1943, Li, Evans and Simpson[4] obtained a protein with considerable adrenocorticotrophic activity from the pituitary glands of sheep, and, simultaneously, Sayers, White and Long[5] reported the isolation of a similar preparation from pig pituitaries. Both groups of workers considered that they had prepared pure adrenocorticotrophic hormone. The two preparations exhibited similar chemical, physical and physiological properties, and it was estimated that their molecular weights were about 20,000.

Li *et al.*[4] found that their protein preparation was stable to heat in acid or neutral solution but not in an alkaline medium. They showed that its activity was destroyed by trypsin but not by pepsin, and they found that 36 per cent. of the protein could be digested without the loss of biological activity. Thus, it became evident that the molecule of the active principle was smaller than was originally believed. Li[6] reported that protein adrenocorticotrophic hormone could be broken down by heat and strong acid, and that a peptide, with a molecular weight of 1200 and possessing

considerable adrenocorticotrophic activity, could be isolated from the products of the reaction.

Cortis-Jones, Crooke, Henly, Morris and Morris[7] prepared adrenocorticotrophic hormone concentrates from the pituitary glands of cattle. They found that their preparations could pass through cellophane membranes impermeable to molecules whose molecular weights were greater than 13,000. Morris and Morris[8] reported the isolation of a polypeptide with 10 times the biological activity of the original protein preparations of Li et al.[4] and Sayers et al.[5]

It appeared, therefore, that the biological activity of the original protein preparations of adrenocorticotrophic hormone resided in a peptide fraction of the molecule. However, Lesh, Fisher, Bunding, Kocsis, Walnszek, White and Hays[9] obtained an adrenocorticotrophic hormone preparation of which the activity was 10 to 15 times as great as that of the Morris and Morris[8] preparation, and of which the molecular weight appeared to be between 2500 and 10,000. Astwood, Raben and Payne[10] showed that active material could be separated from the protein hormone by adsorption on oxy-cellulose. The fact that highly active preparations could be obtained from protein adrenocorticotrophic hormone by processes which did not involve hydrolysis has also been shown by Dixon, Moore, Stack-Dunne and Young.[11] Recent workers have generally based the assessment of the biological activity of their preparations on the ability of adrenocorticotrophic hormone to cause adrenal ascorbic acid depletion in hypophysectomised rats. Stack-Dunne and Young[12] reported the presence of two factors in adrenocorticotrophic hormone. One factor increased the weight of the adrenal glands of hypophysectomised rats and the other caused adrenal ascorbic acid depletion.

Stack-Dunne and Young[12] considered that the ascorbic acid factor was probably a basic peptide which was associated in the pituitary gland with a slightly acidic protein.

THE PHYSIOLOGICAL EFFECTS OF ADRENOCORTICOTROPHIC HORMONE

The physiological actions depend upon its ability to stimulate the adrenal cortices to increase their output of steroid hormones. It has very little effect on adrenalectomised animals and almost all its physiological actions are mediated by the adrenal cortex.

Adrenocorticotrophic hormone produces changes in the adrenal cortex which are associated with an increased secretion of adrenocortical hormones. The elevated blood level of adrenocortical steroids is responsible for the production of effects which are widespread throughout the body and which include actions on protein, fat, carbohydrate and salt metabolism, growth, lymphoid tissue and many others. In this review it is proposed to consider mainly the *direct* effects of adrenocorticotrophic hormone on the adrenal cortex.

The adrenocortical and metabolic changes which follow the subjection of animals to various types of stress stimuli have occupied the attention of physiologists for many years. Only comparatively recently has it become

possible to explain some of the observations and to correlate the experimental findings with a knowlege of the pituitary-adrenal relationship.

The subjection of a normal animal to various types of harmful stimuli (e.g. cold, heat, hæmorrhage, trauma, injections of toxins or drugs) results in adrenal hypertrophy,[13] and depletion of the sudanophilic lipides,[14] cholesterol[15] and ascorbic acid[16] in the adrenal cortex. These adrenocortical changes in response to stress are not produced in hypophysectomised animals.[17] However, the injection of adrenocorticotrophic hormone causes the changes in both normal and hypophysectomised animals.[4,14,16,18,19] Sayers and Sayers[16] claimed that the injection of adrenocorticotrophic hormone into hypophysectomised rats produced changes in the adrenal glands identical with those which occur in the adrenals of normal animals subjected to stress. The same authors showed, in normal animals, that the increase in adrenal weight and depletion in adrenal cholesterol and ascorbic acid concentration caused by stress was proportional to the severity of the stress. Sayers, Sayers and Woodbury[20] found, in hypophysectomised animals, that the fall in adrenal ascorbic acid concentration produced by adrenocorticotrophic hormone was proportional to the amount of hormone injected. From these and other similar investigations it was concluded that stress results in increased pituitary adrenocorticotrophic activity, and that the increased secretion of adrenocorticotrophic hormone is responsible for the changes in the morphology, histology and chemistry of the adrenal glands. The changes in adrenal cholesterol and ascorbic acid concentrations are probably concerned with the synthesis and release of adrenocortical hormones.

Sayers[17] attempted to draw up a classification of adrenocortical responses to stress into various types. In general, all kinds of stress cause adrenal hypertrophy except when the stress stimulus is maintained for only a short period. Any stress except a very mild form (e.g. pregnancy or fasting) causes depletion of sudanophilic lipides, cholesterol and ascorbic acid in the adrenal glands. The sudanophilic lipide, cholesterol and ascorbic acid levels return to normal in time, except in prolonged intense forms of stress ending in death.

The important part played by the pituitary gland in the response of the organism to stress was indicated by the work of Tyslowitz and Astwood[21] and many others who showed that hypophysectomised animals, like adrenalectomised animals, were extremely sensitive to various types of harmful stimuli. Tyslowitz and Astwood[21] demonstrated that adrenocorticotrophic hormone and adrenocortical extracts increased the resistance of hypophysectomised rats to cold.

It has been firmly established that the adrenocortical response to stress is governed by the adrenocorticotrophic activity of the pituitary gland. However, the adrenal cortex cannot be entirely dependent upon the pituitary gland for its functional activity. Adrenalectomised animals die whereas hypophysectomised animals can survive. Although hypophysectomy results in adrenal atrophy, the cortices are apparently still capable of secreting a quantity of steroids sufficient to maintain life.

A considerable amount of evidence is beginning to accumulate that adrenocorticotrophic hormone causes the adrenal cortex to secrete mainly 17-hydroxycorticosterone (compound F) with smaller quantities of corticosterone and 11-dehydro-17-hydroxycorticosterone (cortisone or compound E). Mason[22] isolated 17-hydroxycorticosterone from the urine of human subjects who had received injections of adrenocortico-trophic hormone or who had been subjected to the stress of surgery. Sprague, Hayles, Power, Mason and Bennett[23] prepared 17-hydroxycortico-sterone from the urine of a patient with Cushing's syndrome. Cope, Boyson and McCrae[24] found, in normal human subjects, that increased adrenocorticotrophic activity caused increased excretion of cortisone and 17-hydroxycorticosterone. Stress caused no increased excretion of these steroids in a patient with panhypopituitarism. Zaffaroni, Burton and Keutmann[25] detected cortisone and 17-hydroxycorticosterone in small quantities in normal human urine and Schneider[26] isolated cortisone from the same source.

Methods are gradually being developed for the detection of adreno-cortical steroids in adrenal effluent blood—a technique which was first developed by Vogt.[27] Nelson, Reich and Samuels[28] found that the principal steroid in the blood leaving the adrenal glands of dogs, which had received injections of adrenocorticotrophic hormone, was 17-hydroxy-corticosterone. Bush[29,30] analysed adrenal effluents in various animal species by a paper partition chromatographic method. He found that stress or adrenocorticotrophic hormone resulted in a tremendous increase in the secretion of corticosterone and 17-hydroxycorticosterone. The ratio of these steriods was characteristic of the species studied and not changed by adrenocorticotrophic hormone.

THE ASSESSMENT OF PITUITARY ADRENOCORTICOTROPHIC ACTIVITY

Until comparatively recently, attempts to measure the concentration of adrenocorticotrophic hormone in blood have met with little success. Sayers, Burns, Tyler, Jager, Schwartz, Smith, Samuels and Davenport[31] showed that, following injection into human subjects, it disappeared very rapidly and very little was excreted. Greenspan, Li and Evans[32] demonstrated that, when injected intravenously into rats, it disappeared completely from the blood stream in 20 minutes. Taylor, Albert and Sprague[33] were unable to detect adrenocorticotrophic hormone in the blood of normal human subjects. Reiss, Badrick, Halkerston and Plaice[34] reported its extreme lability in blood *in vitro*, and Pincus[35] considered that it was destroyed rapidly by an enzyme present in blood. However, it should be mentioned that, recently, Geschwind and Li[36] found that it was *not* inactivated by rat plasma although it was rapidly destroyed by incubation with tissue slices and homogenates. On the other hand it was found to be unstable in rats' whole blood in this laboratory. Its instability in blood probably explains the failure of several workers to demonstrate its presence in normal serum.

Methods are gradually being developed for the direct detection of increased blood levels of adrenocorticotrophic hormone in various

experimental and clinical conditions. Direct estimations of the concentration in blood were first made by Golla and Reiss,[37] who found that extracts of pregnant mares' serum caused increases in the weight of the adrenal glands of hypophysectomised rats. Cooke, Graetzer and Reiss[38] demonstrated adrenocorticotrophic hormone activity in acetone precipitates of human plasma and Taylor et al.[33] detected increased levels in unfractionated serum from patients with Addison's disease. Bornstein and Trewhella[39] and Bornstein, Gray and Parrott[40] measured adrenocorticotrophic hormone activity in human plasma. Parrott[41] found elevated concentrations in human blood in Cushing's syndrome and pregnancy, and considerably decreased levels in the blood of patients suffering from Simmonds' disease.

At the present time, direct methods for the determination of the concentration in blood are not often used. More frequently indirect methods are employed as indices of the rate of secretion. The most commonly used methods depend upon (1) the effects which it produces on the weight and histology of the adrenal cortex and on the cholesterol and ascorbic acid content of the gland, (2) the fall in blood eosinophil and lymphocyte counts caused by increased secretion of adrenocortical hormones and (3) the increased urinary excretion of "corticoids" (adrenocortical steroids estimated biologically or adrenocortical-like steroids estimated chemically) caused by adrenocorticotrophic hormone.

The influence of adrenocorticotrophic hormone on the weight, histology and chemical constituents of the adrenal glands has already been considered. Fluctuations in adrenal ascorbic acid concentration are used most widely as indices of adrenocorticotrophic activity. However, there is a considerable amount of conflicting evidence concerning the part played by ascorbic acid in the synthesis of the hormones of the adrenal cortex. Sayers[17] maintains that changes in adrenal ascorbic acid concentration are reliable indices of adrenocortical, and hence of adrenocorticotrophic, activity in acute experiments in healthy rats.

Dougherty and White[42] showed that the circulating lymphocytes were under the regulatory control of the adrenal cortex and that the lymphocytopenic response to a variety of stress stimuli was due to increased putuitary adrenocorticotrophic activity resulting in an increased secretion of adrenocortical hormones. They demonstrated that neither stress nor adrenocorticotrophic hormone caused lymphocytopenia in adrenalectomised animals.

The number of circulating eosinophils is also dependent upon the activity of the pituitary-adrenocortical system. This fact has been made the basis of a convenient method for assessing adrenocortical and pituitary adrenocorticotrophic activity. Forsham, Thorn, Prunty and Hills[43] showed that the administration of adrenocorticotrophic hormone to human subjects caused a prompt fall in the number of circulating eosinophils. The fall in the eosinophil count was twice as great as the simultaneous fall in the lymphocyte count. They considered that the eosinopenic response to adrenocorticotrophic hormone was less variable than the lymphocytopenic response. Stress has been found by many

workers to cause eosinopenia, and the literature concerning this effect of stress was reviewed by Hills, Forsham and Finch.[44]

The assessment of adrenocorticotrophic and adrenocortical activity by means of eosinophil and lymphocyte tests has been of great clinical advantage. In man the administration of adrenocorticotrophic hormone or the application of stress (e.g. surgery) is normally followed by a decrease in the lymphocyte and eosinophil counts. The absence of lymphocytopenic and eosinopenic responses is generally indicative of a failure of some part of the pituitary-adrenocortical system. In·addition to their clinical applications, tests for pituitary adrenocorticotrophic activity based on eosinopenic and lymphocytopenic responses are frequently used in experimental work on laboratory animals. McDermott, Fry, Brobeck and Long[45] found that the results they obtained in tests using eosinopenic responses as indices of pituitary adrenocorticotrophic activity were in agreement with those obtained using changes in adrenal ascorbic acid concentration. These workers used eosinophil tests in their investigations on the part played by adrenaline in the production of pituitary adrenocorticotrophic activity in rats. Colfer, de Groot and Harris,[46] investigating the mechanism by which the secretion of adrenocorticotrophic hormone is controlled, used lymphocyte tests in their work on rabbits.

The presence of adrenocortical hormones ("corticosteroids") in human urine can be demonstrated by biological or chemical methods. The administration of adrenocorticotrophic hormone or the application of stress stimuli causes an increased secretion of adrenocortical hormones with the result that larger quantities appear in the urine. Thus the urinary output of corticoids is a measure of adrenocortical activity and hence of pituitary adrenocorticotrophic activity. Sayers[17] reviewed the various methods for the biological and chemical determination of urinary corticosteroids and illustrated the general correlation between adrenocorticotrophic activity and urinary corticosteroid excretion. He considered that the amount of corticosteroids, estimated biologically, in the urine gave a good index of adrenocorticotrophic activity. Chemical corticosteroid estimations provided a less satisfactory measure. Recently Cope et al.[24] found that there existed no parallelism between the urinary output of corticoids, estimated biologically, and corticoids, estimated chemically, in human subjects receiving adrenocorticotrophic hormone or ephedrine.

Increased pituitary adrenocorticotrophic activity also results in an increased urinary excretion of 17-ketosteroids. Sayers[17] listed his reasons for considering that 17-ketosteroid excretion was a very unreliable index of adrenocortical and adrenocorticotrophic activity. He stated that there existed no correlation between 17-ketosteroid excretion and adrenocorticotrophic activity as illustrated by other measures.

THE CONTROL OF PITUITARY ADRENOCORTICOTROPHIC ACTIVITY

It has been firmly established that the secretion of the adrenal cortex in stress is almost entirely governed by the adrenocorticotrophic hormone of the anterior pituitary gland. However, the exact mechanism by which the secretion of adrenocorticotrophic hormone is produced is not yet fully

understood. At the present time it is believed that the adrenocorticotrophic activity of the pituitary gland may be controlled in at least three ways (a) by the level of cortical hormones in the blood, (b) by the level of adrenaline and noradrenaline in the blood, and (c) by some neural or neuro-humoral mechanism.

(a) *Control of Pituitary Adrenocorticotrophic Activity by the Level of Cortical Hormones in the Blood.* The results of experiments performed in many laboratories indicate that the adrenocorticotrophic activity of the pituitary gland is controlled by the blood level of adrenocortical hormones. Ingle, Higgins and Kendall[47] and Ingle and Kendall[48] found that atrophy of the adrenal cortex was one of the results of the chronic administration of adrenocortical extracts to rats. Selye[49] showed that desoxycortico-sterone acetate caused adrenal cortical atrophy in the rat and the mouse. Ingle[50] demonstrated that the adrenal hypertrophy, which occurred in rats after a 12-hour period of muscular exercise, could be prevented by pre-treating the animals with adrenocortical extracts. Selye and Dosne[51] found that the previous administration of desoxycortone acetate to rats partially prevented the increase in adrenal weight caused by stress. Winter, Silber and Stoerk[52] found, in rats, that the administration of large doses of cortisone caused adrenal atrophy. The histological changes in the atrophied adrenals involved the zona fasciculata and zona reticularis but not the zona glomerulosa. Similar findings have been reported by Stebbins.[53] Since Deane and Greep[54] showed that hypophysectomy resulted in adrenal atrophy, which was confined to the zona fasciculata and zona reticularis, it was probable that this effect of cortisone was due to the inhibition of pituitary adrenocorticotrophic activity. Sayers and Sayers,[55,56] Long[57] and Gershberg, Fry, Brobeck and Long[58] demonstrated, in rats, that the fall in adrenal ascorbic acid concentration, which followed the application of various types of stress stimuli, could be prevented by pre-treatment of the animals with adrenocortical extracts.

Sayers and Sayers[56] put forward the theory that the adrenocorticotrophic activity of the pituitary gland was controlled by the concentration of adrenocortical hormones in the blood. They considered that the anterior pituitary gland elaborated adrenocorticotrophic hormone at a rate inversely proportional to the blood level of adrenocortical hormones. They suggested that stress caused increased adrenocortical activity by increasing the requirement of the organism for cortical hormones. This increased demand for cortical hormones was satisfied by pre-treatment with adreno-cortical extracts and in this way the necessity for the adrenal cortices to increase their activity was obviated. Sayers and Sayers[56] found that the mobilisation of endogenous adrenocorticotrophic hormone in response to stress could be completely or partially blocked depending upon the dose of cortical hormones administered. If the intensity of the stress applied was increased, the amount of cortical hormones required to inhibit the adrenocorticotrophic activity of the pituitary gland was increased. Similar findings have been made in this laboratory. Sayers and Sayers[56] attempted to explain the quantitative relationship by assuming that the rate of secretion of adrenocorticotrophic hormone varied according to the

requirement of the organism for cortical hormones. They suggested that the pituitary-adrenocortical system attempted to maintain the tissues in a state of well-being in regard to adrenocortical hormones, to which condition they gave the name "eucortism." They considered that, under optimal environmental conditions, the peripheral tissue cells required small amounts of cortical hormones, and the level in the blood was sufficient to depress the adrenocorticotrophic activity of the pituitary gland. In conditions of stress the demand of the peripheral cells for cortical hormones was increased, with the result that the concentration of cortical hormones in the blood was reduced temporarily. The pituitary gland responded by increasing its output of adrenocorticotrophic hormone which resulted in increased activity of the adrenal cortex. In this way the increased demand of the peripheral cells for cortical hormones might be satisfied. The pituitary-adrenocortical system remained active until the stress was removed or until adaptation occurred when the requirement of the peripheral cells for cortical hormones was diminished.

There exists, therefore, a considerable amount of evidence that the adrenocorticotrophic activity of the pituitary gland is controlled by a "peripheral-humoral" mechanism. It should be emphasised, however, that several workers have been unable to demonstrate the pituitary inhibitory effect of adrenocortical steroids. Moya and Selye,[59] Hall, Finerty, Hall and Hess[60] and Gershberg et al.[58] have all been unable to show that desoxycortone acetate could prevent the release of adrenocorticotrophic hormone in response to stress. Fortier, Yrarrazaval and Selye[61] were unable to demonstrate the pituitary inhibitory effect of cortisone. In this laboratory it was confirmed that both desoxycortone acetate and cortisone acetate were effective in preventing the stress-induced secretion of adrenocorticotrophic hormone.

Although it was a relatively simple matter to demonstrate, in laboratory animals, that an increased concentration of cortical hormones in the blood prevented the increased pituitary adrenocorticotrophic activity which normally followed stress, very little experimental evidence has been produced to support the theory that low blood levels of adrenocortical hormones cause increased pituitary adrenocorticotrophic activity. However, Taylor et al.[33] demonstrated the presence of adrenocorticotrophic hormone in the blood of patients with Addison's disease, but were unable to detect the hormone in the blood of normal subjects. Gemzell, Van Dyke, Tobias and Evans[62] showed that removal of the adrenal glands from rats caused a considerable increase in the concentration of adrenocorticotrophic hormone in the blood. These findings added some support to the "peripheral-humoral" theory for the control of pituitary adrenocorticotrophic activity.

There is little doubt that the adrenocorticotrophic activity of the pituitary gland is controlled by a peripheral-humoral mechanism. However, there is still some confusion in the literature concerning the ability of desoxycortone acetate and cortisone acetate to depress the increased secretion of adrenocorticotrophic hormone normally caused by stress.

(b) *Control of Pituitary Adrenocorticotrophic Activity by the Level of Adrenaline and Noradrenaline in the Blood.* There exists a considerable amount of evidence that the sympathetic nervous system plays some part in the activation of the pituitary-adrenocortical system. Vogt[63] found that adrenaline stimulates the secretion of the adrenal cortex. She demonstrated the presence of increased amounts of adrenocortical hormones in the blood leaving the adrenal glands of cats and dogs after intravenous infusions of adrenaline. She also found that adrenaline produced these effects in a decapitated dog and she suggested that adrenaline might act directly on the adrenal cortex. Further evidence for a direct action of adrenaline was provided by Hungerford,[64] who showed that adrenaline caused lymphocytopenia in hypophysectomised rats, and by Spiers and Meyer,[65] who found that it produced eosinopenia in hypophysectomised mice.

On the other hand Vogt[66] found that the histological changes and weight increases in the adrenal glands, caused by the chronic administration of adrenaline to rats, were prevented by removal of the pituitary gland. Long and Fry[67] showed that depletion in adrenal cholesterol and ascorbic acid concentration followed the injection of adrenaline into normal rats. These changes did not occur after hypophysectomy.

It is probable, therefore, that the effects of adrenaline on the adrenal cortex are mediated by the pituitary gland. However, recently Pickford and Vogt,[68] working on hypophysectomised dogs, have produced more evidence that adrenaline can exert a direct effect on the adrenal cortex.

Long[57] considered that the release of adrenaline by the sympatho-adrenal system, in conditions of stress, was the most important factor in the production of pituitary adrenocorticotrophic activity. He suggested that all forms of stress caused an increased concentration of adrenaline in the blood which stimulated the pituitary gland to secrete adreno-corticotrophic hormone. Sayers and Sayers[16] considered that the action of adrenaline was an indirect effect on the pituitary gland and was due to its ability to increase the rate of utilisation of cortical hormones, thus producing a decreased concentration of the hormones in the blood.

Gershberg et al.[58] showed that small doses of adrenaline, which they considered to be within the physiological range, caused significant falls in the concentration of ascorbic acid in the adrenal glands of rats. They claimed that similar effects caused by cold, trauma and haemorrhage were due to the release of endogenous adrenaline. These workers confirmed that the effects could be prevented by cortical hormones and were abolished by hypophysectomy. Gellhorn and Frank[69] found that stress caused lymphocytopenia in normal, but not in adrenal-demedullated, rats. McDermott et al.[45] compared adrenocortical activity in normal rats with that of those unable to secrete adrenaline as a result of demedullation of the adrenal glands, transection of the spinal cord or electrolytic diencephalic lesions. They found that the eosinopenic responses in these animals when subjected to stress were smaller than in normal animals. Adrenal-demedullated rats and animals, in which the neural pathway responsible for the secretion of adrenaline had been interrupted, no longer responded to mild stress stimuli but showed a response to more severe stress. These

workers showed that, in normal animals, a marked response was obtained within 1 hour of the application of the stress, and that a maximal effect was obtained within 4 hours. In animals in which adrenaline secretion had been prevented, they found that stress caused no response within 1 hour, but some effect within 4 hours of its application. For this reason they suggested that the secretion of adrenocorticotrophic hormone was controlled by a double mechanism: (1) a rapid autonomic mechanism depending upon the secretion of adrenaline, and (2) a slower metabolic mechanism independent of the secretion of adrenaline.

In another publication[70] the same authors reported that both adrenaline and hypertonic saline solution, when injected into hypophysectomised rats with pituitary transplants in the anterior chamber of the eye, caused marked falls in the eosinophil count. An eosinopenic response was produced when very small doses of adrenaline were instilled into the eyes with the pituitary transpants, but not when injected into the other eyes. They concluded that adrenaline caused increased adrenocorticotrophic activity by direct stimulation of the pituitary gland.

The work which has been described so far indicates that endogenously secreted adrenaline has some function in stimulating an increased secretion of adrenocorticotrophic hormone when an animal is subjected to stress stimuli. However, there exists a considerable amount of evidence that adrenaline does not play such an important part in the production of increased pituitary adrenocorticotrophic activity in response to stress as Long and his colleagues believe. Sayers[17] pointed out that the completely sympathectomised animal may resist stress as well as a normal animal. Colfer et al.[46] found that the lymphocytopenic response which followed immobilisation or mild electric shock stimuli, in rabbits, was the same whether the adrenal glands were denervated or not. Tepperman and Bogardus[71] demonstrated that neither dibenamine nor tetraethyl-ammonium bromide prevented the fall in adrenal ascorbic acid in rats subjected to stress. Recant, Hume, Forsham and Thorn[72] found that formaldehyde injections caused eosinopenia in completely sympathectomised dogs. Vogt[73] showed that the depletion of lipides in the adrenals of rats or cats subjected to various types of stress was not prevented by denervation of the glands. Gordon,[74,75] Nasmyth,[76] Fortier[77] and Vogt[78] have all shown that the adrenocorticotrophic response to stress is not prevented by adrenal demedullation. Experiments carried out in this laboratory have produced similar results.

It seems probable that the secretion of the adrenal medulla is not of fundamental importance in the production of pituitary adrenocortico-trophic activity, and that other mechanisms are capable of stimulating the adrenocorticotrophic response in animals in which the secretion of adrenaline is prevented.

(c) *Control of Pituitary Adrenocorticotrophic Activity by a Neural or Neuro-Humoral Mechanism.* The adrenocorticotrophic hormone is liberated rapidly from the pituitary glands of animals subjected to stress. It was shown, in this laboratory, that changes in the ascorbic acid concentration in the adrenal glands of rats could be detected within a few

minutes of the application of stress. The rapidity of the pituitary adreno-corticotrophic response suggested to many workers that a neural or neuro-humoral mechanism might be involved in the process controlling the secretion of adrenocorticotrophic hormone.

Colfer et al.[46] showed that emotional stress stimuli caused lymphocytopenia in rabbits. The response was abolished by hypophysectomy but still occurred in hypophysectomised rabbits which received injections of adrenocorticotrophic hormone. De Groot and Harris[79] showed that lymphocytopenia could be produced in rabbits by electrical stimulation of parts of the tuber cinereum or mamillary body. Stimulation of other regions of the hypothalamus or pituitary gland had no effect on the lymphocyte count. They found that, in rabbits with electrolytic lesions in the hypothalamus and pituitary gland, the lymphocytopenic response to stress was abolished in animals in which the zona tuberalis, the posterior part of the tuber cinereum or the mamillary body were destroyed or damaged. They concluded that, in rabbits, the secretion of adrenocortico-trophic hormone in response to some stress stimuli is regulated by the hypothalamus. A similar conclusion was reached by Hume[80] who showed, in dogs, that the eosinopenic response of the pituitary-adrenal system to stress was abolished by certain lesions of the hypothalamus.

On the other hand Uotila,[81] Recant et al.[72] Tang and Patton,[82] Cheng, Sayers, Goodman and Swinyard,[83] and Fortier and Selye[34] have performed experiments, on animals with transected pituitary stalks and hypophysec-tomised animals with pituitary transplants, the results of which indicated that neural or vascular connections between the pituitary gland and the hypothalamus were not essential for the immediate secretion of adreno-corticotrophic hormone in acute stress.

Fortier[85] recently demonstrated that the adrenocorticotrophic response to emotional stress (e.g. sound, immobilisation) was dependent upon the existence of neural and vascular connections between the pituitary gland and the hypothalamus. However, he found that the response to systemic stress stimuli (e.g. trauma, drugs) was independent of the existence of an intact pituitary stalk.

No definite conclusion can yet be reached with regard to the exact mechanism which controls the output of adrenocorticotrophic hormone by the pituitary gland. It is probable that the mechanism controlling a steady secretion of adrenocorticotrophic hormone in optimal environ-mental conditions may differ from the mechanism governing increased adrenocorticotrophic activity in conditions of stress, Again, the manner in which increased blood levels of adrenocorticotrophic hormone are reached is probably influenced by the nature of the stress.

THE BIOLOGICAL STANDADRISATION OF ADRENOCORTICOTROPHIC HORMONE

The discovery of the existence of this hormone naturally led to attempts to devise methods for the determination of adrenocorticotrophic activity in anterior pituitary extracts. Almost all the methods developed so far for the biological standardisation of this hormone have been inaccurate and

difficult to perform and no really satisfactory technique has yet been described. Most of the bioassay methods employed have depended upon the changes which it produces in the adrenal glands of normal and hypophysectomised animals.

