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SYMPOSIUM

WOUND HEALING AND DRESSINGS

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The skin is the barrier between the outside world and the organism, it is the pliable, water vapour permeable container in which we live. Its very situation makes it the most vulnerable of all tissues to injury and infection, but in the course of evolution it has developed amazing reparative powers. In the Edwin Smith[1] Surgical Papyrus, discovered in Egypt by Smith in 1878, and believed to have been written somewhere about 1500 years B.C., there is frequent reference to materials used as wound dressings and the importance of certain aspects of treatment. Drainage of deep or contaminated wounds had been found to be essential and to keep the wound open raw fresh meat was applied for at least the first 24 hours, followed by the application of an ointment consisting of honey and ibex grease which was bound in position by bandages. The bandages which were of linen or papyrus were of the open wove type used at that time by the embalmers. There is mention of absorbent lint to remove discharge from wounds. The edges of superficial clean wounds were held together by strips of adhesive plaster which was probably made from linen bandages and glue. A similar treatment of wounds was practised by the Greek surgeons. Hippocrates describing the treatment of a head wound says: "it should not be moistened nor should it be bandaged; after cleaning the wound as soon as possible, one should dry the wound . . . for what is soonest dried up . . . thereby most readily separates from the rest of the tissue which is full of blood and life". Coming to more recent times, Joseph Lister, in a treatise "On the antiseptic principle in the practice of surgery"[2] which he read before the British Medical Association in Dublin on August 9, 1867, stated that "all the local inflammatory mischief and general febrile disturbance which follow severe injuries are due to the irritating and poisoning influence of decomposing blood or sloughs. In conducting the treatment, the first object must be the destruction of any septic germs which may have been introduced into the wound, either at the moment of the accident or during the time which has since elapsed". He achieved this by introducing phenol of full strength into all accessible recesses of the wound by means of a piece of rag held in dressing forceps and dipped into the liquid. He also applied a piece of lint dipped in phenol, overlapping the sound skin to some extent, covered with a tin cap which was daily raised in order to touch the surface of the lint with the antiseptic.

The object of the tin cap was to prevent evaporation. If it was desirable not to disturb the dressing in contact with the wound, but at the same time to keep it moist with antiseptic, a putty of common whitening (carbonate of lime) mixed with a solution of one part of carbolic acid in four parts of boiled linseed oil was applied. So long as the discharge continued, the paste was changed daily, but the lint was left in position. When the discharge ceased, the paste was discontinued, but the original lint was left adhering to the skin until scabbing was completed. He found that "if a perfectly healthy granulating sore be well washed and covered with a plate of clean metal, such as block tin (also sheet lead from tea chests was used) fitting its surface pretty accurately and overlapping the surrounding skin for an inch or so in every direction and retained in position by adhesive plaster and a bandage, it will be found, on removing it after 24 or 48 hours, that little or nothing that could be called pus is present". . . . "The clean metallic surface presenting no recesses, like those of porous lint, for the septic germs to develop in, the fluid exuding from the surface of the granulations has flowed away undecomposed, and the result is absence of suppuration". He made the point that the mere contact of a foreign body does not cause granulations to suppurate, whereas the presence of decomposing organic matter does.

Joseph Gamgee[3] on February 8, 1880, at the Queen's Hospital, Birmingham, gave a clinical lecture entitled "Absorbent and Antiseptic Surgical Dressings". He stated that "clinical experience has demonstrated the great value of absorbent materials. Discharges drain through them so rapidly that wounds are kept clean and the surrounding parts dry". He demonstrated Gamgee tissue which consists of a layer of absorbent cotton wool between two layers of absorbent gauze, combining the powers "both of compression and absorption". According to Gamgee the invention of absorbent dressings was due to Dr. Mathias, Mayor of Lausanne, but it was Gamgee's idea to combine absorbent cotton wool with the compressing gauze and it was he who first insisted that the material should be manufactured in an antiseptic manner. In his own words he states "the soothing surgical pressure is like that which you interchange with the hand of a lady, your hand adapts itself to hers and tenderly presses it wherever it can touch it, but nowhere squeezes it for fear of offending".

Paul Gerson Unna[4] was another who contributed much to the improvement of surgical dressings. In 1881, he described Unna's paste: gelatin 3 parts, zinc oxide 3 parts, glycerine 5 parts and water 9 parts. He stated that "the application was protective and soothing, excluding the air and lessening the chances of scratching and secondary infection, it provides a well-fitting covering which is easily applied and easily removed; incorporated in a bandage the paste forms an excellent support for varicose ulcers".

At the present time minor skin injuries are a major problem, both in industry and in the home. It is impossible to determine the working time lost and the inconvenience caused by puncture wounds, incisions, lacerations, and small burns. It has been estimated by Squire[5] that in this country nearly half a million skin injuries occur each day which need at

least a first aid dressing. Well over 50,000 of these injuries require attention in factory surgeries. Each year at least twenty-thousand industrial workers receive compensation for septic injuries and each worker loses on an average from two to four weeks working time.

A wound dressing is the most commonly used therapeutic agent. The variety available shows that the ideal dressing has not been produced and while it may never be possible to achieve the ideal, criteria for such a dressing can be established. These criteria are dependent on an appreciation of the anatomy and physiology of the skin, the mechanisms of tissue repair, the bacteriology of wounds and sepsis and the properties of natural and synthetic materials.

Structure of Normal Skin

Skin consists primarily of two layers (Fig. 1), the outer one being in part alive and in part dead. This outer layer or epidermis is attached to the under-lying inner layer or dermis by downward projections or rete

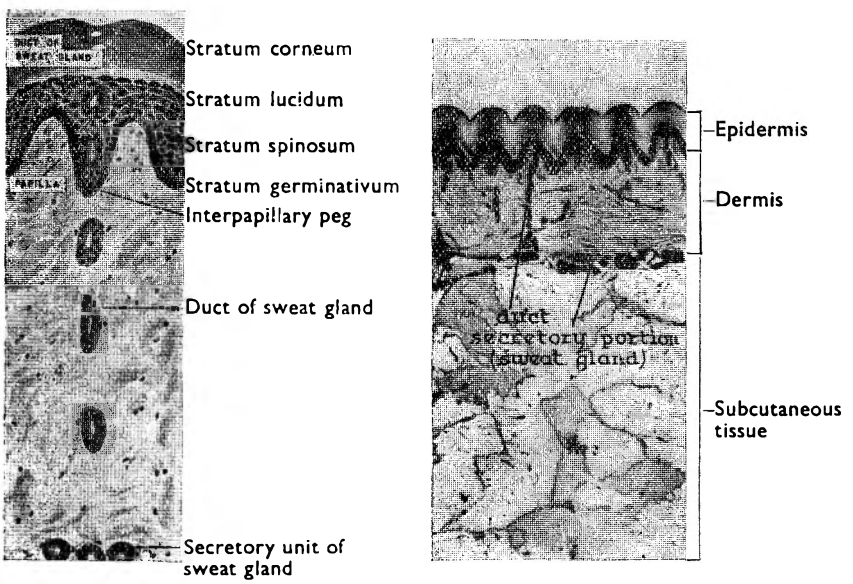

FIG. 1. *a*. Section of skin. Duct of sweat gland enters on interpapillary peg and is constituted by the cells of the different layers of epidermis through which it passes.

b. Low power photomicrograph of section of thick skin from sole of foot (from "Histology", A. W. Ham, Lippincott, London).

pegs, the upward projections of the dermis being known as the dermal papillae. This arrangement (i) allows the epithelial tissue, which consists entirely of cells, to stretch. If the reserve of cells in the rete pegs were not present, the epidermis would split, as it has little tensile strength. (ii) It allows for a reservoir of repair cells; (iii) allows an increased total contact surface between the epidermis and the dermis, and (iv) prevents

the epidermis from sliding on the dermis. The effect of the epidermis sliding on the dermis is well known to those who develop blisters after digging in the garden.

The epidermis is entirely cellular and has no blood supply or sensory endings, its metabolism being dependent on diffusion of tissue fluids from within outwards. Thus the cells nearest the source of supply of tissue fluid, that is the basal cell layer, have a satisfactory environment and are able to multiply. Above this layer the nutritional requirements of the cells cannot be met and mitotic division does not normally occur. Towards the periphery of the prickle cell layer the cells are on the verge of starvation and changes occur in their protoplasm, starting at the periphery of the cell, which result in the formation of the protein called keratin. This process is not understood but it is believed that the utilisation of glycogen is important for the proper formation of keratin. These keratin forming cells appear to have an excess of glycogen compared with other cells in the body. Finally, keratinisation is completed with death and dehydration of the cell, the keratin and cellular debris then being rubbed from the surface of the skin. Evaporation of water vapour from the skin surface, to allow full dehydration to take place, is essential. The keratin layer protects the cells beneath from bacterial infection and in its normal dehydrated state does not provide a nutrient medium for bacterial growth to occur However, being a protein it can imbibe water and swell, producing the typical macerated condition found under waterproof dressings and around soggy, wet and infected wounds. The hair follicles are in the dermis while the sweat glands extend to the subcutaneous tissue. The dermis has a rich vascular and sensory supply.

Mechanism of Wound Repair

Assume that a wound passes through the epidermis and the dermis into the subcutaneous tissue and that a small piece of the epidermis and the dermis has been lost. With penetration of the dermis there will be damage to capillary loops with bleeding which should be allowed to continue for a short time providing the bleeding is not from major vessels. The initial break in the continuity of the skin becomes plugged by a fibrin clot which is nature's first aid dressing. It prevents further loss of blood and tissue fluid, infection of the tissues and, as it dries out, contracts and pulls the skin edges together. Having plugged the hole, continuity of the skin has to be restored. This is accomplished by two distinct processes: the first, fibrous tissue repair of the dermis, and the second, migration of the epidermis. Until there is a good foundation of healthy vascular granulation tissue, free from sepsis, epithelium cannot cover the defect. If a hard thick clot plugs the wound, the contraction of the fibrous tissue in the deeper layers is retarded. Until recently it was thought that it was the basal cell layer of the epidermis which was responsible for the repair of the epidermal layer. However, it has been shown by Hartwell[6] that these cells play a secondary role in the healing process. His work has been carried out on pigs and man, the pig being used because its skin and subcutaneous tissue are similar to that of man.

While the two processes of wound healing are distinct they are intimately related and proceed simultaneously. At the edge of the wound the epithelial cells in the middle layer of the prickle cell layer migrate towards the defect: they become amoeboid. As the cells at the edge of the wound move, so the cells adjacent to them change their shape and move in the same direction. This process continues for some distance away from the wound. Hartwell[6] has shown that these mobile cells have entirely different staining and morphological characteristics to the stationary cells. Gradually, as this moving membrane of epidermal cells extends, the basal cells supply an increasing number of cells to the prickle cell layer. Any islets of epidermis which may have been left behind, such as a hair follicle or sweat duct, proliferate. Providing the repair of the dermis is continuing to keep step with the epidermis all is well. The lower cells of the migrating membrane form the new basal cell layer and these cells in turn divide and produce more cells to thicken up the epidermis (Fig. 2). If, however, the wound is infected with pathogenic organisms and there is a mass of stagnant and necrotic debris, the process is impeded. Should the granulation tissue formation be too rapid, migration of the epidermal membrane cannot take place. These granulations have then to be "burnt down" with, for example, silver nitrate. This overgrowth of granulation tissue occurs if the area of epithelial loss has been too great, when there is continual trauma of the epidermal layer, or infection. One important cause of delayed healing is the dressing. If this covers a mobile part, such as a finger, then, every time movement occurs, there will be abrasion of the delicate film of epidermal tissue. Even more important is the adhesion of the dressing to the wound. The upper layer in the migrating sheet must lose water if proper keratinisation is to occur. This is also essential for the control of bacterial growth. However, if the dressing pad dries out completely and adheres to the surface of the wound, or is of a fibrous nature so that the fibres become incorporated in the tissues, then each time the dressing is changed damage will result. A continual battle is therefore waged between those who dress the wound and the reparative powers of the

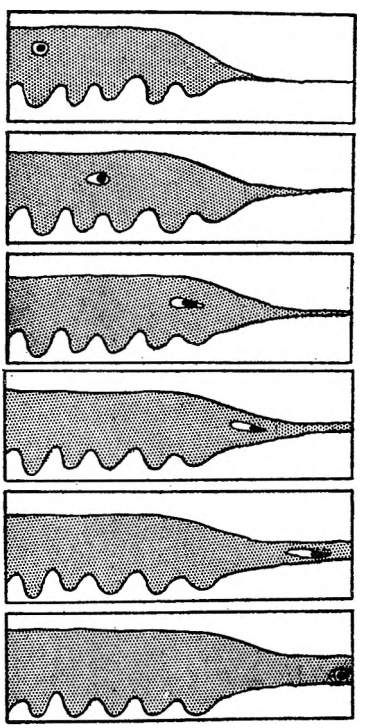

FIG. 2. Diagrams to show how a "prickle cell" in the epithelium bordering a wound can become a "basal cell" in the healing extension membrane of epithelial cells. (From "Mechanism of Healing in Human Wounds", S. W. Hartwell, Thomas, Illinois, U.S.A.)

patient. A dressing must be such that it will absorb by capillarity excess secretions and allow the evaporation of water vapour, but will not adhere to the wound or exudate. It is essential that all dressings which are stuck to wounds be moistened with saline. To protect the healing wound, particularly in exposed areas, such as the hand or foot, a certain minimum thickness is required. There must be a compromise, therefore, between what is required for protection and absorption, and what can be tolerated by the patient if he is to continue his occupation. Again, immobility of a wound is desirable, but this is rarely possible particularly in minor injuries of the hand. The pressure exerted on a wound by the dressing pad is important provided that the surface in contact is of a smooth nature. Cells are able to migrate more readily if they are able to move between two planes, rather than on one. Ideally the pad should not be of a fibrous construction, but be a continuous film. Thus the primary functions of a dressing emerge. They are, to absorb tissue fluid, tissue breakdown products, and blood, to protect the wound from injury during healing, to prevent infection, and to allow the evaporation of water vapour from beneath the dressing.

Wound Infection

We come now to the exceedingly complex problem of wound infection, the only full and exhaustive study of which being probably that of Williams and Miles[7]. They found that 20 per cent of wounds, swabbed within six hours of injury, were infected with *Staphylococcus aureus*. Contamination was commoner in those patients who normally carry the *Staph. aureus* on the skin—60 per cent of those with staphylococcal infection were the result of contamination from the patient's own skin. They investigated the bacterial flora of 487 septic wounds and other septic lesions that required operative treatment and found that *Staph. aureus* and *Streptococcus pyogenes* were the chief organisms causing infection. 86 per cent of these septic wounds yielded *Staph. aureus* and 22 per cent *Str. pyogenes*. While the staphylococcus appeared early in the wound, the streptococcus was a late infector and was usually the result of cross infection at some stage during treatment. More than 35 per cent of clinically healthy wounds yielded *Staph. aureus* on culture.

Wound Cleansing Agents and Medicaments

A great deal has been written and much publicity is still given to various agents which are said to kill organisms in a wound. While tests *in vitro* may show bacteriostatic or bactericidal properties, their effects on the tissues may limit their use *in vivo*. In 1942, Barnes[8] described the antiseptic properties of cetrimide. Williams and others[9] found that it was bactericidal to Gram-positive cocci, for example, the staphylococcus, *in vitro*, although Baker, Harrison and Miller[10] had stated that this activity was impaired in the presence of proteins and certain fats. Swabs taken from normal skin treated with cetrimide are found to be sterile; in addition it is a good detergent removing dirt and oil very effectively without causing the patient pain. Jacoby[11] showed that the surface layer of cells

treated with cetrimide is destroyed. Thus, in healing wounds, the delicate layer of migrating epithelial cells, which is endeavouring to cover the granulating surface, may be damaged. Squire[12] and others have observed dermatitis after its continual use, and it is, therefore, better to restrict it to the initial cleaning of a wound. At subsequent treatments either a dry swab, or one moistened, but not dripping, with saline should be used. While the local application of these agents may reduce the bacterial flora, the damage which they do to the tissues, either in causing its immediate death or inducing a hypersensitivity or allergy, may be of greater significance. Sensitisation after the use of sulphonamides and penicillin have been seen frequently. Williams and Miles[7] have stated that "drug treated wounds appear to heal slowly and in two small burns repeated drug treatment was associated with considerable sloughing of granulations in the absence of any demonstrable infection. Repeated applications of powders often lead to hard caking in the wound, but this could be avoided by the use of impregnated lint dressings". By repeated small doses, the drug resistant organisms may dominate the picture. It is important to remove necrotic material and debris from wounds at an early stage and allow free drainage. Whether bactericidal agents carried by the dressing are of use is doubtful, other than for the sterilisation of the dressing itself.

Importance of Porosity of Dressing

Wound dressings should allow the evaporation of water vapour from the wound area. Beneath a dressing a considerable quantity of water may accumulate. This arises from the sweat glands of the skin surrounding the wound; from normal insensible water vapour loss through the epidermis and from the dehydration of epidermal cells; and from tissue fluid lost from the wound itself.

Burch and Winsor[13] showed that approximately 1·63 g. of water are lost per sq. metre of the body surface every ten minutes, the relative humidity of the surrounding atmosphere being 50 per cent and at an ambient temperature of 75° F. This represents 235 g./sq. metre of body surface per 24 hours, or assuming the average adult body to have a surface area of 1·8 sq. metres, 423 g./24 hours. They also found that the loss of tissue fluid from the floor of a blister raised by cantharides was nearly ten times as great. Thus from their figures, a dressing would need to have a porosity of approximately 2340 g./sq. metre/24 hours. This high porosity is, of course, only necessary for that area of the dressing which corresponds to the area of the wound and for the period when the wound is actively losing tissue fluid. Bull, Squire and Topley[14] reported on experiments with a plastic film dressing—Nylon, I.C.I. type 8—which had a porosity of 600 g. of water vapour per sq. metre per 24 hours, at 100° F. with a differential water vapour pressure of 55 mm. of mercury across the film. In preliminary experiments on normal skin it was found that, if a Nylon dressing was left *in situ* for three days or more, *Staph. aureus* could not be recovered from beneath the window of the uncoated area of the dressing. The only organisms that could be cultured from the covered area were of the *Staph. albus* group. This reduction of bacterial flora and

the absence of *Staph. aureus* was not due to any chemical effect of the Nylon film. Their work demonstrated that the skin, if it were not continually reinfected, would, in fact, be free of pathogens. If a non-porous waterproof dressing covers the skin, there is a quantitative increase in the staphylococcal flora. Why there should be a difference under porous and non-porous cover is not clear. Both types of dressings prevent contamination of the skin by micro-organisms under normal conditions. Brann[15] found that human hair rarely carries pathogenic organisms. He suggested that the fatty acids of the scalp might be bactericidal. Marchionini and others[16,17] demonstrated the importance of the "acid mantle" for skin protection from bacterial invasion. Burtenshaw[18] showed that the sterilising effects of the skin depended mainly on the unsaturated fatty acids in the sweat and sebum. Ricketts, Squire and Topley[19] found that it was the long chain fatty acids, particularly oleic acid, which were responsible. Under both the micro-porous and occlusive dressings the fatty acids are retained. Incomplete dehydration of the keratin coupled with a change in the gaseous environment under a non-porous dressing may be responsible for the difference in bacterial flora.

Experimental Work on Wound Healing

Some interesting work on the comparison of wound healing with and without dressings was carried out by Heifetz, Lawrence and Richards[20] on clean surgical wounds of the anterior abdominal wall of rabbits. They found that well coapted wounds heal equally well whether they are covered by cotton gauze dressings or not. They thought that the absence of a dressing allows quick drying of the surface coagulum and this gives an early protection to the wound. When they repeated their work with the surgical wounds in human subjects, they found the following requirements had to be fulfilled if a dressing was not applied. There must be no local anaesthesia of the wound, sharp dissection and minimal undermining of the tissue planes, gentle handling of the tissues at operation, almost complete haemostasis with no dead space, insertion of non-absorbable sutures, absence of contamination and debris in the wound, accurate coaptation without strangulation of tissues, no drains, and absence of interference with the wound by the patient.

Bacterial counts taken from clinically aseptic wounds showed statistically no significant differences whether or not the wound was dressed, although the data suggested that the bacterial counts were, in fact, lower in those wounds where gauze dressings had not been applied. Körlof[21] investigated different methods of treating pyocyaneus infected burns of guinea pigs. He used polymyxin B, an antibiotic produced by a strain of *Bacillus polymyxa* and effective against pyocyaneus *in vitro*, phenoxetol, also effective in dilution 1 in 500 in broth, and chlorophyll. He found that the mortality of the animals was considerably lower if no dressing or a porous dressing was used, than in those cases in which a thick occlusive dressing had been applied; there was also a diminution of the bacterial growth in those wounds exposed to the air. The therapeutic agents employed had no effect on the pyocyaneus infection or on the rate

of healing of these experimental wounds. Baron[22–25] in a series of papers from 1951 to 1955, has studied the effects of textiles on standard wounds in guinea pigs. He believes that the open air treatment of wounds should be used only for wounds of a superficial nature, that compression by a plate of Cellophane prevents loss of tissue fluid and congestion of the surrounding tissues, and that textile drainage allows the removal of wound secretion with a reduction of infection of wounds. His experiments show that the indiscriminate use of wound textiles, without regard to the number of layers used or character of the fabric, increases the mortality rate of experimental animals. The finishing processes applied to wound textiles have an undesirable effect on wounds[26], the use of titanium dioxide and optical whiteners being frequently the cause of delayed healing. Non-delustered viscose rayon wool which has a yellowish tinge and is glossy has a favourable influence on wound healing when compared with the delustered white viscose rayon wool. All agents which are added to give a pleasing appearance to the material and which serve no useful purpose should be avoided.