The earliest methods of Moon[86,87] and Bates, Riddle and Miller[88] depended upon the increases in adrenal weight which a series of injections caused in rats and chicks. The results of such methods, obtained using normal instead of hypophysectomised animals, were practically valueless since the subjection of intact animals to any environmental change causes increased pituitary adrenocorticotrophic activity with subsequent adreno-cortical changes. In hypophysectomised animals the mobilisation of endogenous adrenocorticotrophic hormone is prevented and the effects produced are due entirely to the activity of the samples under test. Bioassay methods using hypophysectomised rats are more sensitive and more accurate than methods employing normal animals. Most of the methods using hypophysectomised animals are performed on rats.

Methods based upon the changes produced in the weight and histology of the adrenal glands of hypophysectomised animals fall into two main groups: (1) adrenal "repair" tests,[3,5,19,89] (2) adrenal "maintenance" tests.[5,19,89,90] In the repair tests, rats were hypophysectomised and their adrenal glands were allowed to regress. Several days later the animals were given frequent injections of adrenocorticotrophic hormone. The dose necessary to restore the weight and histology of the adrenals to normal was determined. In the maintenance tests hypophysectomised rats were given frequent injections commencing immediately after hypophysectomy. The dose necessary to maintain the weight of the adrenal glands at the normal level was determined. Repair methods generally utilised histological changes in the adrenal cortex and maintenance tests were usually based upon changes in adrenal weight.

In 1948, interest in adrenal repair and maintenance methods waned as a result of the development of the adrenal ascorbic acid depletion technique by Sayers et al.[20] These workers showed, in hypophysectomised rats, that the adrenal ascorbic acid depletion produced by the intravenous injection of adrenocorticotrophic hormone was proportional to the dose administered. They developed this finding into an extremely sensitive and accurate method of bioassay. Hypophysectomised rats were used 24 hours after the removal of their pituitary glands. The left adrenal glands of the animals were excised and injections of the hormone were administered. Later the animals were killed and their right adrenal glands were removed. The ascorbic acid concentrations in the adrenals were determined. The response was expressed as the difference between the concentration of ascorbic acid in the left adrenal, removed immediately before the injection, and the concentration of the acid in the right adrenal, removed 1 hour after the intravenous administration of the hormone. Sayers et al.[20] found that a rectilinear relationship existed between the adrenal ascorbic acid depletion and the logarithm of the dose. The authors claimed that the adrenal ascorbic acid depletion method possessed several advantages over methods employing changes in the weight and histology of the adrenal

glands. It was rapid, more accurate and possessed a greater degree of sensitivity. They suggested that the method was specific for adreno-corticotrophic hormone since they found that extracts of animal tissues, except pituitary glands, caused no adrenal ascorbic acid depletion.

Munson, Barry and Koch[91] modified the Sayers technique. They carried out no unilateral adrenalectomy before the injection but compared the mean total adrenal ascorbic acid concentration in an injected group with the mean of a control group. Sayers *et al.*[20] reported that, using this modification, it was necessary to use twice the number of animals to obtain the same accuracy as their method provided.

Morris,[92] using certain modifications of the adrenal ascorbic acid depletion technique for the biological standardisation, obtained results as accurate as those of Sayers *et al.*[20] Similar results have been obtained by Clayton and Prunty.[93] On the other hand Reiss *et al.*[89] found that, in their laboratory, the method did not always give satisfactory results. Green-span, Li, Simpson and Evans[94] also found that the adrenal ascorbic acid depletion method provided them with results the accuracy of which was not as great as that achieved by Sayers *et al.*,[20] although they were able to confirm the existence of a linear relationship between the logarithm of the dose of adrenocorticotrophic hormone injected and the adrenal ascorbic acid depletion.

Their disappointment with the accuracy they obtained using the adrenal ascorbic acid depletion technique stimulated Reiss *et al.*[89] to investigate other methods of biological standardisation. They found that the adrenal glands showed an increased uptake of phosphorus-32 in conditions of increased activity. They demonstrated, in hypophysec-tomised rats, that if adrenocorticotrophic hormone and phosphorous-32 were injected at the same time there existed a dose-response relationship between the amount of hormone injected and the percentage of phosphorus-32 taken up by the adrenals.

Rheinhardt and Li[95] found that the values obtained in the assessment of adrenocorticotrophic hormone activity depended upon the method of bioassay employed. They showed that the results produced using adrenal maintenance tests differed widely from those obtained employing the adrenal ascorbic acid depletion technique. Their findings indicated that more than one factor existed in some preparations of the hormone. Stack-Dunne and Young[12] have actually separated two factors. One is the "adrenal weight factor" which, they suggested, is concerned with the synthesis and deposition of adrenal cholesterol, and the other is the "ascorbic acid factor" which is probably associated with the release of cortical hormones. The existence of at least two factors in adrenocortico-trophic hormone makes it advisable to utilise at least two bioassay methods in assessing the adrenocorticotrophic activity of commercial preparations of the hormone. An adrenal repair or maintenance method should be used in addition to the ascorbic acid depletion method. However, it should be mentioned that the clinical effectiveness of the hormone is apparently most closely related to its potency in terms of ascorbic acid factor.

The methods of biological standardisation, which have been considered so far, all depend upon the direct effects which the hormone produces on the adrenal cortex. Other methods have been developed which are based upon the effects of the increased secretion of adrenocortical hormones which adrenocorticotrophic hormone causes. Clayton and Prunty[93] described a method which depends upon the antagonistic effect of adreno-corticotrophic hormone to wound healing in mice. Recently, Bruce, Parkes and Perry[96] have developed a bioassay method which depends upon the fact that the hormone causes involution of the thymus gland. Nestling rats are used in this method, which is considered to be specific, since they are incapable of the mobilisation of endogenous adrenocorticotrophic hormone in response to stress.

Hypophysectomy renders animals very sensitive to stress. Tyslowitz and Astwood[21,97] found that adrenocorticotrophic hormone increased the resistance of hypophysectomised rats to cold. Li, Simpson and Evans[98] showed that the hormone increased the resistance of hypophysectomised and normal rats to cold, starvation and anoxia. Reiss *et al.*[89] found that it increased the resistance of normal mice to insulin. It is probable that these and many similar actions could be developed into quantitative methods for the bioassay. However, the accuracy and specificity of such methods are not likely to be great unless hypophysectomised animals are used. In this event, no advantage is gained, and it is probably more reliable to measure the direct effects on the adrenal cortex.

The technique of hypophysectomy in the rat is not easy, and complete success is difficult to attain except after considerable practice on the part of the operator. For this reason, attempts are being made in this laboratory to modify the Sayers' technique by substituting, for hypophysectomised animals, rats in which pituitary adrenocorticotrophic activity has been "blocked" with cortical hormones. The investigations are still in progress but, so far, a method has been devised which is at least as sensitive as the Sayers' method although its accuracy has yet to be ascertained. Such a method has the advantage that it is less time consuming and readily performed by comparatively unskilled workers.

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RESEARCH PAPERS

THE TOXICITY OF THE DINITRO-CRESOLS

PART II. THE FORMATION AND TOXIC PROPERTIES OF SOME NITRO-COMPOUNDS DERIVED FROM *meta-* AND *para-*CRESOLS

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INTRODUCTION

IN Part I of this series (Harvey[1]) it was shown that some commercial samples of 4:6-dinitro-*o*-cresol* (DNOC) were relatively pure and that their LD50 values for rats were similar to that of the pure substance. This observation appeared to exclude the possibility that significant quantities of other nitro-cresols might be formed in side reactions during the manufacture of dinitro-*o*-cresol.

Molnar's[2] report of two unidentified dinitro-cresols has stimulated further research on the possible identity of these compounds. This has led to a fuller investigation of the original reaction of Nolting and Salis[3] for the preparation of dinitro-*o*-cresol from *o*-cresol. In addition, this reaction has been applied to *m*- and *p*-cresols, both of which might occur as "natural" contaminants of *o*-cresol.

The toxicities of the main products of the various reactions have also been studied as single substances and as mixtures.

The nitration of o-, m- and p-cresols

Heilbronn and Bunbury[4] and Beilstein[5] list respectively 10 and 8 known dinitro-cresols out of a theoretical 18 isomers. Of these only 2 are prepared by direct methods of nitration. They are the well known 4:6-dinitro-*o*-cresol and 2:6-dinitro-*p*-cresol (DNPC), also known as Victoria Yellow or Victoria Orange, and originally incorporated in a mixture containing the ammonium salts of the two compounds. This was used as a colouring agent for foodstuffs until its poisonous nature was discovered.

The first stage in the preparation of dinitro-*o*-cresol is the sulphonation of the *o*-cresol. The structure of the resulting product depends on several factors. These include the temperature at which the reaction is conducted and the quantity of sulphuric acid used. 4:6-Disulphonic-*o*-cresol is the main product if excess of sulphuric acid is employed and if the reaction is carried out in a temperature range of 80° to 100° C. Limited quantities of sulphuric acid and variations of temperature result in at least three *o*-cresol monosulphonates (Datta and Mitter[6], Claus and Jackson[7],

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* The descriptions of all chemical substances described in this paper have been made to conform with the terminology that describes DNOC as 4:6-dinitro-*o*-cresol and *not* 3:5-dinitro-*o*-cresol.

Engelhardt and Latschinow[8]). Subsequent treatment of the disulphonate by nitric acid (Nolting and Salis, *loc. cit.*) or by nitrous fumes (Datta and Varma[9]) leads to the formation of dinitro-*o*-cresol in good yield and purity. According to Datta and Varma, *loc. cit.*, nitration of the 5- or 6-monosulphonates by nitrous fumes leads to the formation of 5:6-dinitro-*o*-cresol. Claus and Jackson, *loc. cit.*, and Nevile and Winther[10] claim that concentrated nitric acid reacts with the 4- and 6-mono-sulphonates to yield dinitro-*o*-cresol.

Sulphonation and nitration of *m*-cresol yields 2:4:6-trinitro-*m*-cresol[11] (TNMC). This compound is also known as cresylite, an explosive with similar properties to picric acid.

2:6-Dinitro-*p*-cresol is readily formed by several simple methods of nitration. These include treatment of *p*-cresol by nitric acid in acetic acid (Frische[12]) by nitric and sulphuric acids (Borsche and Fiedler[13]) and the action of nitric acid on 2:6-disulphonic-*p*-cresol (Nolting and Kohn[14]). Datta and Varma, *loc. cit.*, have prepared dinitro-*p*-cresol by treating the sulphonate with nitrous fumes.

Other methods can be used for the preparation of dinitro-*o*-cresol, trinitro-*m*-cresol and dinitro-*p*-cresol, but they are of academic rather than practical interest. They include the treatment of the cresotinic acids (Datta and Varma, *loc. cit.*) and of the toluidines (Datta and Varma[15]) by nitrous gases. The main reactions and products involved in the "full" nitration of the cresols are summarised in Figure 1. The reactions and products specially studied in this communication are printed in thick type.

<center>EXPERIMENTAL</center>

As the main object of the experiments was to apply the Nolting and Salis reaction to the three cresols and to isolate and identify the main products no attempt was made to examine each reaction exhaustively, or to isolate any very small quantities of possible by-products. The method employed for nitrating the cresols was essentially as follows.

A mixture of the cresol (1 mol.) and concentrated sulphuric acid (A.R. approx. 2·2 mols.) was heated on a boiling water bath for 2 hours. The resulting mixture was poured into water to give a 5- to 10-fold dilution and treated with nitric acid (A.R. approx. 2·2 mols., sp.gr. 1·42) in small additions with good shaking or stirring. As all the reactions studied were variably exothermic the temperature was raised to 100° to 105° C. in order to obtain uniformity of heat conditions in all preparations. After cooling, the separated solid was filtered off, well washed with cold water, suspended in hot water, again cooled, filtered, and finally recrystallised from ethanol (96 per cent.). Melting points were determined by capillary tube method and were uncorrected. Micro-analyses for carbon, hydrogen and nitrogen were carried out by Drs. Weiler and Strauss of Oxford.

Fractional crystallisation of suspected mixtures proved to be laborious and unsatisfactory, therefore chromatographic separation on active alumina was employed. Ultra-violet absorption spectra data and curves

of the chief compounds were obtained by Dr. E. R. Holiday of the Spectrographic Unit, Medical Research Council. Visible absorption spectra curves were made on a Unicam spectrophotometer.

LD50 values on the products were obtained for mice in a manner similar to that described by Harvey, *loc. cit.*, for testing commercial dinitro-*o*-cresol samples. Blood decay rates were determined by administering single 20 mg./kg. doses of solutions of the substances
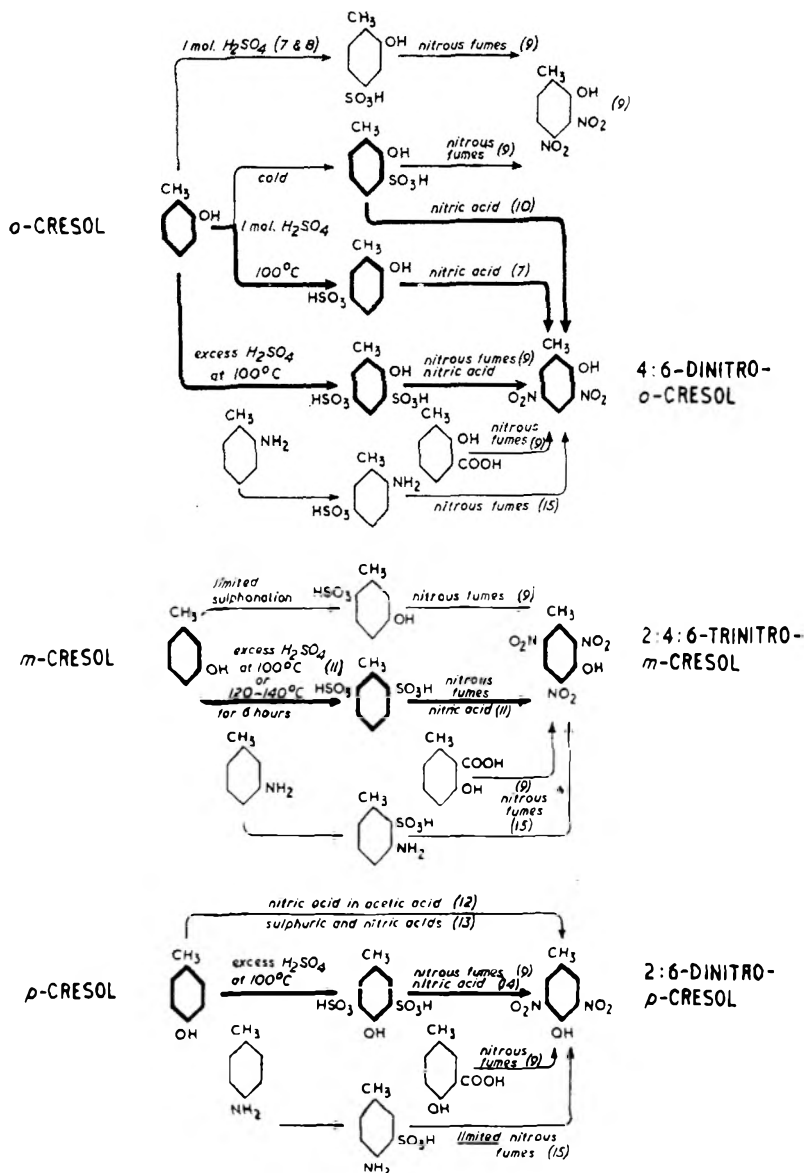
FIG. 1. Nitro compounds derived from *o*-, *m*- and *p*-cresols.

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TABLE I

NITRATION PRODUCTS OF THE CRESOLS PREPARED ACCORDING TO THE NOLTING AND SALIS REACTION

Serial No.	Cresols or mixture	Yield		Melting point °C.		Analysis for once recrystallised material						Empirical formula	Identity
						Calculated:			Found:				
		Cresol/product g.	per cent.	Crude	Once recrystallised	C per cent.	H per cent.	N per cent.	C per cent.	H per cent.	N per cent.		
1	o-Cresol	27/36·5	73	87·0	86·0	42·4	3·03	14·1	42·9	3·2	13·7	$C_7H_6N_2O_5$	4:6-Dinitro-o-cresol (d)
2	m-Cresol	27/26·5	44	104·5	106·0	34·5	2·05	17·2	35·2	2·3	16·7	$C_7H_5N_3O_7$	2:4:6-Trinitro-m-cresol, Cresylite (d)
3	p-Cresol full nitration (a)	27/5·0	10	78·0	80·0	42·4	3·03	14·1	42·9	3·3	13·8	$C_7H_6N_2O_5$	Both products are 2:6-dinitro-p-cresol, Victoria Yellow (d)
4	limited nitration	27/16·5	33	79·5 (d)	81·0	42·4	3·03	14·1	43·0	3·04	13·4		
5	8:1:1 o-, m-, p-cresols "80 per cent." mixture (b)	100/99	(b)	78·5 (d)	79·5	—	—	—	—	—	—	—	Approximately: 85 per cent. of dinitro-o-cresol; 10 per cent. of trinitro-m-cresol; 5 per cent. of dinitro-p-cresol
6	1:1:1 o-, m-, p-cresols "33 per cent." mixture (b)	30/12	(b)	40—<50	—	—	—	—	—	—	—	—	Approximately: 60 per cent. of dinitro-o-cresol; 30 per cent. of trinitro-m-cresol; 10 per cent. of dinitro-p-cresol (c)

NOTES:
(a) Full nitration carried out at 105° C.; limited nitration at lower temperature (50° C.).
(b) Mixed products have "mixed" molecular weights so percentage yield is not given.
(c) Different runs gave varying yields according to the dilution (high dilution—5 to 10-fold—high yield and vice versa) and temperature of reaction (when maintained at about 105° C.—lower yields).
(d) See Table VI for ultra-violet absorption spectra data.

to mice by intraperitoneal injection and sacrificing equal groups at 4 and 24 hours later in order to obtain the blood concentrations of the nitro-cresols. The concentrations of the compounds in blood were estimated essentially by the method of Parker[16] for serum dinitro-o-cresol as modified by Harvey[17] and by King and Harvey[18] for dinitro-o-cresol in whole blood of man and of animals. Minimum transmission values were obtained for the sodium salts of the compounds in methyl ethyl ketone at appropriate wavelengths (see Table III, last column, and Fig. 3).

Application of the Nolting and Salis reaction to o-, m- and p-cresols

The nitration of the three cresols by the method of Nolting and Salis results in three main compounds, dinitro-o-cresol from o-cresol, dinitro-p-cresol from p-cresol and trinitro-m-cresol from m-cresol (Table I).

Although limited quantities of sulphuric acid were used in the sulphonation of o-cresol (Datta and Mitter, *loc. cit.*, Claus and Jackson, *loc. cit.*), subsequent treatment of the product with nitric acid appeared to result in 4:6-dinitro-o-cresol and no 5:6-dinitro-o-cresol could be identified (cf. Datta and Varma, *loc. cit.*). Preliminary steam distillation to remove any unchanged o-cresol reduced the yield of dinitro-o-cresol in the mother liquor, but treatment of the steam distillate, containing unchanged o-cresol, with nitric acid at 100° C. for 8 hours and for 7 days at room temperature yielded further quantities of dinitro-o-cresol (Table II). Excessive dilution (>10-fold) of the sulphonated mixture made it necessary to heat the nitration mixture, but if dilution was about 3-fold the reaction became too violent, cooling was necessary and some product was completely destroyed. The formation of trinitro-m-cresol required that the mixture should be raised to about 105° C., otherwise the yield of separated material was low. p-Cresol was readily nitrated (*vide supra*), and it was found that cautious addition of nitric acid to the sulphonated mixture caused the precipitation of a bulky mass of yellow crystals at relatively low temperatures (less than 50° C.). On recrystallisation this product proved to be identical with a recrystallised commercial specimen of 2:6-dinitro-p-cresol. If the temperature was allowed to exceed 80° C. the reaction became violent and much of the yellow separated dinitro-p-cresol was destroyed, leaving only a small yield of the substance. Table III summarises some properties of the nitro-cresols.

Separation of the nitro-cresols: a simple test for their differentiation

Benzene solutions of the nitro-cresols or their mixtures gave definite colour bands on benzene pre-treated alumina columns. Three colour bands were formed: top layer—bright yellow trinitro-m-cresol, middle layer—orange-red dinitro-p-cresol, and the lowest band yellow dinitro-o-cresol. Some commercial samples (Harvey, *loc. cit.*) gave a small indefinite layer of brown material on top of the column, but this was not further investigated. Analysis of the extracts of the three colour bands yielded substances which were impure (intermixed) specimens of trinitro-m-cresol, dinitro-p-cresol and dinitro-o-cresol (Fig. 2). The melting points were low with the exception of those obtained for dinitro-o-cresol

TABLE II

THE NITRATION OF o-CRESOL

Conditions of sulphonation			Pre-nitration treatment (a)	Product yield per cent.	Melting point °C.		Probable identity of product (d)
mols. H₂SO₄	Temperature °C.	Duration: hours			Crude	Once recrystallised from ethanol	
1·2	Cold 0 to 4	24	Steam distillation	21	87·5	87·5	4:6-Dinitro-o-cresol
			Dilution	66	84·5	87·0	,,
1·2	Hot: boiling water bath 100	10	Steam distillation	74	86·5	86·0	,,
			Dilution	77	87·5	86·0	,,
2·2	Hot: boiling water bath 100	2	(i) Steam distillation	8	—	87·5	,,
			(ii) Steam distillate from (i) (b)	25	—	87·5	,, (c)
			(iii) Dilution	50–70	—	87·5	,,

NOTES:

(a) With the exception of (b) below only mother liquors were nitrated.

(b) The steam distillate, 200 ml., was treated with concentrated nitric acid, A.R., 100 ml. heated on boiling water bath for 8 hours, allowed to stand at room temperature for 7 days before separating off the product and recrystallising.

(c) Analysis figures for the 3 products are as follows:

   (i) Steam distillation (mother liquor); Found: C, 42·4; H, 3·1; N, 13·6 per cent.

   (ii) Steam distillate from (i)   C, 42·1; H, 3·3; N, 13·8 per cent.

   (iii) Dilution   C, 42·6; H, 3·1; N, 13·7 per cent.

   Calculated for $C_7H_6N_2O_4$ C, 42·4; H, 3·03; N, 14·1 per cent.

(d) See Table VI for ultra-violet absorption spectra data.

that were as high as any recorded, and these specimens appeared to be fairly pure. Such colour bands did not occur when chromatographic cellulose powder was used. The explanation of this probably lies in the fact that some salts (e.g., sodium) of the nitro-cresols studied have characteristic colours, and the obvious salts in this separation would be those formed by reaction with aluminium oxide.
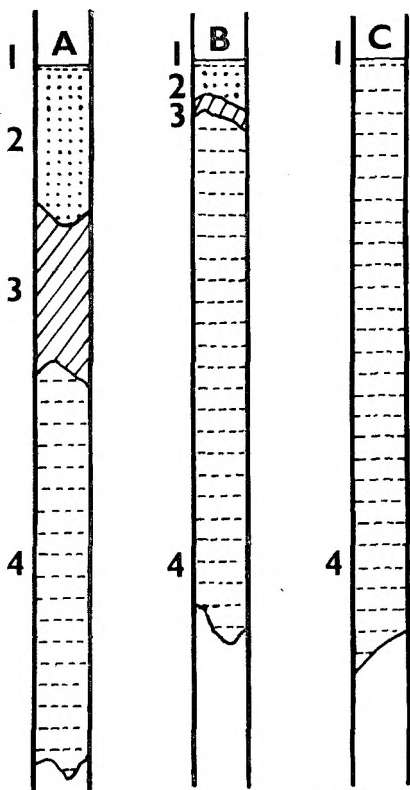
Fig. 2. Absorption of dinitro-o-cresol, trinitro-m-cresol and dinitro-p-cresol on alumina :—

A. From 33 per cent. mixture. 1. Dark brown. 2. Bright yellow. Mainly trinitro-m-cresol, m.pt. (recryst.) 95° to 97° C.; found, $N = 16.2$ per cent.; Calc., $N = 17.2$ per cent. 3. Orange-red. Mainly dinitro-p-cresol, m.pt. (recryst.) 62° C.; found, $N = 14.5$ per cent.; Calc., $N = 14.1$ per cent. 4. Yellow. Mainly dinitro-o-cresol, m.pt. (recryst.) 87.5° C.; found, $N = 14.6$ per cent.; calc., $N = 14.1$ per cent.

B. From 80 per cent. mixture. 1. Dark brown. 2. Trinitro-m-cresol. 3. Dinitro-p-cresol. 4. Yellow. Mainly dinitro-o-cresol, m.pt. (recryst.) 87.5° C.: found, $N = 15.3$ per cent.; calc., $N = 14.1$ per cent.

C. From commercial dinitro-o-cresol. 1. Dark brown. 4. Yellow. Dinitro-o-cresol m.pt. (recryst.) 87.5° C.: found, $N = 14.5$ per cent.; calc., $N = 14.1$ per cent.

This differentiation of the colour bands might prove to be useful initially in testing the purity of a dinitro-o-cresol sample when contamination by trinitro-m-cresol or by dinitro-p-cresol is suspected. For example, it was found that 25 ml. of a benzene solution containing 249 mg. of dinitro-o-cresol and 1 mg. of contaminating dinitro-p-cresol or trinitro-m-cresol was readily differentiated into two coloured bands on an alumina column approximately 9 mm. in diameter.

The depths of the bands are an approximate indication of the proportionate concentration of each substance present. Obviously this cannot be accepted as an accurate measure since the flow rates of the three substances are different and are in the order of speed, dinitro-o-cresol, dinitro-p-cresol, trinitro-m-cresol. The depths of the bands increased disproportionately on continued elution.

No other nitro compounds have been mixed deliberately with the 3

TABLE III

SOME PROPERTIES OF THE THREE NITRO-CRESOLS

Substance	Appearance	Solubility in water (a) per cent.	Melting point °C.	Colour 10 cm. depth of 1 per cent. alkaline solution (Na, Al, etc.)	Polarogram values Half-wave potential of 10 μg./ml. in 0·1N sodium hydroxide (v.)	Minimum transmission in visible spectrum (e) mμ
Dinitro-o-cresol ..	Yellow needles or rhombo-hedrons	0·018 to 0·024 (b)	86·5 to 87·0	Yellow-orange	−0·530	430
Trinitro-m-cresol ..	Pale yellow needles	0·094	106 to 109	Bright yellow	−0·550 (c)	415
Dinitro-p-cresol ..	Yellow to yellow-brown needles or rhombohedrons	0·029	80 to 81	Red-orange	−0·455 to −0·475 (d)	475

NOTES:
(a) Determined by shaking up solid material in water at 18° C. and estimating the dissolved material by Parker's method.
(b) See Harvey².
(c) At 10 μg./g. there was a tendency to form several waves.
(d) The values obtained are −0·455 v. for commercial sample and −0·475 v. for sample prepared by limited nitration.
(e) See Figure 3 for visible absorption spectra, Figure 4 for ultra-violet absorption spectra and Table VI for ultra-violet absorption spectra data.

TABLE VI

ULTRA-VIOLET SPECTROGRAPHIC DATA OF THE NITRO-CRESOLS (M/100 SODIUM HYDROXIDE)

Substance	Reference	Ultra-violet spectrographic data					
		λ_{max}. mμ	E mol. × 10^{-4}	λ_{max}. mμ	E mol. × 10^{-4}	λ_{max}. mμ	E mol. × 10^{-4}
Dinitro-o-cresol, pure (authentic) ..	—	370	1·52	263	0·72	222	1·20
" (Noting and Salis) ..	Serial No. 1, Table I	370	1·53	265	0·76	218	1·17
" (cold sulphonation) ..	Serial No. 1, Table II	368	1·10	262	0·48	225	0·71
" (cold sulphonation) ..	Serial No. 1, Table II	368	1·39	263	0·64	225	0·74
Dinitro-p-cresol, pure (authentic) ..	—	446	0·64	259	0·63	225	1·84
" (full nitration) ..	Serial No. 3, Table I	446	0·68	259	0·66	225	1·90
" (part nitration) ..	Serial No. 4, Table I	446	0·70	259	0·67	225	1·93
Trinitro-m-cresol, pure ..	Serial No. 2, Table I	348	1·21	260	0·848	215	1·44

studied in this communication. Also, it is obvious that the presence of additional contaminants cannot be excluded completely. However, examination of melting points, and the ultra-violet absorption spectra data suggests that it is unlikely that such substances, if they are formed, will occur in significant quantities.

Physiological tests

LD50 values for pure dinitro-o-cresol and for pure dinitro-p-cresol were similar and contamination of the former with the latter or with trinitro-m-cresol caused relatively little reduction in the toxicity or any obvious changes in the symptoms (Table IV). The symptoms exhibited

TABLE IV

LD50 VALUES AND SOME PHYSIOLOGICAL EFFECTS OF DINITRO-o-CRESOL, TRINITRO-m-CRESOL, DINITRO-p-CRESOL AND THEIR MIXTURES

Substance (a)	LD50 mg./kg.	Effects
Pure dinitro-o-cresol	24·2	Sweating, increased respiration, thirst, "stretching" of the body, diminished physical activity, death accompanied by marked rigor. Fully described by many authors
"80 per cent." Dinitro-o-cresol (b)	22·9	Ditto
"33 per cent." Dinitro-o-cresol (b)	32·5	Ditto
Dinitro-p-cresol	24·8	Ditto
Trinitro-m-cresol	168·0	Animals tend to "bunch" up, hair becomes erect giving a porcupine-like appearance, marked "shivering" or fibrillating movements, occasional spasms followed by great nervous activity, i.e., running very rapidly round the cage. Death not accompanied by any marked rigor

NOTES:
(a) Given as 0·5 per cent. solutions in 0·5 per cent. NaCl, 0·5 per cent. NaHCO₃ by intraperitoneal injection.
(b) Prepared by nitrating mixtures of 8:1:1 and 1:1:1 o-, m- and p-cresols (see Table I).

by animals poisoned by dinitro-o-cresol and dinitro-p-cresol were different from those poisoned by trinitro-m-cresol. The marked "fibrillation" or shivering resulting from trinitro-m-cresol was in marked contrast to the reduction of physical activity caused by the dinitro-cresols. Blood levels of the three substances taken at 4 and 24 hours following dosing gave similar reduction values, i.e., 76 to 82 per cent. (Table V).

TABLE V

ELIMINATION OF NITRO-CRESOLS BY MICE

Substance (a)	Blood levels μg./g.		Reduction over 4 to 24 hours per cent.
	4 hours after injection	24 hours after injection	
Dinitro-o-cresol (b)	33, 36, 43, 35 Mean 37 ± 4·5	5, 8, 5 Mean 6 ± 0·6	80
Trinitro-m-cresol	66, 60, 46, 60, 64, 54 Mean 58 ± 7·3	12, 12, 10, 12, 14, 1 Mean 10 ± 4·6	82
Dinitro-p-cresol	31, 32, 38, 53, 27, 28 Mean 35 ± 8·9	8, 7, 9, 7, 8, 8 Mean 8 ± 0·32	75

NOTES:
(a) Given as single intraperitoneal injection of 20 mg./kg. of substance in 0·5 per cent. NaCl, 0·5 per cent. NaHCO₃ solution.
(b) Figures from a wider investigation which will be reported elsewhere.
(c) Means (± S.D.) to nearest μg.

Spectrum analysis of the nitro-cresols

Absorption curves in the visible spectrum were prepared by examining the sodium salts of the nitro-compounds in methyl ethyl ketone at concentrations of 5 μg./ml. These are shown in Figure 3. Transmissions at the minimum values were used in the estimation of the 3 compounds in blood (dinitro-*o*-cresol 4300 Å., trinitro-*m*-cresol 4150 Å. and dinitro-*p*-cresol 4750 Å.).

Ultra-violet absorption spectra of dinitro-*o*-cresol, trinitro-*m*-cresol and dinitro-*p*-cresol are shown in Figure 4. Details of the ultra-violet spectra of authentic compounds and of other substances prepared in the course of this investigation are given in Table VI.

Molnar, *loc. cit.*, has recorded curves of several nitro-compounds, including his unidentified Dinitro-cresols I and II. Summarised comparisons between Molnar's values and those obtained in the present investigation are as follows:
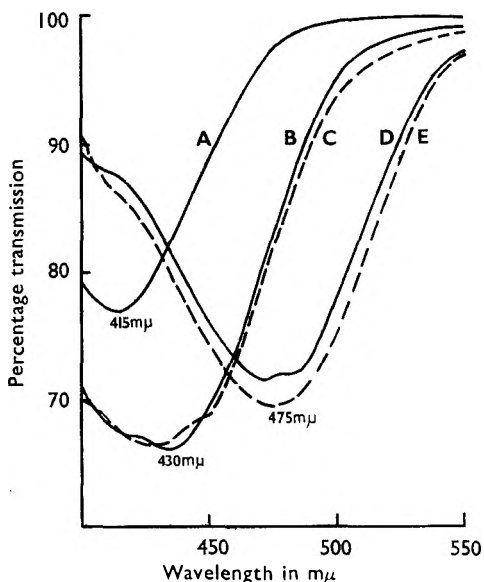

FIG. 3. Visible absorption spectra of the sodium salts of dinitro-*o*-cresol, trinitro-*m*-cresol and dinitro-*p*-cresol. All in methyl ethyl ketone.

A. Trinitro-*m*-cresol (recrystallised).
B. Dinitro-*o*-cresol (crude).
C. Dinitro-*o*-cresol (recrystallised).
D. Dinitro-*p*-cresol (once recrystallised).
E. Dinitro-*p*-cresol (twice recrystallised).

| | Maximum value mμ | |
	Molnar	Present investigation
Dinitro-*o*-cresol, pure	—	368–70
Dinitro-cresol I	360	—
Dinitro-*p*-cresol, pure	440	446
Trinitro-*m*-cresol	360	348

There is good agreement between Dinitro-cresol I and pure dinitro-*o*-cresol and reasonable agreement between the dinitro-*p*-cresol values except in the shorter wavelengths. Agreement is not good between the trinitro-*m*-cresol samples. No curves have been obtained in the present investigation that correspond precisely to Molnar's Dinitro-cresol II. It is interesting to note that the molar extinction coefficients of the second two samples of dinitro-*o*-cresol (see Table VI) are low, although the forms of their spectra are indistinguishable from the authentic specimen obtained essentially by the method of Nolting and Salis (see Tables I

and II).   Dinitro-*p*-cresol specimens prepared by limited nitration at low temperatures or by full nitration at higher temperatures give spectra which are identical with authentic recrystallised dinitro-*p*-cresol.

No explanation can be advanced for the low molar extinction coefficients of the second two dinitro-*o*-cresols (Table VI), but the similarity of the spectral forms of all four compounds leaves little doubt that the identity of these compounds is 4:6-dinitro-*o*-cresol.
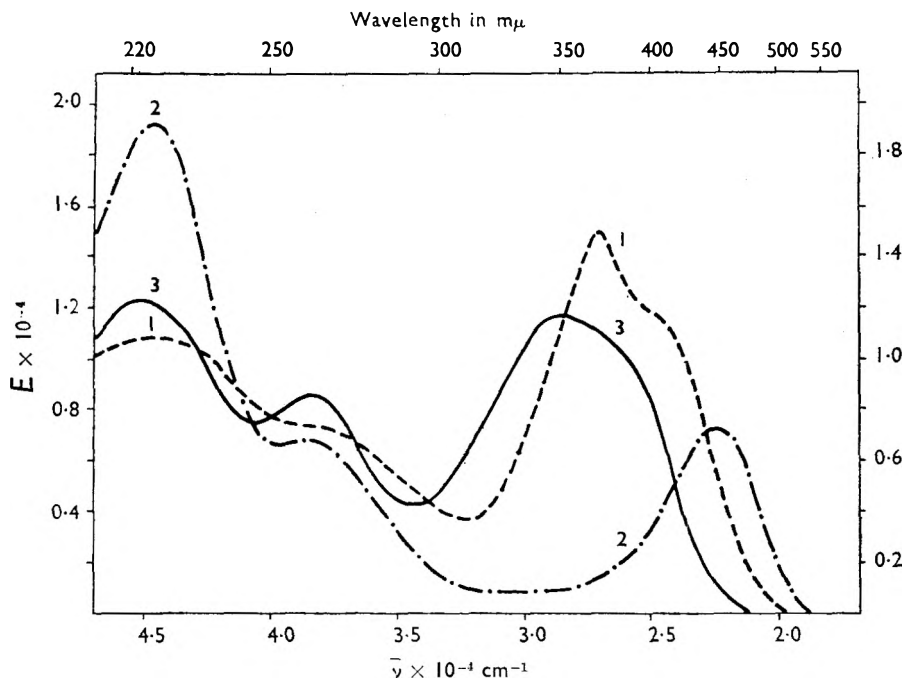
FIG. 4.   Absorption spectra of dinitro-*o*-cresol, trinitro-*m*-cresol and dinitro-*p*-cresol.   Solvent 0·01N sodium hydroxide.

1. 4:6-Dinitro-*o*-cresol.
2. 2:6-Dinitro-*p*-cresol.
3. 2:4:6-Trinitro-*m*-cresol.

DISCUSSION

If the nitration of *o*-cresol is carried out in the presence of excess of sulphuric acid as a nitrating agent, 4:6-dinitro-*o*-cresol is the only product. Provided that the *o*-cresol used is a good grade, significant contamination by the *m*- and *p*-isomers is unlikely.   Nevertheless, if *p*-cresol is present 2:6-dinitro-*p*-cresol may contaminate the final product to a minor extent, but if the temperature has been maintained above 80° C., then it is likely that the final yield of dinitro-*p*-cresol will be low since it is destroyed in the presence of excess of nitric acid or nitrous fumes at high temperatures.

It is clear that fairly wide limits in the experimental conditions can be observed in the preparation of dinitro-*o*-cresol without causing any major changes in its yield or purity.   In any case, if the reaction is carried

out at high temperature, i.e., $>80°$ C., the vigorous conditions will favour destruction of formed substances rather than the formation of a new one. This is illustrated by the preparation of dinitro-*p*-cresol.

It is interesting to compare this with the other findings of Datta and Varma (*loc. cit.*), who have stated that excess of nitrous gases on *p*-cresol sulphonate gives rise to some oxalic acid and practically no nitro-compounds, and that the presence of free (unsulphonated) cresol causes the formation of much tarry material.

The chromatographic separation of the three nitro-compounds could serve as the basis for a simple colour test in checking the purity of samples of dinitro-*o*-cresol when contamination by dinitro-*p*-cresol or trinitro-*m*-cresol is suspected, since its sensitivity is $0·5$ per cent. or less on a few mg. of crude material. Because this method has not been extended to other nitro-cresols their presence cannot be excluded, although the slightly lowered melting points of the second and third groups of the preparations recorded in this paper suggest a general homogeneity of substance.

Although Molnar's Dinitro-cresol I and pure dinitro-*o*-cresol have similar, if not identical ultra-violet absorption curves, the melting points are at variance (Molnar gives 105° C. for Dinitro-cresol I and 85·5° C. for Dinitro-cresol II). The value of 105° C. corresponds most closely to 2:6-dinitro-*m*-cresol, melting point 101° C. (Will[20]), or 4:6-dinitro-*m*-cresol, melting point 97° C. (Gibbs and Robertson[21]), both of which are prepared by indirect means. If *m*-cresol was a contaminant in the preparation of the two Dinitro-cresols reported by Molnar, then trinitro-*m*-cresol, and not a dinitro-*m*-cresol, would be the resulting compound.

It has been stated by Oetlingen[19] that dinitro-*o*-cresol is sometimes contaminated by dinitro-*p*-cresol. The experiments reported in this paper suggest that dinitro-*p*-cresol is about as toxic as dinitro-*o*-cresol towards mice. The visible symptoms of animals treated with the two compounds are very similar. Therefore any minor contamination is unlikely to reduce the gross toxic effect. However, examination of several commercial samples manufactured in this country (Harvey, *loc. cit.*) did not reveal any contaminating dinitro-*p*-cresol. The alumina test appeared to be quite sensitive and there was no suggestion of any orange-red band dinitro-*p*-cresol appearing on the columns used for the analysis of the commercial samples. If any trinitro-*m*-cresol is present in the sample it will not lower the effective total toxicity to any extent. It is interesting to record that a "33 per cent." sample, containing about 30 per cent. of trinitro-*m*-cresol is about 60 per cent. less toxic than pure dinitro-*o*-cresol, wheras trinitro-*m*-cresol itself has about 12 per cent. of the toxicity of pure dinitro-*o*-cresol. This suggests that trinitro-*m*-cresol does not depress the toxicity of the other two compounds to any extent. All 3 compounds appear to be hyperthermic, and according to Molnar the dinitro-cresols are more toxic and hyperthermic than *o*-nitro-*p*-cresol and 2:4:6-trinitro-*m*-cresol. The identity of the two dinitro-cresols described by Molnar has not been established, although one compound has some properties similar to dinitro-*o*-cresol. They

appear to be metabolic stimulants and produce similar toxicological effects, although to a differing degree.

The differences in the symptoms of the 3 compounds are interesting and possibly significant, and the similar rates of their elimination from mice suggest that in other species there is the possibility of some cumulative action of dinitro-*p*-cresol and trinitro-*m*-cresol. Arguing by analogy with the action of dinitro-*o*-cresol (King and Harvey[22]) it is likely that trinitro-*m*-cresol and dinitro-*p*-cresol will accumulate in man if absorbed in small quantities at frequent, i.e., daily, intervals. Thus, in manufacturing or using dinitro-*p*-cresol or trinitro-*m*-cresol, the same rigorous safety precautions should be adopted as already recommended for handling dinitro-*o*-cresol (Hunter[23]).

SUMMARY AND CONCLUSIONS

1. The preparation of 4:6-dinitro-*o*-cresol by the method of Nolting and Salis has been studied and it has been demonstrated that if pure *o*-cresol is sulphonated by limited or excess quantities of sulphuric acid, and nitrated by excess of nitric acid at temperatures $>80°$ C., only dinitro-*o*-cresol will result.

2. Dinitro-*p*-cresol and trinitro-*m*-cresol are likely to be formed if the *o*-cresol is contaminated by the *m*- and *p*-isomers.

3. Dinitro-*o*-cresol and dinitro-*p*-cresol appear to be equally toxic to mice and result in similar symptoms.

4. Trinitro-*m*-cresol has about 12 per cent. of the toxicity of dinitro-*o*-cresol and results in different symptoms.

5. A few properties of dinitro-*o*-cresol, dinitro-*p*-cresol and trinitro-*m*-cresol have been summarised.

6. Attention has been drawn to the use of alumina for separating and identifying the 3 nitro-compounds studied. This has been suggested as a simple preliminary check for testing the purity of dinitro-*o*-cresol samples.

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APPLICATIONS OF FLAME PHOTOMETRY TO THE ANALYSIS OF ALKALIS AND CALCIUM IN SALINE SOLUTIONS FOR INFUSION

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THE practical use of flame photometry for the determination of the alkali and the alkaline earth metals in different materials has widely increased during the last few years. Even though a great number of other elements can now be determined with the flame photometer, the application of flame photometry to the alkali analysis was greeted with special enthusiasm. The analytical chemist has always been faced with long, tedious "wet" methods for sodium and potassium, often involving time-consuming separations. Rapid determination of sodium and potassium in plasma, urine, etc., has become of increasing importance not only in research, but also in diagnosis and treatment. By the technique of flame photometry the clinician can obtain data on sodium and potassium concentrations in these materials in a few minutes. The superior rapidity of the flame photometric methods has made them very advantageous for routine analyses. The great number of papers dealing with the practical aspects of flame photometry published during the last few years in various scientific journals indicate that flame photometry has assumed an important position among the practical sciences. In a previous paper the author has given a review of flame photometry, involving the developments in instruments and techniques and the limitations and advantages, which are inherent in the method[1].

The theoretical connection between the concentration of the element in the flame and the intensity of its emission is not yet elucidated. Therefore quantitative flame photometric analysis must be based upon an empirical comparison of the test solution with standard solutions. Besides the instrumental errors, which are exclusively dependent on the instrument used, the interferences, caused by the effect of dissolved substances on the emission of the test element, are the most troublesome aspects of flame photometry. These interferences are as yet so little known that no generalisations can be made. The observations on interferences made by different authors have often been divergent, probably due to different instrumental and other measuring conditions. The investigations described in this paper were carried out in order to serve as a basis for the application of flame photometry to the routine control of saline solutions in this laboratory.

APPARATUS

A Beckman DU spectrophotometer with the flame photometer attachment model 9200 was used. The instrument is exhaustively described in the manufacturers instruction manual[2], and therefore no detailed description of it will be given here.

This new Beckman flame photometry attachment incorporates an all-metal combination burner-atomiser in which the sample is sucked into a vertical palladium capillary and is sprayed directly into the flame. A concentric channel provides oxygen to operate the atomiser and the flame. An additional concentric channel provides acetylene or hydrogen for the flame. In this atomiser the spray is introduced directly into the flame and no condensing chamber is used. Since no pre-mixing of gases is necessary, backfiring is eliminated. The burner consumes 1·5 to 2 ml. of liquid per minute. Figure 1 shows the construction of the burner-atomiser.
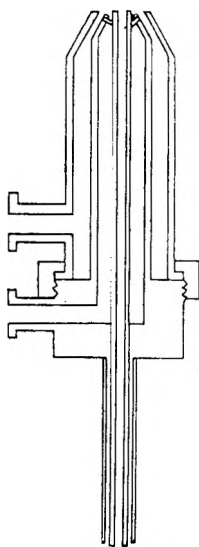
The flame photometer is equipped with pressure regulators on a control panel.

A base assembly unifies the DU spectrophotometer and the attachment into a single instrument. The burner housing is fixed with hinge pins in sockets on the base assembly. When not in use the housing can be easily swung out of the way to permit the use of a lamp. A resistance box assembly on the phototube housing permits the quick interchange of resistor for emission and absorption work. For flame photometry the model DU spectrophotometer is provided with a 10,000-megohm phototube load resistor to increase the sensitivity 5 times above that normally available with the standard 2000-megohm resistor. According to Gilbert[3] this is the practical limit of the increase of sensitivity.

By the use of the spectrophotometer in the flame photometer the combination of adjustable slit, sensitivity control, and wavelength drum enables the operator to select the most favourable ratio between background and analytical line radiation and more effectively to isolate the radiation due to the element analysed.

Fig. 1. Integral atomiser–burner unit.

Examination of Instrumental Conditions

The instrumental conditions for reproducibility and stability were considered first. The instrument, used in this work, had an acetylene burner. The burner was supplied with tank acetylene and oxygen. At an oxygen pressure of 20 lb./sq.in. the burner consumed 1·5 ml. of liquid per minute. The manufacturer recommends an atomising rate of 1·5 to 2 ml. of liquid per minute. The suction pressure in the capillary will then be about 100 cm. of water. With the oxygen pressure at 20 lb./sq.in. an acetylene pressure of 4 lb./sq.in. gave a flame of desired height and appearance. These pressure settings, i.e., oxygen 20 lb./sq.in. and acetylene 4 lb./sq.in. pressure, were used for all measurements of sodium potassium and calcium described in this paper.

The instrumental conditions at these pressure settings were examined in respect to flame background, stability and sensitivity, and furthermore the reproducibility of the settings was tested.

Flame background. The flame background consists of the spectrum of the flame itself. This was established by setting the instrument to zero with the shutter closed, and then rebalancing the galvanometer circuit with the shutter open by rotating the transmission knob. In Figure 2 these readings are plotted against the wavelength. All measurements were made with the sensitivity control at the midpoint.
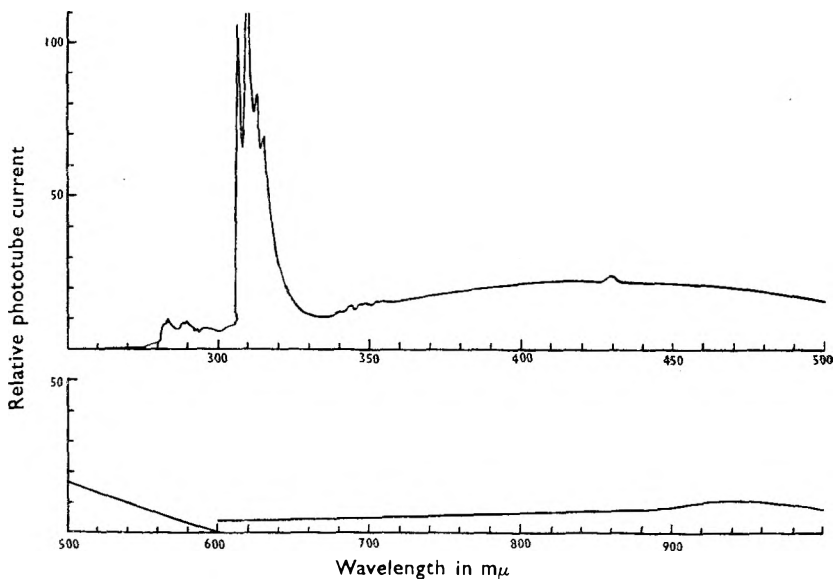
FIG. 2. Flame background.

Between 250 and 600 mμ the blue sensitive phototube is used. Slit width 0·2 mm.
Between 600 and 1000 mμ the red sensitive phototube is used. Slit width 1 mm.

The acetylene-oxygen burner had strong emission bands within the wavelength range 260 to 350 mμ belonging to the OH spectrum. The background radiation decreased then continuously towards the infra-red. A weak maximum could, however, be noticed in the near infra-red after increasing the width of the slit.

The background radiation as a function of the width of the slit is shown in Figure 3 at 554 mμ (calcium), 589 mμ (sodium) and 768 mμ (potassium). Here, too, the sensitivity was held constant with the sensitivity control at the midpoint. At this setting 1 galvanometer scale division is generally equivalent to 1 transmission scale
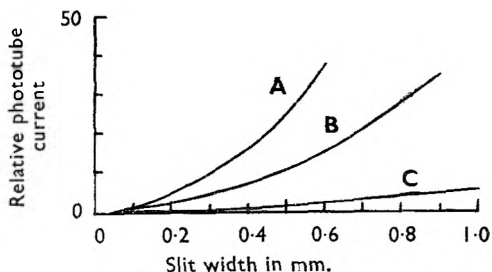
FIG. 3. Flame background as a function of slit width. Wavelengths shown as parameters.
A. 554 mμ.
B. 589 mμ.
C. 768 mμ.

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division with selector switch at 0·1. The measurements were made with distilled water spraying, which decreased the background radiation somewhat.

Flame conditions. A number of measurements were carried out on solutions of sodium and calcium in order to give a view of how the radiation intensity varied with the gas pressure. The results are graphically shown in Figures 4 and 5. Figure 4 shows relative photo-currents for the sodium lines at 589 mμ and the calcium band at 554 mμ as a function of acetylene pressure at the oxygen pressure of 20 lb./sq.in.
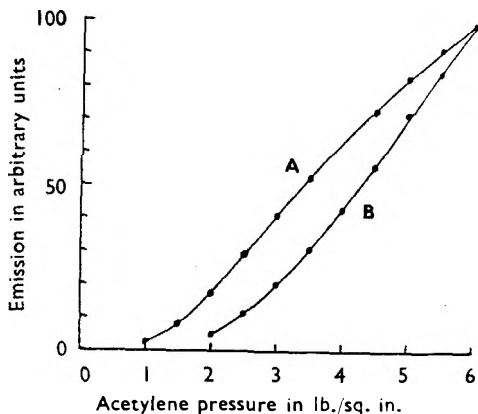
FIG. 4. Curve A. Sodium emission at 589 mμ. Oxygen pressure 20 lb./sq.in. Slit width 0·05 mm. Concentration of sodium 100 p.p.m.
Curve B. Calcium emission at 554 mμ. Oxygen pressure 20 lb./sq.in. Slit width 0·1 mm. Concentration of calcium 500 p.p.m.
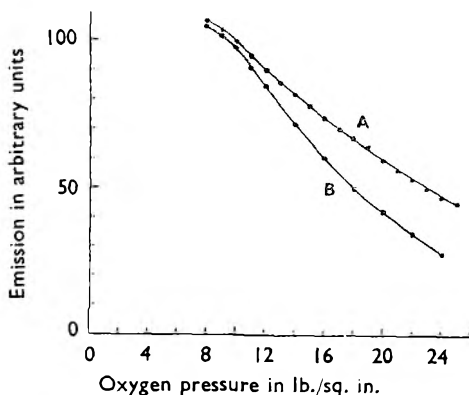

FIG. 5. Curve A. Sodium emission at 589 mμ. Acetylene pressure 4 lb./sq.in. Slit width 0·05 mm. Concentration of sodium 100 p.p.m.
Curve B. Calcium emission at 554 mμ. Acetylene pressure 4 lb./sq.in. Slit width 0·1 mm. Concentration of calcium 500 p.p.m.

Figure 5 shows readings at the same wavelengths as a function of oxygen pressure at the acetylene pressure of 4 lb./sq. in. Net readings (with background subtracted) are plotted against pressure settings.

The luminosity showed no maximum or plateau with respect to acetylene and oxygen pressure. For a given oxygen pressure the luminosity increased steadily with the acetylene pressure.

Flame stability. The Beckman flame spectrophotometer is designed for standardisation with external standards. This direct intensity procedure assumes that the flame is a constant, steady source of light and that the rate of atomisation is constant. The stability of the flame, obtained with above stated standard settings of gas pressure, was tested by taking series of readings on a solution containing 100 p.p.m. of sodium. Operating conditions for the flame spectrophotometer were: wavelength at 589 mμ, transmission at 100, slit at 0·05 mm., and selector switch at 0·1. Between each reading the test solution was lowered from the capillary

and the photocell shutter was closed. In each series 10 readings were taken. 3 series of readings gave mean deviations from the average readings of 0·3, 0·2 and 0·4 per cent. The results showed that the stability of the flame was satisfactory. In practice, in routine analyses, the stability of the flame is further checked by repeated standardisations during the measuring of a series of test solutions.

The reproducibility of gas pressure settings. In order to check the performance of the gauges the following measurements were carried out. 30 readings were serially taken on a solution containing 100 p.p.m. of sodium. The instrumental conditions were the same as described above under flame stability. Between each reading the test solution was lowered from the capillary after closing the photocell shutter, the oxygen and acetylene pressures were changed and then restored exactly to their original values. No other adjustments were made. These readings showed a mean deviation from the average reading of 0·5 per cent. The reproducibility of the pressure settings was consequently very good.

Sensitivity. The investigation of the sensitivity for sodium, potassium, and calcium described below, was not intended to establish the absolute efficiency of the instrument in this respect. It may only be considered as an examination of the possibilities in practice under the instrumental conditions used here. With further adjustments of pressure settings, etc., in measuring the different elements the sensitivity can probably be increased.

All measurements were carried out with the sensitivity knob at the midpoint and the selector switch at 0·1. With 1 p.p.m. of sodium at 589 mμ and with a slit width of 1 mm., a net reading (background subtracted) of 70 transmission scale divisions was obtained. With 10 p.p.m. of potassium at 768 mμ and slit at 1·5 mm., the net reading was 85 scale divisions. 10 p.p.m. of calcium at 554 mμ and with a slit width of 0·8 mm. gave a net reading of 31 scale divisions.

INVESTIGATION OF INTERFERENCES

The term "interference" covers various effects of dissolved substances on the light produced by the test substance. Many authors have studied these interference effects. Published results are apparently greatly dependent upon instrumental and other measuring conditions and cannot directly be applied. For that reason experiments were carried out to elucidate the importance of interferences in the determination of sodium, potassium and calcium in saline solution for infusion. These solutions contain sodium, potassium and calcium as salts of various acids, and the metals are often present together in various ratios.

Compounds used in interference tests

The metal salts used in the experiments were for the most part of reagent purity. Before use they were dried at 105° C. to constant weight. In some cases chemicals of Swedish Pharmacopœia purity were used. Solutions of lactates and acetates of sodium and potassium were prepared from the acids and the metal hydroxides. In all calcium salts the content

of calcium was determined by titration with the disodium salt of ethylene-diaminetetra-acetic acid[4].

Sodium and potassium

Anion interference. A study was made on the interference effects of a number of anions, several of which are often met with in the saline solutions. This study was carried out as a comparison of sodium chloride with the sodium salts mentioned in Table I and of potassium chloride with the potassium salts in Table II. The relation of concentration of test element to the anion interference was also studied by determining the interference effect at two different concentrations of the metals. The solutions containing 100 p.p.m. of the test element were prepared from the solutions containing 1000 p.p.m. by dilution with distilled water.