Criteria of an Ideal Dressing

Although the type and severity of wounds vary widely and the dressing may be required for a variety of purposes and may be a composite structure, it should possess the following properties (Scales[27]). It should:

(1) have a porosity to water vapour of at least 1400 g./sq. metre/24 hours, measured at 37° with a relative humidity of 75 per cent (Patra Tentative Standard Method[28]);

(2) absorb wound exudate but not adhere to a granulating surface or allow the penetration of capillary loops;

(3) not produce a tissue reaction when applied to normal skin or granulating surfaces, nor induce a state of allergy or hypersensitivity;

(4) not allow the passage of micro-organisms;

(5) not allow penetration of fluid from outside;

(6) be capable of following the contours around a joint during movement, e.g., flexing of a finger;

(7) not be affected by domestic or industrial fluids, e.g., detergents and oils;

(8) be smooth on both surfaces;

(9) have adequate tensile strength;

(10) have constant physical properties between 0° and 40°;

(11) be non-inflammable;

(12) be capable of being sterilised;

(13) prevent excessive movement of the wound;

(14) not become readily soiled;

(15) be available at low cost; and

(16) be capable of being sealed to the skin by an agent which is both unaffected by all solvents used in industry and at the same time easily removable.

So far these criteria have not been satisfied in any one dressing.

The most commonly used dressing, the sterile gauze pad covered with cotton wool and a bandage, will allow the evaporation of water vapour and will absorb exudate. However, it adheres to wounds and, if it becomes damp, allows the passage of micro-organisms. Various fats and oils, e.g., tullegras, can be used to prevent adhesion between the wound and the dressing, although these often appear to delay healing. The passage of organisms through a dressing has been demonstrated by Colebrook and Hood[29] using a "mock limb" which consisted of a metal tube, perforated at one place to represent a wound. The perforations were covered by a pad which could be soaked in sterile broth. The "wound" was dressed in the traditional manner with gauze, cotton wool and bandage and the whole sterilised. The wound would remain sterile provided the dressing was not dampened with serum containing organisms. In a very short time after such treatment the "wound" became infected.

This type of dressing is often difficult to apply and is completely unsuitable for domestic and industrial injuries, both because of its bulk and the impossibility of its remaining dry and, in industry the risk of a loose bandage catching in machines. The development of the fabric elastic adhesive dressing was an endeavour to provide a covering which would be easier to apply coupled with a degree of compression, depending on the method of application, and at the same time, because of its continuous film of rubber adhesive, would prevent the passage of organisms across the dressing. The form of the weave gave elasticity to the dressing, with consequent increase of freedom of movement of the part. It has proved only partially successful. The adhesive hinders the evaporation of water vapour and the subsequent drying of the wound, especially in those areas where marked sweating occurs.

Owens[30] showed that when a rayon fabric dressing with a fibre of 30 denier and a warp and weft of 114 $\times$ 114 is applied to a burn, there is little disturbance of the underlying granulation tissue and pain and bleeding are diminished and infection reduced. The dressing had a high porosity and a pore size of less than 8 μ. The size of capillary buds is approximately 8 μ. Although granulation tissue did not penetrate the fabric, adhesion to the wound occurred and it was necessary to moisten the dressing with saline to prevent reopening of the wound.

With the development of synthetic materials during the war, plastic films became available which appeared to have many desirable properties, although for all practical purposes they had no water vapour permeability. These early materials were types of plasticised polyvinyl chloride. It was thought by some workers that a waterproof dressing provided with an absorbent pad would be satisfactory, as the initial high loss would be absorbed by the pad. In the chemical industry and under the Chromium Plating Regulations the provision of a waterproof dressing has become compulsory. It was soon found, however, that these dressings had disadvantages; the skin became macerated, the edges of the wound gaped, with subsequent delay of healing, and the plasticiser was migratory and was removed from the film by various organic solvents.

Bloom[31] reported the use of water wettable Cellophane in the treatment of burns in the Army. The material was the wrapping from blood transfusion sets and was sterilised by autoclaving. The Cellophane formed a semipermeable covering—not an occlusive dressing. The Cellophane was porous, yet prevented secondary infection; it was smooth and partially flexible and allowed the wound to be seen. When dry, however, Cellophane is brittle and relatively inelastic and after a few days it cracks at flexures. It is easily torn, and a sheet 1½ thou. in. in thickness, gives little protection to the wound. Another film material, polyvinyl alcohol, was investigated, but had no advantages over Cellophane. Once a film of serum forms over the surface of these materials, the permeability is markedly reduced.

Bull, Squire and Topley[14] reported experiments with another plastic film—Nylon I.C.I. type 8, 3 thou. in. thick. The use of this film as an industrial wound dressing was investigated by Schilling and others[32] and Engel[33]. In the clinical trial, Schilling used a standard waterproof dressing as a control. Healing times were:—

Nylon type 8 dressings	6·04 days.
Waterproof dressings	8·39 days.

It seemed that this film would be useful in the control of secondary infection of bedsores and tuberculous sinuses and it was for this reason that its use was investigated by the Institute of Orthopaedics. It was found that the porosity of the samples of film obtained from the manufacturers was about 200 g. of water vapour/sq. metre/24 hours at 37°, relative humidity 75 per cent (using the Patra[28] technique), that adhesive was difficult to apply and that the seal was even more difficult to maintain, due to the lack of elasticity of the film. Further, maceration of the skin occurred around sinuses and bedsores due to the accumulation of exudate which was retained beneath the film in contact with the tissues. Whilst there was evaporation of water vapour, the osmotic pressure of the discharge increased and this resulted in a subsequent increase of exudate. When removing the backing from a Nylon adhesive dressing it was difficult to prevent the film from curling. The work of Bull, Squires and Schilling, however, once more demonstrated the advantages of porous materials. The Nylon film dressing used by Schilling did not have a pad, but this is necessary to protect the wound from injury and to absorb blood and exudate particularly if the dressing is of the first aid type.

One of the functions of a dressing is to absorb secretions. It must be able to absorb fluid rapidly and the approximate value of this property can be obtained from the sinking test which is described in the British Pharmaceutical Codex. It is dependent on the physical properties of the surface of fibres and on the construction of the yarn if the fabric be knitted or woven. The rate of absorption is not, however, necessarily related to the quantity of fluid which the dressing is capable of absorbing. Work has been done on this problem by Savage, Bryce and Elliott[34]. They thought it better to replace the term "absorbing capacity" of a

dressing by the term "water retention coefficient" in order to avoid confusion with absorbency or rate of absorption of fluids into a dressing. They have defined the water retention coefficient as the number of grams of water absorbed per gram of dressing. Equal weights of cotton and rayon wool dressings were able to retain a greater weight of water than woven forms of surgical dressings. These were followed by paper pulp, cellulose wadding, lint, open gauzes, B.P.C. and hospital qualities, and the finer gauzes used in other countries, in decreasing order of efficiency. As was to be expected, it was found that all these materials absorbed less as they were compressed under increasing loads. It is important to remember this point, since the amount of fluid which a dressing is capable of absorbing will depend on how the dressing has been treated before it is used, i.e., during storage and sterilisation, and under what tension or compression it is applied to the wound. While the rate and the volume of fluid absorbed by the dressing is important, it is equally necessary that the dressing does not remain saturated and that it loses water vapour in the shortest possible time and allows a coagulum to form to which it does not adhere.

Spray-on Dressings

Wallgren[35] investigated the flora under five spray-on plastic film dressings. The following were used:—

1. Aeroplast, which is an ethyl acetate solution of co-polymers of hydroxyvinyl chloride-acetate and sebacid acid 9·3 per cent by weight, and modified maleic resin ester 3·1 per cent by weight with fluorochlorohydrocarbon gas as a propellant.
2. Bonoplast, which consists of an acrylic resin dissolved in ethyl acetate, a plasticiser being added.
3. An improved form of this material is now being manufactured by the same company under the trade name of Nobecutan. This dressing consists of a 4 per cent solution of polymethacrylic acid ester with an ether bridge in the alcohol component. The solvent is ethyl acetate. A fluorohydrocarbon is used as a propellant. The solution used abroad contains 0·5 per cent by dry weight of tetramethyl thiuramide disulfide. (The preparation available in Great Britain is a sterile solution containing no added bacteriostat or bactericide.)
4. Newskin, which consists of pyroxylin 6·9, camphor 0·6, castor oil 3·0, butyl alcohol 5·0, ethyl acetate 57·3 and ethyl alcohol 25·0 per cent, is supplied in tubes, not as an aerosol.
5. Portex plastic skin consists of phenyl salicylate 10, resorcinol 5 and eugenol 25 per cent, and a methyl ester of an acrylic resin.

The experiments were conducted mainly on blood agar plates and on intact human skin. All the products listed were found to be sterile. One loopful of bacteria, approximately 6×10^8 ml., was mixed with 1 ml. of the plastic solution and samples were taken at fixed intervals. Table I indicates the maximum intervals after which bacterial growth could no longer be obtained. It was found that only Nobecutan gave a zone of inhibition when a test culture on a blood agar plate was half covered by a

plastic film. The bacteriostatic agent was tetramethyl thiuramide disulfide. They found, however, that *Staph. pyogenes* could sometimes be grown when swabs were taken from underneath the Nobecutan film. The authors believed that the bacteriostat was in some way inhibited by the constituents of the plastic film. They found that if a film was sprayed on to a culture plate, both proteus and coli organisms could be recovered from beneath the film when the exposed surface of the film was inoculated.

TABLE I

BACTERICIDAL ACTIVITY OF SPRAY-ON DRESSINGS[35]

	Staph. aureus	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Escherichia coli*
Aeroplast	5 min.*	1 min.		1 min.
Bonoplast (smear) ..	17 days	1 min.	1 hour	1 min.
Bonoplast (spray) ..	17 days	1 min.	1 hour	1 min.
Newskin	2 min.	1 min.	1 min.	1 min.
Nobecutan (spray) ..	15 min.	1 min.	1 min.	2 min.
Portex	1 min.	1 min.	1 min.	2 min.

* The times indicate the maximum intervals after which bacterial growth could no longer be shown after the addition of approximately 6×10^8 organisms per ml. to the plastic solution.

Therefore, there must have been holes in the film produced during spraying of 0·8 μ–1·0 μ in size. A film thickness of 60 μ to 90 μ which required four to five coatings by spraying, would prevent through-growth. They found that when cultures were taken underneath films applied to normal skin 24 hours previously, the number of colonies recovered decreased by 50 to 100 per cent, compared with normal skin. Portex plastic skin was not suitable as the film would not remain whole and was difficult to remove. When the thickness of the plastic film was increased, the bacterial flora increased. Wallgren[35] has now treated over 4500 patients with "spray-on" dressings and although infections have occurred, the incidence has been below the three to five per cent, reported by Beekman and Sullivan[36] and Hirschfeld[37] to be the normal incidence of wound infection in sterile surgery. He thinks that Nobecutan and Aeroplast are superior to other film type dressings which he has tested. These films, however, cannot be used in the treatment of a wound which is bleeding or infected, nor are they suitable for wounds such as those on the hand, which are subject to continuous flexion and trauma. For first aid purposes the adhesive dressing is certainly more convenient.

Microporous Polyvinyl Chloride Film

With the difficulties experienced with a variety of dressings and the properties of an ideal dressing in mind, it was decided to investigate a physically porous membrane—Porvic which is used for battery separator plates. It allowed the passage of electrolytes and had the advantage that the base material was one of the cheapest plastics, this being a major consideration in an investigation of this type. No matter how advantageous

a material may be for a dressing, if the cost is three or four times above the cost of existing dressing materials, then it is doubtful if any manufacturer would be willing to carry out the necessary experimental work which is required in this field.

Our initial experiments were carried out with plasticised battery separator plates having a porosity of about 4000–5000 g. of water vapour/sq. metre/24 hours at 37°, relative humidity 75 per cent (Patra tentative standard method[28]). This material was not a bacterial filter or barrier. It had, however, the elasticity and plasticity required. Ulcers and wounds which had been slow to heal improved or healed in a short space of time when these dressings were used. It was soon found that a non-migratory plasticiser was needed, as the physical properties of the film altered during storage and the plasticiser migrated into the adhesive with a change in its characteristics. Migratory plasticisers are quickly removed by certain industrial solvents with a resulting change in the physical properties of the film.

The manufacture of a physically porous material which is required to act as a bacterial filter presents many technical problems. The staphylococcus has a minimum measurement of approximately 0·7 μ. Whether the filter must thus have a pore size of less than 0·7 μ still remains to be determined. It is known that organisms and membranes carry electrostatic charges. The part these play, especially in the presence of detergents or serum which reduce surface tension is not known.

Over 350 batches of film material have been produced and examined as well as many formulations of adhesive spread tested.

As a result of a number of clinical trials, a microporous dressing (M.P.F.) is now available[38], which allows the passage of 1400 to 1800 g. of water vapour per sq. metre/24 hours at 37° with a relative humidity of 75 per cent or a water vapour pressure gradient across the film of 46·6 millibars. The film carries an adhesive, spread in a diamond pattern. The pad, which is of cotton stockinet, is impregnated with 0·1 per cent domiphen bromide B.P.C. Although a cotton pad has been used, a continuous filament viscose rayon pad would be more suitable, since the risk of leaving fibres in the wound would be reduced. Clinical trials using a standard waterproof (O.F.) dressing as a control which also carried a cotton stockinet pad impregnated with 0·1 per cent domiphen bromide, were made on the normal skin of patients in hospital and on patients who attended a surgery of the London Transport Executive for first aid treatment of minor injuries.

Normal Skin—Bacteriological Studies

The normal skin flora of both forearms of 39 male and female hospital patients was determined by daily swabbing for four or five consecutive days. Three dressings of each type were then placed on the forearm of these patients. One of each kind was removed at 24 hour intervals, the skin beneath the pad being swabbed. The results are shown in Tables II and III, and Figure 3.

Minor Injuries—Bacteriological Studies

Complete studies were possible only in 60 cases. The results are given in Table IV.

It can be seen from Tables III and IV and Figure 3 that, when normal skin and wounds are covered with a microporous film carrying a pad impregnated with 0·1 per cent domiphen bromide, the no-growth recovery rate is increased. The healing time for finger wounds was reduced under microporous dressings compared with occlusive dressings.

TABLE II

BACTERIOLOGICAL STUDIES OF NORMAL UNCOVERED SKIN

No. of patients swabbed	Total No. of swabs examined	No growth	Scanty	Heavy
39	346	25 (7·2 per cent)	220 (63·6 per cent)	101 (29·2 per cent)

TABLE III

NO GROWTH RECOVERY RATE EXPRESSED AS A PERCENTAGE

	24 hours	48 hours	72 hours
Normal skin	7·2	—	—
Porous dressing ..	59	82	74
Non-porous dressing ..	15·3	2·55	10·2

TABLE IV

BACTERIOLOGICAL STUDY OF MINOR INJURIES

Type of dressing	Initial swab		24-hour swab		48-hour swab	
	Growth	No growth	Growth	No growth	Growth	No growth
M.P.F., 29 cases ..	18	11 (37·5 per cent)	20	9 (30 per cent)	16	13 (44 per cent)
O.F., 31 cases ..	14	17 (55 per cent)	26	5 (16 per cent)	27	4 (13 per cent)

Pads in Contact with the Wound

The replacement of the traditional cotton pad is being investigated. The use of a cotton pad may, in fact, be a bad procedure. Once the cotton has become wet it retains water, resulting in sogginess and maceration of the skin. No dressing film, no matter how porous, will allow drying out of a cotton pad within a reasonable time. The cotton fibre is of short length, irregular in cross section and it has a rough crystalline surface. Artificial fibres are available as continuous filaments of regular cross section and having a smooth surface. They can be obtained free of contaminants. Preliminary experiments using a standard adhesion technique with serum have shown that the following factors have to be considered in choosing a fabric which is to come into contact with a wound.

The composition of the fibre, its surface and any residual agent left in the fibre. The size of the fibre. The construction of the yarn, and the construction of the fabric.

With rayon, which is a regenerated cellulose fibre, a continuous filament of regular cross section and having a smooth surface can be obtained. Experiments which we have carried out at Stanmore suggest that continuous filament rayon fabrics adhere less to wounds than cotton fabrics.

Fixation of Dressing to Skin by Adhesives

Even if the dressing has all the desirable properties, the maintenance of the seal between dressing and skin is all important.

Fixation of a dressing is best achieved by pressure-sensitive rubber-based adhesives. As porosity is a feature of the resultant dressing, the application of an adhesive should impair this property to a minimal degree. With a window dressing, with a non-porous adhesive spread, an appreciable area is rendered non-porous and the bacterial flora beneath the adhesive increases; the conventional pad is difficult to fix, and production is cumbersome and costly. The ideal dressing will need to carry a porous adhesive spread. Immediate "tack" and strong adhesive are two distinct properties which must be combined in a pressure sensitive adhesive. Moreover, the adhesive must remain integral with the dressing film so that the film does not slide on the adhesive. Careful application of the dressing to the patient is vital. If there are creases or unstuck areas where fluid can penetrate, or if the seal is not maintained, then the main object of the dressing, i.e., prevention of infection coupled with porosity to water vapour will be defeated.

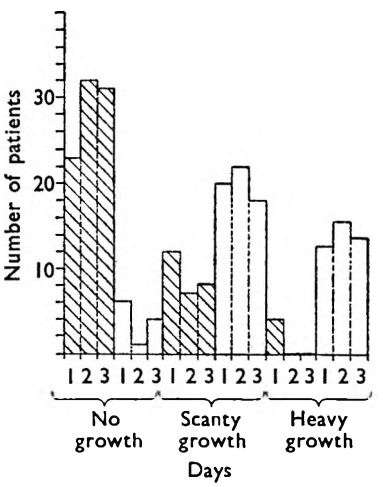

FIG. 3. Results of bacterial studies on normal skin.

Shaded area, porous dressing, M.P.F.
Non-shaded area, non-porous dressing, O.F.

Preparation of the Skin

To maintain an adhesive dressing *in situ*, the skin must be dry. Many worker's hands become covered with water and oils during the course of a shift. It has been found that when dressings are applied towards the end of a shift it is almost impossible to maintain a seal unless the area to which the dressing is to be applied has been adequately cleaned and dried with methylated ether. Fingertip injuries present a particularly difficult problem as the cleft between the nail and the skin invariably allows leakage of fluids to occur. A fingerstall type of dressing requires to be devised but this presents many difficulties.

Have wound dressings been improved in the last 3500 years? I think the answer is yes, but many of the problems have yet to be solved. At

the present time the best dressing is but a compromise and is a poor substitute for an intact healthy skin.

I would like to thank the Medical Research Council, who are assisting with a grant, work on certain aspects of wound dressings.

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RECENT DEVELOPMENTS IN SURGICAL DRESSINGS

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The existing dressings which are available to the medical and surgical practitioners and to the general public have been evolved over a number of years, and during this time many have been little improved. Surgical dressings are either by-products of, or based on materials made available by, the textiles and plastics industries and only in rare instances have materials been specifically designed for the purpose of a wound covering.

Part V of the 1954 British Pharmaceutical Codex covers some dozen types of materials classified as surgical dressings, and many differ widely in physical characteristics. It seems reasonable to postulate broad division between those dressings which are "self-adhesive" and those which require supplementary fixation and here termed "miscellaneous". Major developments have taken place in the "self-adhesive group" and these will be considered in detail. There has also been some advances in the "miscellaneous group" and these will be treated individually.

The Self-adhesive Group of Surgical Dressings

This group includes three kinds of dressing listed in the Codex. They are Zinc Oxide Elastic Self-Adhesive Bandage (Fully Spread, Half Spread, Ventilated and Porous), Self-Adhesive Plasters, and Standard Dressings Nos. 3–6 (Elastic Adhesive Wound Dressings). All consist of a self-adhesive mass or pressure sensitive adhesive coated on a textile supporting material. The steadily increasing variety of self-adhesive first aid dressings and strappings based on plastic films which are available to the general public are also included under this heading.

Characteristics of Pressure Sensitive Adhesive Masses

Although this kind of adhesive has been known both in medicine and industry for some 50 years, it is only within the last 15 years that any real understanding has been gained of its possibilities and limitations. The everyday user is perhaps not sufficiently aware of the limitations to enable him to be selective in choosing the pressure sensitive product most suited to his purpose.

In the current British Pharmaceutical Codex, pressure sensitive adhesives are termed "self-adhesive masses" and are said to "consist of a mixture of cohesive agents, tackifiers, plasticisers and fillers". Examples of cohesive agents are stated to be "best Pará rubber, first quality pale crepe rubber and first quality smoked sheet rubber; polyisobutylene or other synthetic higher polymers with similar properties or mixtures of such polymers are also suitable. Tackifiers include various resins such as colophony and its derivatives".

It is desirable to elaborate the above statement to understand some of the underlying reasons for the possibilities and limitations of self-adhesive masses.

Pressure sensitive adhesives form the basis of self-adhesive tapes as well as the self-adhesive surgical products, and all possess similar qualitative compositions. They differ from the conventional type of adhesive in containing a material which renders them cohesive. This material is sometimes termed a "cohesive agent" and is either natural rubber or a synthetic polymer which has rubbery properties, examples of which are polyisobutylenes, polybutadienes, polyisoprenes and polyvinyl ethers. These rubbers can be rendered tacky by plasticisation with a variety of materials, examples of which are the higher paraffins and the ester plasticisers, for example phthalates, sebacates and lanolin. Adhesive strength is given to the mixture by the inclusion of resins, the most common being colophony or its esters, either unsaturated or hydrogenated. Fillers provide internal strength to the pressure sensitive adhesive, examples are zinc and titanium oxides. Examples of pressure sensitive adhesives without fillers are those used for the manufacture of transparent cellulose adhesive tapes. Frequently included in these mixtures are antoxidants and preservatives. The preservatives used are those required to prevent degradation of the polymer, and also for the preservation of unvulcanised rubber stocks, examples being Flectol H* (a polymer of 2:2:4-trimethyl-1:2-dihydroquinoline), Santovar A* (2:5-di-*tert.*-amylhydroquinone), salts of dialkyldithiocarbamic acid[1] and metal chelating agents like the salts of ethylenediaminetetra-acetic acid[2].

While there is considerable freedom of choice of ingredients for pressure sensitive adhesives required for industrial purposes, the choice of adhesives for surgical products is limited. The adhesive is normally coated on the supporting material from organic solvents, generally light petroleum, or as a hot melt. The bond or "key" between the adhesive and the textile supporting material should be adequate to ensure freedom from "off-setting" (detachment of the adhesive from the base material) in use. The bond between a pressure sensitive adhesive and the plastic base materials can be adversely affected by migration of plasticiser from the base or by inherent lack of polar affinity between the base and adhesive. The former defect has been largely overcome by the use of "plasticiser-free" films by non-migratory polymeric plasticisers in the formulation of the plastic film, the latter by the use of primer or "tie-coats". These are pressure sensitive adhesives with low tack and adhesive properties, the rubber portion of which is of a "polar type" such as an acrylonitrile:butadiene copolymer.

This improved keying of the adhesive on the commercially available plastic first aid dressings has been apparent to the everyday user within recent years and is a big advance in the manufacture of these dressings.

Major problems which are associated with surgical adhesives are, the limited powers of adhesive or tack; skin reactions; lack of porosity to air or moisture vapour; and the susceptibility of the adhesive to attack by organic solvents which limits the industrial use of adhesive first aid dressings. Attempts to overcome these defects have been made.

* Monsanto Chemical Co.

Attempts to Improve Performance of Pressure Sensitive Adhesives

Natural rubber probably still remains the most effective cohesive agent. Its recognised disadvantage of susceptibility to oxidative degradation stimulates a continued search for additives with improved antoxidant power, coupled with freedom from effect on human skin. There are few resins other than the natural resins which will impart the necessary tack-forming properties to rubbers to provide the adhesive powers which are desirable.

Skin Reactions to Pressure Sensitive Adhesives

Dermatologists have in recent years directed their attention to skin reactions which develop beneath self-adhesive dressings. Peck and others in the United States[3–5] were the first to make a systematic study of the problem and more recently, in this country, Russell and Thorne[6] have differentiated between the types of reactions which may develop. These are stated to be: trauma of removal; mechanical irritation by the adhesive; retention of sweat and serous discharges; disturbance of bacterial flora by antibacterial action of a constituent of the adhesive; and sensitisation by a substance in the adhesive.

These five reactions were considered separately and in detail by these workers, and they disposed of the first by the recommendation of a "removal solvent" like ether or propylene glycol ethyl ether.

Mechanical irritation from the adhesive is stated to be due to stimulation of the formation of keratin. Seeking a quantitative relation between adhesiveness and this type of irritation, Russell and Thorne, carried out patch tests with adhesive plasters having varying adhesive powers and showed some relation between adhesive strength and incidence of irritation. An obvious deduction from these observations would seem to be that the most desirable plaster from the point of lack of irritation would be one which possesses no adhesive property!

Retention of Sweat

Russell and Thorne[6] found sweat and serous retention to lead to maceration, infection, and infectious eczema, and described the effects of fully spread and porous plasters on this reaction. They found it to be slightly reduced by the use of porous dressings and quoted work by Scheffler and Lindner[7] reporting that porous plasters were tolerated best.

Porous adhesives are produced by a variety of methods which generally consist of a discontinuous spreading technique[8,9] or are based on the "blowing" of a fully spread adhesive by the application of air pressure through the interstices of the supporting textile base material[10].

The need to maintain waterproofness in some kinds of first aid dressings, particularly those used for the covering of hand and finger wounds in certain chemical processes and in those affected by the Food Hygiene Regulations, has stimulated the search for waterproof yet porous base materials to which a discontinuous coat of pressure sensitive adhesive can be applied.

Disturbance of Bacterial Flora

Russell and Thorne[6] attribute this to the antibacterial activity of the adhesive composition. On these grounds, as well as those of possible allergic effects, they recommend the elimination of causal ingredients from pressure sensitive adhesives.

Sensitising Constituents

Although allergy to pressure sensitive adhesives is well recognised, it is fortunately a comparatively rare phenomenon and Russell and Thorne[6] state that it is the least common of the five types of reaction they describe. Practically any ingredient is capable of causing an allergic or sensitising reaction and the detection of the culprit and its elimination requires collaborative work between the adhesive technologist, the pharmacologist and the clinical investigator.

Russell and Thorne, in collaboration with Bavin and James[6] classified some of the common plaster ingredients in order of sensitising power, colophony being shown to have sufficient activity to justify a serious search for alternatives. Some reactions to natural rubber were attributed to its protein content, deproteinised rubber showing no such reactions in patients already sensitive to crepe or smoked sheet rubber.

It is an unfortunate coincidence that many of those materials which exhibit sensitising properties often have the most useful performance as adhesive ingredients, and it is only by constant search and screening of materials that new adhesive formulae will be evolved which will maintain skin reactions at minimal proportions.

Supporting Materials for Pressure Sensitive Adhesives

The most commonly used supporting or base materials are textiles constructed of cotton or rayon or their mixtures. These are used both in the rigid and elastic forms, elasticity being achieved by the use of highly twisted warp or weft yarns or recently by the use of crimped filament yarns[11].

The use of calendered, extruded or cast plastic films as a replacement for textiles is clearly an outstanding development which for certain purposes, particularly first aid dressings and strappings, possess marked advantages over their textile counterparts. Many attempts have been made to increase the porosity to moisture vapour of plastic films, and these have been adequately described by Scales and probably the most suitable material which has been provided to date is the microporous polyvinyl chloride film termed "Porvic"*[12].

An alternative method of increasing the porosity of plastic films and sheetings applicable to porous first aid dressings is that of mechanical perforation.

Materials Used for Direct Application to the Wound

The self-adhesive component of a surgical dressing normally fulfils the function of supporting a wound dressing or pad consisting of one of a

* Pritchett & Gold and E.P.S. Ltd.

variety of materials which are commonly used for the manufacture of conventional surgical dressings, like gauzes, cotton wools and lints. The preferred covering should be non-adherent to the wound yet allow absorption of wound exudate and free passage of air and moisture vapour. No completely satisfactory covering has so far been evolved although a wide variety of materials have been examined. Paraffin gauze dressings or tulle gras are probably the simplest form of non-adherent dressing. Their disadvantages have been already commented upon by Scales and in addition they are unsuitable as a first aid dressing pad, but the need for a non-adherent dressing for the covering of large wounds justifies continued investigation of tulle gras-like materials.

The use of very thin films as a direct covering of wounds has been investigated and the application of perforation techniques to films has overcome one of their main disadvantages, namely, their inability to allow absorption of wound exudate. The combination of such perforated films with absorbent materials have been studied in Germany, the United States and this country. An example of such a commercially available product is the "Telfa" dressing[13] which consists of a perforated polyester film backed by absorbent cellulose, the perforations being sufficiently small to prevent penetration by granulation tissue—one of the main causes of adhesion. Polyester film can be produced in very thin gauges and can be sterilised by conventional methods; it is probable that a dressing using this type of material could approach the criteria for the ideal covering stated by Scales.

Miscellaneous Group of Surgical Dressings

Cotton Wools, Gauzes, Lints

Cotton remains the most favoured material for the construction of dressings in this group and undoubtedly will continue to hold the leading place for a number of years, for in addition to cheapness, it is readily sterilised and durable with repeated usage.

Following the lead of the textile industry in general, rayon has been suggested as a substitute for cotton for the manufacture of surgical dressings and in the 1957 Supplement to the British Pharmaceutical Codex, there exist monographs for absorbent rayon gauze and absorbent rayon lint. Although it is doubtful whether these rayon products possess any performance advantages over their cotton counterparts, they can, for most most purposes, be regarded as acceptable alternatives.

It is surprising that lint still retains popularity as a surgical dressing for as a covering for direct application to wounds, it appears to possess few, if any, of the criteria of the ideal dressing.

Scales has referred to the work of Baron who claims that surgical dressings based on rayon are less adherent to wounds than those based on cotton. This does not seem to have been fully confirmed by other workers although it is probable that in the continuous filament form, rayon may behave in a somewhat analogous manner to other filament materials, for example, cellulose acetate, polyamide and polyester, which in the woven

form have been found by Bavin (personal communication) to adhere less than cotton to wounds.

X-Ray Opaque Surgical Swabs

The increased use of machine-made swabs in surgery has prompted the search for X-ray markers which can be conveniently incorporated into the swab and which would enable it to be located in the body if forgotten. Plastic filaments, strips, and a rayon thread loaded with heavy metallic salts have been utilised for this purpose, but thin metallic wire and strips have not been found to be entirely satisfactory.

Finishing Agents

Reference has been made by Scales to finishing agents which may have an undesirable effect on wounds and outstanding examples are the optical brightening agents (fluorescent dyes) which have come into common use in the laundering of textile goods within recent years. "Blues" have been used for decades in the finishing of surgical dressings and cotton wool and some of these have recently been replaced by the fluorescent agents. These agents cannot fulfil any useful function in the performance of a wound dressing, and consideration is being given to restricting their use to comply with the standards of the British Pharmaceutical Codex.

Plaster of Paris Bandages

This surgical dressing has specialised application in orthopaedics, and also in other branches of surgery.

The use of Plaster of Paris as a wound dressing was introduced by Trueta[14] during the Spanish Civil War. The method consists of applying an occlusive Plaster of Paris bandage to a wound after thorough cleansing with antiseptics. Healing is then allowed to proceed under the occlusive plaster and it is modern practice to give the patient simultaneous antibiotic treatment.

The conventional type of bandage is produced by the coating of a Plaster of Paris slurry containing an adhesive to a textile supporting material. The incorporation of a melamine formaldehyde resin into this slurry gives a bandage which yields casts with higher mechanical strengths and resistance to water. It is unfortunate that melamine formaldehyde resin has been shown to be a skin sensitising agent and these bandages should be used with care.

Sterilisation and Storage of Wound Dressings

In Appendix XII of the British Pharmaceutical Codex, instructions are given for sterilising surgical dressings composed chiefly of cotton, rayon or other cellulose materials. These processes are based on the conventional procedures of steam sterilisation and are effective for the common types of dressing. With materials sensitive to heat it is necessary to investigate less drastic methods of sterilisation for although it is common practice in the United States for self-adhesive first aid dressings to be supplied in sterile form, this type of product is by no means improved by

heat sterilisation. Other methods, like exposure to ethylene oxide or ethylene oxide:carbon dioxide mixtures, are used. Radiation methods for sterilisation will no doubt find a place in the field of surgical dressings in future[15].

At one time, simple paper or cardboard wrappers were considered adequate for the purpose of protecting surgical dressings but the advent of more specialised types of products and, in particular, the necessity for ensuring safe transport and keeping under tropical conditions, has led to the investigation of new materials such as the flexible plastics. Heat sealed polythene packs are an example and these may be supplemented by an outer protection of metal or heavy cardboard. Elaborate arrangements are necessary to ensure knowledge of the behaviour of dressings under tropical conditions and most manufacturers now possess in their laboratories means of simulating extremes of climatic conditions. Even with this additional laboratory facility, it is usually considered necessary to institute routine examinations of materials which have been in climatic extremes for varying periods. One of the present unsolved problems which beset manufacturers of dressings is the lack of a comparatively short-term test which will provide adequate information about the behaviour of a dressing over a long period under extreme climatic conditions.

Antibacterial Agents

The inclusion of antibacterial agents in wound dressings is still the subject of controversy. The development of new antibacterial agents with a wide spectrum, such as that possessed by some antibiotics and newer synthetic substances, has provided the manufacturer with agents which can be used in antiseptic wound dressings. It is probable that the function of these agents is to give a reasonable assurance that the dressings are free from bacterial contamination rather than to reduce the bacterial flora of the wound itself or to accelerate wound healing. In the current Supplement to the British Pharmaceutical Codex, medication on the pad of the Standard Dressing No. 3 has been extended to allow inclusion of bismuth subgallate, aminacrine, euflavine and domiphen bromide as well as boric acid. Other agents will almost certainly come into use in the future but the same limitations will probably apply, namely, that they primarily sterilise the dressing rather than act as medications in themselves.

Spray-on Dressings

No review on recent advances in the field of surgical dressings would be complete without reference to the development of spray-on dressings. They are described in the accompanying paper and were referred to at the Symposium of this Conference held in 1955. They are established and important members of the group of surgical dressings but have limited usefulness and a considerable technological advance is necessary before they are likely to supplant the older and more conventional types.

Industry is fully aware of the necessity for improving existing means of covering wounds and is doing its best to take advantage of scientific

advances in many fields to achieve this end. In fairness, it should be pointed out that the mass production of new types of wound dressings is by no means a simple matter. Reference to a paper by Squire[16] and to a symposium on "Plastics in Surgery and Medicine" held at the British Plastics Convention in 1951[17], will show the amount of large scale development work necessary to transfer production to the factory. Production in the laboratory of a few gross of dressings for a clinical trial is comparatively easy, but transference to full manufacturing scale involves the design of new machines and the scrapping of old.

Dr. Scales has already emphasised the comparative lack of knowledge of the fundamental processes underlying wound healing and until these are fully understood the ideal dressing is unlikely to be achieved. It is hoped that if and when such a dressing is produced, it will be found that the advances which have been made within recent years, and which have been partially described in this paper, were steps in the right direction.

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17. Seymour, *ibid.*, p. 272.

DISCUSSION

Dr. Maxwell Savage (Barnet). In spite of past controversy, it was untrue that the flavine antiseptics were so strongly adsorbed to cotton that they were not released in plasma. Plasma was capable of eluting flavines which inhibited the growth of pathogenic anaerobes at concentrations as low as 1 in 1 million. New drugs administered by injection or by mouth were evaluated on their efficiency by comparison with their predecessors, but little progress would be made in the development of new dressings until a new attitude was adopted to their cost.

Dr. Scales agreed that 1 in 1 million was a satisfactory bacteriostatic concentration of flavine *in vitro* but it was difficult to assess its action in a wound. There were reports of sensitivity to the flavines. The cost of clinical trials was high because it was necessary to do the tests on a large number of cases.

Mr. Griffiths (Coventry). The adhesion of dressings to wounds might be either chemical or physical. Little appeared to be known

about the relative importance of these two factors. It was suggested that continuous filament rayon fabrics adhered less to wounds than cotton fabrics, and that film materials such as polyesters adhered less than cotton or rayon to wounds. A comparison has not been made between the same material in filament and staple form. The findings of Baron that delustred rayon fibre with titanium dioxide caused delayed healing seemed unlikely.

DR. SCALES agreed that both physico-chemical and "mechanical" adhesion occurred. Chemical adhesion was the more complex. He preferred fibres containing no additives.

The PRESIDENT. No comment had been made on the formation of scar tissue. To what extent was the irritant action of the dressing on the margins of the wound responsible for the production of granular tissue? The removal of a suture rarely caused trauma, whilst permanent scarring was frequently seen after incision or injury?

DR. SCALES. Keloid formation often occurred in epithelial scars. There were sometimes pockets of sub-clinical infection around sutures. Some people formed fibrous tissue very readily.

MR. S. G. E. STEVENS (London). Was the increase in sensitivity to colophony caused by the use of a resin different from that used twenty-five years ago?

MR. SEYMOUR. Colophony was now purer. Derivatives of colophony, the acidity of which had been reduced by esterification, and oxidation avoided by hydrogenation were in use; the hydrogenated esters of colophony were less irritant.

DR. J. W. FAIRBAIRN (London). Attempts had been made recently to increase the yield of colophony by treating trees with bacteria and sulphuric acid, and there may have been changes in the composition of the resin.

MR. S. DURHAM (Sheffield). Was anything done to preserve the sterility of gauze after opening the packet in the home?

MR. J. D. WIMBORNE (Wanstead). Gauze packed in individual $\frac{1}{4}$-yard "Cellophane" wrappings was available; sufficient for one dressing could be withdrawn without contaminating the rest.

DR. SCALES preferred individual packing.

MR. SEYMOUR doubted the value of sterile dressings for general use applied under non-aseptic conditions.

MR. J. A. MYERS (Bradford) suggested the patient should be given antibiotics by injection, and after the wound had been cleaned, warm, dry, sterile air under positive pressure should be applied.

DR. SCALES. It had been shown that if the temperature were raised as much as 10° by passing warm, dry, sterile air over a wound there was more rapid healing. Normally the wound temperature was below that of the body. The treatment suggested could only be carried out in hospital.

DR. K. R. CAPPER (London) wondered about the function of some B.P.C. dressings. Mr. Seymour had expressed doubt about the value of lint. He himself had even more doubt about boric lint. Boric acid might have certain physical properties which rendered it useful in a dressing, but if it were included as a bactericide he doubted whether it was of any value. He also questioned the use of bismuth subgallate. Had progress in the treatment of minor wounds advanced further than the treatment of major wounds? It might be advantageous if the Codex included a commentary on each dressing as it did with drugs. Why was there a "hospital quality" cotton wool? Was it that the B.P.C. standards were too high, or were there separate and distinct uses for B.P.C. and for "hospital quality" cotton wool? It was true that monographs had now been included for rayon lint and gauze, but he doubted the wisdom of copying cotton dressings in rayon. It might have been wiser if those developing rayon surgical materials had concentrated on dressings where rayon had advantages over cotton. It was difficult to say whether titanium dioxide reduced wound healing or not. The object of delustering was to prevent slip between warp and weft. Was rayon a suitable material for an absorbent gauze? It was stated that very fine weave rayon material seemed to have the advantage of not adhering to the wound to the same extent as cotton. It was gratifying to read the strong statement by Dr. Scales about the inadvisability of adding foreign materials. He confirmed that optical fluorescent agents were being added to dressings yet little seemed to be known about the toxicity of these materials. Although stilbene derivatives, they were not oestrogenic, but there was little information other than that reported by Baron on their effect on wounds. The B.P.C. was not without blame, it included a number of loose statements about the addition of dyes to dressings. It would be interesting to know the effect of those dyes and whether the Codex had not been too lenient in its specifications.

MR. SEYMOUR. A great deal of work had been carried out, but without success, to assess the value of boric acid in a wound dressing. It was certainly a poor antiseptic. He wondered why a pink dyestuff was added to boric lint. Bismuth subgallate had some styptic action. A substance which he had used a great deal was domiphen bromide. Other quaternary compounds were equally effective. The use of second quality materials was a question of economics. Cotton wool was used in hospitals for many purposes. It was a mistake to copy cotton products in rayon. Rayon could be produced in filament form whereas cotton could not, and a great deal of work needed to be done on filament dressings. He had investigated the optical brightening agents and they were not oestrogenic; they were widely used domestically in washing materials, and no doubt the risks involved in that way, if any, were far greater than in surgical dressings.

DR. SCALES agreed that commentaries on dressings ought to be included in the Codex, particularly if one could recommend the best dressing for a particular type of wound.

DISCUSSION

MR. HUMPHREY JONES (Liverpool) referred to the treatment of wounds in his young days when in the absence of modern dressings and antiseptics emplastrum saponis fuscum, ung. resinae and cobwebs were used. Perhaps his generation had developed an immunity to infection which the modern generation did not have.

MR. H. S. GRAINGER (London) deplored the term "hospital quality dressings"; it suggested that hospitals could make do with inferior materials. The treatment of wounds was much studied during war, when substitute materials had to be provided in abundance for serious war wounds. The reason for the use of hospital quality, so called, instead of the B.P.C. quality, arose from the fact that the standards were empirical. There was really no evidence that the dressing of a wound required a specific quality material. Another factor was that the choice of materials and the dressing of the wound followed traditional practice. The pharmacist was the only officer in the hospital who had any technical knowledge of surgical dressings. The vapour permeable film type of dressing seemed to offer an answer to the problem of applying a thin layer to the larger surface wounds.

DR. SCALES agreed that the pharmacist was the person who should be responsible for the dressings used in hospitals. The perforated film dressing promised a considerable advance over the traditional type of gauze pad for operative wounds. Whether it could be used for first aid purposes was not yet clear, but for the abdominal wound and possibly in plastic surgery the dressing was a good one.

MR. C. E. TURNER (Stoke-on-Trent). Is it possible to ascertain the reactions of patients' skins to elastic dressings?

MR. SEYMOUR. It could be done by patch tests.

MR. T. D. WHITTET (London). In University College hospital three older dressing coverings, jaconet, battiste and oil silk, had been replaced by plastic materials.

MR. G. SYKES (Nottingham) suggested that bacteria in a wound prevented healing. An appropriate antiseptic in a dressing might be helpful, and the acridines had the unique property of not losing, and possibly gaining, in activity in the presence of blood. He was surprised at the view that the sterility of dressings did not matter very much. Ethylene oxide was a reliable sterilising agent. It would kill organisms which were not protected in any way, but if they had any mucoid or serous protection or were in fibrous material they might not be killed.

DR. SCALES. It was probably in the early history of a wound that micro-organisms were important. There was no evidence to show that after the first five days of injury a wound could be reinfected. That was noted in 1900 when pus was rubbed into wounds 4–5 days after injury without producing clinical sepsis.

MR. J. R. ELLIOTT (London) said that boric lint was coloured pink because Lister coloured his boric lotion pink with litmus to distinguish

it from his phenol lotions. Subsequently other medicated dressings were coloured with artificial dyes. Lint meant different things in different ages. In 1400 it was not the fluffed up surface of a material, as it was known to-day, but the actual fluff which was removed from cloth. That early forerunner of cotton wool was used to stuff into wounds. Even as late as 120 years ago there were lints on the market which were not woven fabrics. They were all warped threads which had been fluffed up and wadded together.

MR. A. R. G. CHAMINGS (Horsham) suggested that in time the use of antibiotics and corticosteroids in treatment of minor disorders might accentuate the wound healing problem.

MR. D. F. SMITH (Bournemouth). Much production time was lost by industrial injuries, and any improvement in dressings which could reduce the period of healing might well play an important part in the nation's economy. It had not been his experience that surgeons had been influenced by the cost of any form of treatment which they adopted.

SCIENCE PAPERS AND DISCUSSIONS

LOSSES OF BACTERIOSTATS FROM INJECTIONS IN RUBBER-CLOSED CONTAINERS

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The losses of bacteriostats from rubber-closed multidose containers of injections are confirmed, and the mechanism of these losses by absorption, diffusion and volatilisation is discussed. The present B.P. methods of pretreating rubber closures are inadequate, and methods for their more satisfactory equilibration with various bacteriostatic solutions are given. From measurements of the partition ratios of the B.P. bacteriostats and of benzyl alcohol between rubber and water, phenol and benzyl alcohol appear to be the most stable and promising and phenylmercuric nitrate the least stable. The value of using closures equilibrated for phenol and benzyl alcohol is demonstrated and methods for preventing the diffusion losses are discussed.