TABLE I

INTERFERENCE BY ANION ASSOCIATED WITH SODIUM

Compound	Change of emission in per cent. at 589 mμ compared with sodium chloride	
	1000 p.p.m. Na	100 p.p.m. Na
Sodium lactate	0	0
Sodium citrate	0	0
Sodium bicarbonate	0	0
Sodium acetate	−5	0
Sodium nitrate	−4	0
Sodium iodide	−3	0
Sodium sulphate	−8	0
Disodium phosphate	−6	0

It was observed that only at the higher concentration of sodium the light output in some cases was affected by the difference in the anions associated with the metal. The lactate, citrate and bicarbonate gave also at the higher concentration the same readings as the chloride. In all cases the measurements of the solutions containing 100 p.p.m. of sodium were interference free. Solutions containing 10 p.p.m. of sodium were also assayed. No interference was noticed.

Similar results (recorded in Table II) were obtained on repeating the investigations with potassium instead of sodium.

From these findings it is clear that flame photometric determinations of the sodium and potassium salts of the saline solutions can be made with the chlorides as standards.

TABLE II

INTERFERENCE BY ANION ASSOCIATED WITH POTASSIUM

Compound	Change of emission in per cent. at 768 mμ compared with potassium chloride	
	1000 p.p.m. K	100 p.p.m. K
Potassium lactate	0	0
Potassium acetate	0	0
Potassium bicarbonate	0	0
Potassium iodide	−2	0
Potassium nitrate	−3	0
Potassium sulphate	−4	0
Monopotassium phosphate	0	0

Interference effect of some acids. An excess of free acid is not often found in the saline solutions. For that reason less attention was given to the effect of free acids on the determination of sodium and potassium. The observations made on the interferences engendered by smaller amounts of some acids are recorded in Table III.

TABLE III

INTERFERENCE BY ACIDS WITH SODIUM AND POTASSIUM

Acid	Concentration of acid	Change of emission in per cent.	
		Sodium at 589 mμ 100 p.p.m.	Potassium at 768 mμ 500 p.p.m.
Hydrochloric acid	0·1N	0	0
Sulphuric acid	0·01N	0	0
	0·1N	0	−4
Nitric acid	0·1N	0	0
Phosphoric acid	0·01N	0	0
Citric acid	0·1N	0	0
Lactic acid	0·01N	0	0

A number of sodium chloride and potassium chloride solutions were prepared containing the metals and acids in amounts stated in Table III. These solutions were then analysed flame photometrically for sodium and potassium, using pure sodium and potassium chloride solutions of the same metal content as comparison standards. It is observed that the presence of 0·7 per cent. (0·1N) of citric acid did not affect the determination of sodium or potassium.

Interference of calcium with sodium and potassium and interference of sodium and potassium with each other. Several authors have shown that the alkali metals enhance each other, and that the enhancement is not proportional to their concentration. In order to investigate the magnitude of these interferences under the instrumental conditions used in this laboratory, quantitative measurements were made. The results of these

TABLE IV

MUTUAL INTERFERENCE OF SODIUM AND POTASSIUM AND INTERFERENCE OF CALCIUM WITH SODIUM AND POTASSIUM

Interfering element	Weight ratio of interfering element to test element	Change of emission in per cent.			
		Sodium at 589 mμ		Potassium at 768 mμ	
		100 p.p.m.	10 p.p.m.	200 p.p.m.	50 p.p.m.
Sodium	1:10	—	—	0	0
	1:2	—	—	0	0
	1:1	—	—	+4	0
	5:1	—	—	+12	+7
	10:1	—	—	+16	+15
Potassium	1:10	0	0	—	—
	1:2	0	0	—	—
	1:1	+1	0	—	—
	5:1	+5	+2	—	—
	10:1	+8	+3	—	—
Calcium	1:10	0	0	0	0
	1:2	0	0	0	0
	1:1	0	0	0	0
	5:1	+4	0	+6	+3
	10:1	+6	+1	+8	+5
Slit width		0·05 mm.	0·15 mm.	0·2 mm.	0·4 mm.

measurements are recorded in Table IV. With transmission scale at 100 (or at 90 when the enhancement was greater than 10 per cent.) and selector switch at 0·1, the flame photometer was standardised with a solution containing the test element only. Then readings were taken on solutions containing the same quantity of the test element but with various amounts of interfering element added. The effect of dilution on the interference was studied. At first the stronger solutions were assayed, then they were diluted and assayed at the lower concentration range. The chlorides of the metals were used.

It is evident from the findings that the presence of potassium in the same (or larger) concentration as sodium gave a positive interference in the determination of sodium at the 100 p.p.m. concentration. The effect of calcium on sodium was smaller. The interference of sodium with potassium was stronger than *vice versa*, while calcium increased the emission of potassium in about the same degree as the emission of sodium. In all cases dilution decreased the interference, even though the ratio of interfering element to test element remained constant.
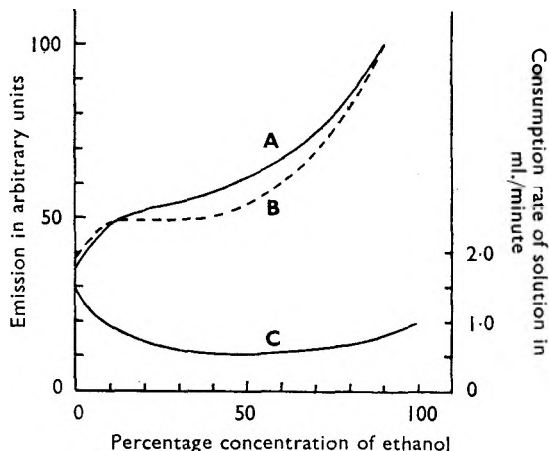
Since the saline solutions generally contain less potassium and calcium than sodium, most determinations of sodium in these solutions can be made using a standard solution containing only sodium chloride.

In connection with the measurements of interference described above, experiments were carried out to ascertain if the increased readings included also a continuum interference, i.e., an increased background radiation due to continuous spectral emission by the interferant. For that reason solutions containing only the interfering metal, in the same quantities as the solutions assayed above, were measured. No significant increase of the background was observed in any case.

FIG. 6. Effect of ethanol on the emission of sodium and potassium.
Curve A. Sodium emission at 589 mμ. Slit width 0·05 mm. Concentration of sodium 100 p.p.m.
Curve B. Potassium emission at 768 mμ. Slit width 0·1 mm. Concentration of potassium 500 p.p.m.
Curve C. Consumption rate of the sodium solution as a function of ethanol concentration.

Effect of ethanol and dextrose on the determination of sodium and potassium. It has been reported[5],[6],[7] that light emission is actually increased by the addition of ethanol. The cause of this interference is not known. This observation was verified by experiments. The results are given in Figure 6, which shows the readings of sodium and

potassium at different concentrations of ethanol.   The solutions of sodium and potassium used in these experiments contained the metals as iodides.

Osborn and Johns[8] consider that this interference is a physical effect resulting from an increased rate of atomisation owing to the lowered surface tension of the test solution.   In order to check this hypothesis the rate of atomisation was measured at different concentrations of ethanol.   The results of these measurements are recorded in Figure 6 in the form of a curve showing the rate of atomisation in ml./minute as a function of the concentration of ethanol in the test solution.   From the curves in Figure 6 it is evident that the interference of ethanol cannot be attributed to an increased rate of atomisation. The highest rate of consumption was obtained with the pure water solution.

Dextrose is reported[9,10] to cause negative errors in the determination of sodium and potassium with an instrument containing a condensing chamber in the atomiser. On the other hand, Weichselbaum and Varney[11] found that a concentration of 4 per cent. of sugar did not effect the readings.   In the Weichselbaum atomiser the spray was introduced directly into the flame and no condensing chamber was used.   The presence of dextrose or sugar changes the viscosity of the solution.   From these findings it might be concluded that the atomiser with condensing chamber is more readily affected by changes in viscosity resulting in changed spray rate.

As mentioned before the

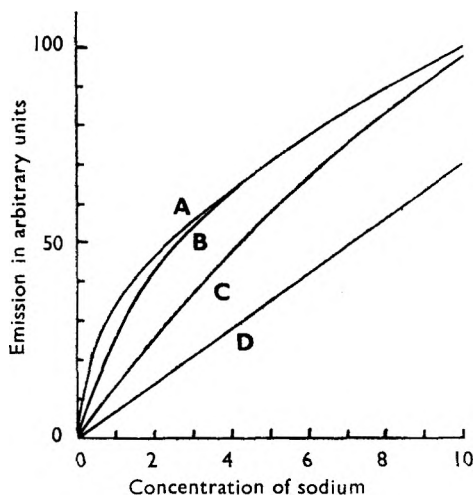
FIG. 7.   Calibration curves for sodium at 589 mμ.   Linear co-ordinates.
A.   0 to 1000 p.p.m.
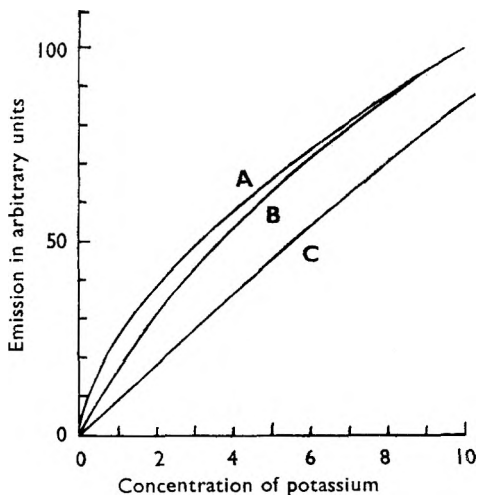B.   0 to 100 p.p.m.
C.   0 to 10 p.p.m.
D.   0 to 1 p.p.m.

FIG. 8.   Calibration curves for potassium at 768 mμ.   Linear co-ordinates.
A.   0 to 1000 p.p.m.
B.   0 to 100 p.p.m.
C.   0 to 10 p.p.m.

atomiser in the Beckman instrument sprays the solution directly into the flame. Measurements of the rate of consumption by atomising solutions of sodium chloride containing 0, 1, 2 and 3 per cent. of dextrose showed that these quantities of dextrose did not change the spray rate.

Calibration curves. Figures 7 and 8 show the relative photocurrents for sodium and potassium in different ranges of concentration. These curves are actual working curves. The net readings are plotted as ordinates.

The sodium lines at 589 mμ show, at higher concentration of the metal, a pronounced upward convexity due to self absorption. At the 0 to 1 p.p.m. range the curve is linear.

With potassium at 768 mμ and 0 to 10 p.p.m. range, the curve obtained was nearly linear.

Bills *et al.*[7] showed that, in the determination of sodium and potassium, reduction in interference, both mutual and from foreign solutes, was effected by working at low concentration ranges. The results of Tables I, II and IV also confirm this finding. There is, too, another advantage of standardising for a low concentration range. At, for example, the 0 to 1 p.p.m. range, the relation between concentration and dial reading is linear. Hence no calibration curve is required. But certain disadvantages are also associated with the measurements of solutions containing very small quantities of the metals. The light output is of course lower, and therefore an increase in the slit width is necessary. This increase in slit width has certain undesirable effects in that the spectral band width is increased, resulting in an increased background and possible inclusion of lines of other elements in the sample. The reproducibility of the readings, too, becomes less satisfactory. Furthermore, it is very important to handle and preserve very diluted solutions of alkali with care to avoid the possibility of accidental contamination with the metal to be determined. The accidental introduction of sodium or potassium, as from a particle of dust, tobacco smoke, or fingerprint, can seriously upset the analysis. Glass containers too may contribute important quantities of sodium or potassium. These sources of practical error make the work with very diluted solutions in routine analyses less reliable.

For reasons discussed above, and with the guidance of the results obtained by the investigations of interference, the concentration ranges 0 to 100 p.p.m. for sodium and 0 to 200 p.p.m. for potassium are generally used in the analyses of the saline solutions in this laboratory. Because the saline solutions contain comparatively large amounts of the alkalis, the use of these relatively high concentration ranges also reduces the work of preparing the solutions to be atomised. The amounts of interfering substances in the solutions are known, and therefore radiation interferences are eliminated by including the proper amounts of the interfering substances in the standards, which is the most effective remedy for radiation interference.

For sodium the wavelength setting at 589 mμ was used measuring the radiation of the sodium lines at 589·0 and 589·6 mμ. The determination of potassium was based on the radiation of the potassium

lines at 766·5 and 769·9 mμ. The calibration curves were found to be reproducible.

Calcium

The examination of the measuring conditions for the determination of calcium by flame photometry was, for various reasons, given little room in this investigation. In the last few years rapid and sensitive chemical methods for the determination of calcium have been described in the literature[4]. Furthermore, calcium is met with in only a small number of saline solutions for infusion. The experiments described below might, however, show that several causes of interference complicate the flame photometric determination of calcium. Above all, the high concentration of sodium to calcium in saline solutions renders this particular determination most difficult.

All determinations of calcium have been carried out by measuring the radiation of its spectral emission band at 554 mμ. At this wavelength the flame background (Fig. 3) is higher than at the wavelength settings used in the determination of sodium and potassium.

Previous studies[12] showed that the band spectra of calcium and the other alkaline earths are affected by the nature of the anion present in the solution. This is to be expected since the band system originates from the thermal excitation of calcium-containing molecules (probably the oxide or chloride). Measurements of this anion interference were carried out by comparing solutions of 4 calcium salts containing the same content of calcium. The solution of calcium chloride was used as standard. The results are recorded in Table V.

TABLE V

INTERFERENCE OF ANION ASSOCIATED WITH CALCIUM

Compound	Change of emission in per cent. at 554 mμ compared with calcium chloride			
	1000 p.p.m. Ca	500 p.p.m. Ca	100 p.p.m. Ca	50 p.p.m. Ca
Calcium bromide ..	−32	−16	0	0
Calcium gluconate	−28	−21	−7	−3
Calcium lactate	−17	−17	−5	−4

When the concentration was decreased the interference effect decreased rapidly. From these results it is clear that the standards used in the flame photometric determination of calcium must be prepared from the same compound or compounds in the same ratio as that contained in the sample being analysed.

The presence of an excess of hydrochloric acid changed the luminous output from calcium (as chloride). Thus, solutions of calcium chloride containing 500 p.p.m. of calcium, which were 0·01N and 0·1N in respect to hydrochloric acid, gave 3 to 9 per cent. higher readings than a solution of only calcium chloride with the same content of the metal. Since the solutions were diluted 10 times (to 50 p.p.m. of calcium), no interference was observed.

Sodium and potassium increased strongly the emission radiation of

calcium. In order to investigate the effect of these cations, series of readings were taken to compare a pure solution of calcium chloride with solutions containing the same amount of calcium chloride, but with various quantities of sodium or potassium as chlorides added. The results are recorded in Table VI.

TABLE VI

INTERFERENCE OF SODIUM AND POTASSIUM WITH CALCIUM AT 554 Mμ

| | | | 200 p.p.m. Calcium | |
Interfering element	Weight ratio of interfering element to test element	Background radiation in scale divisions	Change of reading in per cent.	Change of emission of calcium in per cent.
Sodium	0	5·1	—	—
	1:10	5·5	0	0
	1:2	5·8	−5	−5
	1:1	6·0	−9	−10
	5:1	8·2	−18	−20
	10:1	11·0	−14	−20
Potassium	0	5·1	—	—
	1:10	5·2	+1	+1
	1:2	5·4	0	0
	1:1	5·9	−3	−4
	5:1	7·3	−15	−17
	10:1	9·1	−22	−25
Slit width		0·2 mm.		

At a concentration of 200 p.p.m. of calcium, 200 p.p.m. (or more) sodium depressed the light output of calcium. Similar results were obtained with potassium in a concentration of 200 p.p.m. or more. Table VI also shows the results of background readings obtained by measuring the light output, when solutions containing only the interfering metal were atomised. A pronounced increase of the background radiation was observed.

Calcium gave for the concentration range 0 to 1000 p.p.m. a slightly S-shaped calibration curve. At the 0 to 100 p.p.m. range a linear curve was obtained. Figure 9 (p. 524) shows these curves. The net readings are plotted against the concentrations.

EXAMPLES OF DATA FOR VARIOUS TYPES OF DETERMINATIONS

The procedures for the analyses of various types of saline solutions, which were arranged with the guidance of the results obtained by the investigations described above, were applied to about 100 solutions of varying composition. These solutions, prepared in Swedish pharmacies, might be a representative material of saline solutions for infusion used in this country. The solutions contained sodium as chloride, bicarbonate, phosphate, lactate and citrate, potassium as chloride, lactate and phosphate, and calcium as chloride. Solutions of the alkalis containing dextrose were also analysed. Determinations of the sodium and potassium salts based on their anions were made, and for the sake of comparison the results of these determinations are also recorded in the tables below. Sodium and potassium were not assayed with chemical methods. Tables VII to XIII show some of the results.

TABLE VII

SOLUTIO NATRII CHLORIDI PHYSIOLOGICA PHARM. SVECICA ED. XI

This solution contains 9·0 mg./ml.

Sample No.	Sodium chloride mg./ml. determined	
	Argentimetrically as chloride	Flame photometrically as sodium
1	8·9	9·0
2	8·9	9·0
3	8·9	9·0
4	8·9	9·0
5	8·1	8·1
6	8·9	9·0
7	9·1	9·2
8	9·3	9·4
9	8·7	8·8
10	9·0	9·0
11	9·1	9·1
12	9·1	9·1

TABLE VIII

INJECTABILE NATRII BICARBONATIS 1·3 PER CENT.

Sample No.	Sodium bicarbonate mg./ml. determined	
	Acidimetrically as bicarbonate	Flame photometrically as sodium
1	12·9	12·9
2	13·2	13·0
3	12·9	12·9
4	12·9	13·0
5	12·8	13·0
6	13·0	13·2
7	12·9	12·9
8	12·9	12·9
9	13·0	13·0
10	13·3	13·2
11	13·0	13·0

TABLE IX

SOLUTIONS OF SODIUM CHLORIDE WITH GLUCOSE

Sample No.	Glucose mg./ml.	Sodium chloride mg./ml.		
		Stated	Determined	
			Argentimetrically as chloride	Flame photometrically as sodium
1	16·3	6·0	6·0	6·0
2	18·0	6·0	6·1	5·9
3	23·4	4·5	4·4	4·5
4	24·5	4·5	4·5	4·6

TABLE X

SOLUTIONS OF SODIUM CITRATE

Sample No.	Sodium citrate mg./ml.		
	Stated	Determined	
		As citric acid	Flame photometrically as sodium
1	30·0	29·7	30·2
2	30·0	31·0	30·8
3	38·0	39·3	39·2
4	38·0	37·5	37·8

TABLE XI

SOLUTIONS OF SODIUM CITRATE WITH CITRIC ACID AND GLUCOSE

Sample No.	Glucose mg./ml.	Citric acid mg./ml.	Sodium citrate mg./ml.		
				Determined	
			Stated	As citric acid	Flame photometrically as sodium
1	23	7·5	21·0	21·6	22·2
2	22	6·3	21·0	20·0	20·6
3	23	8·0	22·0	22·2	21·7
4	24	8·3	22·0	22·3	22·0
5	16	5·0	13·2	12·9	13·4

Solutions containing different salts of sodium. The determinations of sodium in these solutions were all made in the following manner. The saline solution was diluted with distilled water to a sodium content of about 100 p.p.m. With wavelength scale at 589 mμ, transmission scale at 100, selector switch at 0·1 and slit at 0·05 mm., the flame spectrophotometer was standardised with a solution of sodium chloride containing 100 p.p.m. of sodium, and then the intensity of the unknown was determined. The standard solution was freshly prepared every day from a stock solution. A calibration curve for the 0 to 120 p.p.m. range was used to convert the ratio of readings to the ratio of concentrations.

Solutions containing both sodium and potassium. These solutions (Table XII) had the following compositions:—

Darrow's solution:—Potassium chloride 2·0 g., sodium chloride 3·0 g., 1 molar solution of sodium lactate 40 ml., sterile water 710 ml.

Butler's solution:—Sodium lactate 2·2 g., sodium chloride 0·6 g., potassium chloride 1·0 g., dipotassium phosphate 0·5 g., sterile water to 50 ml.

Inject. natrii et kalii lactat.:—Potassium chloride 3·8 g., 20 per cent. stock solution of sodium lactate 51·5 g., monosodium phosphate 0·3 g., disodium phosphate 1·2 g., sterile water 950 g.

FIG. 9. Calibration curves for calcium at 554 mμ. Linear co-ordinates.
A. 0 to 1000 p.p.m.
B. 0 to 100 p.p.m.

With the exception of Inject. natrii et kalii lactat. the sodium content of these solutions was determined as above described, using sodium chloride as standard. In the analysis of Inject. natrii et kalii lactat. the proper amount of potassium was included in the sodium standard

in order to compensate for the interference of potassium with sodium. Potassium was added as potassium chloride.

All determinations of potassium were carried out with self-compensating standards containing potassium and sodium in the same ratio as the test solutions. The standard solutions were prepared from sodium chloride

TABLE XII

Sample	Potassium mg./ml.		Sodium mg./ml.	
	Stated	Found	Stated	Found
Darrow's solution	1·40	1·37	2·£0	2·86
,, ...	1·40	1·38	2·£0	2·86
Butler's solution	15·0	14·7	13·7	13·7
,, ...	15·0	14·8	13·7	14·0
Inject. natrii et kalii lactat. ..	1·99	2·00	2·47	2·50

and potassium chloride. The saline solutions were diluted to a potassium content of 200 p.p.m. With transmission scale at 100 the flame spectro-photometer was standardised with the standard. The standard solutions were freshly prepared every day from stock solutions. The measurements were made at 768 mμ with a slit width of 0·2 mm. A calibration curve for the 0 to 220 p.p.m. range was used to convert the ratio of readings to the ratio of concentrations.

Solutions containing sodium, potassium and calcium

Ringer's solution:—This saline solution contained, in each 1., 9·0 g. of sodium chloride, 0·42 g. of potassium chloride and 0·24 g. of calcium chloride ($CaCl_2,6H_2O$).

Determination of sodium. The determination of sodium was made as described above, using sodium chloride as standard.

Determination of potassium. Sodium and potassium were included in Ringer's solution in the ratio of 16 to 1. The comparatively large content of sodium affected strongly the light output of potassium, and therefore a standard solution of potassium containing the proper amount of sodium had to be used for the standardisation of the instrument, and furthermore, the determination was carried out at a lower concentration range than usually. The saline solution was diluted 4 times. The solution, to be assayed, then contained 55 p.p.m. of potassium. The small amount of calcium did not affect the potassium light. The calibration curve was obtained in the following way. With the transmission scale at 100 and slit at 0·5 mm., the instrument was standardised with the standard solution. Then a number of solutions containing the same amount of sodium as the standard but with different quantities of potassium added were measured. No increase of the background radiation was observed at 768 mμ, when a solution containing only sodium in the same concentration as the standard (885 p.p.m.) was assayed.

The effect of small variations of the concentration of sodium on the determination of potassium in Ringer's solution was also tested. When the concentration of sodium was changed within ±10 per cent. no change of the readings of potassium was noticed, but the light output decreased

about 1 per cent. when the concentration of sodium was decreased 20 per cent. and increased about 1 per cent. when the content of sodium was increased 20 per cent. This result demonstrates the principle underlying the use of radiation buffers to reduce interference[13]. At higher concentrations of interfering materials small variations in the amount of the interferant have relatively little effect on the analysis.

Determination of calcium. Ringer's solution contained calcium, potassium and sodium in the ratio of 1 to 5 to 80. The possibilities of determining calcium flame photomet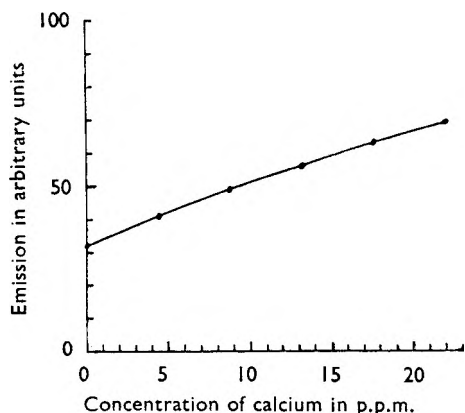rically in the presence of the relatively large amounts of sodium and potassium were investigated. The saline solution was diluted with an equal volume of water ($= 22$ p.p.m. of calcium) and the measurements were carried out at 554 mμ with slit at 0·4 mm. The calibration curve (Fig. 10) was obtained in the same way as the potassium curve above. With transmission scale at 100 and slit at 0·4 mm., the instrument was standardised with a standard solution of the same composition as the test solution.

FIG. 10. Calibration curve for calcium in Ringer's saline solution.

Then a number of solutions containing the same amount of sodium and potassium as the standard but added different quantities of calcium were measured. The concentration is plotted against the net reading (the radiation of the flame fed with pure water is subtracted).

As Figure 10 shows, the relatively high concentration of the alkali metals caused a pronounced increase of the background radiation. Therefore the ratio between background and band radiation of calcium was unfavourably low. The reproducibility of the readings was less satisfactory. The accuracy of these measurements could be estimated to about ±5 per cent. Further, it was found that a little change in the content of alkali metals affected the reading. Thus an increase or a decrease of the concentration of the alkali metals of 10 per cent. increased or decreased the reading about 5 per cent., giving an error of ±10 per cent. in the determination of calcium. This effect was due to a change of the background radiation. The background radiation made up about 50 per cent. of the reading obtained (Fig. 10). This error can of course be reduced. At first, flame analyses are made of sodium and potassium, and then the proper amounts of the metals shown in these analyses are added to the standard for the determination of calcium.

In Table XIII results of some analyses of Ringer's solutions are recorded. Calcium was also determined by chemical means with ethylenediamine-tetra-acetic acid[4].

With the application of flame photometry the assay of saline solutions for infusion could be organised on a rational basis in this laboratory. The cost of this analytical control could be reduced considerably by using these rapid and labour-saving methods. It is evident, too, that

TABLE XIII

SOLUTIO RINGERI PHARM. SVECICA ED. XI

Sample No.	Sodium as NaCl mg./ml.	Potassium as KCl mg./ml.	Calcium as $CaCl_2,6H_2O$ mg./ml. determined	
			Volumetrically	Flame photometrically
1	9·0	0·42	0·243	0·25
2	9·0	0·42	0·236	0·25
3	9·0	0·42	0·243	0·25
4	9·0	0·42	0·246	0·24

flame photometry can successfully be applied to many other materials in pharmaceutical analytical work. The Swedish Pharmacopœia Commission has recently proposed flame photometry for the assay of saline solutions for infusion.

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SOME QUANTITATIVE STUDIES ON A BACTERIAL PYROGEN

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THE quantitative estimation of bacterial pyrogens has not, until recent years, been seriously attempted. This is mainly because interest in bacterial pyrogens has hitherto centred in ensuring their absence from solutions for injection, rather than in seeking to estimate the amount present. All the qualitative tests of any value are biological—inadequate knowledge of the chemistry of these substances having precluded the development of chemical tests. The work about to be described represents an attempt to establish a quantitative approach to the study of bacterial pyrogens, and is based upon the methods used in the older qualitative tests.

Biological tests for the presence or absence of pyrogens depend on whether the test material, when administered intravenously to rabbits or dogs, produces a rise in temperature[1,2,3,4,5,6], or a change in the number of circulating leucocytes[7,8,9,10]. But only those tests dependent on the temperature response of the rabbit have come into general use.

An essential prerequisite for quantitative studies on the temperature response of the rabbit to pyrogens is an active and stable pyrogen preparation. A number of impure solutions, and dry extracts, containing bacterial pyrogens have been described by previous workers[5,8,11,12,13,14,15,16], some of whom have attempted, with varying success, to make quantitative observations upon them. Some have claimed that impure solutions are stable[5,21,22], but others have shown that this is not always so[15]. Others again have stated—but without submitting their evidence—that even highly purified preparations are unstable[17]. In the absence, therefore, of any generally accepted stable pyrogen preparation, it was decided to prepare and study, in the first instance, a crude, dry, pyrogen-containing extract.

Part I of this paper describes the preparation of this extract, and the different methods used to measure the temperature response of rabbits to it. Part II gives an account of the various experimental studies subsequently undertaken. These were carried out over a period of 5 years and are described in the order in which they were undertaken. As the successive studies constitute a logical series the results of each are fully discussed before proceeding to the next.