THAT rubber-closed containers of injections containing phenolic bacteriostats lose substantial quantities of these on storage has long been known. As early as 1923 Masucci and Moffat[1] reported the loss of 50 to 70 per cent of cresol and 20 to 40 per cent of phenol from rubber-capped vials of solutions which had been stored for eighteen months at room temperature, and in the late 1930's similar observations were made in these laboratories; we also showed considerable differences in the uptake of phenol, cresol and chlorocresol by different types of rubber closures when they were heated with successive quantities of the respective bacteriostatic solutions, but unfortunately all of these records were lost in 1941. We still have the records, however, of work by a colleague who in the period 1945 to 1947 examined vials of insulin returned from tropical countries and which showed losses of from 60 to 70 per cent of cresol after storage for 12 to 18 months in these areas.

More recently, Berry[2] has adequately summarised the lack of information on this topic in his contribution to the symposium on Containers and Closures at the British Pharmaceutical Conference in 1953. Since then Weiner[3] has discussed the absorption of thiomersalate by rubber, and there have been contributions by Wing[4–6] on the absorption of phenol and chlorocresol by rubber and the effects of varying the chemical composition of the rubber mix on this absorption.

Since 1952 we have made many tests with various types of rubber closures used for injectable products. Our results with phenol and chlorocresol are in broad agreement with other workers in this field, but our data also include observations with cresol, phenylmercuric nitrate and benzyl alcohol, and this does not appear to have been covered elsewhere.

Most of the tests were made with rubber diaphragms or plunger components used in medical cartridges, and they involved storage periods up to three years.

EXPERIMENTAL

The B.P. Treatment of Rubber Closures

The British Pharmacopoeia, 1953, states on page 850 that rubber caps are "either boiled under a reflux condenser for thirty minutes, or stored for not less than forty-eight hours, in a solution containing the same bacteriostatic in the same concentration, or preferably in twice the concentration used in preparing the injection". The uncertainty of the method is implied, for, apart from the fact that it permits two strengths of solution for treatment which will obviously give different results, it states on the same page "rubber so treated is liable to continue to absorb bacteriostatic from the injection".

TABLE I

THE ABSORPTION OF PHENOL, CRESOL AND CHLOROCRESOL BY RUBBERS

	Concentration per cent of bacteriostat remaining in solution in contact with different rubber components					
	Phenol with		Cresol with		Chlorocresol with	
Time of contact	Red rubber	White rubber	Red rubber	White rubber	Red rubber	White rubber
Hot treatment						
Initial	1·00	1·00			0·20	0·20
5 min.	0·96	0·97				
15 min.	0·91	0·92				
30 min.	0·87	0·90			0·12	0·14
1 hr.	0·83	0·88			0·10	0·12
2 hr.	0·80				0·09	0·10
4 hr.	0·77	0·80				
6 hr.	0·77	0·78			0·08	0·09
Cold treatment						
Initial	1·00	1·00	0·32	0·32	0·20	0·20
1 day	0·90	0·95	0·26	0·31	0·09	0·14
2 days	0·88	0·93	0·21	0·29	0·06	0·12
6 days					0·05	0·08
7 days	0·84	0·89				
9 days			0·19	0·27		
14 days	0·82	0·86				
21 days	0·80	0·84				

That the treatment is inadequate can easily be shown by experiment. For the present work it was demonstrated with phenol, cresol and chlorocresol by treating three types of rubber components for different periods. In each there was one component weighing approximately 0·33 g. to each 1 ml. of bacteriostatic solution. For the hot treatments, separate sealed containers were used, the containers being heated together in flowing steam and one container being removed at each appointed time for analysis of the residual solution. For the cold treatments, one large container was used, the ratio of component to residual solution being kept constant by removing the appropriate number of components at each sampling. The phenols were estimated by the normal bromination method, and the results of these tests are given in Table I.

Effect of Repeated Treatments

In further examination of the hot treatment, red rubber and white rubber components were subjected to a number of successive treatments each of 30 minutes at 100° and each time with a fresh solution. The first few heatings were on the same day and thereafter they were at varying time intervals up to several weeks. When the treatments were on the same day the components were rinsed and dried each time and immediately put back into fresh solution, but where the interval between heatings was of a day or more they were kept at room temperature during this period. Again one component was used to each 1 ml. of solution, and the residual bacteriostat was estimated by bromination. From this estimate, the uptake of the phenol by the rubber was calculated, and the results are given in Table II.

TABLE II

UPTAKE OF PHENOLS BY RUBBER AT SUCCESSIVE HEAT TREATMENTS

	Uptake of bacteriostat by rubber (mg./g.) from:—					
	Phenol, 1 per cent		Cresol, 0·3 per cent		Chlorocresol, 0·2 per cent	
	Red component	White component	Red component	White component	Red component	White component
Successive heatings for 30 min. at 100° on same day:—						
1st	3·0	2·1	2·4	1·5	3·3	2·7
2nd	1·2	1·2	0·9	0·6	2·1	1·8
3rd	0·3	0·6	0·6	0·6	1·2	0·9
4th	Nil	0·3	0·6	0·6	0·3	0·3
Subsequent heatings for 30 min. at 100° on:—						
day 2	0·9	0·9			2·1	1·8
day 4			1·2	0·6	1·8	1·5
day 11					1·5	1·5
day 18			0·6	0·6	1·8	0·9
day 22	2·1	1·5				

The near approach to apparent equilibration after four treatments on the same day followed by further substantial uptakes of bacteriostat when the components are retreated after standing for some time can have at least two explanations: (i) the bacteriostat is absorbed on the surface layers only of the rubber, and so gives an impression of quick saturation during the initial treatments, but subsequently it diffuses into the deeper layers, thus freeing the surface layers to absorb more (a point already noted by Sykes[7]); and (ii) the bacteriostat is lost to the atmosphere by the normal process of volatilisation.

To investigate this, red and white rubber components were treated with a 1 per cent solution of phenol by the B.P. hot method, after which they were rinsed and dried. The phenol was then extracted from these components, some immediately and some after exposure to the atmosphere for six weeks, by heating *in water* in a sealed container for 30 minutes at 100°. Untreated rubber controls were also subjected to the same extraction process. The following figures were obtained:—

LOSSES OF BACTERIOSTATS FROM INJECTIONS

	Red rubber	*White rubber*
Phenol absorbed (mg./g. of rubber) during initial treatment	3·0	2·1
Phenol extracted (mg./g. of rubber) by immediate water treatment	2·4	1·5
Phenol extracted (mg./g. of rubber) after six weeks storage	2·0	1·3
Control: phenol extracted from untreated rubber	0·0	0·0

These show clearly that even after six weeks exposure most of the absorbed phenol can still be extracted and, therefore, that the main reason for the continued absorption of the phenol is due to its diffusion and consequent further dissolution in the rubber.

Partitioning of Bacteriostats between Rubber and Water

The acknowledged solubilities of phenols and other bacteriostatic substances in rubber suggests that in a rubber-water system, as in all systems containing two immiscible solvents, a normal partitioning of the bacteriostat takes place between the rubber and water and that in each example a partition ratio might be calculated. Because one of the solvents is a solid the time taken to reach equilibrium may be considerable, depending on the rate of diffusion of the substance throughout the rubber.

TABLE III

THE PARTITIONING OF BACTERIOSTATS BETWEEN RUBBER AND WATER

Bacteriostat per cent	Approximate distribution per cent between rubber and water	
Benzyl alcohol 1	15	85
Phenol 0·5	25	75
Cresol 0·3	33	67
Chlorocresol 0·2	85	15
Chlorbutol 0·5	80–90	10–20
Phenylmercuric nitrate 0·002 ..	>95	<5

Wing[4,5] has already drawn attention to this in relation to phenol and chlorocresol and has shown that with chlorocresol the equilibration time may be as long as 58 days at 2° or 23 days at 37°. In general he used about 2 g. of rubber to each 10 ml. of solution and showed that the partition coefficient varies with different types of rubber, but that in each the value for chlorocresol is much greater than that for phenol. Thus, there is always a much higher proportional absorption of chlorocresol than of phenol into any type of rubber.

On similar lines, we have made a large number of tests with several different rubbers and using varied proportions of rubber and bacteriostatic solution. The bacteriostats examined were benzyl alcohol and those recommended in the B.P. 1953, and a ratio of 1 g. of rubber to 3 ml. of solution was employed, one month being allowed at room temperature for substantial equilibrium to be reached. From such tests the partition ratios as given in Table III were obtained. These values were all about the same for the several rubbers examined, although there was one exception, an oil-resistant rubber, which absorbed some three to four times as much phenol as did other rubbers. Our findings with phenol and chlorocresol were very similar to those of Wing[5], in that (*a*) he found uptakes of

chlorocresol between 73 and 80 per cent for latex rubbers and up to 91 per cent for other rubbers against our average value of 85 per cent, and (*b*) he obtained partition ratios for chlorocresol "which are about 20 times greater than for phenol", whilst our value was about 17.

Tests with Equilibrated Rubbers

From the foregoing observations it is clearly possible to equilibrate any rubber closure with any bacteriostat in solution. But in view of the large differences in the partitioning of bacteriostats and the time required, it is necessary to consider each type of rubber component separately. It is for these reasons that the treatments at present prescribed in the B.P. are inadequate.

From the evidence available it requires about one month at room temperature to allow of adequate diffusion of a bacteriostat into rubber, and using this as a basis equilibration treatments were worked out for red and white components for phenol, chlorocresol and benzyl alcohol. Different concentrations of bacteriostatic solution and different volumes of solution in relation to the amounts of rubber were examined, and the following treatments were found satisfactory:—

To equilibrate with phenol, 0·5 per cent	a 1 per cent solution using 1 ml. for each component;
To equilibrate with chlorocresol, 0·2 per cent	a 1 per cent suspension, using 1 ml. for each component;
To equilibrate with benzyl alcohol, 1 per cent	a 2 per cent solution using 1 ml. for each two components.

The efficacy of these equilibrations was demonstrated by filling cartridge tubes with the appropriate bacteriostatic solution, closing them with either (*a*) components treated as above, (*b*) components treated by the B.P. process, using twice the bacteriostatic concentration, or (*c*) untreated components, and then storing them at room temperature. At intervals up to one month the solutions were assayed for their phenolic or benzyl alcohol* content. The results (Table IV) show practically no change in concentration of the bacteriostats in contact with rubbers subjected to the equilibration treatment, but significant losses in those untreated or treated by the B.P. process. Thus it is possible in practice to ensure that a bacteriostat will remain in an injection at the required concentration for at least a few weeks, and such conditions obtain with injections prepared extemporaneously or in normal hospital practice.

But from the pharmaceutical manufacturers point of view this is not the complete answer, for a bacteriostatic stability for one, two or even more years is required.

* The benzyl alcohol estimations were made spectrophotometrically in the Physical Assay Division of the Standards Department, Boots Pure Drug Co. Ltd.

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In this connection, attention has already been drawn to the fact that bacteriostats are not only absorbed by rubber, but they also diffuse through the rubber and are ultimately lost by volatilisation. In a multi-dose container, therefore, there is a continuous slow diffusion of the bacteriostat from the injection through the rubber to the atmosphere.

TABLE IV

THE EFFICACY OF "EQUILIBRATED" RUBBER COMPONENTS IN CARTRIDGES CONTAINING BACTERIOSTATIC SOLUTIONS

Period of storage	Concentration of bacteriostat remaining in solution		
	Untreated components	Components treated by B.P. cold method	"Equilibrated" components
Phenol, 0·5 per cent, in 1 ml. cartridges			
0 days	0·51	0·51	0·51
2/3 days	0·46	0·50	0·52
1 week	0·45	0·48	0·51
2 weeks	0·43	0·46	0·51
1 month	0·42	0·45	0·51
Chlorocresol, 0·2 per cent, in 1 ml. cartridges			
0 days	0·21	0·21	0·21
2/3 days	0·11	0·20	0·21
1 week	0·08	0·16	0·22
2 weeks	0·07	0·12	0·21
1 month	0·06	0·10	0·19
Benzyl alcohol, 1 per cent, in 2 ml. cartridges			
0 days	1·00	1·00	1·00
1 week	0·97	0·98	1·02
2 weeks	0·93	0·96	1·00
1 month	0·90	0·95	1·01

The rate of this diffusion and loss will depending on the volatility and concentration of the bacteriostat, the size, thickness and type of the rubber closure, the amount of solution in relation to the amount of rubber and the storage conditions, and the effects of some of these factors can be seen in the results of some experiments quoted in Table V. In these tests, different sizes of containers were filled with a 0·3 per cent solution

TABLE V

LOSSES OF CRESOL FROM DIFFERENT RUBBER-CLOSED CONTAINERS

Period of storage	Concentration per cent of cresol remaining in			
	2·2 ml. cartridge with 1 red diaphragm and 1 white plunger (0·66 g. rubber)	5 ml. vial with brown latex plug, metal rimmed (0·4 g. rubber)	10 ml. vial with white waxy plug, metal rimmed (1·8 g. rubber)	5 ml. vial with obsolete red rubber cap (0·7 g. rubber)
Nil	0·30	0·30	0·30	0·30
1 week	0·23	0·26	0·27	0·25
1 month	0·21	0·24	0·27	0·23
3 months	0·17	0·22	0·26	0·15
6 months	0·15	0·18	0·25	0·10
1 year	0·11	0·12	0·23	0·04

of cresol and the cresol contents estimated after different periods of storage. None of the closures used had been subjected to any pretreatment, and so it may be assumed that the losses recorded during the first month or so are due to absorption effects whilst those occurring later give a measure of the diffusion and volatilisation. The high rate of loss with the obsolete red rubber cap can be readily attributed to its thin

construction and comparatively large surface area, whereas the low rate of loss from the 10 ml. vial is due not only to the larger volume of solution in the vial, but also to the different quality of the rubber containing a high proportion of wax and to the protective metal seal over part of the closure. The cartridge pack, in spite of the small volume of solution it contains and its rubber seal at both ends, assumes an intermediate position on account of the small surface area of the rubber plug and its thickness.

Curtailment of Diffusion Losses

An obvious method of preventing diffusion losses is to seal the rubber surface with some less penetrable seal, and several attempts have been made to do this. There are, however, other factors to be considered such as the resistance of the seal to needle puncture, the possibility of needle blockage by the sealing material, the shedding of particles into the medicament, and the possible reaction of the seal with the medicament on long-term storage, and it is for these reasons that several of the more obvious solutions are rejected.

TABLE VI

EFFECT OF A PARAFFIN WAX SEAL IN PREVENTING LOSS OF BACTERIOSTATS FROM RUBBER-CLOSED CONTAINERS

Period of storage	Concentration of bacteriostat remaining in 1 ml. cartridges with					
	Phenol, 0·5 per cent, and			Chlorocresol, 0·2 per cent, and		
	Untreated components	Equilibrated components	Equilibrated components + paraffin wax seal	Untreated components	Equilibrated components	Equilibrated components + paraffin wax seal
Nil	0·51	0·51	0·50	0·21	0·21	0·20
1 month	0·42	0·51	0·51	0·06	0·19	0·20
2 months	0·41	0·49	0·51	0·05	0·17	0·18
3 months	0·40	0·46	0·50	0·05	0·14	0·18
6 months	0·35	0·41	0·48	0·04	0·11	0·15
9 months	0·32	0·36	0·45	0·03	0·09	0·15
1 year	0·26	0·31	0·41	0·02	0·07	0·15
1½ years	0·20	0·23	0·39	0·02	0·06	0·14
2 years	0·16	0·17	0·32	0·02	0·05	0·13
3 years	0·09			0·01		

Viscose, paint, paraffin wax, sputtered metal and various metal overseals and resin-coated foils were all tried in turn. Of these, the metal overseals effected some reduction in losses, but the results were variable and seemed to depend on the rimming procedure. The most successful results were obtained with a paraffin wax coating, but this, as pointed out by Berry[2], is liable to cause needle blockage. With this material the rate of initial loss of the bacteriostat was markedly reduced, but not eliminated, and typical results with phenol and chlorocresol are quoted in Table VI. It is evident that the paraffin seal, as with other seals, has only a delaying effect and that after a sufficient passage of time the overall losses will be the same.

Benzyl Alcohol

Benzyl alcohol is now used as a bacteriostat in several preparations administered parenterally at concentrations ranging between 0·9 and 2

per cent. Earlier in this communication the favourable partition ratio of benzyl alcohol between rubber and water was noted (Table III), and consequently the slow uptake by rubber from an aqueous solution (Table IV), and for these reasons a series of long term storage tests was made with cartridge packs containing a 1 per cent solution of benzyl alcohol. The cartridges were stored for 2 years at normal room temperature and the residual benzyl alcohol concentrations determined at varying intervals with the results recorded in Table VII. Even at the end of the test period a substantial amount of the benzyl alcohol remained and in this respect it compares favourably with phenol, the best of the pharmacopoeial bacteriostats.

TABLE VII

COMPARATIVE RATES OF LOSS OF BENZYL ALCOHOL AND PHENOL FROM RUBBER-CLOSED CONTAINERS

	Concentration per cent of bacteriostat remaining			
	Benzyl alcohol, 1 per cent, in 2 ml. cartridges with		Phenol, 0·5 per cent, in 2 ml. cartridges with	
Period of storage	Untreated components	Equilibrated components	Untreated components	Equilibrated components
Nil	1·00	1·00	0·51	0·51
1 month	0·90	1·01	0·42	0·51
2 months	0·87	0·97	0·41	0·49
3 months	0·80	0·90	0·40	0·46
6 months	0·69	0·83	0·35	0·41
9 months	0·66	0·78	0·32	0·36
1 year	0·61	0·72	0·26	0·31
1½ years	0·48	0·57	0·20	0·23
2 years	0·39	0·48	0·16	0·17

Phenylmercuric Nitrate

Reference is made in Table III to the large absorption of phenylmercuric nitrate by rubber, a point which was commented upon by one of us a few years ago[8]. The estimation of phenylmercuric nitrate in low concentration is a complex analytical procedure, but for the present purposes comparative observations only were required and these were obtained by a simple microbiological technique. It consisted briefly in preparing serial twofold dilutions of the test solutions in a diluted nutrient broth and finding the level in each case at which a test organism (*Pseudomonas pyocyanea*) would just grow. Using this technique, the uptake of phenylmercuric nitrate from a 0·002 per cent solution was assessed for both red and white rubber components. The numbers of components and the amounts of solution were varied to give ratios of 1 component to each 1, 2, 3, 5 and 10 ml. of solution, and measurements were made at intervals of 1, 3 and 7 days at room temperature.

With the red components, at least $\frac{7}{8}$ths of the available phenylmercuric nitrate was absorbed and lost within 3 days even with only 1 component to each 10 ml. of solution, and with the white components similar losses were found after 7 days. On this basis, phenylmercuric nitrate is not a satisfactory bacteriostat in rubber-closed containers, especially in medical cartridges, because the amount absorbed by the rubber is much too great.

Discussion

No complete solution to the problem of the losses of bacteriostats from multidose containers of injections is as yet in sight. There is no rubber or other suitable elastomer closure which will completely prevent such losses. However, it is possible to reduce them substantially by selecting rubbers with a relatively low absorbency and to design the closures so that they have a maximum thickness and present a minimum surface area to the bacteriostatic solution.

The initial absorption of a bacteriostat from an injection can be dealt with by a proper equilibration treatment, details of which can be worked out for any particular closure. The subsequent diffusion and volatilisation losses are more difficult to prevent, but they can be partially overcome by some form of sealing. Further work with nonvolatile or less volatile preservatives may also be of advantage.

With regard to the bacteriostat itself, choice can be made from those with a more favourable partitioning between rubber and water and with small subsequent diffusion losses. On this basis, phenol and benzyl alcohol both come high on the list and phenylmercuric nitrate is contraindicated; in fact it would seem that none of the phenylmercuric salts is suitable as a bacteriostat for any injection in a rubber-closed container.

It might be possible to extend the shelf life of an injection by including where possible a higher concentration of the chosen bacteriostat than that normally recommended. But this is not always feasible for various reasons; also the advantage may be temporary since the higher the concentration of a bacteriostat in a solution the greater is its diffusion loss.

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DISCUSSION

The paper was presented by MR. A. ROYCE.

MR. C. L. J. COLES (Greenford). Was latex or a synthetic mix used?

MR. G. R. WILKINSON (London). Was the composition of the rubber given by the makers? Phenylmercuric nitrate and rubber might react to form metallic mercury under certain conditions. Had lacquered plugs been tested?

MISS A. E. ROBINSON (London) suggested that ultra-violet absorption techniques would be more satisfactory than the bromination method for estimating phenol.

MR. E. ADAMS (Plymouth). Did solutes affect the loss of bacteriostats; had flexible collodion been tried to reduce diffusion?

MR. R. L. STEPHENS (Portsmouth). Had the use of a rubber mix containing phenol been considered?

MR. HOBBS (Bristol). Had any other method of pre-treatment been tried?

MR. A. G. FISHBURN (Rochdale). Some rubber stoppers, after as many as twenty separate boilings with water, still yielded an extractive which gave a high titration figure with permanganate.

DR. L. M. ATHERDEN (Sunderland). Had sealed ampoules stored under the same conditions for the same length of time been used as controls for decomposition of the bacteriostats?

MR. T. D. WHITTET (London) confirmed that phenylmercuric nitrate reacted chemically with rubber; it seemed to be a surface reaction.

MR. J. R. ELLIOTT (London). How was the 1 per cent suspension of chlorocresol made?

DR. H. DAVIS (London). Were "expiry date" and "shelf life" synonymous?

MR. D. F. SMITH (Bournemouth). Did the rate of loss change after the cap had been punctured?

MR. F. STOWELL (Liverpool). Had the inclusion of a barrier, like nylon, within the rubber cap been considered?

DR. L. SAUNDERS (London) suggested that the partial polymerisation of phenol might reduce its volatility and rate of diffusion.

MR. A. ROYCE replied. Plugs of latex and red rubber mixes were used. The formulae for some of the mixes were known, but no special approach had been made to the makers. There was a chemical reaction between rubber and phenylmercuric nitrate. No experiments were made with lacquered plugs. Some were made with plugs coated with flexible collodion, but no difference had been observed. The bromination method for estimating phenol was used, as the equipment for physical methods was not available in his own laboratory. An assessment of the effect of solutes had not been made. Rubbers containing 0·5 per cent and 0·75 per cent phenol were used, but phenol was still lost by diffusion. No other pre-treatment had been tried. They had not used ampoules as suggested. The chlorocresol was not specially suspended. When a mixture of 1 per cent chlorocresol in water is in contact with rubber the undissolved chlorocresol goes rapidly into solution as the rubber absorbs the chlorocresol from the solution. All tests were made on intact closures. The barriers used had been put on top and underneath the plug.

MR. G. SYKES added that if a bacteriostat was going to be lost from a multidose container of an injection, the product should have an expiry date. He had not considered that there was any difference between an "expiry date" and "shelf life".

THE PURITY OF CHLOROFORM B.P.

By A. C. Caws and G. E. Foster

From the Wellcome Chemical Works, Dartford

Received June 11, 1957

The "chloro-compound", isolated during previous work on a source of error in the assay of strychnine salts and preparations[1], has been identified as strychnine chloromethobromide. Its identity has been established by the preparation of authentic strychnine chloromethobromide and the corresponding nitrate and iodide. Chlorobromomethane occurs in chloroform B.P. used for extraction of strychnine during the assay. The presence of this impurity in chloroform has been confirmed by gas chromatography which also revealed the presence of methylene dichloride as an additional impurity.

At the Dublin meeting of the British Pharmaceutical Conference we presented a paper on a source of error in the assay of strychnine salts and preparations containing strychnine[1]. It was concluded that the chloroform, used for extraction of the strychnine during the assay, reacted with some of the alkaloid and caused an error in both gravimetric and volumetric estimations. A chloro-compound, assumed to be formed by combination of chloroform and strychnine, was isolated but although samples gave consistent figures when subjected to ultimate analysis we were unable to suggest a formula in harmony with the accepted structure of strychnine. Moreover, our analytical figures were not in agreement with those reported by Klemperer and Warren[2] for a similar compound claimed to be strychnine dichloromethochloride.

To investigate the chloro-compound further sufficient was prepared for a full examination. For this purpose strychnine was dissolved in chloroform and the solution boiled continuously under a reflux condenser, it being expected that the chloro-compound, which commenced to separate from the solution after about one hour, would continue to be formed and the procedure would afford a simple means of preparation. It was found, however, that although the chloro-compound continued to separate from the boiling solution its rate of formation decreased and practically ceased after several days boiling. If the chloroform were recovered from the solution at this stage and used for a repetition of the experiment very little, if any, chloro-compound was obtained. This evidence suggested that the formation of the chloro-compound was due to reaction between strychnine and an impurity present in the chloroform.

To investigate the purity of the chloroform B.P. used in our work it was arranged for samples, before and after treatment with strychnine, to be examined by gas chromatography in the Department of the Government Chemist. Evidence of the presence of methylene dichloride was obtained. On reacting methylene dichloride with strychnine a crystalline addition compound was formed which, however, was not identical with the compound isolated during our work.

More careful examination of our compound revealed that it contained both chlorine and bromine, the latter being the ionisable halogen. Analytical figures were in complete agreement with the compound being strychnine chloromethobromide, $C_{22}H_{24}O_2N_2ClBr$, presumably formed by reaction between strychnine and chlorobromomethane. By interaction between strychnine and authentic chlorobromomethane a crystalline compound was readily obtained and this had the characteristics of our "chloro-compound" and appeared identical with it in all respects although, owing to the substances decomposing on heating, it was not possible to carry out a mixed melting point determination. The presence of chlorobromomethane in chloroform B.P. was confirmed by gas chromatography.

Experimental

Chlorobromomethane. The preparation of chlorobromomethane was analogous to that used by Dougherty[3] for dichlorobromomethane.

Methylene dichloride (127 ml.) was mixed with ethyl bromide (148 ml.) and finely powdered anhydrous aluminium chloride (10 g.) added. Most of the solid matter dissolved and the reaction mixture assumed a pale brown colour. After standing at room temperature for 48 hours the mixture was shaken successively with 5 per cent hydrochloric acid (100 ml.), water (100 ml.), 5 per cent sodium hydroxide solution (100 ml.) and two portions (100 ml.) of water. The product was dried over anhydrous calcium chloride and distilled, the fraction boiling between 66° and 71° being collected. By redistillation a portion boiling at 68–69° was obtained. Yield 25 g. Found: total halogen, estimated gravimetrically and calculated as chlorine, 63·0, CH_2ClBr requires 63·3 per cent.

Chloro-compound from strychnine. Strychnine (10 g.) was dissolved in chloroform B.P. (500 ml.) and the solution boiled under a reflux condenser for 10 hours. The chloro-compound which separated from the solution was filtered off, washed with several portions of chloroform and dried at 105°. The yield varied with the sample of chloroform B.P. but was usually about 2 g. and could be increased by continued boiling of the reaction mixture. After recrystallisation from water the compound was obtained as colourless needles which had no definite melting point; at 270° it darkened in colour and at about 300° decomposed. Found on material dried at 105°: C, 57·2; H, 5·35; N, 6·46; Cl, 7·75; Br, 17·4; ionisable Br, 17·1 per cent. $[\alpha]_D^{20} = +14{\cdot}4°$ (*c*, 2·0 in water).

Strychnine chloromethobromide. Strychnine (3 g.) was added to chlorobromomethane (10 ml.) and the mixture stirred. The alkaloid began to dissolve but before solution was complete the reaction product began to separate from the mixture which soon became a solid mass. Methanol (50 ml.) was added and the mixture boiled under a reflux condenser for 2 hours, after which the insoluble matter was filtered off and washed with several portions of methanol. After recrystallisation from water 1·8 g. of colourless needles was obtained. Found, on material dried at 105°: C, 56·7; H, 5·4; N, 6·6; Cl, 7·56; Br, 17·4; ionisable Br, 17·1. $[\alpha]_D^{20} = +15{\cdot}2°$ (*c*, 2·0 in water). $C_{22}H_{24}O_2N_2Cl.Br$ requires C, 57·0; H, 5·22; N, 6·04; Cl, 7·65; Br, 17·23; ionisable Br, 17·23 per cent.

Strychnine chloromethochloride. Strychnine (3 g.) was dissolved in methylene dichloride (30 ml.) and the solution boiled under a reflux condenser for 10 hours. The insoluble matter was removed by filtration, washed with a small amount of chloroform and dried at 105°. Yield 1·8 g. Recrystallisation from aqueous acetone afforded colourless needles. Found on material dried at 105°: C, 62·6; H, 5·94; N, 7·01; Cl, 16·5; $C_{22}H_{24}O_2N_2Cl_2$ requires C, 63·0; H, 5·77; N, 6·68; Cl, 16·9 per cent.

Strychnine chloromethonitrate. Strychnine chloromethobromide (2 g.) was dissolved in hot water (100 ml.) and, after cooling, concentrated nitric acid (1 ml.) was added with stirring. After standing for 2 hours the precipitate was collected, washed with a little water and recrystallised from water. 1·2 g. of colourless or pale yellow needles was obtained.

Found on material dried at 105°: C, 59·4; H, 5·64; N, 9·95; Cl, 7·83; $C_{22}H_{24}O_5N_3Cl$ requires C, 59·2; H, 5·42; N, 9·4; Cl, 7·95 per cent.

The nitrate prepared in the same way from the strychnine chloro-compound and nitric acid gave the following analytical results. Found: C, 59·6; H, 5·98; N, 9·86; Cl, 7·92 per cent.

Strychnine chloromethoiodide. Strychnine chloromethobromide (3 g.) was dissolved in water (60 ml.) by warming and to the hot solution 10 per cent potassium iodide solution (25 ml.) was added with stirring. A precipitate formed almost immediately and, after cooling the mixture, was separated and recrystallised from water. The salt obtained as crystalline needles, contained three molecules of water of crystallisation which were removed by drying *in vacuo* over phosphorus pentoxide. It melted with decomposition at about 260°. Yield 3·5 g. Found, on anhydrous material: C, 51·0; H, 5·14; N, 5·80; total halogen (as Cl), 18·2; I, 24·6; $C_{22}H_{24}O_2N_2Cl$ I requires C, 51·7; H, 4·73; N, 5·49; total halogen (as Cl), 18·3; I, 24·8 per cent.

The iodide prepared similarly from the strychnine chloro-compound gave the following results. Found: C, 51·5; H, 5·15; N, 5·78; total halogen (as Cl), 18·1; I, 24·6 per cent. It melted with decomposition at about 260°.

Ultra-violet Absorption

A critical comparison of the strychnine chloro-compound with strychnine chloromethobromide was made by measurement of their ultra-violet absorption with a "Uvispek" spectrophotometer using 0·002 per cent w/v solutions of the compounds in water. The results are shown in Figure 1, which also includes the corresponding absorption curve of strychnine.

Gas Chromatography

Samples of chloroform B.P. and of chloroform, prepared by boiling a solution of strychnine in chloroform B.P. until no more chloro-compound separated and recovering the solvent, were subjected to examination by gas chromatography. This work was carried out by Dr. B. A. Rose and Mr. A. J. Blake, A.R.I.C., at the Government Laboratory, and we are very much indebted to their department for permission to include their

results in the present paper. The apparatus employed was similar to that described by Scott[4] using a flame detector.

Detection of Methylene Dichloride in Chloroform

The samples were examined by gas chromatography on columns filled with (*a*) polyethylene glycol 400, (*b*) liquid paraffin and (*c*) cetyl alcohol, all operating at 100°; there was no significant difference between the chromatograms obtained from the two samples.

It was decided to attempt to concentrate any trace impurity by fractionation to facilitate its detection. Accordingly 25 ml. of each sample was distilled through an air-jacketed twelve-plate column and the distillate collected at a speed of one drop in approximately 5 seconds. Fractions consisting of a few drops each were collected in weighed receivers and examined chromatographically on the cetyl alcohol column at laboratory temperature; the lower temperature was chosen to retard the passage of the volatile samples through the column and improve the separation.

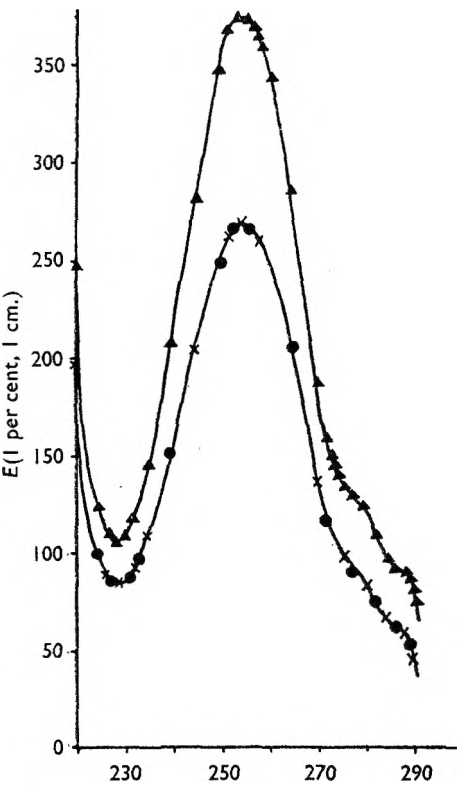

FIG. 1. Ultra-violet absorption curves.
▲ = 0·002 per cent strychnine in 0·001N HCl.
× = 0·002 per cent "chloro-compound" in water.
● = 0·002 per cent strychnine chloromethobromide in water.

In all cases, a peak was obtained indicating the presence of an impurity with a lower retention volume than chloroform; this peak became smaller in successive fractions and was also smaller in the fractions from the strychnine-treated chloroform than in the corresponding fractions from the untreated samples.

Identification of impurity. The impurity had a retention volume corresponding to methylene dichloride; no other substance has been found to have the same retention volume on cetyl alcohol and also on liquid paraffin, on which our diagnosis was checked.

Estimation of the amount of impurity (CH_2Cl_2). Mixtures of methylene dichloride and chloroform in varying ratios were prepared and examined

on the cetyl alcohol column at laboratory temperature and the ratio of the peak heights $CHCl_3$: CH_2Cl_2 measured. From these figures the concentration of methylene dichloride in the weighed distillation fractions was calculated and hence the methylene dichloride content of the samples.

The figures obtained from measuring peak heights on the chromatograms are set out in Tables I—standard mixtures ($CH_2Cl_2/CHCl_3$), II—"untreated" $CHCl_3$, III—strychnine treated $CHCl_3$.

TABLE I

CHROMATOGRAPHIC DATA: STANDARD MIXTURES, CH_2Cl_2 AND $CHCl_3$

Per cent CH_2Cl_2 v/v	$CHCl_3$ Peak height	CH_2Cl_2 Peak height	Peak height ratio $CHCl_3$: CH_2Cl_2
1	3·30 in.	0·2 in.	16·5
3	2·12 ,,	0·35 ,,	6·06
5	2·08 ,,	0·58 ,,	3·60
10	1·80 ,,	0·80 ,,	2·25
15	1·90 ,,	1·24 ,,	1·53
20	1·75 ,,	1·60 ,,	1·09

Some of the chromatograms obtained are also shown in Figure 2.

The chloroform B.P. contains 1–2 per cent of ethanol. Using cetyl alcohol as the fixed phase the ethanol was to be expected to have a longer retention time than the chloroform. In fact there were indications of the ethanol to the right of the chloroform peak.

The fractional distillation and chromatographic examination of the fractions was repeated using portions of the samples which had been washed four times with water and dried with sodium sulphate. The purpose was to ensure that the alcohol (1–2 per cent) contained in Chloroform B.P. was not causing any interference. The figures for methylene chloride content of the washed samples are lower than for the unwashed samples presumably because methylene dichloride has a higher solubility than chloroform in water.

The figures obtained are set out in Tables IV and V.

Detection of Chlorobromomethane in Chloroform

50 ml. of the liquid with a small addition of *p*-xylene was slowly fractionated through a column (25 theoretical plates) over more than 1 working day. Towards the end 16 small separate fractions (each of 0·1–0·2 ml.) were collected between the point where the b.p. showed a slight sign of rising and the final point of rise towards *p*-xylene. Several of these small fractions were then examined by gas chromatography. Fractions 13, 14 and 15 gave chromatograms (in duplicate) showing a small but distinct shoulder near the bottom of the right-hand slope of the chloroform peak. Made up mixtures of pure chloroform with $\frac{1}{2}$–1 per cent of chlorobromomethane gave closely similar graphs and the comparison indicates the presence of between 0·5 and 1 per cent by volume of the bromo-compound in these small fractions. An approximate estimate is that 0·004 ml. of the bromide was present in the 3 small fractions 13, 14 and 15 which totalled *c.* 0·5 ml.

Figure 3 shows chromatograms obtained during these experiments.

Discussion

The object of the work was the determination of the chemical structure of the "chloro-compound" isolated during our investigations on the assay of strychnine salts[1]. The compound is shown to consist of strychnine chloromethobromide. The unexpected detection of bromine made it essential to obtain confirmatory evidence. Accordingly, an authentic specimen of strychnine chloromethobromide was prepared by reaction between strychnine and chlorobromomethane. Both the "chloro-compound" and strychnine chloromethobromide melt with decomposition and, on this account, their identity could not be established by a mixed melting point. Their ultimate analyses and ultra-violet absorption spectra were, however, identical and their corresponding nitrates and iodides possessed respectively identical characteristics. The ultra-violet absorption curve of our "chloro-compound" is similar in shape to that of strychnine and practically identical with it if calculated in terms of molecular extinction instead of E(1 per cent, 1 cm.), as in Figure 1. This evidence supports the view that the strychnine structure remains intact in the molecule and that the "chloro-compound" is, in fact, strychnine chloromethobromide.

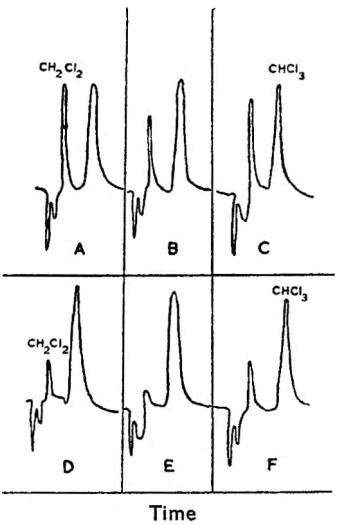

FIG. 2. Chromatograms on cetyl alcohol column at laboratory temperature.

A, Chloroform B.P. fraction 1.
B, Chloroform B.P. fraction 2.
C, 20 per cent CH_2Cl_2–80 per cent $CHCl_3$.
D, Chloroform, strychnine treated fraction 1.
E, Chloroform, strychnine treated fraction 2.
F, 10 per cent CH_2Cl_2–90 per cent $CHCl_3$.

The substance isolated by Klemperer and Warren[2] during their work on *Strychnos henningsii* and described by them as strychnine dichloromethochloride has again engaged our attention. The analytical figures given by these authors for carbon, hydrogen and nitrogen are in good agreement with our own but without knowing how their halogen determinations were carried out it is not possible to comment upon their published figures for total and ionisable chlorine.

The formation of strychnine chloromethobromide when strychnine is dissolved in chloroform led to an investigation of the purity of this solvent. The gas chromatograms reproduced in Figures 2 and 3 afford evidence of the presence of both methylene dichloride and chlorobromomethane in the sample of chloroform B.P. examined. These impurities were much reduced in chloroform, which had been recovered from a solution of strychnine in chloroform previously boiled for some hours.

It must be accepted that the bromine present as chlorobromomethane arises from the chlorine, used directly or indirectly, in the manufacture

TABLE II

CHROMATOGRAPHIC DATA: DISTILLATION FRACTIONS FROM "UNTREATED" $CHCl_3$ (25 ML.)

Fraction No.	Wt. (g.)	Vol. (ml.)*	Peak height ratio $CHCl_3:CH_2Cl_2$	Per cent v/v CH_2Cl_2	Vol. CH_2Cl_2 (ml.)
1	0·067	0·044	1·00	20	0·009
2	0·095	0·063	1·52	12	0·0075
3	0·102	0·085	2·80	6	0·005
4 to 6	0·345	0·23	8·04	2	0·004

Total CH_2Cl_2 = 0·0255 ml. = 0·1 *per cent v/v in original sample* approx.

* Assuming specific gravity of 1·5.

TABLE III

CHROMATOGRAPHIC DATA: DISTILLATION FRACTIONS FROM STRYCHNINE-TREATED $CHCl_3$ (25 ML.)

Fraction No.	Wt. (g.)	Vol. (ml.)*	Peak height ratio $CHCl_3/CH_2Cl_2$	Per cent v/v CH_2Cl_2	Vol. CH_2Cl_2 (ml.)
1	0·084	0·056	2·79	6	0·0034
2	0·097	0·064	7·96	2	0·0013
3	0·107	0·070	10·8	1	0·0007

Total CH_2Cl_2 = 0·0054 ml. approx. = 0·02 *per cent in original sample* approx.

* Assuming specific gravity of 1·5.

TABLE IV

CHROMATOGRAPHIC DATA: DISTILLATION FRACTIONS FROM "UNTREATED" WASHED $CHCl_3$ (14 ML.)

Fraction No.	Wt. (g.)	Vol. (ml.)*	Peak height ratio $CHCl_3/CH_2Cl_2$	Per cent v/v CH_2Cl_2	Vol. CH_2Cl_2 (ml.)
1	0·076	0·05	2·14	10	0·005
2	0·083	0·055	3·38	5·3	0·003
3	0·105	0·07	6·6	2·5	0·002
4	0·097	0·065	13·4	1·5	0·001

Total CH_2Cl_2 = 0·011 ml. = 0·08 *per cent on washed sample* approx.

* Assuming specific gravity of 1·5.

TABLE V

CHROMATOGRAPHIC DATA: DISTILLATION FRACTIONS FROM STRYCHNINE TREATED, WASHED $CHCl_3$ (18 ML.)

Fraction	Wt. (g.)	Vol. (ml.)*	Peak height ratio $CHCl_3/CH_2Cl_2$	Per cent v/v CH_2Cl_2	Vol. CH_2Cl_2 (ml.)
1	0·073	0·048	6·38	2·75	0·0013
2	0·087	0·058	8·8	2	0·0011
3	0·074	0·05	20	1	negligible

Total CH_2Cl_2 = 0·0024 ml. = 0·013 *per cent on washed sample* approx.

* Assuming specific gravity of 1·5.

of the chloroform. Very little analytical information is available regarding the purity of commercial chlorine and, as far as we are aware, no international specification is available for this product. It has been stated that impurities totalling 0·2 per cent occur in chlorine[5] and that these consist of hexachlorethane, carbon tetrachloride, chloroform, bromine and ferric chloride, while carbon dioxide, hydrogen chloride, oxygen, carbon monoxide, nitrogen and hydrogen have also been reported.

Chlorinated hydrocarbons may rise from reaction between chlorine gas and lubricants used on valves, etc., and these together with some other impurities may be removed by liquefaction and subsequent distillation of the chlorine. This procedure, however, cannot be relied upon to remove bromine.

Chloroform may be made by treatment of alcohol, acetaldehyde or acetone with bleaching powder, and also by chlorination of methane or reduction of carbon tetrachloride[6]. Most chloroform, we believe, is manufactured in this country today by the acetone – bleaching powder process although chlorination of methane may also be used. The latter process gives rise to a mixture of methylchloride, methylene dichloride, chloroform and carbon tetrachloride which are then separated by fractionation and chloroform, so produced, may contain traces of methylene dichloride. There is also no doubt that brominated products would result when chloroform is made from acetone and bleaching powder, containing bromine, but we have insufficient knowledge to indicate the course of the reaction. We believe that most impurities would be removed by distillation during the final stages of the manufacture of commercial chloroform but that chlorobromomethane, with a boiling point similar to chloroform, would be difficult to eliminate. Although we have carried out no accurate quantitative work, it seems, from the yields of strychnine chloromethobromide, that up to 0·5 per cent may be present in chloroform B.P. Samples of chloroform B.P. from four British manufacturers have been examined and chlorobromomethane detected in all by the strychnine reaction.