PART I

MATERIALS AND METHODS

Preparation of the Pyrogen Test Material

The pyrogen test preparation is a dry powdered material obtained from *Proteus vulgaris*. The method of preparation was essentially that of Robinson and Flusser[12] modified so as to give a good yield of active

material without the use of elaborate purification procedures. It makes use of the fact that bacterial pyrogen is soluble in water but insoluble in acetone[12], and that greater amounts of pyrogen appear to be produced when the bacteria are grown on natural rather than on purely synthetic media[18,19].

1 ml. of a 24-hour subculture of a strain of *P. vulgaris* (N.C.T.C. No. 6821) grown in standard Lab-Lemco medium, was diluted with 7 ml. of normal saline solution, and the whole used to inoculate a culture bottle containing solid agar medium. This medium was prepared by adding 3 per cent. of agar to the standard Lab-Lemco medium. The large volume of the inoculum suffices to moisten the surface of the medium and subsequent emulsification of the growth is facilitated. 24 bottles were used for preparing a batch of material, the medium in each bottle having an effective surface area of about 200 sq. cm. The inoculated culture bottles were incubated for 48 hours at 37° C. At the end of this period the luxuriant bacterial growth was collected as an emulsion in the smallest possible volume of normal saline solution. To keep the emulsion volume to a minimum 3 successive quantities of 20 ml. of normal saline solution were used to emulsify the growth in the first bottle; these 3 emulsions were then used, in the same order, to emulsify the growth in the second bottle; the process being continued from bottle to bottle. When the first emulsion contained approximately 9×10^9 organisms/ml. its further use was found unprofitable, so it was reserved, and the second and third much weaker ones were used as the first and second, respectively, and a fresh 20 ml. of saline solution was introduced as the third. This procedure was continued until the whole of the growth from the 24 bottles had been emulsified. The volume of the pooled emulsions from a batch was approximately 175 ml. and contained about 6×10^9 organisms/ml. The pooled emulsion was gently agitated with glass beads for 1 hour, incubated for 24 hours at 37° C., again agitated for 1 hour, and reincubated for a further 24 hours. The bacteria were then removed by centrifuging at 6000 r.p.m. for 2 hours, and filtering the supernatant liquor through a seitz filter. The filtrate, which was pale buff in colour and slightly opalescent, was immediately poured into 10 volumes of acetone containing 0·4 per cent. of acetic acid[12]. A colloidal precipitate formed immediately. This soon coagulated and was removed after 24 hours on a sintered glass filter. Since the colour of the precipitate changes from cream to brown on exposure to the atmosphere, the filtration was carried out rapidly and the precipitate immediately transferred to a vacuum desiccator. The significance of the colour change is not known but it seemed advisable to reduce atmospheric exposure to a minimum. After drying for 7 days the precipitate became hard and brittle. The exterior was brown but the interior, on fracture, was a pale buff. Several batches have been prepared by this method and the mean yield was 1·78 g. The dried material from several batches was reduced to No. 90 powder, mixed, and packed in dry glass ampoules which were sealed by fusion of the glass. This material constitutes Pyrogen Test Preparation No. 1. It has been stored between

0° and 2° C. Preliminary tests established that this preparation contained a potent pyrogen.

Methods of Measuring Pyrogenic Response

Two different methods were used, in each of which the effect of Pyrogen Test Preparation 1 on the temperature of rabbits weighing more than 1·75 kg. was measured.

Method 1. The rabbits, deprived of food for 12 to 18 hours, were weighed and placed in holders at 09.30 hours. The holder—a modified form of a type previously described[20]—consisted of a base board to which was attached an adjustable neck stock and body straps. The rabbit was placed prone on the base board, and secured by the neck-stock and the body straps, so that the hind legs overhung the end of the base board. The rectal temperature of rabbits restrained in this way can be taken by clinical thermometers with minimum disturbance, and it is only on exceptional occasions that rabbits show resentment to the restraint. In each experiment the temperature of each rabbit was taken at 11.15 hours and 11.45 hours, by inserting a thermometer in the rectum to a standard depth of 6·0 cm., and the mean of the two readings was taken as the normal for that rabbit on that occasion. The appropriate dose of Pyrogen Test Preparation 1, dissolved in 5 ml. of normal saline solution, was given through the ear-vein at 12.00 hours. At 12.15 hours a clinical thermometer was inserted in the rectum of each rabbit and left in position for 4 hours. The thermometers were retained in position by means of light elastic slings. At the end of this period they were removed and the temperature recorded on each was noted. The thermometers were re-inserted and the temperature of each animal again taken to ascertain whether it had passed the maximum. Temperature readings were continued at intervals until the remaining increase in temperature was less than half the difference between the normal and the maximum recorded. The difference between the pre-injection normal and the post-injection maximum temperature in each rabbit was used as the measure of the response.

Method 2. Electrical thermometry was used in this method so it was possible to use a simpler holder and to follow the temperature changes in detail.

Rabbits, deprived of food for 12 to 18 hours were weighed and placed in holders at 09.30 hours. The holder consisted of a base board to the front of which was attached an inclined board in which was cut a vertical slot. The rabbit was placed in the holder in a normal sitting posture with its neck in the slot. Apart from the neck-slot no restraint was placed on the rabbit. The base board was covered with wire mesh to provide a secure foothold. The thermometers were inserted in the rectum to a depth of 6 cm. and each rabbit's temperature was recorded at 12-minute intervals from 11.00 hours onwards. The mean of the first 5 temperature readings for each animal was taken as the normal for that animal on that occasion. The appropriate dose of Pyrogen Test Preparation 1 dissolved in 5 ml. of normal saline solution was given through the ear-vein at 12.00 hours. Temperature readings were

continued until the temperature for each rabbit had passed its maximum and had declined to a value less than half-way between the pre-injection normal and the post-injection maximum. The response was measured in two ways: (a) as for method 1; (b) as the total temperature effect.

In the latter, the differences between the individual observations of the post-injection temperature and the normal for each animal were plotted against the time from injection until the temperature had passed its maximum and returned to a value half-way between the pre-injection normal and the post-injection maximum. A line perpendicular to the abscissa was drawn through this point. The measure of the response was obtained from the area enclosed by the graph, abscissa and perpendicular (see Fig. 1). The use of an arbitrary criterion of when the
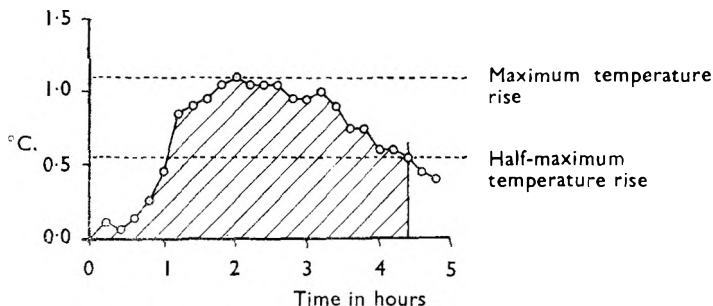

Fig. 1. Showing a typical temperature change following intravenous administration of Pyrogen Test Preparation No. 1 in a rabbit. Ordinate: temperature rise above normal. Abscissa: time in hours from injection. The shaded portion gives the measure of the total temperature effect. For further explanation see text.

response had terminated is necessary, because, no matter for how long the temperature readings continue to be taken, the temperature rarely returns to the pre-injection value. Extensive experiments established that there is a diurnal variation in the normal temperature of rabbits subjected to the experimental procedure, being minimal at 12.00 hours, and rising about $0.2°$ C. by 18.00 hours. This variation occurs in control experiments whether an injection of pyrogen-free saline solution is given or not. Hence, as all injections were given at 12.00 hours the "true" normal temperature for each rabbit gradually increases as the experiment continues. Whilst the mean increase with time is known, that of individual rabbits cannot be predicted with sufficient certainty, at any particular time, for it to be taken into account in determining the end of the pyrogenic response.

PART II

EXPERIMENTAL STUDIES ON PYROGEN TEST PREPARATION No. 1

In seeking to establish quantitative methods for the estimation of bacterial pyrogen it is clear that two points have first to be determined. The first is the relationship between dose and response of the pyrogen preparation; and the second, whether the pyrogen preparation, and

solutions of it, are stable. The first two studies about to be described were undertaken to obtain information on these points in relation to Pyrogen Test Preparation No. 1. It was apparent from the very variable results obtained that unknown factors of fundamental importance were affecting the responses to pyrogen. Attempts were made to ascertain the nature of these factors and these attempts form the subject-matter of the two further studies described here.

1. *Relationship of Dose to Response of Rabbits*

Several series of experiments designed to study the dose/response curves, when the response is measured in the two ways described in Part I, have been done. For reasons which appear later in the paper the results are open to such criticism as to render them useless for the purposes originally envisaged. While there is thus no point in describing them, they nevertheless establish that the magnitude of the temperature response increases with increasing doses, and that the minimum effective dose of this preparation in the rabbit is less than 0·02 μg./kg.

2. *Stability of Solutions*

3 solutions, each containing 200 μg./ml. of Pyrogen Test Preparation 1, were prepared at widely different times and their activities compared after varying periods of storage between 0° and 2° C. The comparisons of activity were made by administering standard volumes of each solution per kg. of body weight and determining whether there were any significant differences between the mean responses to the different solutions. As all 3 Solutions were originally made up to contain the same amount of Pyrogen Test Preparation 1, equal volumes of the solutions should be equi-active if the preparation, and solutions of it, are stable. Two series of comparisons were made:—(i) Solutions A and B, 17 months and 3 months, respectively, after preparation. (ii) Solutions A, B and C, 32 months, 18 months and 1 month, respectively, after preparation. As each series of comparisons took several weeks to perform the age of each solution is given as its age halfway through the experimental series.

(i) *Comparison of solutions A and B*

12 rabbits, which had not been given pyrogen for several months, were used for the experiments. They were divided into two equal groups containing equal numbers of each sex, and the experiments were so arranged that on the first occasion solution A was given to one group and solution B to the other, the solutions being interchanged between groups on the second occasion. 3 such crossover tests were made using the same group of rabbits, the experiments being done at 3- or 4-day intervals. The volume of solution given to each rabbit was 0·005 ml./kg. In order to give such a small dose a dilution of each solution was used. These dilute solutions were prepared just before the injections were given, on each experimental day. Experimental method 1 was used.

The experimental data, and an analysis of them, are given in Tables I and II. As the analysis showed no significant differences between the

various interaction mean squares, their sums of squares have been pooled for the calculation of the error mean square. From the large probability for the test of significance between solutions it is concluded that there is no significant difference between their activities. The differences between rabbits and between tests are judged to be significant.

TABLE I

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A AND B OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 17 AND 3 MONTHS RESPECTIVELY

| | Response measured as maximum temperature rise in ° C. above normal | | | | | |
| | Test I | | Test II | | Test III | |
Rabbit No.	Solution A	Solution B	Solution A	Solution B	Solution A	Solution B
5	0·27	0·61	0·17	0·56	0·39	0·11
6	1·44	2·70	1·12	0·78	0·61	1·00
10	0·72	0·94	0·95	0·22	0·33	0·44
41	1·22	0·83	0·78	0·67	0·44	0·67
27	0·44	0·56	0·00	0·56	1·17	0·56
20	1·12	0·83	2·00	1·17	0·50	1·22
16	0·72	0·95	0·17	0·56	0·56	0·11
29	0·33	0·44	0·06	0·06	0·33	0·06
34	0·61	1·00	0·78	0·50	0·67	0·50
11	0·27	0·33	0·33	0·95	0·89	0·78
17	1·38	1·38	0·00	0·27	0·39	0·44
43	0·89	0·61	0·38	1·12	1·78	0·72
Mean response to each solution in each test	0·78	0·93	0·56	0·62	0·67	0·55
Mean response in each test	0·86		0·59		0·61	
Mean response to each solution	Solution A = 0·67 Solution B = 0·70					

TABLE II

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE I

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	"t"	P
Main effects:						
Between solutions ..	0·01389	1	0·01389		0·2957	0·7–0·8
Between rabbits ..	6·09457	11	0·55405	3·4883		<0·001
Between tests ..	1·06470	2	0·53235	3·3516		0·05–0·01
Interactions:						
(Error)	9·05371	57	0·15883			
Totals	16·22687	71				

(ii) *Comparison of solutions A, B and C*

6 rabbits of each sex, which had not been given pyrogen during the previous 8 months, were used. Each rabbit was given each of the 3 solutions in a complete test; and the whole group was used in 3 such tests. The solutions were given randomly within the restrictions that 2 rabbits of each sex received the same treatment on each day; each rabbit received each treatment once in a complete test; the order in which the solutions were administered differed from day to day, and from animal to animal. The experimental design is given in Table III. The volume of the appropriate solution given in each experiment was

0·005 ml./kg.; fresh dilutions of each solution being prepared on each day as described above. Method 2 was used for these experiments.

The data obtained when the response is measured in terms of the maximum elevation in temperature are given in Table IV. From the mean response to each solution given in Table IV, and the analysis

TABLE III

ORDER IN WHICH SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 WERE GIVEN DURING EXPERIMENTS TO COMPARE THE ACTIVITIES OF THE 3 SOLUTIONS AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

Sex		♂						♀				
Rabbit No.		6	9	11	20	27	43	5	8	10	34	51	52
Test	Day												
I	1	C	C	A	B	A	B	C	B	A	C	A	B
	2	B	A	C	A	B	C	A	C	C	B	B	A
	3	A	B	B	C	C	A	B	A	B	A	C	C
II	1	A	B	B	A	C	C	B	C	B	A	C	A
	2	C	C	A	B	B	A	A	B	C	B	A	C
	3	B	A	C	C	A	B	C	A	A	C	B	B
III	1	C	C	A	B	B	A	A	B	B	C	C	A
	2	B	A	C	C	A	B	C	A	C	B	A	B
	3	A	B	B	A	C	C	B	C	A	A	B	C

TABLE IV

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

		Response measured as maximum temperature rise in ° C. above normal								
Solutions		A			B			C		
Tests		I	II	III	I	II	III	I	II	III
Rabbit No. 6		1·03	0·75	0·78	1·18	1·03	0·73	1·65	1·15	0·83
9		0·98	0·73	0·95	0·98	0·80	0·80	1·30	0·53	0·80
11		0·75	0·80	0·80	0·85	0·90	0·60	0·85	0·88	0·88
20		1·38	0·95	0·98	1·80	0·90	1·00	1·40	1·28	1·20
27		1·75	0·63	0·70	1·45	0·40	0·48	0·95	1·15	0·78
43		0·90	0·90	1·25	1·45	1·08	0·90	1·20	1·43	0·85
5		0·90	0·50	0·68	0·45	0·68	0·20	1·20	0·65	0·78
8		1·00	1·00	0·80	1·30	0·80	0·93	1·08	1·10	1·05
10		1·15	0·80	0·68	1·03	1·00	0·90	1·30	0·90	0·93
34		0·58	0·55	0·53	0·78	0·58	0·55	1·10	0·75	0·83
51		2·10	1·80	1·55	2·13	1·60	1·45	1·90	1·93	1·38
52		1·20	0·88	1·13	1·50	0·90	0·78	1·15	0·98	1·05
Mean for each solution in each test		1·14	0·86	0·90	1·24	0·89	0·78	1·26	1·06	0·95
Mean for each solution		0·97			0·97			1·09		

TABLE V

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE IV

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between solutions	0·3432	2	0·1716	4·4113	0·01–0·05
Between rabbits	8·0683	11	0·7335	18·856	<0·001
Between tests	2·3477	2	1·1739	30·1774	<0·001
Interactions:					
(Error)	3·5763	92	0·0389		
Totals	14·3355	107			

in Table V, it is clear that solutions A and B are equi-active, whilst solution C is significantly more active than either A or B. Again there are very significant differences between rabbits and between tests. The data obtained when the response is measured in terms of the total temperature effect are given in Table VI. The tests of significance given in Table VII indicate that there are no significant differences between solutions, or between rabbits, but that the differences between tests are significant.

TABLE VI

DATA FROM EXPERIMENTS TO COMPARE THE ACTIVITIES OF SOLUTIONS A, B AND C OF PYROGEN TEST PREPARATION NO. 1 AFTER STORAGE FOR 32, 18 AND 1 MONTHS RESPECTIVELY

| | Response measured as the total temperature effect (see Fig. 1) | | | | | | | | |
| Solutions | A | | | B | | | C | | |
Tests	I	II	III	I	II	III	I	II	III
Rabbit No. 6	2·07	1·17	2·50	1·99	2·56	1·66	5·69	2·58	1·95
9	1·83	2·35	2·25	2·23	1·88	2·19	5·19	1·19	2·76
11	2·21	2·35*	3·63*	1·79	2·95	2·11*	1·68	3·09*	2·99*
20	2·89	1·80	3·31*	5·36	1·96	2·78	2·62	2·52	2·96
27	4·93	2·63*	2·24*	2·89	1·17	2·33*	2·15	2·94	2·19*
43	1·83	1·93	2·81	5·55	2·78	2·44	2·14	3·48	1·84
5	1·65	1·55*	2·76	0·85	2·18	0·59	4·65	1·12	2·73*
8	2·20	2·70	2·04	3·81	1·95	2·16	2·31	2·83	3·30
10	3·03	1·73	1·61	2·08	2·37	2·71	3·29	2·01	1·99
34	1·08	1·20	1·47	1·56	1·25	1·49	4·00	1·88	2·75*
51	3·74	2·17	1·88	3·03	2·01	1·66	2·60	6·40	2·07
52	2·47	1·44	2·15	3·70	1·38	1·61	1·96	1·66	1·92
Mean for each solution in each test	2·49	1·92	2·39	2·89	2·04	1·98	3·02	2·64	2·46
Mean for each solution	2·27			2·30			2·71		

* These observations are of doubtful accuracy because the temperature was returning to normal so slowly that the duration of action could not be precisely determined.

TABLE VII

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE VI

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between solutions	5·4612	2	2·7306	2·8770	0·1–0·05
Between rabbits	11·2710	11	1·0246	1·0795	>0·2
Between tests	9·5116	2	4·7556	5·0106	0·01–0·001
Interactions:					
(Error)	87·3253	92	0·9491		
Totals	113·5691	107			

Discussion. When the response was measured in terms of the maximum elevation in temperature, no differences in the activity of solutions A and B were found in either of the two series of tests; but solution C was found to be significantly more active than either of the others. An examination of the ages of each of the solutions in the two tests reveals that solution B was already 3 months old when it was first compared with solution A, whereas solution C was only 1 month old when it was compared with solutions A and B. This suggests that loss in activity occurs in solutions immediately after preparation, but that the rate of inactivation rapidly diminishes, so that after 3 months it has become negligible. Whether the loss in activity is due to chemical decomposition of the pyrogen,

or to physical factors, is not known; but as the solutions were stored in 2-ml. glass ampoules some adsorption on the glass may have occurred.
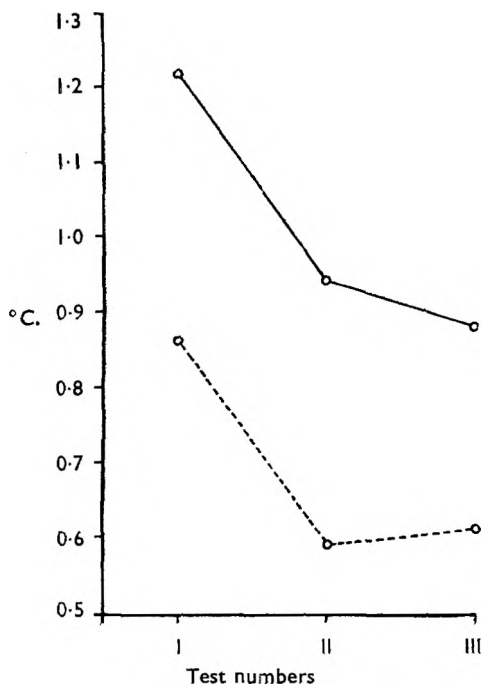

Calculation shows that for the adsorption of half the Pyrogen Test Preparation 1 contained in an ampoule, the concentration at the surface of the glass would only be 1.5×10^{-5} g./sq. cm., so that adsorption may account for the whole of the observed loss in activity.

In the second series of experiments the response was also measured in terms of the total temperature response. No significant differences between the activities of the 3 solutions were found by this method. But these results are less valuable than those obtained when the response was measured in terms of the maximum elevation of the temperature above normal, because the standard deviation obtained when the total temperature response was measured was ± 44 per cent. of the mean as against only ± 20 per cent. when the maximum temperature elevation was used. Further-

FIG. 2. Showing the mean responses in each test of two series of experiments comparing the activities of Pyrogen Test Preparation No. 1 solutions after storage. Ordinate: mean temperature rise above normal in ° C. Abscissa: test number. The broken line shows the mean responses when solutions A and B were compared using method 1 (see Table I): solid line those when solutions A, B and C were compared using method 2 (see Table IV).

more, temperature readings must be taken for more than 6 hours after the injection in order to measure the total response, and quite often the temperature returns to normal so slowly that it is not possible to determine the end of the response with any precision (see Table VI). There is no evidence in these experiments of any loss in the activity of the Pyrogen Test Preparation No. 1 stored in the dry form.

3. *Effect of Repeated Administration on the Response of the Rabbit*

The finding that there were consistent and significant differences in the response from test to test in both series of experiments (see Tables II and V) was unexpected, so the data were critically examined to see whether the cause of this could be determined.

The mean response for each test has been plotted in Figure 2 and it is obvious that in each series of experiments there is a progressive reduction

in the response as test succeeds test. The regularity of this reduction in both series of tests seems to preclude any possibility of it being attributable to random variation. To determine whether the reduction was also consistent from day to day, the daily means from the second series of experiments were plotted (Fig. 3) —a procedure which is justified in virtue of the experimental design. The regular reduction during the first few days, after which the response is fairly constant, is very striking. An examination of the data shows that the effect is common to all the rabbits.

The most obvious explanation of the regular reduction in response is either (a) that solutions

FIG. 3. Showing the mean responses to a standard quantity of Pyrogen Test Preparation No. 1 given repeatedly to each of two groups of rabbits at 3- or 4-day intervals. Ordinate: mean temperature rise above normal. Abscissa: experimental day. The solid line shows the mean response, on each experimental day, in the series of experiments comparing by method 2, the activities of solutions A, B and C: it may be derived, by the use of Table III, from the data in Table IV. The broken line shows the mean responses on each experimental day for the data given in Table IX.

rapidly lose their activity, or (b) that rabbits exhibit tachyphylaxis to pyrogen. The data were examined to determine whether either of these views was tenable.

The mean responses to each solution in each test in the second series of experiments are given in Table IV and have been plotted in Figure 4. It is obvious from Figure 4 that a similar reduction in response occurs for each solution. Hence, if this is due to progressive loss in activity,

TABLE VIII

TESTS OF SIGNIFICANCE FOR DIFFERENCES BETWEEN MEAN RESPONSES TO SOLUTIONS A AND B IN THE FIRST TEST AND SOLUTION C IN THIRD TEST IN EXPERIMENTS COMPARING THE ACTIVITIES OF THE 3 SOLUTIONS AFTER DIFFERENT PERIODS CF STORAGE. DATA TAKEN FROM TABLES IV AND V

Solution A, first test
Solution C, third test $\Big\}\, t_{(92)} = \dfrac{1 \cdot 14 - 0 \cdot 95}{\sqrt{0 \cdot 0389(^{1}/_{12} + {}^{1}/_{12})}} = 2 \cdot 3567 \quad P = 0 \cdot 02$

Solution B, first test
Solution C, third test $\Big\}\, t_{(92)} = \dfrac{1 \cdot 24 - 0 \cdot 95}{\sqrt{0 \cdot 0389(^{1}/_{12} + {}^{1}/_{12})}} = 3 \cdot 5971 \quad P = <0 \cdot 001$

then each solution must be losing activity at approximately the same rate. The mean ages of solutions A, B and C, at the time of the tests, were 31 months, 18 months and 1 month, respectively. Now if these 3

solutions, of widely differing ages but stored under identical conditions, were all losing activity at the same rate at the time their activities were compared, it is reasonable to infer that they have all been losing activity continuously at the same rate throughout their lives. But, if this is so, the response elicited by solutions A and B during the first test would be expected to be less, not greater, than the response to solution C in the third test. That the responses to solutions A and B in the first test were significantly greater than the response to solution C in the third test is shown in Table VIII. Thus the data do not support the hypothesis that the reduction in response in successive experiments is due to loss in activity.

The data are, however, compatible with the hypothesis that the effect is due to a progressive reduction in sensitivity when pyrogen is given repeatedly to rabbits.

To confirm this conclusion another group of 12 rabbits, which had never before been given pyrogen, was submitted to a series of experiments in which the same dose of solution B as had been used in the earlier experiments was given. The experiments were performed at 3- or 4-day intervals, and method 2 was used to determine the temperature responses. The data and analysis of variance are given in Tables IX and X. There are again significant differences between the responses on different experimental days. That these differences are due to a progressive

TABLE IX

DATA FROM EXPERIMENTS TO DETERMINE THE EFFECT OF REPEATED ADMINISTRATION OF A STANDARD DOSE OF 0·005 ML./KG. OF SOLUTION B OF PYROGEN TEST PREPARATION NO. 1 AT INTERVALS OF 3 OR 4 DAYS ON THE TEMPERATURE RESPONSE OF RABBITS

	Response measured as maximum temperature rise in ° C. above normal							
Experimental days	1	2	3	4	5	6	7	8
Rabbit No. 010	1·23	1·15	0·95	1·03	0·73	0·88	0·70	0·70
259	1·85	1·60	1·40	1·35	0·95	1·13	0·83	0·80
277	1·53	1·40	1·75	1·78	1·60	1·58	1·38	1·18
453	1·33	0·95	1·18	0·95	0·90	0·60	0·88	0·63
468	1·45	0·90	1·10	0·88	1·05	0·75	0·88	0·60
662	1·78	1·50	1·38	1·48	1·20	1·33	1·15	0·85
274	1·90	1·55	1·43	0·93	0·73	0·93	0·80	1·05
457	1·23	1·00	1·35	1·08	0·95	0·83	0·73	0·63
458	0·95	0·95	1·15	0·78	0·80	0·63	0·73	0·58
460	1·90	1·63	1·18	1·10	0·88	1·10	0·93	0·78
467	1·60	1·70	1·75	1·23	1·08	1·15	1·18	0·78
651	1·25	0·70	0·80	1·00	0·38	0·60	0·70	0·13
Means	1·50	1·25	1·29	1·13	0·94	0·96	0·91	0·73

TABLE X

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE IX

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Main effects:					
Between experimental days	5·2870	7	0·7553	25·6034	<0·001
Between rabbits	5·1943	11	0·4722	16·0068	<0·001
Interaction:					
(Error)	2·2727	77	0·0295		
Totals	12·7540	95			

reduction in the mean response is evident from Figure 3. Examination of the data from individual rabbits again shows that the effect was common to all the rabbits (see Table IX).

These confirmatory experiments were begun 2 months after finishing the second series of comparisons of activity. Hence, if the reduction in response found in the second series was due to loss of activity in the solutions, the mean response on the first day of the confirmatory series should not be greater than the mean response to solution B on the
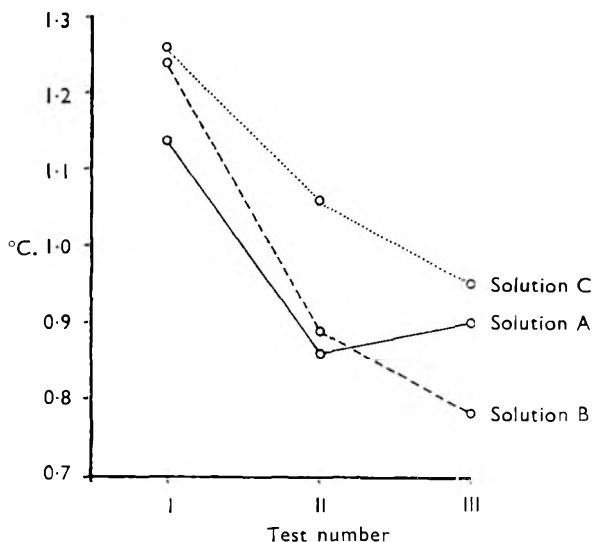
FIG. 4. Showing the mean response in each of the 3 tests of the series comparing solutions A, B and C by method 2 (see Table IV). Ordinate: mean temperature rise above normal. Abscissa: test numbers.

last day of the second series, and should be significantly less than the response to solution B on the first day in the second series. On the other hand, if the reduction in response is due to reduction in sensitivity resulting from the repeated administration of pyrogen, then the mean responses on the first day in both series should not differ significantly; but the mean response on the first day of the confirmatory series should be significantly greater than the mean response on the last day of the previous series. The appropriate data have been taken from Tables IV and IX and are given in Table XI. The analysis of variance reveals that there are significant differences between days, but it is evident that the mean responses on the first day in each series do not differ significantly, whereas the mean response on the last day of the second series is significantly less than either of these obtained on the first day of either series. It is thus impossible to escape the conclusion that there is a significant reduction in the response to bacterial pyrogen when it is given repeatedly at short intervals to the same rabbits.