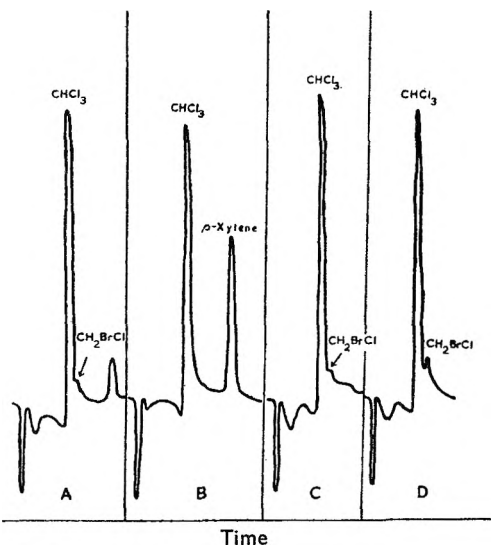

FIG. 3. Chromatograms on polyethylene glycol 400 column at 100°.
A, Chloroform B.P. fraction 14.
B, Chloroform, strychnine treated, fraction K.
C, 1 per cent CH_2BrCl in $CHCl_3$.
D, 2 per cent CH_2BrCl in $CHCl_3$.

There remains the problem of preparing chloroform suitable for alkaloidal assays. From our experiments, the presence of methylene dichloride and chlorobromomethane will give rise to error in strychnine assays[1]. Chloroform, purified by strychnine treatment, gave a substantially smaller analytical error in strychnine determinations but the error was not entirely eliminated and we are unable to state, at this stage,

whether or not reaction occurs between strychnine and pure chloroform. We hope, in due course, to describe how chloroform suitable for analytical purposes may be prepared.

We wish to thank the Government Chemist, Dr. G. M. Bennett, F.R.S., for his interest in this work and for providing facilities for gas chromatography in his department. We are also indebted to Mr. F. J. McMurray for the micro analyses.

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DISCUSSION

The paper was presented by MR. A. C. CAWS.

MR. C. A. JOHNSON (Nottingham). The gas chromatographic evidence indicated that chlorobromomethane was present, but in much smaller amount than methylene dichloride. Strychnine was shown to form additional compounds with both methylene dichloride and chlorobromomethane, yet the analytical figures agreed closely with those for strychnine chloromethobromide. With different samples of chloroform containing varying amounts of impurities, some variation in the analytical figures for the precipitate would be expected.

MR. S. G. E. STEVENS (London) thought there would be little difference between the spectra of strychnine chloromethochloride and chloromethobromide. Had the chloroform which had been refluxed for some time been examined for decomposition?

DR. W. MITCHELL (London). How much methylene dichloride was present in the chloroform examined? Since methylene dichloride reacted readily with strychnine, the reaction products could be assumed to contain both derivatives. Was the preparation of Klemperer and Warren a mixture of the two derivatives?

DR. J. B. STENLAKE (Glasgow). What was the stability and toxicity of chlorobromomethane?

DR. N. J. HARPER (London). Had any attempt been made to titrate the compounds in non-aqueous media? High purity chloroform could be obtained from chloral hydrate.

DR. D. C. GARRATT (Nottingham). An indication of the error which could arise in strychnine determinations would be helpful.

MR. H. E. BROOKES (Nottingham). Were the impurities found in chloroform consistent from sample to sample?

DR. R. E. STUCKEY (London). Had any attempt been made to find an alternative solvent to chloroform?

MR. A. G. FISHBURN (Rochdale). Were any of the samples of chloroform supplied as suitable for anaesthesia?

MR. D. J. DRAIN (Ware). Had the authors tried reacting chloroform with a less complex but possibly more reactive tertiary base than strychnine?

The AUTHORS in reply said that no great accuracy was claimed for the results obtained by gas chromatography. The precipitate obtained was contaminated with a substance containing chloride ions, as yet not isolated. The analytical figures were consistent because the precipitate was recrystallised from water, so separating the chlorobromide compound from the methylene dichloride compound. The molecular absorption of the two compounds would be the same, but the *E* (1 per cent, 1 cm.) would be different. The figure for content of methylene dichloride in chloroform given by gas chromatography was 0·1 per cent. The reaction between that concentration of methylene dichloride and strychnine was very slow; chlorobromomethane reacted much more readily. Klemperer and Warren stated that their substance was recrystallised from water, so it seemed unlikely that it was a mixture. They had no information about the toxicity of chlorobromomethane, but it seemed to be stable. The compounds had not been titrated in non-aqueous solvents. Experiments had been carried out using chloroform B.P. and chloroform previously treated with strychnine; by evaporation without the addition of alcohol the error was reduced from just over 1 per cent to about 0·5 per cent. The amounts of impurity in chloroform were not consistent. Some samples contained as much as 0·4 to 0·5 per cent and others were nearer 0·1 per cent. An alternative solvent would be welcome, but strychnine was not an easy substance to dissolve. One of the four samples examined was labelled "Chloroform for Anaesthesia". Another sample from the same manufacturer labelled "Chloroform B.P." contained about the same amount of bromo compound. Only one tertiary base had been tried, and that was dimethylaniline. It appeared to react more slowly than strychnine, and did not give a solid derivative.

SOME PROPERTIES OF MIXED SOLS OF LECITHIN AND LYSOLECITHIN

By L. Saunders

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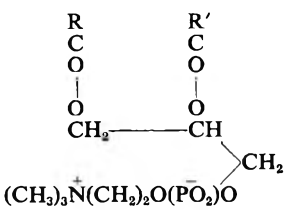
Received June 19, 1957

The preparations of pure crystalline lecithin and lysolecithin are described. Mixed sols of these phosphatides have been prepared and their viscosities, stabilities to salts, and haemolytic activities have been examined. The viscosity results indicate a marked interaction between the two compounds in the aqueous sols. The salt stability and haemolysis results are used to support a theory of cell membrane formation.

Lecithin no longer seems to have any direct application in pharmacy; the monograph on lecithin was removed from the last edition of the British Pharmaceutical Codex. As an emulsifying agent it is expensive and its therapeutic value is dubious. However, since the phosphatides, and lecithin in particular are important constituents of cell membranes their physical properties are of interest in elucidating the structure of such membranes. The soap-like structure of lecithin with its bi-ionic polar head group (I)



(I) (R,R′ are hydrocarbon chains, C_{13} up to C_{23} or higher)

renders it dispersible in water, and from the aqueous sols stable fatty films having some of the properties of simple cell membranes can be obtained at a boundary formed between the aqueous sol and a suitable salt solution[1].

The main difficulty in work with phosphatides is that of purifying the materials used. The physical chemistry of the lecithins was reviewed recently by Saunders and Elworthy[2] and it was pointed out that all the earlier work was carried out with samples of lecithin which contained varying amounts of lysolecithin (lysolecithin has the formula (I) with one of the fatty acid radicals RCO replaced by H). It was not until Lea and Rhodes developed their chromatographic purification on a silicic acid-Celite column in 1954[3] that complete removal of the lyso-compound from lecithin became possible. This removal causes a considerable change in the properties of aqueous lecithin sols particularly in their stability to salts and in the conditions necessary to form films from the sols[4]. The lyso-compound is invariably associated with lecithin from

natural sources and the present investigation has been undertaken to examine the interaction between the two phosphatides in water.

For this purpose pure synthetic materials would be ideal but the synthesis of quantities of lecithin[5] is difficult. The synthetic lecithins with saturated fatty acid groups also show considerable differences in their behaviour with water from the unsaturated material prepared from natural sources[6]. The synthetic compounds do not form stable sols.

The yolks of hen's eggs is an abundant source, containing up to 10 per cent dry weight of lecithin. But the extensive purification process results in a low yield. Lecithin so obtained has a mixture of fatty acid radicals in which palmitoyl, oleyl, and linoleyl radicals are the main components. In water, the phosphatides form large micelles in which all the fatty acid radicals are likely to be mixed in a random manner.

Experimental

Preparation of Lecithin

The yolks of twelve eggs were dried by extraction with 500 ml. of acetone, and the resulting solid filtered and extracted with 1 l. of warm ethanol in three successive portions. This extract was shaken with successive portions of alumina powder until it no longer gave a colouration with ninhydrin solution on warming. This treatment removed cephalins and other phosphatides containing primary amine groups. It was then evaporated to dryness at 40° under vacuum and the residue extracted with 150 ml. of ether. Crude lecithin was precipitated by adding to 500 ml. of cold acetone. The precipitate was dissolved in 40 ml. of 1 : 4-methanol-chloroform. The resulting solution was put on a column containing 75 g. of Mallinckrodt chromatographic silicic acid and 20 g. of Celite filter aid and the column developed with 1 : 4-methanol-chloroform. The first fraction contained some coloured compounds and a trace of ninhydrin reacting material which was rejected. The subsequent colourless effluent was collected until nearly all the lecithin had been eluted (about 1·5 l.), lysolecithin being firmly retained by the column, and was then centrifuged to remove any silicic acid and evaporated to dryness at 40°. The product was recrystallised three times from warm methyl-ethyl-ketone to give a white solid which showed parallel extinction under the polarising microscope. Finally it was dissolved in ethanol to give a 10 per cent solution which was stored in a nitrogen filled desiccator at −5°. We have found that although it is impossible to keep the solid in the dry state without decomposition, in ethanol solution it can be kept for many weeks without apparent change in its properties. The final yield was 5–6 g. of solid which had a nitrogen content of 1·74 per cent, a phosphorus content of 3·8 per cent and an iodine value of 73. The mean molecular weight calculated from the nitrogen and phosphorus contents was 810.

Preparation of Lysolecithin

This compound was prepared from the lecithin by a modification of Hanahan's method[7]. Five g. of lecithin was dissolved in 500 ml. of ether

and 5 ml. of an aqueous solution containing 10 mg. of Russell viper venom was added and the mixture shaken for a minute. After half an hour the liquid became turbid due to the hydrolysis of lecithin catalysed by the enzyme lecithinase A in the venom. This enzyme is a highly specific catalyst for the removal of one fatty acid radical from the lecithin molecule, to give lysolecithin which is insoluble in ether.

The lysolecithin was allowed to settle and the clear supernatant liquid was decanted. 50 ml. of acetone was added to granulate the slimy precipitate which was washed with acetone. The precipitate was dissolved in a minimum amount of chloroform and reprecipitated by adding it to 6 times its volume of ether. This procedure was repeated five times using a minimum volume of chloroform in each case. The final amorphous product was washed thoroughly with acetone, dried and dissolved in a small amount of warm ethanol. The solution was slightly cloudy owing to traces of proteinous matter from the venom and was centrifuged keeping the solution warm. The clear solution was then set aside for the lysolecithin to crystallise; the crystallisation was repeated twice. The author has added this crystallisation step to Hanahan's process which stopped at the amorphous material, in order to ensure removal of traces of venom from the product. The lysolecithin is a white solid of nitrogen content 2·8 per cent and phosphorus content 5·5 per cent, iodine value 4 (approx.), mean molecular weight from nitrogen and phosphorus 535, yield 2 g.

The low iodine value of the lysolecithin indicates that the venom removes the unsaturated fatty acid radicals preferentially from the lecithin; the molecular weight estimates indicate that the mean molecular weight of the acid removed was 275.

The lysolecithin was stored under ethanol.

Preparation of Aqueous Sols

Mixed sols of lecithin and lysolecithin were prepared by mixing ethanol solutions containing known amounts of the two phosphatides and evaporating to dryness, the residue being shaken with warm distilled water until dispersion was complete. Dispersion was checked by centrifugation and the sol was passed through an ion exchange column containing a mixture of the Amberlite resins IR-120 and IRA-400. These removed traces of small ions present as impurities, a fact checked by electrical conductivity measurements. The column was then washed with water and the combined effluent made up to the required volume. The loss of phosphatides in this step was small.

Properties of the Mixed Sols

Appearance. Pure lecithin sols although turbid in appearance were stable for long periods. Introduction of lysolecithin reduces the turbidity and at a weight fraction lysolecithin to total phosphatide of 0·35, the sols become optically clear.

Viscosity. The sols showed anomolous viscosities; for comparative purposes their times of flow through an Ostwald viscometer were divided

by the time of flow for a pure lecithin sol. The results for 0·5 per cent total phosphatide sols at 25° are shown in Table I.

Pure lecithin and lysolecithin sols both had viscosities similar to that of water. Introduction of lysolecithin into the lecithin sol caused a big rise in viscosity and at the maximum the sols were very thick liquids. These results indicate a very considerable interaction between the two compounds, in the mixed sols.

Stability to electrolytes. Film formation from a phosphatide sol is known to be related to the stability of the sol to electrolytes[4]. A salt solution of concentration such that it causes precipitation of the sol also gives films of measurable strength at a boundary formed between the sol and the salt solution.

TABLE I

RELATIVE VISCOSITIES OF MIXED LECITHIN AND LYSOLECITHINS SOLS AT 25° RELATIVE TO A PURE LECITHIN SOL. ALL SOLS CONTAINED 0·5 PER CENT TOTAL PHOSPHATIDES

Weight fraction of lysolecithin	Relative viscosity	Weight fraction of lysolecithin	Relative viscosity
0·0	1·0	0·5	2·5
0·2	1·1	0·7	2·9
0·3	1·4	0·8	2·2
0·35	1·7	0·9	1·6
0·45	2·4	1·0	1·0

Sol stabilities were examined by setting up a series of tubes each containing 1 ml. of 0·5 per cent total phosphatide sol. To these, small volumes of salt solutions were added from an Agla microsyringe. The resulting mixtures were thoroughly stirred and set aside overnight. It was found that pure lysolecithin sols were stable to all the concentrations of sodium and calcium chlorides examined. It was not until the lysolecithin weight fraction of a mixed sol was reduced below 0·4 that precipitation by calcium chloride was observed. At a weight fraction of 0·33 lysolecithin the phosphatide was completely precipitated by 10^{-4}M calcium chloride but not by 0·1M sodium or potassium chlorides. Pure lecithin sols were precipitated by 2×10^{-5}M calcium chloride and also by 0·01M sodium and potassium chlorides. Addition of the lyso compound to lecithin sols can produce any required degree of stability to salts.

Haemolytic properties. Lysolecithin owes its name to its ability to dissolve the membranes of erythrocytes, releasing their contents into the surrounding medium. If the theory of cell membrane formation given in the discussion is correct it is to be expected that the addition of lecithin to lysolecithin would remove the lysing action of the latter. To test this the effect of introducing lecithin into the lyso-sols was examined using rabbit's blood containing sodium citrate as an anticoagulant.

Blood, 0·5 ml., was mixed in a centrifuge tube with 0·1 ml. of 0·1M sodium chloride solution in which lysolecithin was dissolved to give a 0·5 per cent sol, in addition varying quantities of lecithin were present in this sol. Each final mixture therefore contained a lysolecithin concentration of 0·08 per cent.

After mixing, the tubes were centrifuged for one minute and the extent of haemolysis estimated from the colour of the supernatant liquid. Table II shows the results, the amount of lecithin being shown as a weight fraction of total phosphatides.

It is seen that at a lecithin weight fraction between 0·53 and 0·68, that is a lysolecithin weight fraction between 0·47 and 0·32, the lysing action of the sol is lost.

TABLE II

HAEMOLYTIC EFFECTS OF MIXED SOLS. 0·5 ML. OF RABBITS BLOOD WITH 0·1 ML. OF SOL, 0·1M WITH RESPECT TO NaCl AND 0·5 PER CENT WITH RESPECT TO LYSOLECITHIN

Lysolecithin in final mixture per cent	Lecithin weight fraction	Lysolecithin weight fraction	Appearance of supernatant liquid
0·00	0	1	No colour
0·08	0	1	Deep red, complete lysis
0·08	0·37	0·63	Deep red, complete lysis
0·08	0·53	0·47	Pink, slight lysis
0·08	0·68	0·32	No colour

DISCUSSION

The pronounced interaction between lecithin and lysolecithin in aqueous sols, shown by the viscosity results, has not previously been reported. At a lysolecithin weight fraction of 0·35 to 0·4, important changes in the properties of the sols occur; below this range, the sols are precipitated by salts and any required degree of stability to salt solutions can be obtained by adjusting the proportion of the lyso-compound in the sol. Below the 0·35 to 0·4 range, the sols lose their lysing effect on erythrocyte membranes. It should be noted that a lysolecithin weight fraction of 0·39 corresponds to an equimolecular mixture of the two phosphatides.

Pure lecithin sols are unstable to 0·01M sodium and potassium chlorides and so would be precipitated by salt solutions equivalent to the electrolyte composition of erythrocyte contents (mainly alkali metal chlorides total concentration $>0{\cdot}1$M). Lysolecithin invariably occurs in association with lecithin from natural sources and this is to be expected since the former is the first decomposition product of the latter, and the specific enzyme for the conversion, lecithinase, is widely distributed in living systems. If sufficient lysolecithin is present in the internal fluid of a cell, it can stabilise the lecithin to the monovalent metal salts present, but can still permit precipitation of a phosphatide membrane when this fluid meets a solution containing divalent metal ions such as plasma (calcium ion concentration $>0{\cdot}001$M). This membrane would be stable since the lysolecithin in the cell contents would not be lytic at the weight fraction necessary to give precipitation. In addition, the mixed phosphatide sol would have a high viscosity even without the presence of other colloidal material and so could be formed into units which would cohere until fatty membranes were formed around them.

In the development of a cell membrane the many other components present in the fluids will of course play a part, but it is suggested that this phosphatide balance in the cell contents is the important factor in the

primary formation of the cell membrane which is then probably strengthened by adsorption of proteins and modified by other lipids.

Acknowledgement. The author thanks Dr. A. C. White for the gift of Russell viper venom.

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4. Elworthy and Saunders, *J. chem. Soc.*, 1957, 330.
5. Baer and Kates, *J. Amer. chem. Soc.*, 1950, **72**, 942.
6. Saunders, *2nd International Congress on Surface Activity*, London, 1957.
7. Hanahan, Rodbell and Turner, *J. biol. Chem.*, 1954, **206**, 431.

DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The author was nearly dealing with the stoichiometric properties of lecithin; hydrolysis, as was suggested, took place from the ester group on the primary carbon atom, but it was also possible to break off the fatty acid grouping from the secondary carbon atom as well.

How widely was lecithinase A distributed in the body?

MR. H. J. BRAGG (Folkestone). Had lecithin from soya bean been tried? There might be some natural antoxidants in soya bean which would help stabilise the lecithin. Chloroform might be a better volatile solvent than ethanol. The sols, although turbid, were said to be stable for a long period. For how long were they examined?

DR. J. B. STENLAKE (Glasgow). The viscosity measurements were made at 25° and he presumed that the other work was done at that temperature. Were the sols stable at 37° and at higher temperatures? What was the effect of other non-ionic substances on the stability of the sols towards electrolytes?

DR. G. E. FOSTER (Dartford). Were the lecithin and lysolecithin single substances? Was the hydroxyl group in the lysolecithin in the α or β position, or was it a mixture of the α and β esters?

MR. N. J. VAN ABBÉ (Loughborough). Was it known that lysolecithin was present *in vivo* with lecithin? Could such substances as rutin and hesperidin affect cell permeability by preventing the formation of lysolecithin in some way?

MISS A. E. ROBINSON (London). Did lysolecithin dissolve, disperse or solubilise the membrane? The erythrocyte membrane was thought to be a mosaic structure. It was stated in the paper that the phosphatides form large micelles in water, and the term "dissolve" seemed a vague one to use. Had the work of Rideal and Taylor on permeability of the cell been considered?

DR. SAUNDERS replied. A solvent which preserved the material for a reasonable period met his needs; ether free from peroxides was probably the best. The completely dispersed sol did not settle out for about a

week so long as it was protected from decomposition. At temperatures higher than 25° the sols in contact with air took up oxygen more quickly. Non-ionic surface-active materials like ether oxides increased the stability to electrolytes. Lecithin and lysolecithin were not single substances. All preparations of phosphatides from natural material were fractions. Single substances could be obtained only by synthesis. This was difficult. He believed Hanahan had found the OH group in lysolecithin to be in the α position. There was no analytical procedure to prove lysolecithin was present with lecithin *in vivo*, but it was the first decomposition product when lecithin was hydrolysed by lecithinase in the tissues. Lecithinase A occurred in a large number of body tissues. Viper venom was used because it was a concentrated source. Lysolecithin dissolved the fatty part of the erythrocyte membrane, which then broke up. It was behaving as a soap in its haemolytic effect. An inconclusive attempt had been made to estimate micelle size in the sols by diffusion studies.

THE APPLICATION OF INFRA-RED SPECTROPHOTOMETRY TO THE EXAMINATION OF ESSENTIAL OILS

PART I. CINEOLE IN LAVENDER OIL

BY A. H. J. CROSS, A. H. GUNN and S. G. E. STEVENS

From Smith Kline and French Laboratories Ltd., London

Received May 15, 1957

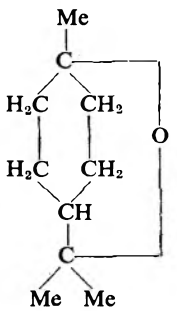
Lavender oils in capillary films, in the range 5000 cm.$^{-1}$ to 650 cm.$^{-1}$ show peaks at 1310, 1220, 1085 and 855 cm.$^{-1}$ of diagnostic value for cineole. A quantitative method based on the cineole peak at 1085 cm.$^{-1}$ in carbon disulphide solution, and using an "Absorbancy Difference" method provides a truer assessment of the cineole content of lavender oils than do the methods currently described. The *o*-cresol method is inapplicable to oils containing cineole in the presence of esters, and alcohols. The cineole contents of English and spike lavender oils are lower than the published figures based on the formation of complexes. The official, French, English and spike lavender oils might be classified in the pharmacopoeias by their cineole content.

LAVENDER oil varies in its composition depending on the botanical source, the environmental conditions during the plant growth, the time of harvesting and the manner in which the oil is isolated. At the present time, the commercial oils are derived mainly from *Lavandula intermedia*, *L. officinalis*, and *L. latifolia*[1,2] and are characterised by their varying amounts of esters, alcohols, and cineole which influence the odour of the oils.