When the experimental methods used in this work were first defined it was assumed, from the reports of previous workers, that a constant response could be repeatedly elicited by a standard dose of bacterial pyrogen. From the large body of data collected in the U.S.P. Commission's *Collaborative Studies on Pyrogens*[5,11,21], the writers noted that

"there was a slight trend to a lower response" when a large dose of pyrogen was given repeatedly, but that no such effect was observed when a sequence of different doses was given. That the "trend" was regarded as unimportant is shown by the fact that the test designed during that work and subsequently included in the U.S.P., permits the repeated use of the same rabbits. Evidence has been given[17] from which it was

TABLE XI

Observation	Response in ° C. to 0·005 ml./kg. of solution B		
	Data reconstructed from Tables III and IV (Rabbits previously used but rested for 8 months)		Data from Table IX (New rabbits)
	Test I First day	Test III Last day	First day
1	1·80	0·80	1·23
2	1·45	0·60	1·85
3	1·30	1·45	1·53
4	1·50	0·20	1·33
5			1·45
6			1·78
7			1·90
8			1·23
9			0·95
10			1·90
11			1·60
12			1·25
Means	1·51	0·76	1·50

Analysis of variance

Items	Sums of squares	Degrees of freedom	Mean squares	Variance ratios	P
Between days	1·6977	2	0·8489	6·9355	0·01–0·001
Within days	2·0808	17	0·1224		
Total	3·7785	19			

suggested that the reduction in response observed in the U.S.P. studies was due, at least in part, to the rabbits becoming accustomed to the procedure, the implication being that rabbits thoroughly experienced in the procedure would not exhibit this trend. Wylie and Todd[15], on the other hand, found no change in the magnitude of the response when rabbits were used repeatedly over long periods.

Such conclusions are not in agreement with ours*, but insufficient data are presented by some of these workers[15,17] to enable one to judge whether their results are capable of a different interpretation. Welch et al.[21], however, in the U.S.P. studies, present a comprehensive summary of their data, analysis of which reveals that what they describe as a "trend" is in fact a very highly significant shift in response level. Furthermore, their statement that after an interval of 3 to 4 weeks the "response was approximately equal to the initial" is an over-statement, for it was intermediate between the first and the later responses, and still significantly less than the first one. Full recovery of sensitivity clearly does not occur

* *Note added in press:* The writer's attention has been drawn to a paper by Beeson (*J. exp. Med.*, 1947, **86**, 29) who reports that the response, when measured as the total temperature effect, suffers a progressive reduction when the same rabbits are repeatedly used. This seems to be the first reference to this phenomenon.

in 4 weeks. That the effect is only temporary, however, is established by our work, for rabbits which had been given much pyrogen were found after a rest of 8 months to give the same response as rabbits which had never had any (see Table XI).

The fact that each succeeding response is affected by the previous experimental history of each rabbit does not invalidate any of the conclusions drawn in this paper, because in the experiments described here the doses used did not vary widely, and because the design of the experiments was such that the magnitude of the variation from this source was known. In experiments where a series of widely differing doses is used a very different situation exists, and the drawing of any but the most general conclusions from such work is open to criticism. For this reason our own early results on the dose response curve of rabbits to pyrogen have been discarded as virtually useless, and new studies on this subject, which make use of the facts just described, and of others yet to be discussed, are in progress at the time of writing.

4. Effect of Varying the Experimental Method on the Magnitude of the Rabbit's Response to Pyrogen

In method 1 the holders used are such that the rabbits are subjected to severe restraint. These holders were adopted because it has been stated that in completely immobilised animals the normal temperature settles to a lower steady value than that otherwise obtained and that the magnitude of the response to pyrogens is correspondingly increased[17]. Clinical thermometers were used in method 1 as a temporary measure, pending the availability of electrical thermometers. In the meantime, however, doubts had arisen as to the advisability of subjecting the rabbits to such severe restraint. Hence, when the electrical thermometers were ready, the opportunity of using holders in which the rabbits were subjected to less restraint was utilised. These are the conditions described in method 2. Thus the methods differ in two major respects—in the degree of restraint imposed on the rabbits, and in the type of thermometer used.

From different experiments with each method it appears that the magnitude of the response is considerably greater with method 2. This is illustrated in Figure 2, which shows the results for two series of experiments in each of which a different method was used, although the doses given were the same. It was not, of course, known how much each of the two factors which had been deliberately changed was contributing to the apparent difference in the magnitude of the response. Furthermore, it was not known if variations in other unknown factors might also have contributed to the difference, since the various experiments using the two methods were done at widely separated times. It was therefore desirable to determine, by experiments conducted simultaneously, whether the apparent difference was real, and, if so, what was the contribution of each of the two variable factors to it.

There are four possible ways in which the two factors can be combined: (a) rigid restraint + clinical thermometers; (b) slight restraint + electrical

TABLE XII

DATA FROM EXPERIMENTS TO DETERMINE THE MAGNITUDE OF THE RABBIT'S TEMPERATURE RESPONSE TO PYROGEN TEST PREPARATION NO. 1 WHEN DIFFERENT EXPERIMENTAL METHODS ARE USED

Groups of rabbits	Rabbit No.	I Experimental days			II Experimental days			III Experimental days			Totals of residual sums of squares
		1	2	3	4	5	6	7	8	9	
1 ♀	71	(a) 0·72	(c) 1·63	(b) 1·43	(b) 1·22	(c) 1·47	(a) 0·56	(a) 1·00	(c) 0·71	(b) 1·03	
	72	(c) 0·91	(b) 1·59	(a) 0·56	(a) 0·89	(b) 1·35	(c) 1·04	(b) 1·32	(a) 0·78	(c) 0·97	
	73	(b) 1·39	(a) 1·28	(c) 0·73	(c) 0·73	(a) 0·78	(b) 0·70	(c) 0·87	(b) 1·27	(a) 0·39	
Residual sums of squares		0·0699			0·0374			0·0170			0·1243
2 ♀	74	(b) 1·26	(a) 1·11	(c) 1·15	(a) 0·39	(b) 1·55	(c) 1·09	(c) 1·34	(b) 1·37	(a) 1·11	
	75	(c) 0·83	(b) 1·75	(a) 1·22	(b) 1·16	(c) 1·07	(a) 0·78	(a) 1·00	(c) 0·89	(b) 1·17	
	76	(a) 0·89	(c) 1·11	(b) 0·98	(c) 0·87	(a) 0·89	(b) 0·74	(b) 0·73	(a) 0·39	(c) 0·70	
Residual sums of squares		0·1385			0·2097			0·0192			0·3674
3 ♂	77	(b) 1·17	(c) 1·33	(c) 0·19	(c) 0·72	(a) 0·67	(b) 1·00	(c) 0·77	(a) 0·94	(a) 0·94	
	78	(c) 1·06	(b) 0·90	(b) 1·50	(b) 2·35	(c) 1·81	(c) 1·89	(b) 2·00	(c) 1·50	(c) 1·62	
	79	(a) 1·51	(a) 1·81	(a) 0·81	(a) 0·44	(b) 1·31	(a) 0·63	(a) 0·89	(b) 0·81	(b) 1·07	
Residual sums of squares		0·4224			0·0756			0·0300			0·5280
4 ♂	80	(c) 1·06	(a) 1·28	(b) 1·28	(a) 0·94	(b) 1·44	(c) 1·26	(b) 1·32	(c) 1·45	(a) 1·06	
	81	(a) 0·67	(b) 1·84	(c) 1·31	(c) 1·40	(a) 1·50	(b) 1·66	(c) 1·67	(a) 1·67	(b) 1·72	
	82	(b) 1·98	(c) 1·29	(a) 0·61	(b) 1·37	(c) 1·27	(a) 0·78	(a) 1·22	(b) 1·49	(c) 1·36	
Residual sums of squares		0·2900			0·0434			0·0052			0·3386
Total residual sums of squares		0·9208			0·3661			0·0714			
Mean responses to each method		(a) 0·96			(a) 0·88			(a) 1·00			
		(b) 1·52			(b) 1·32			(b) 1·29			
		(c) 1·05			(c) 1·11			(c) 1·10			

Letters in parenthesis indicate the method used, and the following figures are the measures of the responses—for full explanation see text.

thermometers; (c) rigid restraint + electrical thermometers; (d) slight restraint + clinical thermometers. Of these combinations, (d) involves handling of the rabbits each time the temperature is observed, and as this would constitute a further variation in technique, the combination was regarded as impracticable. Thus it was not possible to design an experiment which would give precise information on the contribution of each factor.

The fundamental unit of the design adopted was a 3 × 3 latin square, in which the rows were rabbits, the columns days, and the letters methods.

TABLE XIII

ANALYSIS OF VARIANCE OF DATA GIVEN IN TABLE XII

Replicate sets	Items	Degrees of freedom	Sums of squares		Mean squares	Variance ratios	P
I	Total between methods	8	2·5442		0·3180		
	Main effect between methods	2		2·0620	1·0310*	12·0023	<0·001
	Interactions between methods within groups	6		0·4822	0·0804		
	Total between days	8	1·4201		0·1776		
	Main effect between days ..	2		1·1050	0·5525		
	Interactions between days within groups	6		0·3151	0·0525		
	Total between rabbits	8	0·5518		0·0690		
	Residual	8	0·9208		0·1151		
	Residual + interactions ..	20		1·7181	0·0859*		
II	Total between methods	8	1·3935		0·1742		
	Main effect between methods	2		1·1900	0·5950†	16·7135	<0·001
	Interactions between methods within groups ..	6		0·2035	0·0339		
	Total between days	8	0·5840		0·0730		
	Main effect between days ..	2		0·4423	0·2212		
	Interactions between days within groups	6		0·1417	0·0236		
	Total between rabbits	8	3·5365		0·4421		
	Residual	8	0·3661		0·0458		
	Residual + interactions ..	20		0·7113	0·0356†		
III	Total between methods	8	0·6713		0·0839		
	Main effect between methods	2		0·5203	0·2602‡	13·2755	<0·001
	Interactions between methods within groups	6		0·1510	0·0252		
	Total between days	8	0·2172		0·0272		
	Main effect between days ..	2		0·0483	0·0242		
	Interactions between days within groups	6		0·1689	0·0232		
	Total between rabbits	8	2·3054		0·2882		
	Residual	8	0·0714		0·0039		
	Residual + interactions ..	20		0·3913	0·0196‡		

*, †, ‡, indicate the mean squares used in calculating the variance ratios given above.

4 groups of rabbits were used—2 of males, and 2 of females. Each group was used in 3 replicate sets of experiments. The complete design is given in Table XII. The experiments were done at 3- to 4-day intervals, a standard dose of 0·01 ml./kg. of solution C being given each time. The response was measured in terms of the maximum increase in temperature above normal. Experiments using combinations (a) and (b) were conducted as described under methods 1 and 2, respectively. Experiments using combination (c) were conducted as described for method 2, except that the holders used were those described for method 1.

The experimental data are given in Table XII and the analysis of variance in Table XIII.

As the 3×3 latin square was the fundamental unit in the experimental design, the data from each square were analysed separately into sums of squares arising from variations between methods, between days, between rabbits, and the residual portion. It was originally intended to treat the data as from one large experiment by summing the various sums of squares. There are, however, very large and systematic changes in the residual sums of squares from unit to unit, the residual sum of squares consistently becoming less for every group of rabbits in succeeding replicate units (see Table XII). When the residuals are summed over groups for each replicate set there are significant differences between the sums, but when they are summed over replicates for each group there are no significant differences. In consequence of this the combined data from the 4 groups have been treated as a separate set of experiments for each replicate.

TABLE XIV

TESTS OF SIGNIFICANCE FOR DIFFERENCES BETWEEN MEAN RESPONSES FOR EACH METHOD IN EACH REPLICATE SET OF EXPERIMENTS

	Replicate sets		
	I	II	III
Differences due to varying both degree of restraint and type of thermometer (methods (a) and (b))	$t_{(20)} = \dfrac{1 \cdot 52 - 0 \cdot 96}{\sqrt{0 \cdot 0859(^1/_{12} + ^1/_{12})}}$ $= 4 \cdot 6815 \quad P = <0 \cdot 001$	$t_{(20)} = \dfrac{1 \cdot 32 - 0 \cdot 88}{\sqrt{0 \cdot 0356(^1/_{12} + ^1/_{12})}}$ $= 5 \cdot 7135 \quad P = <0 \cdot 001$	$t_{(20)} = \dfrac{1 \cdot 29 - 1 \cdot 00}{\sqrt{0 \cdot 0196(^1/_{12} + ^1/_{12})}}$ $= 5 \cdot 0788 \quad P = <0 \cdot 001$
Differences due to varying type of thermometer, degree of restraint being constant (methods (a) and (c))	$t_{(20)} = \dfrac{1 \cdot 05 - 0 \cdot 96}{\sqrt{0 \cdot 0859(^1/_{12} + ^1/_{12})}}$ $= 0 \cdot 7524 \quad P = 0 \cdot 4 - 0 \cdot 5$	$t_{(20)} = \dfrac{1 \cdot 11 - 0 \cdot 88}{\sqrt{0 \cdot 0356(^1/_{12} + ^1/_{12})}}$ $= 2 \cdot 9866 \quad P = 0 \cdot 01$	$t_{(20)} = \dfrac{1 \cdot 10 - 1 \cdot 00}{\sqrt{0 \cdot 0196(^1/_{12} + ^1/_{12})}}$ $= 1 \cdot 7513 \quad P = 0 \cdot 1$
Differences due to varying the degree of restraint, using the same type of thermometer (methods (b) and (c))	$t_{(20)} = \dfrac{1 \cdot 52 - 1 \cdot 05}{\sqrt{0 \cdot 0859(^1/_{12} + ^1/_{12})}}$ $= 3 \cdot 9291 \quad P = <0 \cdot 001$	$t_{(20)} = \dfrac{1 \cdot 32 - 1 \cdot 11}{\sqrt{0 \cdot 0356(^1/_{12} + ^1/_{12})}}$ $= 2 \cdot 7269 \quad P = 0 \cdot 02 - 0 \cdot 01$	$t_{(20)} = \dfrac{1 \cdot 29 - 1 \cdot 10}{\sqrt{0 \cdot 0196(^1/_{12} + ^1/_{12})}}$ $= 3 \cdot 3275 \quad P = 0 \cdot 01 - 0 \cdot 001$

The various pooled sums of squares for each replicate set, which are given in Table XIII, are each based on 8 degrees of freedom. Of the 8 degrees of freedom for the sum of squares between methods within each replicate set, only 2 are directly concerned with differences between methods, the remaining 6 being interactions. For each replicate set these sums of squares have been appropriately partitioned and, as in no case is the interaction sum of squares significantly different from the residual sum of squares, these 2 have been pooled, providing a residual sum of squares based on 14, instead of 8, degrees of freedom. The pooled sums of squares for differences between days for each replicate set were analysed in the same way, and as none of these interaction sums of squares were found to differ significantly from the appropriate residual sum of squares, these interaction sums of squares were also pooled with the residual sums of squares, thus providing a residual sum of squares based on 20 degrees of freedom. No such partitioning of the sums of squares for differences between rabbits is possible. The details of the analysis are given in Table XIII. From the tests of significance it is concluded that there are significant differences between

the magnitudes of the responses obtained when the experimental method is varied.

Tests of significance for differences between the mean response to each method in each replicate set of experiments are given in Table XIV. It is evident from these tests that the differences in the magnitude of the response obtained in the different experimental circumstances are real, and that both the degree of restraint and the method of taking the temperature affect the response. Although the tests for differences in magnitude of the response caused by varying the method of taking the temperature when the degree of restraint is constant are not all significant (see Table XIV), the differences are all in the same direction; and when the probabilities from these tests are combined by a standard method[23] the differences are highly significant.

When the degree of restraint is minimal, and when electrical thermometers are used for measuring the temperature changes, the response is maximal.

SUMMARY

1. The absence of a stable preparation suitable for use in the development of quantitative methods for the study of bacterial pyrogens is pointed out.

2. A simple method giving a good yield of a highly pyrogenic dry extract from *Proteus vulgaris* is described.

3. Two methods of measuring the pyrogenic response are described.

4. Experimental studies on (i) the temperature response of rabbits to various doses of the pyrogen preparation, (ii) the stability of solutions of the pyrogen preparation, (iii) the effect upon the response of repeated administration of the preparation, (iv) the effect upon the response of varying the experimental procedures, are described and discussed. Standard statistical methods have been used in the analysis of the results.

5. The temperature response increases with increasing doses. The minimal effective dose of the dry extract is less than 0.02 μg./kg.

6. Solutions containing 200 μg./ml. of the pyrogen preparation are still highly active after several years storage, though there is evidence that some loss of activity occurs immediately following their preparation.

7. There is no evidence of loss of activity of the dry extract over a period of 5 years.

8. When bacterial pyrogen is given repeatedly to rabbits, at intervals of a few days, the temperature response diminishes progressively during the first few successive administrations. While the effect is only temporary the rabbits must be rested for considerably more than 4 weeks before it passes off.

9. The magnitude of the pyrogenic response is affected by the degree of restraint imposed on the rabbits, and by the type of thermometer used. The maximum response is obtained when the degree of restraint is minimal and when electrical thermometers are used.

10. The maximum increase in temperature above normal is a more useful measure of pyrogenic effect than is a measure which, in addition, also takes account of the duration of the temperature rise.

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AN ADSORPTION METHOD FOR THE MEASUREMENT OF VITAMIN A STABILITY

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A NUMBER of methods are at present available for the determination of the stability of oils containing vitamin A. One of the earliest, the "Swift" method[1] subsequently modified[2,3,4], depended on passing air or oxygen through vitamin A oils at an elevated temperature, the undecomposed vitamin A being determined after varying periods of time; in the petri dish method[5] the degradation of the oil was produced by placing the oil in a petri dish or similar receptacle and exposing it to air at elevated temperatures in a thermostatically controlled oven. The oxygen absorption method[6,7,8] involved the determination of the amount of oxygen absorbed by an oil during oxidation either directly or by calculation from the pressure change.

It was felt that all these methods had certain disadvantages. Modifications based on the Swift method although reliable, are somewhat tedious and are more suitable for use in laboratories concerned with the stability of vitamin A oils as an everyday routine. The petri dish method is more convenient in operation but has been found, especially with some oils, to give less reliable and less reproducible results. Better results can be obtained if small vessels are used and the complete contents of a vessel are used for each assay, small glass or porcelain rings or cups being used as containers[9]. The direct oxygen absorption method suffers from the disadvantage that actual measurements of vitamin A potencies are not made, the results depending on measurements of oxygen absorption. In the last two methods oxidation takes place mainly at the surface and any skin formation will interfere with the rate of oxidation.

Stability experiments by all 3 methods are generally performed between 37° C., and 100° C., in order to shorten the time involved, although the use of higher temperatures introduces the possibility of thermal decomposition which is unlikely to occur below 25° C. In addition the artificial conditions necessarily adopted may not reflect accurately the stability of the oil in a particular pharmaceutical preparation.

A method was therefore evolved which is simple in operation and specially suitable for the determination of the stability of vitamin A oils when present in tablets or when adsorbed on solids. The vitamin A oil or concentrate was adsorbed on a powder, the mixture being placed in a number of small wide-mouth screw cap bottles which could be closed and stored at 25° C. or 37° C. At suitable intervals the contents of the bottles were weighed, extracted and assayed either spectrophotometrically at 328 mμ or by the antimony trichloride reaction. The method gave a much accelerated rate of decomposition without rise in temperature and was found to be particularly applicable to the determination of the

stabilities of vitamin A oils intended for use in tablets and in certain animal feeding supplements where the vitamin A is adsorbed on a solid medium.

Calcium phosphate, kieselguhr, barium sulphate, and silica were tried as adsorbents. It was difficult to obtain an even distribution of vitamin A oils on available barium sulphate powders; kieselguhr was also unsatisfactory since absorption on this medium caused decomposition of the vitamin A which was too rapid for convenient measurements of stability. Silica and calcium phosphate were both found to be suitable although some difficulty was found in quantitative extraction of low potency oils from silica. It was finally decided after many trials to use calcium phosphate B.P. (primary calcium phosphate has been reported to react as a synergist presumably due to its acidity[10]). The ratio of the weight of vitamin A oil to adsorbent was kept constant at 1:50.

EXPERIMENTAL

Mix 1 g. of oil with 49 g. of calcium phosphate B.P. by grinding together with a glass pestle and mortar; add the calcium phosphate at first in 1, 2, 4 and 8 g. quantities. The whole mixing must be done very rapidly. The efficiency of this stage determines the accuracy of the experiment. Distribute the mixture into 10 or 15 small, wide-mouthed screw cap bottles which are then stored at the chosen temperature. After appropriate time intervals weigh the whole contents of one container into a conical flask and extract the vitamin A with portions of chloroform or cyclohexane by decantation. Make up to volume and filter if necessary. Determine the vitamin A spectrophotometrically or by the antimony trichloride reaction. Table I shows typical results obtained at 25° C. with some high potency vitamin A concentrates.

TABLE I

	High potency (1,500,000 I.U./g.) vitamin A alcohol unstabilised per cent. of original potency	Synthetic vitamin A acetate 1,000,000 I.U./g. per cent. of original potency	High potency (370,000 I.U./g.) natural concentrate per cent. of original potency	Synthetic vitamin A palmitate (1,000 000 I.U./g.) stabilised per cent. of original potency
1 day	2	98	100	100
2 days	—	92	100	100
3 days	—	79	100	100
4 days	—	55	100	100
6 days	—	10	95	98
8 days	—	—	86	94
10 days	—	—	76	89
12 days	—	—	64	82
14 days	—	—	54	74
16 days	—	—	44	66
18 days	—	—	29	48

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ABSTRACTS OF PAPERS PUBLISHED
IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Caffeine, Complexes with Benzoic Acid and Benzoate Ion. T. Higuchi and D. A. Zuck. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 132.) Data from previous studies of the interaction of benzoic acid and caffeine at 0° C. were extended by experiments at 15° C. and 30° C., the partition coefficients of benzoic acid between water and skellysolve-C being used to determine the concentration of benzoic acid and the quantity of benzoic acid complex. Equilibrium constants for the formation of a complex from 1 molecule of caffeine with 1 molecule of benzoic acid and for reaction of this with a further molecule of benzoic acid are of the same order and experimental results agree with the calculated values. The formation of the equimolecular complex is exothermic (3000 cal./mole) corresponding to the formation of a single hydrogen bond. The higher entropy change on the formation of the second complex suggests that it may be a highly ordered triangular type of molecular compound. The degree of interaction between caffeine and sodium benzoate was determined by adding an excess of caffeine to water and a number of solutions containing different concentrations of sodium benzoate at 0°, 15° and 30° C. After shaking for 24 hours and allowing the solids to settle, the concentration of caffeine was determined spectrophotometrically. Results are not in agreement with the formation of an equimolecular complex and one containing 2 benzoate ions to each caffeine molecule. The possible formation of a complex consisting of 2 caffeine molecules to one benzoate ion could not be excluded since the effect of different concentrations of caffeine on the interaction was not investigated.

<div align="right">G. B.</div>

Caffeine, Interactions with Aspirin, *p*-Hydroxybenzoic Acid, *m*-Hydroxybenzoic Acid, Salicylic Acid, Salicylate Ion and Butyl *p*-Hydroxybenzoate. T. Higuchi and D. A. Zuck. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 138.) An excess of aspirin was placed in a number of 125-ml. glass-stoppered bottles with varying amounts of caffeine, 100 ml. of 0·001N sulphuric acid was added to each to suppress dissociation of the aspirin and decrease hydrolysis and the bottles were shaken for 3 hours at 30° C. or 6 hours at 15° C. Aliquot quantities were removed for the determination of the concentration of aspirin in solution. Similar techniques were applied with salicylic acid, *p*-hydroxybenzoic acid, *m*-hydroxybenzoic acid and butyl *p*-hydroxybenzoate (butyl paraben). Salicylate ion was investigated in the manner described previously for benzoate ion. Thermodynamic constants are given for all compounds studied, and indicate that caffeine forms fairly stable complexes with all of them. The equimolecular caffeine/acid complex seems to predominate, but there is also evidence for the combination of 1 molecule of caffeine with 2 of benzoic acid or 2 of salicylic acid or salicylate with 1 of caffeine. No conclusive evidence was found for the formation of complexes of more than 3 molecular units. *p*- and *m*-Hydroxybenzoic acids show greater ability to form complexes than the *o*-compound, and the esterification of a hydroxyl group as in aspirin or butyl *p*-hydroxybenzoate results in the formation of less stable complexes. The heats of reaction range from 1·5 to 7 kg. cal., probably representing formation of several hydrogen bonds.

<div align="right">G. B.</div>

ANALYTICAL

Morphine, Determination in Opium with the Aid of an Adsorption Column. F. E. Klee and E. R. Kirch. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 146.) The following is a precise and simple method for the assay of opium. 0·5 g. is extracted for 3 hours with dehydrated methanol in a continuous extraction apparatus in which the opium extract passes through an adsorption column of florisil to remove interfering substances. The extract is evaporated to dryness and dissolved in water to produce 200 ml. of solution of which 1 ml., representing about 0·25 mg. of morphine, is mixed with 2 ml. of Folin–Ciocalteu reagent, 3 ml. of saturated solution of sodium carbonate and water to 100 ml. and allowed to stand for 5 hours for the development of the colour. The light absorption is measured at 765 mμ, and the result of the assay is calculated from a curve prepared with standard morphine solutions. Results are slightly higher than those obtained by the U.S.P. method, possibly because of losses during extraction by the longer pharmacopœial process. In experiments in which known amounts of morphine were added to opium, good recoveries were obtained. Meconic acid, narcotine, codeine and papaverine do not interfere. With samples containing lactose, the specified sample size should not be exceeded as longer extraction may be necessary; lactose interferes in the assay when the extraction is prolonged.   G. B.

Morphine, Separation from Codeine by Ion Exchange. E. W. Grant and W. W. Hilty. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 150.) Morphine, on account of its phenolic nature is retained on strongly basic anion exchange columns whereas codeine is not. Amberlite XE75 was converted to the hydroxyl form by allowing it to stand overnight in 10 per cent. sodium hydroxide solution, washed with water and packed into columns. A sample of about 0·2 g. of morphine sulphate, dissolved in 15 ml. of water was retained by the column and was not removed by washing with water, methanol, ethanol or acetone. The morphine was completely removed by washing with 50 ml. of 2 per cent. phosphoric acid. Codeine phosphate placed on the column was completely removed by washing with methanol (95 per cent.). For the separation of codeine phosphate and morphine sulphate the mixture was dissolved in 20 ml. of water and passed through the column. Methanol (95 per cent.) was passed through until a total of 100 ml. of eluate was obtained, in which the codeine was determined by titration. The column was washed with water and the morphine removed with phosphoric acid (2 per cent.). The light absorption of this solution was measured at 285 mμ and the content of morphine sulphate calculated from the datum $E_{1\,\mathrm{cm.}}^{1\,\mathrm{per\,cent.}} = 39\cdot8$. Reagent blank tests were performed in each case. Good recoveries of the alkaloids were obtained and the presence of codeine did not interfere with the exchange between morphine and the resin.   G. B.

Polyvinylpyrrolidone, Reactions and Estimation of. S. Camozzo and S. Dal Zotto. (*Ann. Chim. appl. Roma*, 1953, **43**, 113.) This substance in 0·5 and 2·0 per cent. aqueous solution gives precipitates with the following reagents:—palladium chloride 5 per cent., Mayer's, Dragendorff's, Marmé's, Sonnenschein's and Scheibler's reagents, potassium ferrocyanide 10 per cent., potassium ferricyanide 10 per cent., potassium chromate 10 per cent., potassium dichromate 10 per cent., silicotungstic acid 1 per cent., perchloric acid 20 per cent., tannic acid 5 per cent. It gives no precipitate with the following:— chloroplatinic acid 5 per cent., mercuric chloride, zinc iodide 5 per cent.,

potassium thiocyanate 10 per cent., sodium nitroprusside 2 per cent., lead chloride, ferric chloride 5 per cent., uranium nitrate 1 per cent., picric acid 1 per cent. With Bouchardat's reagent it gives no precipitate, but a fine mahogany-red colour, which can be used for estimation. Polyvinylpyrrolidone is soluble in methanol and ethanol and in chloroform, but is insoluble in ether, carbon disulphide, carbon tetrachloride, benzene and toluene. It is hygroscopic. The sample used in the tests was purified by precipitating a concentrated ethanolic solution with benzene and drying *in vacuo* over sulphuric acid to constant weight. The colorimetric estimation was carried out in a Pulfrich photometer on the mahogany-red colour produced by iodine. To prepare the curve of standardisation 1 ml. of Bouchardat's solution (iodine 2 g., potassium iodide 4 g., distilled water to make 100 ml.) is placed in a 100 ml. graduated flask; from 2 ml. to 20 ml. of aqueous solution of polyvinylpyrrolidone containing 1 g./l. is added, made up to 100 ml., mixed, allowed to stand 20 minutes, and the colour compared in 5 mm. cells with a solution of 1 ml. of the iodine reagent in 100 ml. of water, using filter S55. Up to 14 mg. the colour follows the Lambert-Beer law, but above that the curve is no longer a straight line. Another method is to precipitate with silicotungstic acid and determine the excess of the latter with titanous chloride. In a 20-ml. flask put from 2 to 20 mg. of the product and 5 ml. of solution of silicotungstic acid (15 g. of silicotungstic acid, 500 ml. of 2N hydrochloric acid and distilled water to 1 l.) Make up to 20 ml., filter bright, take 10 ml. of the filtrate, add 0·5 ml. of titanous chloride, 10 per cent., and read the colour in a Pulfrich photometer against distilled water, using an S72 red filter. A straight line graph is obtained and the results agree with those obtained with iodine. H. D.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Quercetin and its Glycosides in Leaves of Vaccinium myrtillus. C. H. Ice and S. H. Wender. (*J. Amer. chem. Soc.*, 1953, **75**, 50.) The details of the isolation in pure form of quercetin (3:3′:4′:5:7-pentahydroxyflavone) and 5 of its glycosides from the leaves of the "huckleberry", *Vaccinium myrtillus* are reported. Ion exchange resin (Amberlite IRC-50 (H)) and adsorption chromatography was used in the isolation and separation of the flavonoids. The glycosides were identified as quercetin-3-arabinoside; *iso*quercitrin (quercetin-3-glucoside); quercitrin (quercetin-3-rhamnoside); quercetin-3-gluco-glucoside; and apparently a new quercetin rhamnoside, not identical with quercitrin. A. H. B.

PLANT ANALYSIS

Podophyllotoxin from Juniperus Spp.; Savinin. J. L. Hartwell, J. M. Johnson, D. B. Fitzgerald and M. Belkin. (*J. Amer. chem. Soc.*, 1953, **75**, 235.) Because aqueous suspensions of the dried needles of certain junipers caused hæmorrhage and necrosis of Sarcoma 37 in mice, a search for the active principle was instituted. Successive fractionation with different solvents and chromatography was used, and the distribution of biological activity in the fractions was followed by quantitative bioassay with mice bearing Sarcoma 37. In this way crystalline podophyllotoxin was isolated from certain junipers. From one juniper (savin) a new substance called savinin, inactive towards tumours, was also obtained. Certain physical and chemical data, including infra-red and ultra-violet absorption curves, are recorded for savinin. A. H. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Actithiazic Acid, a New Thiazolidone Antibiotic. W. M. McLamore, W. D. Celmer, V. G. Bogert, F. C. Pennington, B. A. Sobin and I. A. Solomons. (*J. Amer. chem. Soc.*, 1953, **75**, 105.) The antibiotic actithiazic acid, a new *streptomyces* antibiotic effective *in vitro* against *Mycobacterium tuberculosis* was isolated by solvent extraction of the fermentation broth and finally purified by recrystallisation from methanol. It is shown to be (-)2-(5-carboxypentyl)-4-thiazolidone (I)

$$\begin{array}{c} O \\ \parallel \quad H \\ C{-}{-}N \\ \mid \qquad \quad CH(CH_2)_5COOR \qquad (I) \\ H_2C{-}{-}S \end{array}$$
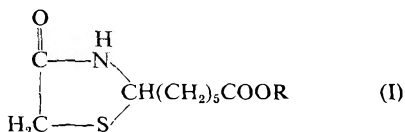
by means of degradation and synthetic studies which are described. A. H. B.

Adrenaline and Noradrenaline in the Human Fœtal Adrenals and Aortic Bodies, Determination of. K. Niemineva and A. Pekkarinen. (*Nature, Lond.*, 1953, **171**, 436.) Adrenaline and noradrenaline have been assayed chemically in human fœtal adrenals and in the Zuckerkandl's bodies. During the last 3 months of the intrauterine period the total catechols of the adrenals averaged 71 μg. and for the aortic bodies 40 μg. and 11 μg., respectively. This is less than the corresponding amount in normal adult adrenals. The greater part of the catechols consisted of noradrenaline, especially in the aortic bodies.

G. F. S.

Glucose, Action of Insulin on the Intestinal Absorption of. M. Lourau. (*C.R. Acad. Sci. Paris*, 1953, **236**, 1376.) Insulin was administered to 2 groups of guinea-pigs. One group also received glucose intramuscularly so as to produce blood-sugar levels similar to those in a control group of guinea-pigs which received no insulin. There was no significant difference between the rate of intestinal absorption of glucose in the control group and in the treated animals having comparable blood-sugar levels. The rate of absorption in the group receiving insulin only was rather less than that indicated by extrapolation of the linear graph relating speed of absorption to logarithm of the blood-sugar level, obtained in previous work. Therefore the hypoglycæmic effect of the insulin was sufficient to account for the increased rate of absorption and there was no evidence that insulin increased the permeability of the mucosa to glucose. Since insulin is known to increase the permeability of the cell membranes in the peripheral tissues, it seems that there is more than one mechanism which regulates the entry of glucose into the cells. G. B.

Œstrogenic Diols, Partition Chromatography of. E. O. Haenni, J. Carol and D. Banes. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 167.) Following the observation that œstrogenic diols may be separated by partition chromatography using sodium hydroxide solution on a celite 545 column, the effects of varying alkali concentration, tightness of packing, mobile solvent, flow rate and temperature were investigated. The use of 0·4N sodium hydroxide on the column with benzene as the mobile phase and a flow rate of 2 to 3 ml./minute are recommended. Separation is more efficient when the temperature is raised but, in collecting the fractions, allowance must be made for the change in partition

coefficient. Details of a short column method for the separation of œstradiols and dihydro-equilins from dihydro-equilenins are given. For the separation of œstradiols, dihydro-equilins and dihydro-equilenins on a long column the first 185 ml. of eluate (plus 14 ml. for each degree below 25° C. or minus 8 ml. for each degree above) is rejected, the œstradiol being eluted in the following 225 ml. (plus 16 ml. for each degree below 25° C. or minus 5 ml. for each degree above). The next 320 ml. (plus 24 ml. for each degree below 25° C. or minus 15 ml. for each degree above) contains the α-dihydro-equilin. The dihydro-equilenins may be removed from the column with ether as solvent. Partition coefficients between the phases used in the columns were determined for β-œstradiol and α-dihydro-equilin and shown to be in agreement with figures deduced from the chromatographic experiments. The partition coefficients for α-œstradiol, β-dihydro-equilin and β-dihydro-equilenin were also determined on the columns.   G. B.

Pituitary Antidiuretic Peptide and Similar Urinary Peptide, Isolation of, by Paper Chromatography. G. C. Arneil and H. E. C. Wilson. (*Lancet*, 1953, **264**, 568.) The unusual peptide which occurs in the urine of patients with nephrosis has been found to be present also in the urine of patients with œdema from other causes and of those who have sustained acute trauma. Excessive antidiuretic activity has been found in the blood of patients with nephrosis and it was thought that the peptide might be associated with anti-diuretic activity. It was also observed that diuresis occurred after administration of corticotrophin and it seemed possible that this might be due to a lessening of the production of pituitary antidiuretic hormone. 8 commercial pituitary extracts were therefore investigated to see whether they contained a similar peptide and if so whether it had antidiuretic activity. Two-dimensional paper chromatographs were prepared from the extracts, using phenol ammonia and butanol acetic acid as solvents. The nephrosis peptide has a high R_F in phenol and a very low R_F in butanol acetic acid. The positions of the amino-acids were ascertained by the ninhydrin reaction, and the part containing the peptide was eluted with water. The urinary peptide was isolated in essentially the same way. Tests on rats showed that the eluates from the pituitary extracts had strong antidiuretic activity. In 22 similar tests on the eluates from the urine of nephrotic patients, a well-marked antidiuretic response was obtained in only 3 instances. In 8 of the 22 tests, convulsions occurred shortly after injection. The urinary peptide therefore differs from the pituitary peptide although it gave the same amino-acids after hydrolysis and resembled it in its behaviour on chromatography and electrophoresis. The same urinary peptide was obtained from the urine of two normal people after antidiuresis had been induced by nicotine. It is suggested that the urinary peptide is the form in which pituitary antidiuretic hormone is excreted.   H. T. B.

3:5:3'-Triiodothyronine; Isolation from Thyroid Gland and Synthesis. J. Gross and R. Pitt-Rivers. (*Biochem. J.*, 1953, **53**, 645.) The unknown radioactive component previously detected in extracts of thyroid gland, plasma, tissues and fæces of animals which had been injected with radioactive iodide or thyroxine labelled with radioactive iodine, has been identified as a 3:5:3' triiodothyronine. Large-scale extraction of ox thyroid glands followed by tryptic digestion yielded a fraction which when submitted to paper chromato-graphy showed spots attributable to the unknown fraction. Elution from large numbers of filter papers yielded still impure material, which could be demon-strated to be acidic, and which was shown by the ninhydrin reaction to contain

an amino group. Its position on the chromatograms compared with the positions of a number of thyroxine peptides indicated that it was likely to possess a diphenyl ether structure; this was supported by the purple colour obtained with diazotised sulphanilic acid. The possibility that this substance might be diiodothyronine having already been excluded by Gross *et al.*, the synthesis of triiodothyronine was attempted. Moniodination of 3:5-diiodo-thyronine with iodine in the presence of ammonia gave triiodothyronine as the main reaction product. The latter was purified by crystallisation as the hydrochloride from aqueous hydrochloric acid, and by partitioning on a column of kieselguhr, using butanol/chloroform and 0·5 N aqueous sodium hydroxide. The synthetic substance behaved chromatographically on paper identically with the radioactive unknown substance. D- and DL-triiodothyronines were obtained similarly. The possibility that triiodothyronine may be derived from thyroxine as an artefact of alkaline hydrolysis was excluded by control experiments. The knowledge of its chemical properties gained during its synthesis made it possible to isolate triiodothyronine in a pure stable form from thyroid gland.   J. B. S.

BIOCHEMICAL ANALYSIS

α-Œstradiol in Œstrogenic Mixtures, Colorimetric Determination of, using Partition Chromatography. E. O. Haenni, J. Carol and D. Banes. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 162.) In preparing extracts of œstrogenic mixtures all but traces of ketosteroids may be eliminated by the method of Carol and Rotondaro, employing the Girard-T reagent. The resulting extract may contain α- and β-dihydro-equilenins, α-dihydro-equilin and œstradiols, as well as impurities which cause interference in assays by charring during the iron-Kober reaction. Dihydro-equilin and œstradiols may be separated from this extract by partition chromatography on a column of celite 545 using 0·4N sodium hydroxide on the column and benzene as the mobile solvent. The first fraction of the eluate, containing most of the non-œstrogenic impurities is rejected, the following fraction containing œstradiols and α-dihydro-equilin being collected and the content of α- and β-œstradiols determined by the spectrophotometric method described in a previous paper. Small amounts of α-dihydro-equilin may be ignored, but for larger quantities a correction should be made. For the determination of α-dihydro-equilin a similar method is used, but the iron-Kober reagent is diluted with 45 per cent. of its volume of N hydrochloric acid, 1 to 3 hours before use, and the optical density of the solution is measured at 472 mμ at intervals until the maximum colour has been reached. A quantity of pure α-dihydro-equilin is submitted to the reaction concurrently and the content of the sample is calculated in terms of the standard. Collaborative tests in 6 laboratories indicate that the method is satisfactory.
                                                                    G. B.

Steroid Hormones, Quantitative Determination of, with 2:4-Dinitrophenyl-hydrazine. A. G. Gornall and M. P. Macdonald. (*J. biol. Chem.*, 1953, **201**, 279.) Steroid hormones are determined quantitatively as 2:4-dinitrophenyl-hydrazones in extracts of urine, blood plasma or tissues, which have been prepared by methods usually considered satisfactory for steroid hormone assay. Suitable aliquots of the extracts to be analysed containing 1 to 20 μg. of cortisone are first evaporated to dryness under nitrogen and the residue dissolved in methanol; 2:4-dinitrophenylhydrazine in acid methanol is added and the mixture heated at 60° C. for 90 minutes. The cold solution is made alkaline,

suitably diluted and the optical density is measured against a suitable blank at 475 mμ. Δ^4-C$_3$-steroid ketones react quantitatively within 5 minutes at 20° C. to give hydrazones which show a peak absorption in alkaline solution at 450 mμ. Heating for 90 minutes at 59° C. causes, in addition, the complete reaction of C$_{20}$ ketones in a dihydroxyacetone side chain, but only partial reactions in monohydroxy side chains. Other ketones react slightly. Molar extinction coefficients for a large range of ketosteroids have been determined under both sets of conditions and the reactivity of the ketone estimate. A C$_{21}$-hydroxyl group causes a specific shift in the wavelength of the absorption maximum for the hydrazone of the C$_{20}$ ketone.   J. B. S.

CHEMOTHERAPY

Amithiozone (Thiacetazone) Analogues from Aralkyl Ketones. W. L. Nobles and J. H. Burckhalter. (*J. Amer. pharm. Ass. Sci. Ed.,* 1953, **42**, 176.) A series of 18 thiosemicarbazones of ketones was prepared by heating the ketone and thiosemicarbazide dissolved in dilute ethanol, with the addition of a few drops of a mineral acid. Satisfactory yields were obtained by heating in a waterbath for 15 to 20 minutes, the thiosemicarbazone being precipitated during the reaction or on cooling. Compounds of the type C$_6$H$_5$·C(CH$_2$R):NNH·CS·NH$_2$ were prepared where R = –H, –CH$_3$, –NHCOCHCl$_2$, –NH$_2$, –CH$_2$N(CH$_3$)$_2$, –OCOCH$_3$, –NH·CO·CH$_3$, or –CH$_2$·CO$_2$H, the phenyl group being *p*-chloro, *p*-nitro, *p*-phenyl, *p*-methoxy or 3:5-dinitro substituted or replaced by 2-thienyl. All these compounds are related to *p*-acetamidobenzaldehyde thiosemicarbazone (thiacetazone) and some also show a structural resemblance to chloramphenicol. The vinylog, C$_6$H$_5$·CH:CH·C(CH$_2$):NNH·CS·NH$_2$ and 3 ring-substituted derivatives were also prepared. Of the 10 compounds submitted to *in vitro* tests against the β-benzoylpropionic acid derivative, 1 was inactive and 8 others showed a certain amount of activity, while *p*-chloroacetophenone thiosemicarbazone was 4 times as active as 4:4′-diaminodiphenylsulphone.   G. B.

Chloramphenicol Analogue. DL-2-**Dichloroacetamido-1-(4-pyridyl)-1 : 3-propandiol.** S. van der Meer, H. Kofman and H. Veldstra. (*Rec. Trav. chim. Pays-Bas,* 1953, **72**, 236.) Several attempts to prepare the pyridine analogue of chloramphenicol failed because of a tendency to intermolecular quaternisation but the synthesis was successfully accomplished in the following stages. The O-*p*-toluenesulphonyl derivative of 4-acetylpyridine was converted to the diacetal of 4-(ω-aminoacetyl) pyridine with potassium ethoxide. Reaction of an ethanolic solution of this compound with benzoic anhydride yielded a stable benzoyl derivative from which the corresponding ketone was obtained by heating with hydrochloric acid. 4-(α-Benzoylamino-β-hydroxypropionyl) pyridine was formed by heating with formaldehyde in the presence of sodium bicarbonate and ethanol, and reduced to the corresponding alcohol with aluminium *iso*propoxide. The benzoyl group was removed by hydrolysis with hydrochloric acid and the pyridine analogue of chloramphenicol, DL-2-dichloroacetamido-1-(4-pyridyl)-1 : 3-propandiol obtained by reaction with methyl dichloroacetate at room temperature. This product and a number of intermediate compounds and derivatives were tested *in vitro* against *Staphylococcus aureus, Escherichia coli, Salmonella typhimurum* and *Eberthella typhi.* The pyridine analogue of chloramphenicol showed only 1 to 3 per cent. of the activity of chloramphenicol against *S. typhimurum* and *E. typhi.*   G. B.

Thiazole Carboxylic Acid Hydrazides, Tuberculostatic Activity of. H. C. Beyerman and J. S. Bontekoe. (*Rec. Trav. chim. Pays-Bas.*, 1953, **72**, 262.) Thiazole-2-carboxylic acid hydrazide was prepared by reaction of the ethyl ester of the corresponding acid with hydrazine acetate, and the 5-carboxylic acid hydrazide was obtained from the corresponding methyl ester. Thiazole-4-carboxylic acid hydrazide was made by the hydrogenation of ethyl 2-bromothiazole-4-carboxylate in the presence of Raney nickel. Thiazole-2-carboxylic acid hydrazide proved to be of the same tuberculostatic activity as the corresponding pyridine derivative, picolinic acid hydrazide, both *in vitro* and *in vivo*. Thiazole-4-carboxylic acid hydrazide was 1/5th as active against BCG and inactive against type D–328 tubercle bacilli. Thiazole-5-carboxylic acid hydrazide, analogous to the inactive nicotinic acid hydrazide was also inactive *in vitro* against type D–328 bacilli and showed only 1/25th of the activity of the 2-substituted isomer against BCG. Thiazole does not give an analogue of *iso*nicotinic acid hydrazide, since the sulphur atom takes the place of the α position of pyridine. G. B.

PHARMACY

NOTES AND FORMULÆ

Hexamethonium Chloride (Methium Chloride). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1953, **151**, 385.) Hexamethonium chloride is hexamethylenebis(trimethylammonium chloride), and occurs as a white, crystalline, hygroscopic powder with a faint odour, m.pt. 289° to 292° C. with decomposition, very soluble in water, soluble in ethanol, and practically insoluble in chloroform and ether; the *p*H of a 3 per cent. solution is 5·0 to 6·5. The diperchlorate obtained by adding a neutral solution of sodium perchlorate to an ethanolic solution of hexamethonium chloride melts at 261° to 265° C., after washing with ethanol and ether and drying at 105° C. for 4 hours. When dried in an Abderhalden-pistol drier over phosphorus pentoxide at 100° C. and 5 mm. Hg for 4 hours, the loss in weight does not exceed 1·5 per cent. It contains 25·4 to 26·5 per cent. of chloride and 72·6 to 75·5 per cent. of hexamethonium ion. The hexamethonium ion is assayed by refluxing with acetic anhydride, dissolving the product in acetic acid, treating with a solution of dry mercuric acetate in acetic acid and titrating with 0·1 N perchloric acid, using a solution of crystal violet in glacial acetic acid as indicator, and titrating to the blue-green end-point. The 0·1 N perchloric acid is prepared by mixing 70 to 72 per cent. perchloric acid with glacial acetic acid and adding acetic anhydride. G. R. K.

Probenecid (Benemid). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1953, **151**, 298.) Probenecid is *p*-(dipropylsulphamyl)benzoic acid and occurs as a white, odourless, crystalline powder, m.pt. 198° to 200° C., soluble in acetone, ethanol, dilute alkalis, and dilute sodium bicarbonate solution, and insoluble in water and dilute acids. When reduced with Raney nickel in alkaline solution, the product yields no colour on diazotisation followed by treatment with sulphamic acid and N-(1-naphthyl)ethylenediamine (distinction from caronamide). A 0·001 per cent. solution in ethanol exhibits ultra-violet absorption maxima at about 2250 and 2480 Å. ($E_{1\,cm.}^{1\,per\,cent.}$, about 336). Probenecid loses not more than 0·1 per cent. of its weight when dried at 105° C. for 4 hours; it yields not more than 0·1 per cent. of ash and complies with limit tests for halides, sulphate, heavy metals, and free acid. It contains 95·0 to 105·0 per cent. of probenecid when determined by measuring the absorption

ABSTRACTS

of a 0·001 per cent. ethanolic solution at 2480 Å., and 4·81 to 5·01 per cent. of nitrogen when determined by semimicro Kjeldahl. Probenecid is used to increase and prolong the plasma concentration of penicillin, sodium amino-salicylate, and other substances, and to promote the elimination of uric acid in the interval treatment of chronic gout.   G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and Noradrenaline, Effect of, on the Activity of Isolated Preparations of the Gut from the Fœtal Guinea-pig. A. F. Munro. (*Brit. J. Pharmacol.*, 1953, **8**, 38.) While adrenaline abolishes the tone and rhythmicity of the longitudinal muscle of the small intestine it contracts the sphincters. Adrenaline and noradrenaline have been shown to cause contractions of isolated segments taken from the two ends of the small intestine of the fœtal guinea-pig while an intervening region was either relaxed or not affected. Atropine potentiated the contraction and reversed the inhibitory responses of adrenaline. In both the fœtal and adult terminal ileum, adrenaline caused a dual response, large doses causing contraction and small doses relaxation. Adrenaline reduced or abolished both the contraction and relaxation caused by noradrenaline in segments from the fœtal ileum. It is suggested that in the fœtal guinea-pig differentiation of function between sphincter and duodenal muscle is as yet incomplete.   G. F. S.

Adrenaline and Noradrenaline Infusions, Effects of, on Respiration in Man. R. F. Whelan and I. M. Young. (*Brit. J. Pharmacol.*, 1953, **8**, 98.) The influence of adrenaline and noradrenaline on the respiratory pattern and gaseous exchange has been examined in conscious human subjects. An infusion of adrenaline or noradrenaline increased the tidal volume, respiratory minute volume and lowered the alveolar carbon dioxide. Both compounds had similar activities in these actions. The respiratory rate was usually increased. Adrenaline increased the oxygen consumption by an average of 32 per cent. while noradrenaline caused no significant change. It is suggested that the hyperventilation is independent of any general increase in the metabolic rate.
                                                                        G. F. S.

Anti-*Jk^b*. A New Blood-group Antibody. G. Plaut, E. W. Ikin, A. E. Mourant, R. Sanger and R. R. Race. (*Nature, Lond.*, 1953, **171**, 431.) This paper reports the finding of the antibody corresponding to the antigen and gene *Jk^b* in the serum of a female, a multipara who had had two miscarriages. The existence of an allelomorphic gene *Jk^b* in the *Kidd* human blood-group had hitherto been assumed. The existence of this anti-body now promotes the *Kidd* system to fourth place in the order of "usefulness" of the 9 blood group systems.   G. F. S.

Anticurare Agents. A. R. Hunter. (*Brit. med. J.*, 1953, **1**, 640.) In view of the fatalities which have occurred after the use of neostigmine as an antidote to the respiratory depressing action of curarising drugs, its dangers and the methods available for their elimination were investigated. The compound was given intravenously together with atropine to 50 patients who had been curarised with various myoneural blocking agents and anæsthetised with nitrous oxide and oxygen and a supplement. As the primary danger is cardiac inhibition, counts of the pulse rate were made every 15 seconds after the injection at first and at intervals of a minute subsequently. The effects on the pulse

rate of giving the atropine before the neostigmine, and of giving a sympatho-mimetic drug in addition, were also determined. In all patients there was a progressive slowing of the pulse rate to a minimum during the first 5 to 10 minutes. The minimum rate depended upon the initial rate; a rate of 120 tended to drop to 70 to 100 while a relatively slow pulse might drop to 40 to 50. Atropine is widely believed to cause a slowing of the pulse by medullary stimulation before its peripheral accelerating action occurs, but doses of up to 1/50 grain at various intervals before the neostigmine produced acceleration immediately the drug reached the heart and there was no change in the pattern of the response to neostigmine even when the latter was given as much as 19 minutes after the atropine. The injection of 30 mg. of ephedrine or 10 to 20 mg. of methedrine with the atropine and neostigmine increased the initial cardiac acceleration but the pattern of the subsequent slowing was unaltered. The use of a vasopressor is therefore unnecessary. On the assumption that a rate of 50 indicated a dangerous degree of bradycardia, it is suggested that 1/50 gr. of atropine should be used when 2·5 mg. of neostigmine is given. Since it is impossible to forecast the exact degree of bradycardia which will be produced by a dose of neostigmine, the patient must be watched for 10 minutes after the injection and atropine given intravenously if necessary.   H. T. B.

Antimalarials, Field Trials of, in West Africa. L. J. Bruce-Chwatt and H. M. Archibald. (Brit. med. J., 1953, 1, 539.) Field trials were carried out in West Africa on the 4 antimalarial drugs chloroquine sulphate, camoquin, pyrimethamine and azacrin (2-methoxy-6-chloro-9-(5′-diethylamino-2′-pentyl) amino-3-aza-acridine). 120 African schoolchildren, aged 5 to 10 years and all of them naturally infected with malaria, were divided into 5 groups, one group receiving no treatment to act as a control, while one of the drugs to be tested was administered to each of the other 4 groups. Dosage given was half that usual for adults. In the chloroquine sulphate and pyrimethamine groups the drug was given in two different regimens. Of those given chloroquine sulphate, half received 0·75 g. in a single dose on the first day, while the other half were given 0·75 g. on the first day followed by 0·25 g. on each of the 2 following days. Of the pyrimethamine group, half received a single dose of 25 mg. on the first day, and the other half had 25 mg. on the first day followed by 25 mg. the next day. The camoquin group were given a single dose only of 0·4 g. The azacrin group received 0·2 g. on the first day, followed by 0·1 g. on each of the 2 following days. Daily blood examinations were carried out and the parasite rate, parasite density and any other malariometrical indices were recorded. All 4 antimalarials were found to be good schizonticides for *Plasmodium falciparum*. The clearance times for trophozoites of *P. falciparum* were similar although camoquin and azacrin appeared to act more rapidly than the other two. None of them is an effective direct gametocide for *P. falciparum*. Infections with *P. malariæ* were too few to give comparable results, but chloroquine and camoquin seemed to act more rapidly than pyrimethamine and azacrin. Two infections of *P. ovale* mixed with *P. falciparum* disappeared within 24 hours of giving camoquin and azacrin. Of the 4 drugs used, pyrimethamine appeared to be the best tolerated, since no complaints of nausea or headache were made. Of the chloroquine sulphate group only 2 children complained of nausea, and 4 of 23 given camoquin complained of headache and nausea within 4 hours of taking the drug. Azacrin was well tolerated on a dose of 0·2 g. but 0·3 g. caused nausea, stomach pains and vomiting in 7 of 10 children.   H. T. B.

ABSTRACTS

Blowpipe Dart Poison from Borneo. J. A. Robinson and H. W. Ling. (*Brit. J. Pharmacol.*, 1953, **8**, 79.) Blowpipe dart poison used by the nomadic Pĕnan of Borneo has been shown to contain a cardiac toxic substance resembling ouabain. In the cat and rabbit it had 20 per cent. and in the mouse 33 per cent. of the toxicity of crystalline ouabain, which suggests that it is not ouabain itself. It has also been shown to differ from aconitine. G. F. S.