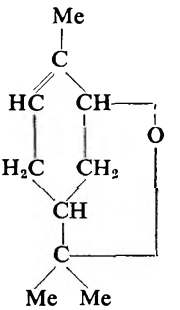
Various methods have been used in the past to determine the cineole contents of lavender oils. Tedesko[3] applied the freezing point method of Kleber and von Rechenburg[4] to spike lavender oil and reported cineole contents of 28·5 to 35·4 per cent but fractional distillation gave only some 10 per cent of cineole. The "cresineol" method of Cocking[5] which gave satisfactory results with eucalyptus oils containing a high proportion of cineole was found inapplicable by Reed[6] to French and spike lavender oils. Others reported that where the cineole content was below 65 per cent, the α-naphthol[7], resorcinol[8] and phosphoric acid[9] complex forming methods did not provide satisfactory answers. Martin and Harrison[10] developed a spectrophotometric method for the estimation of cineole based on a red colour developed with acid *p*-dimethylaminobenzaldehyde, but this was later adversely criticised by Montes[11]. Optical methods, of special value in the detection of adulteration or sophistication were applied by Naves[12] to French and spike lavender oils, but they did not give any useful information on the cineole contents. Presnell[13] and Maennchen[14] published a number of infra-red spectrograms of essential oils as a means of identification, but without quantitative figures.

1:8-Cineole (I) differs from the other constituents of lavender oil in being a cyclic terpene oxide. Two similar compounds pinol (II) and

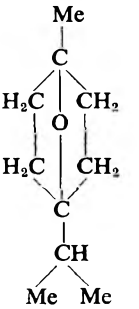
1:4-cineole (III) have been described and while no reference has been found to pinol as a material constituent, the 1:4-cineole has been identified in a few essential oils, notably that from *Piper cubebs*.



1:8-Cineole (I)



Pinol (II)



1:4-Cineole (III)

Since 1:8-cineole is a cyclic ether, its infra-red absorption curve might be expected to show sharp peaks due to the symmetric and asymmetric vibration frequencies of the C–O–C linkage. Barrow and Searles[15] in their study of cyclic ethers showed that the frequencies of the two bands varied with the ring size and with the 6 and 5 membered ring asymmetric vibrations the frequencies approximated to 1100 cm.$^{-1}$ and 1075 cm.$^{-1}$. For the corresponding symmetric vibrations the values were approximately 810 cm.$^{-1}$ and 910 cm.$^{-1}$. From a consideration of the pattern of frequency change with ring size, and the structural formula of cineole, it was expected that its C–O–C bridge would absorb at approximately the above pairs of frequencies. Strong sharp peaks were found at 1085 and 855 cm.$^{-1}$.

Experimental

The instrument was a Hilger H.800 double beam infra-red recording spectrophotometer fitted with sodium chloride optics. The frequency calibration was regularly checked against polystyrene and benzene reference standards and was accurate to ± 3 cm.$^{-1}$. All samples were dried overnight with dried magnesium sulphate.

Screening tests on a range of lavender oils in capillary films, covered the frequency ranges 5000 cm.$^{-1}$ to 2000 cm.$^{-1}$ and 2000 cm.$^{-1}$ to 650 cm.$^{-1}$. Pure cineole, examined in a similar manner showed a number of peaks. From a study of the oils available to us we selected four peaks at 1310 cm.$^{-1}$, 1220 cm.$^{-1}$, 1085 cm.$^{-1}$ and 855 cm.$^{-1}$ as suitable for the detection of cineole in lavender oil. (See Fig. 1.) After some preliminary work a standard concentration of 2·5 per cent w/v of dried oil in carbon disulphide was used for quantitative estimations of cineole. This solvent has a low absorbance in the 1085 cm.$^{-1}$ region. In these experiments the sample cell had a path length of 0·500 mm. whereas the reference cell containing only carbon disulphide was adjusted to a path length of 0·485 mm. to compensate for the volume of oil present and thus to maintain the double beam balance. The solutions were examined over the frequency range

1125 cm.$^{-1}$ to 1040 cm.$^{-1}$ at a scanning speed of 18 cm.$^{-1}$ per minute, a slit setting equivalent to a band width of about 2 cm.$^{-1}$ and a recorder chart speed of 0·5 inches per minute. Four replicate tracings were run for each solution and the average used in computing the cineole content by an "Absorbancy Difference" method.

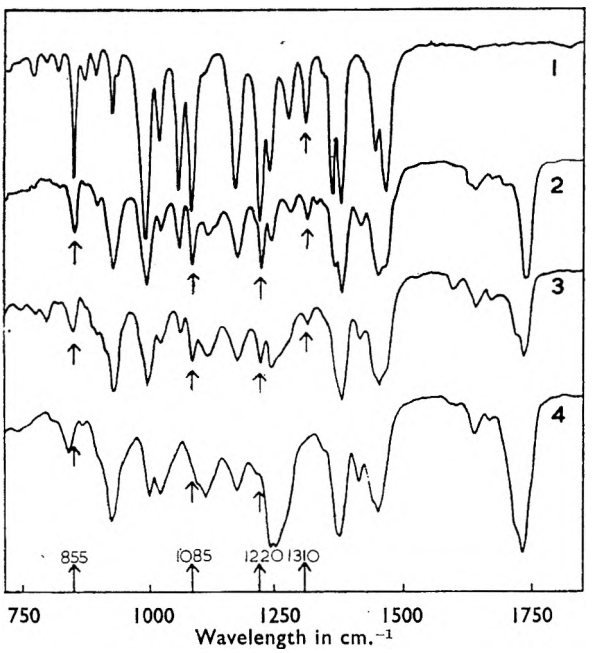


FIG. 1. Infra-red spectrograms, as capillary films from 750 to 1750 cm.$^{-1}$, at approximately 0·02 mm. path length, of cineole and lavender oils.

1. Pure cineole. 2. Spike lavender oil No. 24. 3. English lavender oil No .9. 4. French lavender oil No. 2.

Absorbancy Difference

Since the peak at 1085 cm.$^{-1}$ selected as the basis of a quantitative determination was superimposed on a sloping background absorption due to other components of the oils, the following simplified method was used to correct empirically for this. (See Fig. 2.)

A line BC was drawn across the base of the peak and another line drawn through the peak at A perpendicular to the frequency axis at 1085 cm.$^{-1}$ to cut BC at D. The absorbancy difference used in calculating the cineole content was defined as the optical density at point A minus the optical density at point D. By reference to a standard graph relating cineole to optical density it was possible to estimate the corresponding amount of cineole.

RESULTS

The results obtained with a few oils of known botanical source and history, as well as a range of commercial oils are listed in Tables I and IV.

Standard Cineole Curve, Reproducibility and Recovery Experiments

Solutions of cineole in carbon disulphide were examined over the frequency range 1125 cm.$^{-1}$ to 1040 cm.$^{-1}$ under the operating conditions described above, suitable adjustments being made to the solvent path length in the reference cell to compensate for the amount of cineole in the sample cell.

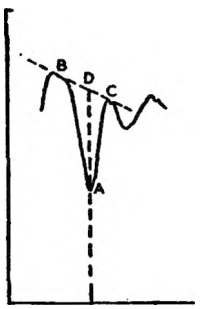


FIG. 2. Base line method for the determination of absorbancy difference at 1085 cm.$^{-1}$.

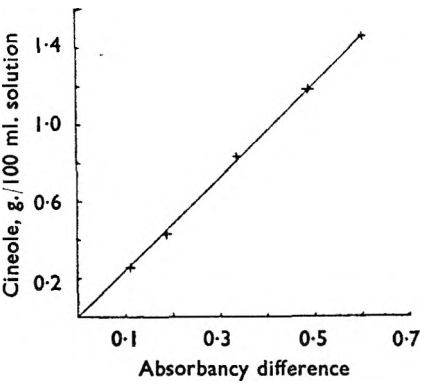


FIG. 3. Standard cineole curve.

When the "Absorbancy Difference" (AD) was plotted against the concentration of cineole (as g./100 ml. of solution), a straight line passing through the origin was obtained indicating that Beer's Law applied over the concentration range tested. The maximum concentration was greater than that likely to be present in genuine lavender oil. (See Fig. 3.)

TABLE I

PHYSICAL CONSTANTS AND SAMPLE CODING OF THE OILS EXAMINED

Sample Number	Supplier	Type of Oil	at n20°	$[\alpha]_D^{20}$ (l = 1dm.)	Wt./ml. at 20°
1	A	French	1·4503	−3·60	0·883
2	B	*French (Basse-Alpes) 1953	1·4633	—	0·877
3	C	French—(extra)	1·4618	−6·40	0·885
4	C	French—(cultivated)	1·4618	−7·40	0·887
5	C	French—(Barreme)	1·4613	−6·80	0·885
6	D	French—(Grasse)	1·4623:	—	—
7	D	French—1956	1·4638	−5·20	0·901
8	D	French (Hautes-Alpes) 1956	1·4633	−6·00	0·893
9	E	*English—1955	1·4689	−10·80	0·880
10	E	*English—1956	1·4706	−12·10	0·880
11	F	*English—1954	1·4744	−10·80	0·885
12	F	*English—1956	1·4733	−9·20	0·876
13	L	English	—	—	—
14		Spike—1954	1·4688	−3·20	0·911
15	G	†Spike—(Cuenca)	1·4673	nil	0·902
16	G	†Spike—(Murcia)	1·4683	+1·00	0·906
17	H	†Spike—(Spanish, extra)	1·4668	−2·20	0·899
18	H	†Spike—(Spanish)	1·4691	+1·50	0·904
19	J	†Spike—(Spanish, extra)	1·4668	−2·50	0·898
20	K	†Spike—(Spanish)	1·4683	—	—
21	H	†Spike—(Spanish)	1·4663	−1·20	0·905
22	J	†Spike—(Spanish, prime)	1·4677	+1·30	0·907
23	J	†Spike—(Spanish, extra)	1·4664	−1·60	0·904
24	G	†Spike—(Cuenca)	1·4654	−1·90	0·900
25	E	*English—Special Variety 1956	1·4608	−6·90	0·893

* Authentic oils. † Samples under 20/-/lb.

A sample of English lavender oil (No. 13) was assayed a total of six times to check the reproducibility of the method. The results obtained varied from 11·2 per cent w/w to 11·6 per cent w/w with an average cineole content of 11·4 per cent w/w.

TABLE II

RECOVERY OF CINEOLE ADDED TO A SAMPLE OF FRENCH LAVENDER OIL

Mix No.	Oil g./100 ml. solution	Cineole g. added/100ml. solution	Total cineole g./100ml. solution	AD* at 1087 cm.$^{-1}$	Cineole g./100ml. solution found	Recovery per cent
1	2·500	nil	0·025	—	—	—
1	2·267	0·224	0·247	0·102	0·245	99·3
2	2·380	0·146	0·170	0·072	0·157	103·0
3	2·145	0·373	0·395	0·164	0·400	101·2
4	1·717	0·815	0·832	0·342	0·825	99·1
5	2·120	0·389	0·410	0·174	0·420	102·4
6	1·669	0·835	0·852	0·346	0·835	98·0

* AD = Absorbancy difference, see text.

A series of determinations based on mixtures prepared from a French lavender oil of a known, one per cent, cineole content and pure cineole gave recoveries ranging from 98 per cent to 103 per cent when assayed as 2·5 per cent w/v solutions in carbon disulphide by the technique described above. (See Table II.)

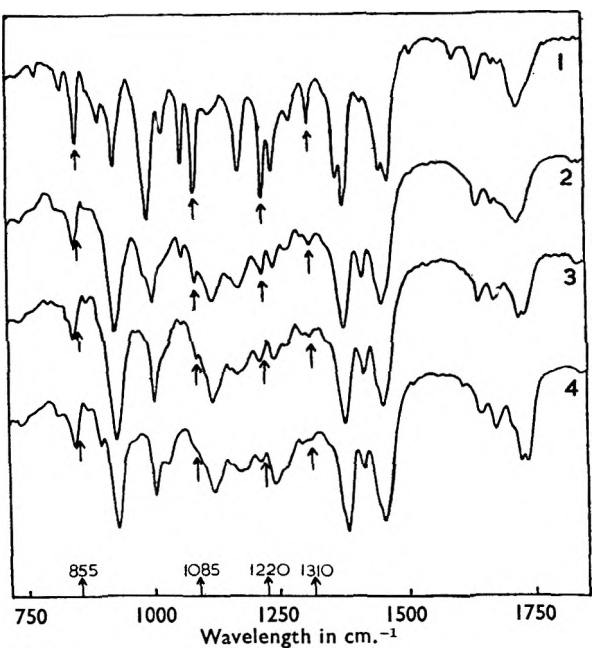


FIG. 4. Infra-red spectrograms, as capillary films from 750 to 1750 cm.$^{-1}$, at approximately 0·02 mm. path length, of fractions from a distilled English lavender oil No. 13.

1. Fraction 3, distilling 170–175°. 2. Fraction 5, distilling 180–190°. 3. Fraction 6, distilling 190–200°. 4. Undistilled residue.

Examination of a Fractionated Oil

One of the methods which had been used for the estimation of the cineole content of lavender oils was to fractionally distil a quantity of the oil and to collect the fraction boiling between 173° and 190°[16]. Subsequent formation of a cineole complex, removal of the liquid non-cineole part of the fraction and recovery of the cineole provided information on the approximate cineole concentration in the original oil.

TABLE III

THE DISTRIBUTION OF CINEOLE DETERMINED BY THE INFRA-RED AND "CRESINEOL" METHODS IN THE FRACTIONS OBTAINED FROM AN ENGLISH LAVENDER OIL (NO. 13) BY DISTILLATION

Fract. No.	Boiling range °C	Per cent w/w of sample	n20°	$[\alpha]_D^{20}$ (l = 1dm.)	Per cent w/w cineole in fraction	
					by infra-red	by "cresineol"
1	95–105°	1	—	—	—	—
2	105–170°	8	1·4598	−1·54°	28·0	27·0
3	170–175°	18	1·4567	−7·04°	40·0	42·5
4	175–180°	8	1·4676	−8·00°	37·5	46·5
5	180–190°	8	1·4687	−11·64°	6·5	26·0
6	190–200°	20	1·4688	−11·64°	1·0	20·5
residue	undistilled	37	—	—	nil	18·5
Original sample		—	1·4680	−9·58°	11·4	27·0

We distilled 100 g. of an English lavender oil (No. 13) at atmospheric pressure through a 6 inch column packed with Dixon Gauze rings and obtained six fractions and an undistilled residue. The first small fraction which consisted almost entirely of water was discarded and the subsequent fractions and the undistilled residue were examined in the form of capillary films from 5000 cm.$^{-1}$ to 650 cm.$^{-1}$, by the "cresineol" method and by the present method.

The capillary film traces conclusively showed that the 2nd, 3rd, 4th and 5th fractions contained varying amounts of cineole whereas the quantities in fraction 6 and in the undistilled residue were extremely small since the four major cineole diagnostic peaks at 1310, 1220, 1085 and 855 cm.$^{-1}$ were barely detectable in fraction 6 and undectable in the undistilled residue. (See Fig. 4.)

The cresineol method used was that described in the British Pharmacopoeia 1953 p. 762 for the determination of cineole in rosemary oil. The apparent cineole contents are shown in Table III. By this method, the recovery was equivalent to about 27 per cent w/w of apparent cineole in the original sample, a value that was almost identical with that obtained from the examination of the unfractionated oil.

By the use of the present infra-red method, an estimation was obtained of the cineole in each fraction and the results are included in Table III. The unfractionated oil was found to contain 11·4 per cent w/w of cineole compared with 13 per cent w/w by calculation.

Estimation of Cineole Content at 1085 *cm.*$^{-1}$ *Peak*

A series of 25 specimens of lavender oils of French, English and spike origin and of different qualities were assayed for cineole by the infra-red

method and the resulting cineole contents are shown in Table IV together with the "apparent cineole" contents where these were estimated by the "cresineol" method.

TABLE IV

PERCENTAGE W/W CINEOLE FOUND IN SAMPLES OF LAVENDER OIL, COMPARED WITH SOME RESULTS USING THE "CRESINEOL" METHOD

Sample No.	Type	Cineole per cent w/w	
		by infra-red	by "cresineol"
1	French	1·0	—
2	French*	less than 1	23
3	French	2·7	—
4	French	less than 1	—
5	French	less than 1	—
6	French	3·4	—
7	French	2·7	—
8	French	2·5	21
9	English*	11·8	28
10	English*	13·0	—
11	English*	9·2	—
12	English*	11·4	—
13	English	11·4	27
14	Spike	23·7	38
15	Spike	25·9	—
16	Spike	31·5	—
17	Spike	22·8	—
18	Spike	22·0	—
19	Spike	26·0	—
20	Spike	24·1	32
21	Spike	29·6	—
22	Spike	22·4	—
23	Spike	24·2	—
24	Spike	28·5	—
25	English-special*	less than 1	—

* Authentic oils.

DISCUSSION

It has long been recognised by the expert that a "good nose" is a useful analytical tool in the grading and identification of lavender oils, but it is of little value for quantitative measurements.

The monograph on lavender oil in the British Pharmacopoeia 1953 specifies certain constants for the English, Commonwealth and French varieties, but makes no reference to the presence or absence of cineole. The British Pharmaceutical Codex 1954 on the other hand, includes the following statement . . . "Cineole occurs in some quantity in English oil, but only in traces in French oil."

The Essential Oil Sub-committee of the Society of Public Analysts[17] studied the *o*-cresol method for the determination of cineole and reported that where the oils contained appreciable amounts of alcohols, esters, aldehydes and ketones the method yielded high results and they recommended that such determinations should be reported as "apparent cineole" content by *o*-cresol. The results in Table III and IV demonstrate the unsatisfactory character of the "cresineol" method when applied to lavender oils and suggest that it is the higher boiling fractions that are mainly responsible for the errors arising from the use of this method. From the fractionation of the English oil reported above, fraction 6 and the undistilled residue, which together represented some 57 per cent of the original oil, contained little or no cineole when examined by the

infra-red method and yet appeared to contain substantial amounts of cineole when tested with *o*-cresol.

In the past the quality of lavender oils has often been based on the ester content, but the results in Table IV show that it is possible to identify also the commercial oils by their cineole content. Thus French oils contain little or no cineole, English oils contain up to 13 per cent and the spike lavender oils from 22 to 31 per cent of cineole. Some doubt has been expressed whether genuine French lavender oil contains any cineole and considerable care needs to be exercised in collecting from genuine plants, free from weeds or other contaminants before this question can be answered. Reports suggest that some French oils offered for sale have been blended with lavender oil obtained from *L. hybrida*, a hybrid derived from *L. officinalis* and *L. latifolia*, and if this were the case then one should expect to identify cineole in the blend. From a consideration of the data in Tables I and IV and the price per lb., it would appear that the cheaper grades of French oil available commercially have been blended to sell at a low price.

We have included in Tables I and IV the results of our examination of a lavender oil (No. 25) specially cultivated in England, which is similar in many respects to a French type oil. This oil, which has some 45 per cent esters appears to be akin to the Old English or Giant Blue variety described by Seager.[18]

Before any general classification on a basis of the cineole content of the official lavender oils can be attempted it will be necessary to examine a larger range of genuine oils.

Reference has already been made to the fact that a peak at 855 cm.$^{-1}$ might serve as a useful diagnostic characteristic for the presence of cineole in lavender oil, but quantitative measurements similar to those used at the 1085 cm.$^{-1}$ peak were found to be unreliable.

Acknowledgements. The authors would like to thank Messrs. Givaudan, Stafford Allen and Yardley who kindly provided the authentic samples used in this investigation.

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DISCUSSION

The paper was presented by MR. S. G. E. STEVENS.

DR. J. W. FAIRBAIRN (London) said as far as was known the French oil was derived from *Lavandula officinalis*, the spike oil from *Lavandula latifolia* and the English oil from *Lavandula intermedia*, which was a hybrid of the other two. If authentic oils behaved similarly it would be an interesting example of taxonomy supporting the analytical results.

MR. A. R. ROGERS (Brighton). Each assay was based on four replicates; what was the variation within those replicates?

DR. J. B. STENLAKE (Glasgow). How reliable did the authors regard their measurements? It seemed from the tracings that the 855 peak appeared to be free from any interference from other peaks and might give a better lead.

MR. S. G. E. STEVENS replied. He would be glad to examine authentic samples. Whether four or six replicates were taken the results, in the case of an English oil, were of the order shown in the text, page 845 where the values were between 11·2 and 11·6 per cent cineole. It was true that as the amount of cineole increased, accuracy in terms of cineole assessment was greater. The peak at 855 cm.$^{-1}$ was not applicable to oils having a low cineole content (Fig. 4) being swamped by extraneous absorption in this area by other components of the lavender oils of French type.

THE VITAMIN B_{12} POTENCY OF PHARMACEUTICAL (INCLUDING DIETETIC) PRODUCTS ESTIMATED BY THE OCHROMONAS METHOD

BY F. WOKES AND M. H. WOOLLAM

From the Ovaltine Research Laboratories, King's Langley, Herts.

Received July 5, 1957

The vitamin B_{12} potencies of a number of pharmaceutical products were estimated microbiologically using *Ochromonas malhamensis*. The low B_{12} contents found in 9 samples of dried milk, compared with the contents of fresh milk samples, indicated losses during normal drying processes. Rather smaller losses occurred during vacuum drying. In 12 samples of infants' and children's foods the vitamin B_{12} contents ranged from nil to 21 mμg./g., excluding one sample fortified with the vitamin. Storage experiments indicated no significant loss of vitamin B_{12} in dried milk, in foods based on dried milk or in non-allergenic foods based on modified cows' milk or on malt and soya fortified with the vitamin. There was, however, a marked and rapid loss of the vitamin in products which also contained ascorbic acid. This could be prevented by stabilisation of the vitamin with gelatin. Storage experiments on capsules and tablets containing vitamin B_{12} and ascorbic acid did not detect losses of vitamin B_{12} during normal storage in this country. There were marked losses of the vitamin in two brands of capsules, but not in one brand of tablets, during a year's storage in the tropics. These all contained ascorbic acid.