Butazolidine, Effect of, on the Excretion of Water and Electrolytes. J. Green and P. O. Williams. (*Lancet*, 1953, **264**, 575.) Preliminary studies of the urinary excretion of sodium and potassium after the intramuscular injection of 1 g. of butazolidine indicated a prompt reduction in the volume of urine and a steep fall in the urinary sodium level. 4 healthy volunteers were then placed on a diet low in sodium and potassium and were given every 2 hours 250 ml. of water and a cachet containing 1 g. of sodium chloride and 0·2 g. of potassium chloride. The daily intake was 2 l. of water, 9 g. of sodium chloride and 4 g. of potassium chloride. After 1 day for stabilisation and 1 day as a control period, 3 of the volunteers were given 1 g. of butazolidine intramuscularly. The fourth volunteer served as a control. Samples of urine were collected 2 hourly during the day and the overnight urine was pooled. The average retention in the 12 hours following the injection was 766 ml. of water, 2·4 g. of sodium, 0·4 g. of potassium and 3·56 g. of chloride. Retention of water and sodium chloride is therefore not merely an occasional toxic effect of butazolidine but a normal result of its use. Œdema does not therefore indicate intolerance and should be controllable by restriction of the sodium intake and perhaps by the use of mercurial diuretics. The effect appears to be due to reabsorption of sodium, and not to a true antidiuretic effect. H. T. B.

Chelating Agent, Effect of, on Urinary Lead Excretion. J. B. Sidbury, J. C. Bynum and L. L. Fetz. (*Proc. Soc. exp. Biol., N.Y.*, 1953, **82**, 226.) A study of the effectiveness of disodium calcium ethylenediamine tetra-acetate, administered by the oral and intravenous route, on 7 patients with symptoms of lead poisoning, was undertaken. Intravenously, 1 g. was given to 5 adults on the first day, followed by 2 g. daily for a total of 5 treatment days. The doses were subdivided and each administered in 250 ml. of 5 per cent. glucose solution given over 1 hour by infusion. 2 children were given 30 mg./kg. in glucose solution twice daily. Orally both adults and children received 30 mg./kg. twice daily. 24-hour urine collections and whole blood specimens were obtained for lead analysis before, during and after the treatment period. Intravenous administration produced a 10- to 40-fold increase in urinary lead excretion on the first day. Subsequent values were generally lower, but never less than 3 times the observed pretreatment level. After oral administration the rise in excretion was more gradual, with a maximum on the third and fourth day, and the effect was less than that of the intravenous method. Side reactions were minimal with the dosages employed. It is concluded from the results that the drug is the most effective agent yet proposed for the treatment of plumbism. J. R. F.

dl-cyclo **Hexyloxy-α-phenylethylamines, Morphine-like Properties of.** A. McCoubrey. (*Brit. J. Pharmacol.*, 1953, **8**, 22.) Evidence is presented that the analgesic effect of these compounds is more nearly related to that of morphine and amidone than to that of phenacetin. Three related amines,

m- and *p*-*iso*propyloxy- and *p*-ethoxyl-α-phenylethylamine were not analgesic and their pharmacological properties were different from those of the *cyclo*hexyl ethers and morphine.   G. F. S.

3:3-Dithienylalkenylamines, Analgesic and Other Properties of. A. F. Green. (*Brit. J. Pharmacol.*, 1953, **8**, 2.) A series of 3:3-dithienylalkenyl- amines have been tested for analgesic, antihistamine and atropine-like properties. 3-Dimethylamino, 3-ethylamino, 3-diethylamino, 3-pyrrolidino and 3-piperidino- 1:1-di-2′-thienylbut-1-ene hydrochlorides showed on the rat and the rabbit analgesic activities between 0·6 and 1·0 times that of morphine, but only 0·1 to 0·4 times the activity of morphine in the dog. Keele has shown these com- pounds to be less active than morphine in man, which suggests that analgesic activities in man and dog are similar. Toxicity tests in mice showed these compounds to be between 0·4 to 0·8 times as toxic as amidone. In mice they caused analgesia, hyperexcitability, mydriasis and respiratory depression leading to death. Respiratory depression was related to analgesic activity. None of these compounds had a mydriatic activity greater than 0·005 times that of atropine in the mouse or an antihistamine activity greater than 0·1 times that of mepyramine on the isolated guinea-pig ileum. While they had a local anæsthetic action on the eye they caused corneal damage. In some respects the actions of these compounds are more closely related to pethidine than morphine.   G. F. S.

Ergometrine Intravenously to Prevent Post-partum Hæmorrhage. J. D. Martin and J. G. Dumoulin. (*Brit. med. J.*, 1953, **1**, 643.) The results in 1000 consecutive obstetric patients given ergometrine intravenously were compared with those obtained in 1000 patients delivered previously who did not receive ergometrine. The volume of blood from the vagina and perineum was measured. Episiotomy under local analgesia was performed if a perineal laceration seemed imminent. The dose of ergometrine in the treated series was 0·5 mg. given intravenously with the crowning of the baby's head. On com- pletion of the third stage 0·5 mg. of ergometrine was given intramuscularly. The intramuscular injection was also given in the control series. Blood losses of 20 fl. oz. or more occurred in 13·1 per cent. of the controls but only in 1·2 per cent. of the treated patients. Secondary post-partum hæmorrhage occurred in 3 of the control patients and in 8 of the treated group. Manual removal of the placenta took place in 11 controls and 30 of the treated group. The duration of the third stage was much less in the treated group and the results confirm the view that there is a direct relation between length of the third stage and blood loss. The treated group showed a slightly higher hæmoglobin level than the controls on the 4th or 5th day of the puerperium. Timing of the injection is of the utmost importance. Intravenous ergometrine acts within 1 minute and should produce a tonic uterine contraction and separation of the placenta while part of the baby still occupies the uterine cavity. The disadvantage of the procedure is the need for an assistant to be present to give the injection at the right moment.   H. T. B.

Hyaluronidase, New Inhibitors of. M. Fabinyi-Szebehely, L. Hahn and J. Szebehely. (*Brit. J. Pharmacol.*, 1953, **8**, 30.) 4 triphenylmethane derivatives, polycondensed trihydroxytricarboxytriphenylmethane, polycon- densed hexahydroxytricarboxytriphenylmethane ("trigentisic acid") and two polycondensed heptahydroxytricarboxytriphenylmethanes, as well as two

diphenylmethane derivatives, polycondensed dihydroxydicarboxydiphenyl-methane and polycondensed tetrahydroxydicarboxydiphenylmethane ("digentisic acid") have been found to have a strong inhibitory effect *in vitro* on hyaluronidases and have been tested for their inhibitory effect *in vivo* by a method based on the action of hyaluronidases on the rate of absorption of urethane injected subcutaneously in mice. All of the compounds possessed marked *in vivo* activity, trigentisic acid being the most active. All 6 compounds had an anti-inflammatory action reducing the artificial œdema produced by the injection of egg white in rats. Toxicity tests showed trigentisic acid to be the least toxic in mice and a daily dose of 8 g. produced no ill effects in human volunteers. G. F. S.

Isoniazid in Pulmonary Tuberculosis. A. J. Proust, E. G. Beacham and H. S. Allen. (*Med. J. Aust.*, 1953, **1**, 179.) 20 patients, of whom 19 were clinically resistant to streptomycin and *p*-aminosalicylic acid, and 18 had far advanced and 2 moderately advanced pulmonary tuberculosis, were treated with isoniazid, 0·15 to 0·2 g. daily, for periods of 21 to 25 weeks. 16 noted a definite improvement in appetite and feeling of well-being. 17 patients gained weight over 2 lb. during the period, the average gain being 12·4 lb. 2 patients showed no gain and 1 lost 0·5 lb. Of 17 patients with significant fever prior to therapy, 11 showed a marked change after treatment, and of 13 patients who produced sputum before treatment, 7 showed no change, 7 showed a definite decrease in amount, and in 4 sputum production was eliminated. The toxic effects of the drug were minimal, but the development of resistant strains of the bacteria has necessitated the addition of streptomycin, *p*-aminosalicylic acid or viomycin to the treatment in almost all cases. J. R. F.

Isoniazid in Treatment of Pulmonary Tuberculosis. Second Report of the Tuberculosis Chemotherapy Trials Committee to the Medical Research Council. (*Brit. med. J.*, 1953, **1**, 521.) Results are reported of 3 months' treatment of 364 patients at 40 hospitals. 142 patients received streptomycin (1 g. daily) with isoniazid (100 mg. twice daily), 102 received streptomycin (1 g. daily) with sodium aminosalicylate (5 g. four times daily), and 120 received isoniazid (100 mg. twice daily) alone. Patients were allocated to one of the three courses of treatment by random selection, and divided into three main groups: acute rapidly progressive disease of recent origin (excluding bilateral disease between the ages of 15 and 30), other forms suitable for chemotherapy, and chronic disease considered unlikely to respond to chemotherapy. Details are tabulated of general clinical conditions, weight and temperature changes, sedimentation rates, radiographic appearances and bacillary sensitivity. Judging solely from the results at 3 months, the combination of streptomycin and isoniazid was found clinically to be the most effective of the three treatments studied, although its superiority to streptomycin and sodium aminosalicylate was not great. Clinical improvement was marked, especially in weight increase, with isoniazid alone, but an important defect in this treatment is the frequent and rapid emergence of bacterial resistance to the drug. After the 3 months' treatment, bacillary resistance to isoniazid was found in 62 per cent. of culture-positive patients treated with isoniazid alone, compared with only 13 per cent. in patients treated with streptomycin and isoniazid. Bacillary resistance to streptomycin was found in 11 per cent. of these cases. For this reason, it is suggested that none of the 3 drugs should be used by itself. Similarly, a combination of 2 of these drugs administered to a patient in whom the infecting bacteria are already resistant to one of the two appears to be tantamount to giving the other alone,

and resistance to the second drug rapidly appears. Wherever possible, the drug sensitivity of the infecting organisms should be assessed before chemotherapy is started so as to avoid an unsuitable combination of drugs, and also on its completion in case more chemotherapy is required later. H. T. B.

Morphine, Antidiuretic Action of. N. J. Giarman, L. R. Mattie and W. F. Stephenson. (*Science*, 1953, **117**, 225.) Morphine sulphate was administered to male adult rats and the pooled urinary output in its natural state or after chemical or physical treatment was dialysed, concentrated to the original volume and assayed for antidiuretic activity in rats. Morphine consistently produced urine with a distinct antidiuretic action although neither pethidine nor methadone did so. Heating the urine under a reflux condenser or in an open vessel for 10 hours at 60° C., heating under a reflux condenser with hydrochloric acid (10 per cent.) for 3 hours at 100° C. or heating for 30 minutes at 100° C. in 2N sodium hydroxide destroyed the antidiuretic principle. Treatment with sodium sulphite at room temperature gave inconsistent results. Similar properties are reported for the antidiuretic hormone of the pituitary gland. Morphine injected into hypophysectomised rats has no antidiuretic effect and this together with the foregoing results suggests that the antidiuretic substance produced by morphine is the antidiuretic hormone which is released by the stimulation of the optico-posterior lobe of the pituitary gland by morphine. G. B.

Nicotinamide, Pharmacological Effects of Massive Doses of. F. Bergmann and L. Wislicki. (*Brit. J. Pharmacol.*, 1953, **8**, 49.) A method is described for the assay of nicotinamide in blood, based on a reaction with alkaline hydroxylamine and ferric chloride after deproteinisation with trichloracetic acid. It was shown that nicotinamide injected intravenously was distributed very rapidly throughout the extracellular fluid but was absorbed only slowly by the tissue cells. Studies of the effects of massive doses of nicotinamide in cats, dogs and rabbits showed that it produced a marked hyperglycæmia and oliguria preceded by a period of anuria which appeared to be due to an action on the tubules. Intravenous injections of very high doses (0·5 to 1·0 g./kg.) caused a fall in blood pressure and an increase in the rate and amplitude of the respiratory movements. G. F. S.

Phenylbutazone (Butazolidine) in Chronic Arthritis. L. Cudkowicz and J. H. Jacobs. (*Lancet*, 1953, **264**, 223.) Courses of phenylbutazone, given either intramuscularly or orally, and ranging from 4 months to a few days, were administered to 34 patients with rheumatoid arthritis and 11 with osteo-arthritis. The daily dose was varied according to the response of the patient but never exceeded 1 g. per day. In rheumatoid arthritis phenylbutazone appears to increase mobility and muscle power, and reduces pain to a significant degree. The changes in erythrocyte sedimentation rate were inconclusive and there were no significant changes in white-cell counts. Of the 11 patients with osteo-arthritis 7 showed objective improvement, and, subjectively, 8 had relief from pain. Toxic effects occurred in 22 pateints and were often quite severe. They included abscess at site of injection, gastro-intestinal symptoms, reactivation of peptic ulcer, melæna, salt and water retention with œdema, and rash. It is concluded that phenylbutazone has a place in the management of chronic joint disease in which the outstanding feature is pain; but careful selection and constant supervision of patients are necessary. A past history of peptic ulceration, or the presence of hypertension, chronic bronchitis and emphysema, or valvular heart disease with a past episode of failure are absolute contra-indications since cardiac failure may follow salt and water retention. S. L. W.

Phenylindanedione as an Anticoagulant. M. Toohey. (*Brit. med. J.*, 1953, **1**. 650.) Phenylindanedione was used as an anticoagulant in 68 patients, most of them suffering from coronary thrombosis or deep venous thrombosis. Prothrombin levels of 10 to 20 per cent. of normal were aimed at and the usual dosage was 100 mg. twice during the first 24 hours followed by 50 mg. twice during the second 24 hours. The full effect is not produced for 36 to 48 hours and only rarely should these doses be exceeded. Only 6 failed to reach an adequate prothrombin level in 48 hours and in only 1 was the level about 40 per cent. Further dosage was based on the response after 36 to 48 hours. In 44 patients the daily maintenance dose was between 75 and 100 mg. and in 5 it was more than 150 mg.; the daily dose was given in 2 equal amounts, morning and evening. While dosage must be based on prothrombin determinations, the amount required is influenced by the weight and condition of the patient. Heavy patients require more, while acutely ill patients, especially those severely shocked, require less until their condition improves. Frail patients also require less. Renal function is particularly important in determining dosage since deficient function causes delayed excretion. No toxic effects were noticed in any of the patients. If an antidote is required, vitamin K_1, even by mouth, has a dramatic effect within 8 to 20 hours. When once a patient's maintenance dose has been ascertained, prothrombin determinations need only be made 2 to 3 times a week. H. T. B.

Procainamide and Hexamethonium Bromide; Combined Use in Controlled Hypotension. A. A. Mason and J. F. Pelmore. (*Brit. med. J.*, 1953, 1, 250.) In a series of 50 unselected surgical cases an average dose of 65 mg. of hexamethonium bromide was given intravenously, preceded by 1 g. of procainamide intravenously in 2 doses. In a control series an average dose of 95 mg. of hexamethonium bromide alone was given. In a further series of 17 cases in which hexamethonium bromide also failed to produce a sufficient fall in blood pressure, the subsequent injection of 1 g. of procainamide produced a quick response. If it is deemed advisable to use the two drugs together, as, for example, with ventricular arrhythmias and maxillo-facial operations, they may be employed without toxic effects. The blood pressure does fall, however, more than with either used separately, and with smaller doses. In other words, the drugs show a true potentiation of each other's hypotensive effects, and when employed together they must be used with extreme caution. Failure of hexamethonium bromide alone seems to occur with the greatest frequency in patients under the age of 30, and it may be anticipated that procainamide will be required most often in this age group. In an addendum the authors report on a further 150 cases, in which the pulse rate has almost invariably been reduced following injection of procainamide. They used the drug in doses of 0·5 to 1 g. in those cases in which the pulse rate remained higher than 110. S. L. W.

Proguanil, Isolation of a Metabolite with High Antimalarial Activity. A. F. Crowther and A. A. Levi. (*Brit. J. Pharmacol.*, 1953, **8**, 93.) It has been suggested that proguanil is modified in the body to form a product which is responsible for antimalarial activity. This paper reports the isolation of a highly active metabolite from the urine of rabbits and from human volunteers which has been shown to be 4:6-diamino-1-*p*-chlorophenyl-1:2-dihydro-2:2-dimethyl-1:3:5-triazine. It was found to have an activity about 10 times that of proguanil against *P. gallinaceum* in the chick and to be active against both the erythrocytic and exoerythrocytic forms. Methods are described for its isolation and identification and a method of synthesis is to be published. G. F. S.

Pyrimethamine in Acute Malaria. T. Wilson and J. F. B. Edeson. (*Brit. med. J.*, 1953, **1**, 253.) The authors report on the use of pyrimethamine in the treatment of 126 patients with acute malaria in Malaya. 80 patients had previously untreated acute falciparum malaria. A single dose of 50 mg. of the drug failed to cure 2 out of 15 patients; a dose of 100 mg. (in 2 doses of 50 mg. each) failed to cure 4 out of 39; and a total dose of 300 mg. (100 mg. on the first day and 50 mg. daily for the next 4 days) failed to cure 7 out of 26. Apart from the failures the action on fever and symptoms was usually slow. The results in 24 proguanil-resistant patients treated with 100 mg. of pyrimethamine were so similar as to suggest that resistance to proguanil does not affect the response to pyrimethamine. It is concluded that pyrimethamine must be regarded as unsuitable for the treatment of acute malaria due to Malayan strains of *P. falciparum*. 11 patients with acute vivax malaria were treated with the 50 mg. single dose and 11 with the 100 mg. dose. Disappearance of parasites was slow, but there were no failures. S. L. W.

Terramycin for Infections in Diabetic Patients. J. B. Walker. (*Lancet*, 1953, **264**, 521.) Infections associated with diabetes are frequently caused by mixtures of organisms, and terramycin, in view of its wide range of activity, is therefore particularly suitable for treating them. The author used terramycin in the treatment of a total of 93 infectious conditions among 70 patients. The majority were infections of the feet varying in severity from simple cases to those that were frankly gangrenous, but other types of infection were treated, including boils and carbuncles, urinary tract infections, respiratory infections and wound infections. Dosage was generally 0·5 g. thrice daily, with or immediately after meals, the total amount required averaging 10 g. In the series of foot infections treated, swelling and pain subsided rapidly, with marked improvement in 48 hours. Toxicity appeared to be low, 11 cases of diarrhœa, one severe, being reported in the 93 treatments. There appears to be little risk of disturbing the diabetic control, and no change of diet was required. Relapse when it occurred was due to arteriosclerosis, but by keeping the infection controlled an active life for the patient could be prolonged. Sensitivity tests seem to be unnecessary, or even misleading, since the organisms were found frequently to be more sensitive clinically than laboratory tests had indicated. Development of resistance to the antibiotic was not observed. H. T. B.

Thiobarbituric Acids, Ultra-short-acting. E. E. Swanson and K. K. Chen. (*Proc. Soc. exp. Biol., N.Y.*, 1953, **82**, 212.) A comparison of four *N*-methyl thiobarbituric acids with sodium 5-allyl-5-(1-methylbutyl)thiobarbiturate and thiopental was made using rats, rabbits, cats and dogs. All were potent anæsthetics by intravenous injection. The duration of anæsthetic and hypnotic action for the *N*-methylated derivatives was found to be shorter than that of the two non-methylated compounds, and less cumulative action was also observed when one-half of the AD50 was injected intravenously. Rabbits and rats were found to be more sensitive to the *N*-methylated derivatives. In cats, all 6 compounds had approximately the same AD50 and the same LD50. 2 of the *N*-methylated derivatives produced less hiccough, sneezing and coughing in the anæsthetised cats than the 2 non-methylated compounds, while the other 2 *N*-methylated derivatives were found to be free from these effects. Dogs showed less sensitivity to the *N*-methylated derivatives, which did not inhibit vagal response but induced slight hypothermia and tachycardia in the anæsthetised animals following an AD50. J. R. F.

Triethylene Melamine in Malignant Disease. E. Paterson, P. B. Kunkler and A. L. Walpole. (*Brit. med. J.*, 1953, **1**, 59.) Clinical trials were carried out with enteric coated tablets, after experiments in dogs had shown that they provided a much more constant response than plain tablets or capsules. In Hodgkin's disease, 0·2 to 0·3 mg./kg., as a single dose, or divided, was suitable for treatment and produced results similar to nitrogen mustards. It proved inadvisable to use triethylene melamine in patients with a low initial poly-morphonuclear leucocyte count or to repeat the treatment unless the count was fully restored. A dosage of 0·1 to 0·2 mg./kg., repeated at intervals of several weeks gave results probably superior to radiotherapy in chronic lymphoid leukæmia, although results in myeloid leukæmia were disappointing. No beneficial effects were observed in tumours originating in epithelial or connective tissue. Oral administration of enteric coated tablets simplifies treatment compared with intravenous injections as used previously, but hæmatological controls are essential because the hæmopoietic system is highly sensitive to triethylene melamine.   G. B.

Triiodothyronine, Biological Activity of. E. G. Tomich and E. A. Woollett. (*Lancet*, 1953, **264**, 726.) Experiments to determine the relative potencies of 3:5:3'-L-triiodothyronine and L-thyroxine were conducted by various methods. A comparison of the effectiveness of the two substances in preventing goitre in thiouracil-treated rats showed L-triiodothyronine to be 7·4 times as active as L-thyroxine. A comparison by a mouse-anoxia method gave an activity ratio of 4·5:1 in favour of L-triiothyronine. Assessments based on the oxygen consumption rates in rats showed L-triiodothyronine to be 5·1 times as active as L-thyroxine (or 5·3 times as active when the drugs were given by mouth). During the course of absorption and excretion studies on rats and cats with L-triiodothyronine it was found that recoveries measured by the Blau isolation technique were similar to those obtained with thyroxine. If exogenous thyroxine is deiodinated to triiodothyronine in the body those who have been using the Blau method for recovering thyroxine from animal tissues may well have been estimating triiodothyronine alone or a mixture of both substances.
                                                    S. L. W.

3:5:3'-Triiodothyronine, Physiological Activity of. J. Gross and R. Pitt-Rivers. (*Biochem. J.*, 1953, **53**, 652.) The activity of 3:5:3'-triiodothyronine relative to thyroxine has been determined by the gastric prevention test in rats and the L-form found to possess 5 times the activity of L-thyroxine. D-Triiodothyronine showed only 7 per cent. of the activity of the L-form, whereas DL-triiodothyronine has about 57 per cent. of the activity of the L-form. Administered orally 3:5:3'-L-triiodothyronine has only about 86 per cent. of the activity exhibited by an equivalent dose given subcutaneously. A daily dose of 1 μg. in thyroid-ectomised rats was sufficient to maintain a rate of growth equivalent to that obtained with 5 μg. daily of L-thyroxine. Large doses of triiodothyronine reduced the rate of growth, causing at the same time enlargement of the kidney cortex, heart and adrenal glands. L-Triiodothyronine was more effective than L-thyroxine in preventing enlargement of basophil and degranulation of acidophil cells in the pituitary gland of the thiouracil-treated rat. It also prevented enlargement of the pituitary gland in the thyro-parathyroid-ectomised rat. It is suggested that 3:5:3'-L-triiodothyronine is the peripheral thyroid hormone and that thyroxine is its precursor.   J. B. S.

BACTERIOLOGY AND CLINICAL TESTS

Bis*iso*quinolinium Salts, Antibacterial Activities of. H. O. J. Collier, M. D. Potter and E. P. Taylor. (*Brit. J. Pharmacol.*, 1953, **8**, 34.) A series of polymethylene bis*iso*quinolinium salts, with chain lengths of 8 to 14, 16, 18 and 20 methylene groups have been prepared and tested for antibacterial activity *in vitro* against *Streptococcus pyogenes*, *Str. fæcalis*, *Staphylococcus aureus*, *Vibrio choleræ*, *Salmonella typhi*, *Shigella shigæ*, *Sh. flexneri*, *Pseudomonas pyocyanea* and *Mycobacterium phlei*. While they showed appreciable activity none was as active as cetrimide. Antibacterial activity increased with chain length but so did toxicity. They were not considered to be of sufficient activity to be useful against bacterial infections in man. G. F. S.

Polymyxin, Absorption of, by Bacteria. A. V. Few and J. H. Schulman. (*Nature, Lond.*, 1953, **171**, 644.) Experiments are described concerning the absorption of polymyxin E by washed suspensions of several Gram-negative and Gram-positive bacteria; the release of soluble cell constituents containing free purines and pyrimidines, initiated by the presence of the antibiotic, was followed concurrently by examination of the absorption maximum at 260 mμ. An assay process was developed, based upon the fact that the polymyxins form stable unimolecular films at the air/water interface when spread upon 70 per cent. (w/w) ammonium sulphate solution. Details of the absorption experiments are given for polymyxin E for 6 organisms together with absorption isotherms, the final solutions being assayed for residual polymyxin and for the presence of released cell constituents absorbing at 260 mμ. Determinations of the sensitivities of the organisms by streaking 24-hour broth cultures upon nutrient agar containing increasing quantities of polymyxin E showed that *Ps. denitrificans*, *B. subtilis* and *E. coli* were all inhibited by 5 μg./ml., whereas *S. aureus*, *P. vulgaris* and *Str. fæcalis* were not inhibited by 100 μg./ml. The initial absorption process was complete at a polymyxin concentration in the supernatant layer of 100 to 150 μg./ml., after which further absorption was slight. Of 4 strains of *E. coli* investigated, 3 showed agglutination phenomena which were maximal at supernatant concentrations of 150 μg./ml. For highly resistant organisms, polymyxin does not significantly increase the release of material showing absorption at 260 mμ, although with sensitive bacteria the presence of polymyxin caused a rapid release of cellular material, complete at low concentrations of polymyxin in the supernatant liquid. With *B. subtilis* the absorption of polymyxin and release of material showing absorption at 260 mμ was rapid and unaffected by incubation times between 5 and 60 minutes. R. E. S.

Tubercle Bacilli; Variations in Virulence Effected by Tween 80 and Thiosemicarbazone. H. Bloch and H. Noll. (*J. exp. Med.*, 1953, **97**, 1.) Tubercle bacilli were grown in the presence of concentrations of tween 80 ranging from 0·05 to 2·1 per cent., and equal numbers of viable bacteria from these cultures were compared in infection experiments in mice, the average survival time of the mice being used as a criterion of the virulence of the bacilli. Reduction of virulence was slight in bacterial suspension from cultures with tween 80 ranging from 0·05 to 1·0 per cent., but was considerable in cultures with 2·1 per cent. Bacteria grown in the presence of 2·1 per cent. of tween 80 gave rise to the same number of colonies, *in vitro*, as bacteria grown in ordinary media,

LETTER TO THE EDITOR

The Melting Point of 4:4'-Diaminodiphenyl sulphone (Dapsone)

SIR,—4 : 4'-Diaminodiphenyl sulphone is required by the British Pharmaceutical Codex to have a melting point as defined in the British Pharmacopœia 1953, page 703, lying between 176° C. and 179° C. (B.P.C. 1949, Supplement 1952, page 19), and material conforming to these limits has been manufactured over a number of years without difficulty. Recently, however, samples have been encountered melting sharply in the region of 180·5° C.; these high melting temperatures were not associated with any known modifications in the method of preparation and it is hardly possible that they represent a purer product. Thus, on frequent occasions, samples having melting points in the region of 178° C. exhibited, after grinding, melting points in the region of 180·5° C., and on a few occasions the same effect was obtained by leaving samples melting at about 178·5° C. in an oven at 50° C. for several days. On the other hand, recrystallisation of the high melting material from water and isolating at 5° C. caused the melting point to fall from 180·5° C. to 178·5° C. and it remained at this level on repeated recrystallisation; recrystallisation from methanol had the same effect.

We conclude from these observations that 4 : 4'-diaminodiphenyl sulphone can be obtained in at least two forms, melting respectively at about 178·5° C. and 180·5° C.; each of these melting points represents material of a high degree of purity, but the material having the higher melting point does not conform strictly to the requirements of the B.P.C. Monograph.

<div align="right">L. T. BUTT.</div>

Imperial Chemical Industries Ltd.,
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but their oxygen uptake was increased. Virulent bacteria grown in the presence of high amounts of tween 80 decolorised methylene blue in a test in which organisms from the same virulent strain but cultured without tween 80 did not reduce the dye (a positive methylene blue test is typical of non-virulent tubercle bacilli). Essentially the same changes occurred when virulent tubercle bacilli were grown in the presence of 0·5 μg./ml. of *p*-formacetanilide thiosemicarbazone; this amount was not sufficient to prevent the growth of bacteria or reduce the number of viable cells in a culture, but it reduced the virulence of bacteria considerably and rendered them capable of decolorising methylene blue. Cord factor, a lipid constituent of virulent bacteria which is toxic for mice, was shown to be present in filtrates from cultures of virulent bacteria when the media contained 2 per cent. tween 80, but could not be recovered from culture filtrates containing 0·05 per cent. On the other hand, no toxic material could be extracted from bacteria grown in the presence of 0·5 μg./ml. of *p*-formacetanilide thiosemicarbazone.   S. L. W.