IN a previous communication[1] from these laboratories to the 1956 Conference describing the stability of different B vitamins in pharmaceutical products, attention was drawn to the lack of data on vitamin B_{12}. Apart from spectrophotometric data on injections presented to the 1953 Conference from these laboratories[2], only microbiological methods of varying degrees of specificity were available. However, in 1956 the Analytical Methods Committee of the Society for Analytical Chemistry published[3] a microbiological assay of vitamin B_{12} using the protozoan *Ochromonas malhamensis*, and based on Ford's method[4], with modifications evolved during an investigation by the S.A.C. Vitamin B_{12} Panel, in which our laboratories collaborated. This S.A.C. Ochromonas method has been applied to a variety of foods, feeding stuffs and other biological materials and found to be more specific than the earlier methods using *Escherichia coli* and *Lactobacillus leichmannii* which have previously been used to estimate the vitamin in pharmaceutical products.

METHODS

The S.A.C. Ochromonas method was followed but the growth of the micro-organism was measured, using a Unicam photoelectric spectrophotometer, by the increase in $E675$ mμ, which is at the peak of the chlorophyll absorption curve, as indicated by lower readings at 650 and at 700 mμ. The average coefficient of variation of a single assay was 5.

RESULTS

Dried milk. The vitamin B_{12} content of 6 different commercial samples ranged from 8 to 17 and averaged 13·3 mμg./g. and of 3 different samples of National Dried Milk ranged from 9·3 to 25 and averaged 17·1 mμg./g.

Dietetic specialities based on dried milk. The vitamin B_{12} content of 13 samples ranged from nil to 21 mμg./g. (excluding one apparently fortified

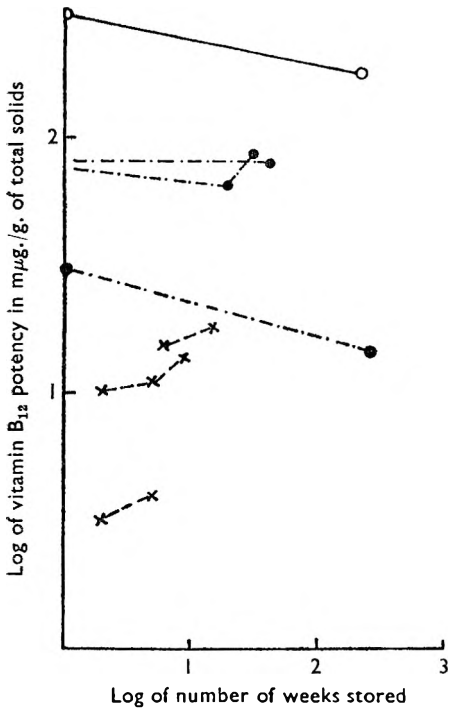

FIG. 1. Stability of vitamin B_{12} under normal storage conditions in dried milk and related foods. Results on dried milk × — — — ×, on non-allergenic foods ● — · — · — ● and on foods deriving vitamin B_{12} from milk and eggs ○——○.

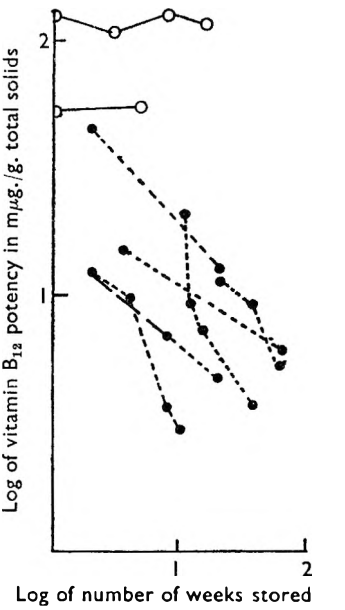

FIG. 2. Stability of vitamin B_{12} under normal storage conditions in foods based on dried milk and containing ascorbic acid. Results before addition of stabilised vitamin B_{12} and stored in closed containers ● — — ●, in open containers ● - - - ●; after addition of stabilised B_{12} ○——○.

containing 46 mμg./g.). In 5 samples also containing eggs the average B_{12} content was 15·3 mμg./g., as compared with an average content of 7·0 mμg./g. in 8 other samples.

Stability of vitamin B_{12} in dried milk and related foods. Figure 1 shows vitamin B_{12} to be reasonably stable in dried milk during storage periods up to 6 months, in dietetic specialities deriving vitamin B_{12} from milk and eggs during storage for more than 3 years and in non-allergenic foods (*vide infra*) during periods of 1 to 4 years, all at room temperature. On the other hand, as shown in Figure 2, marked rapid losses of both added and natural vitamin B_{12} occurred under normal storage conditions in

dietetic specialities containing ascorbic acid. The rate of loss was slower in an unopened container. Vitamin B_{12} treated with gelatin appears to be stable.

In Figures 1 and 2 the results are plotted on logarithmic scales both for potency and for storage periods in order to enable wide ranges to be included. As the precise ages of some of the samples were not known the positions on the time scales of some of the points are approximate only. This does not affect the marked difference between the relatively stable sources of vitamin B_{12} as shown in Figure 1 and the very unstable sources of the vitamin as shown in Figure 2.

Non-allergenic foods. These are given to infants to combat allergies to cow's milk. If the latter is modified by removal of lactose, for treatment of galactosaemia, or of some of the albumen, for treatment of asthma or eczema, considerable losses of different B vitamins may occur. The vitamin B_{12} content may fall to less than 12 or even less than 10 mμg./g. total solids. In 4 commercial samples of non-allergenic foods based on malt and soya which have proved rather more successful in the treatment of these allergies, vitamin B_{12} added at levels of 50 to 85 mμg./g. was found to be reasonably stable during prolonged storage under normal conditions.

Other pharmaceutical products. Vitamin B_{12} occurs in other pharmaceutical products such as liver powder and in various multivitamin preparations including capsules and tablets in which it is supplied either as liver extracts or liver powder or as the pure vitamin. Our tests on a few of these products indicate that the stability of the vitamin is usually satisfactory during normal shelf life in this country. During a year's storage in the tropics losses of 80–100 per cent of the claimed vitamin B_{12} content occurred in 2 brands of multivitamin capsules also containing ascorbic acid, although in tablets containing similar amounts of vitamins B_{12} and C no significant losses were detected.

Discussion

The average vitamin B_{12} content of the samples of dried milk we have examined was only 15 mμg./g. total solids. A recent survey[5] indicated average B_{12} contents of fresh milk ranging from 3 to 6·6 mμg./ml., equivalent to 24 to 50 mμg./g. total solids. Since the vitamin B_{12} in dried milk was found to be reasonably stable, the low B_{12} content of our dried milk samples did not seem likely to be due to losses during storage, but more probably to losses during drying. Some experiments carried out on vacuum drying of milk for 3–4 hours at 40–45° indicated losses of about 10 per cent, and we therefore think that greater losses might occur during drying at atmospheric pressure.

Milk does not normally contain significant amounts of ascorbic acid. If this be added in sufficient quantities to meet human requirements, both natural and added vitamin B_{12} may be rendered unstable and disappear rapidly during storage under normal conditions. Since ascorbic acid reacts at a suitable pH (4–6) with hydroxocobalamin but not with cyanocobalamin[2], the destructive action of the ascorbic acid might be prevented or at least retarded by ensuring that the vitamin B_{12} was all present as

cyanocobalamin. This could be done by combining the cyanocobalamin with a stabilising agent such as protein. We have found gelatin satisfactory for this purpose. It is now being tried for the stabilisation of vitamin B_{12} in multivitamin capsules and tablets containing ascorbic acid. Whilst we encountered losses of vitamin B_{12} in the capsules, though not in the tablets, during storage in the tropics, Campbell and McLeod[6] encountered losses in the tablets but not in the capsules, during storage in Canada.

We are indebted to Mrs. Susan Davis and Miss Jacqueline Lloyd for assistance, also to Messrs. R. P. Scherer Ltd. for a supply of stabilised vitamin B_{12}.

Footnote

After this paper had been prepared for the press Dr. J. E. Ford kindly showed us the typescript of a paper from the National Institute for Research in Dairying, and the Dairy Department, University of Reading, by H. Chapman, J. E. Ford, S. K. Kon, S. Y. Thompson and S. J. Rowland, also E. L. Crossley and J. Rothwell, describing results (which are being published in the *Journal for Dairy Research*) in substantial agreement with our findings on vitamin B_{12} in milk. They indicate vitamin B_{12} contents of 13 to 28 mμg./g. in National Dried Milk and marked losses of the vitamin in the manufacture of condensed and evaporated milk.

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DISCUSSION

The paper was presented by Dr. F. Wokes.

Mr. N. J. Van Abbé (Loughborough). Was roller-dried or spray-dried milk used?

Mr. K. A. Lees (London). Had the results been checked by some comparative technique?

Dr. G. E. Foster (Dartford). What explanation could be offered for the apparent instability of vitamin B_{12} in the presence of ascorbic acid? Why did capsules show loss of vitamin B_{12} whereas tablets did not?

Miss A. E. Robinson (London). It should be possible by differential absorption techniques to determine the relative proportions of scatter and absorption.

Mr. H. J. Bragg (Folkestone). How many determinations were made over the period, and were the capsules and tablets stored in the original containers and accelerated tests carried out?

MR. G. R. WILKINSON (London). The logarithmic scale (Figure 1) compressed what were wide differences. Had the authors any explanation why some samples appeared to increase in vitamin B_{12} content?

MR. K. A. LEES (London). The authors referred to a discrepancy between their results and those of Canadian workers, had the Canadian workers used the same type of capsules?

MR. D. E. WILKS (Bedford). Was the fresh milk, milk straight from the cow or pasteurised; were there any details available about the vitamin B_{12} content of pasteurised milk?

DR. F. WOKES replied. He was not aware how the milk had been dried. They were commercial samples. The limits of reliability were indicated and all the points obtained were in the curves. A Spekker absorptiometer had also been used, and good agreement was found between the two methods. The reaction between ascorbic acid and vitamin B_{12} depended on cyanocobalamin being first converted to hydroxycobalamin. The composition of the capsules and tablets varied and he did not know why deterioration occurred only in the capsules. Opacity methods were accepted methods for measuring growth; if known amounts were taken, and calibrations made, the response was proportional to the log of the concentration of the vitamin. There was a linear response curve. Storage experiments were made on the capsules in the original bottles and packets. An accelerated storage experiment had been made, and it had been found that at above 37° the vitamin became much less stable; also if too high a temperature were used the physical nature of the gelatin altered. If the moisture content were low the gelatin became brittle. In tropical conditions the reverse might happen, and the shell of the capsule might become more permeable. The logarithmic scale was chosen for the graphs in order to include data over a wide range in one diagram. The variations were puzzling. In two or three points in Figure 1 there was an apparent increase. In some cases the rise was rather large for experimental error. Some samples were available five or six years ago, and they were made when crystalline vitamin B_{12} first became available. Of the capsules examined, some were oil-filled and others dry-filled. Tablets which were perfectly dry had also been examined. The samples were of fresh milk. Pasteurised milk contained little vitamin B_{12}.

THE PREPARATION AND ANTIFUNGAL ACTIVITY OF SOME SALICYLIC ACID DERIVATIVES

By L. V. Coates, D. J. Drain, K. A. Kerridge, F. June Macrae and K. Tattersall

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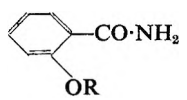
Received June 17, 1957

A series of 2-alkoxy derivatives of benzamide, halogenated benzamides, 3-naphthamide, and *N*-substituted benzamides has been prepared and tested *in vitro* as potential antifungal agents. 2-*n*-Amyloxybenzamide, the most active of the derivatives studied, is a slow-acting fungicide of greater fungistatic potency than undecylenic acid, *N*-*n*-butyl-3-phenylsalicylamide, salicylanilide, Nystatin, phenylmercuric acetate, sodium ethylmercurithiosalicylate and 8-hydroxyquinoline.

During routine screening of compounds for chemotherapeutic activity it was observed that 2-*n*-amyloxybenzamide (I,R=$C_5H_{11}{}^n$) and 2-*n*-hexyloxybenzamide (I,R=$C_6H_{13}{}^n$), originally prepared in the course of a search for new analgesics related to salicylamide[1], had fungistatic properties against certain dermatophytes. Many compounds have since been prepared and tested,



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and those more closely related to the active substances are here reported. 2-*n*-Amyloxybenzamide has been found to have *in vitro* fungistatic activity comparable with substances commonly used in the treatment of dermatomycoses.

Experimental

Chemical

Alkyl ethers of salicylamide and its derivatives. The alkyl ethers of salicylamide and of its nuclear and *N*-substituted derivatives were prepared by treatment of the appropriate salicylamide (1 mole) with an alkyl halide (1 mole) in boiling ethanolic sodium ethoxide (1 mole). After the mixture had been refluxed for 24 hours, the ethanol was removed by distillation to give a residue which was washed with 2N sodium hydroxide. The alkyl ether was collected by filtration or ether extraction and purified by either crystallisation or distillation.

N-*Substituted salicylamides.* Mono-substituted amides were prepared by refluxing together methyl salicylate (1 mole) with the corresponding primary amine (2 mole) for 18 hours. Di-substituted amides were prepared by heating phenyl salicylate (1 mole) with the appropriate secondary amine (2 mole) at 140° for 6 hours. The volatile substances were removed under reduced pressure and the residue either recrystallised or distilled *in vacuo* to give the *N*-substituted salicylamide.

Carboxyalkyl ethers. The ethyl esters of the relevant bromo-acids were used to alkylate salicylamide as described above. The corresponding acids were obtained by hydrolysis of the esters with aqueous 2N sodium hydroxide.

3:5-*Diiodosalicylamide.* Hydrogen peroxide (18 ml. of a 30 per cent solution) was slowly added to a mixture of salicylamide (16 g.), iodine (30 g.), sulphuric acid (8 ml.), and ethanol (80 ml.) at 60°. The product that crystallised out on cooling the reaction mixture was collected by filtration and recrystallised (cf. method of Jurd[2]).

4:5-*Dichlorosalicylamide.* Methyl 4:5-dichlorsalicylate (20 g.), ethanol (50 ml.), and aqueous ammonia (100 ml., d. 0·88) were allowed to stand at room temperature for 7 days. The amide obtained on evaporating the solvent from the reaction mixture was recrystallised from ethanol.

N-n-*Butyl*-3-*phenyl salicylamide.* *N*-*n*-Butyl-3-phenylsalicylamide was prepared by two methods: (*a*) from methyl 3-phenylsalicylate (b.p. 144 to 148°/1 mm.) and *n*-butylamine by the method described by Jules and others[3]; and (*b*) from phenyl 3-phenylsalicylate (m.p. 95°) by heating with *n*-butylamine at 150 to 160° for five hours. The compounds obtained from methods (*a*) and (*b*) both recrystallised from aqueous methanol or light petroleum to give needles, m.p. and mixed m.p. 49·5 to 50·5°. Found: C, 75·7; H, 7·2; N, 5·5. Calc. for $C_{17}H_{19}NO_2$ C, 75·8; H, 7·1; N, 5·2 per cent. Jules records m.p. 71 to 72° for this compound. Comparison with a sample m.p. 71° to 72° kindly supplied by Dr. J. A. Faust, Director of Organic Research, Sahyun Laboratories, gave no depression of melting point on admixture. The product m.p. 49·5° to 50·5°, after melting and seeding with a crystal of the higher melting form had m.p. 71° to 72°.

The pertinent analytical details for new compounds are shown in Table I.

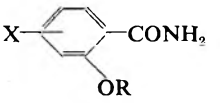
Microbiological

Fungistatic Test. The organisms and media used are shown in Table II. Activity was assayed by the method of Schamberg and Kolmer[4] with the modified inoculation technique devised by Archibald and Reiss[5].

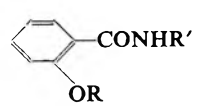
Compounds were dissolved in a 'self-sterilising' solvent consisting of ethanol (70 vols), propylene glycol (5 vols), and distilled water (25 vols), to give a concentration of 1·0 per cent w/v. These solutions were then serially diluted in Sabouraud broth and aliquots transferred to molten malt agar to give final concentrations within the range 50 — 1·5 μg./ml. The highest concentration of ethanol incorporated in the dilution plates was 0·35 per cent v/v which had previously been shown to exert no fungistatic effect. Test plates were inoculated by implanting a small portion of mycelium not exceeding 2 mg. in weight, from a 10 to 17 day old stock culture of the fungus under test. With *M. audouini* and *T. tonsurans* it was necessary to lift the mycelial mat from the agar plate before cutting into small portions. The plates were incubated for 7 days at 25 to 28°,

TABLE I

SUBSTITUTED SALICYLAMIDES



X	R	Physical form	Melting point °C.	Empirical formula	Required per cent			Found per cent		
					C	H	N	C	H	N
H	Bu^{sec}	Prisms[a]	69·5–70·5	$C_{11}H_{15}NO_2$	68·4	7·8	7·3	68·4	7·7	7·4
H	$C_5H_{11}{}^i$	Needles[b]	89	$C_{12}H_{17}NO_2$	69·5	8·3	6·8	69·7	8·2	—
H	$-CH(CO_2H)CH_3$	Prisms[c]	184–185	$C_{10}H_{11}NO_4$	57·4	5·3	6·7	57·5	5·2	6·1
H	$-CH(CO_2H)C_2H_5$	Prisms[c]	179–180	$C_{11}H_{13}NO_4$	59·2	5·8	6·3	59·0	6·0	6·4
H	$-CH(CO_2H)C_3H_7{}^n$	Prisms[c]	156–157	$C_{12}H_{15}NO_4$	60·8	6·3	5·9	60·4	6·3	5·9
5–Cl	Bu^n	Plates[a]	98·5	$C_{11}H_{14}ClNO_2$	58·0	6·2	6·2	58·1	6·1	6·3
5–Cl	$C_5H_{11}{}^n$	Plates[a]	91	$C_{12}H_{16}ClNO_2$	59·6	6·7	5·8	59·4	6·4	5·7
5–Cl	$C_6H_{13}{}^n$	Plates[a]	94	$C_{13}H_{18}ClNO_2$	60·9	7·1	—	61·3	7·2	—
5–Br	$C_6H_{13}{}^n$	Plates[a]	103	$C_{13}H_{18}BrNO_2$	52·0	6·0	4·7	51·9	6·3	4·5
3:5–I_2	H	Needles[c]	199	$C_7H_5I_2NO_2$	21·6	1·3	3·6	22·1	1·5	3·6
3:5–I_2	$C_5H_{11}{}^n$	Needles[c]	147	$C_{12}H_{15}I_2NO_2$	31·4	3·3	3·1	31·5	3·4	2·9
3:5–I_2	$C_6H_{13}{}^n$	Needles[c]	147·5–148	$C_{13}H_{17}I_2NO_2$	33·0	3·6	3·0	33·0	3·6	2·9
4:5–Cl_2	H	Needles[c]	230	$C_7H_5Cl_2NO_2$	40·8	2·4	6·8	40·6	2·6	7·0
4:5–Cl_2	Bu^n	Clusters[a]	137	$C_{11}H_{13}Cl_2NO_2$	50·4	5·0	5·3	50·7	5·2	5·7
4:5–Cl_2	$C_5H_{11}{}^n$	Needles[a]	138	$C_{12}H_{15}Cl_2NO_2$	52·2	5·5	5·1	52·5	5·3	5·1
Benz·*d*	Bu^n	Prisms[a]	144	$C_{15}H_{17}NO_2$	74·1	7·0	5·8	73·8	7·0	6·0
Benz·*d*	$C_5H_{11}{}^n$	Prisms[a]	132	$C_{16}H_{19}NO_2$	74·7	7·4	5·4	74·7	7·3	5·6
Benz·*d*	$C_6H_{13}{}^n$	Needles[d]	123	$C_{17}H_{21}NO_2$	75·3	7·7	5·2	75·2	7·5	5·0



R	R′	Physical form	Melting point	Empirical formula	Required per cent			Found per cent		
					C	H	N	C	H	N
H	$C_6H_{13}{}^n$	Prisms[a]	42·5–43·5	$C_{13}H_{19}NO_2$	70·5	8·7	6·3	70·2	8·8	6·4
Bu^n	Bu^n	Matted needles[a]	40–41	$C_{15}H_{23}NO_2$	72·3	9·3	5·6	72·1	9·4	5·9
$C_5H_{11}{}^n$	Bu^n	Matted needles[a]	39–40	$C_{16}H_{25}NO_2$	73·0	9·6	5·3	73·0	9·8	5·3
$C_5H_{11}{}^n$	$C_6H_{13}{}^n$	Colourless oil[e]	—	$C_{18}H_{29}NO_2$	74·2	10·0	4·8	74·4	10·6	5·0
$C_5H_{11}{}^n$	Ph	Matted needles[b]	44–45	$C_{18}H_{21}NO_2$	76·3	7·5	5·0	76·3	7·6	5·0
$C_6H_{13}{}^n$	Bu^n	Matted needles[a]	36–37	$C_{17}H_{27}NO_2$	73·6	9·8	5·0	73·3	9·7	4·7
$C_6H_{13}{}^n$	Ph	Plates[a]	51	$C_{19}H_{23}NO_2$	76·7	7·8	—	76·3	7·9	—

Analyses by Drs. Weiler and Strauss. M.ps. uncorrected.

[a], Recrystallised from light petroleum; [b], recrystallised from aqueous ethanol; [c], Recrystallised from ethanol; [d], recrystallised from ethyl acetate; [e] (b.p. 172–174°/0·35 mm.).

and the inhibitory level of a compound was taken as the highest dilution which completely prevented fungal development.

An anti-fungal index, being the sum of the minimum inhibitory concentrations divided by the number of dermatophytes tested is incorporated in Tables III and IV.

Antimicrobial activity against yeasts and bacteria was determined by serial dilution of the compounds in the appropriate liquid medium, followed by inoculation and a suitable incubation.

Fungicidal Tests. The method used was similar to that of Golden and Oster[6], except that exposure times were prolonged and the rinsing procedure modified.

TABLE II

Microorganisms	Test medium
Trichophyton mentagrophytes, 6 strains (*a*); *T. rubrum* (*a*); *T. concentricum* (*a*); *Microsporum audouini* (*a*); *T. tonsurans* (*a*); *Sporotrichum schenkii* (*a*); *Epidermophyton floccosum* (*a*); *Allescheria boydii* (*b*); *Hormodendron pedrosoi* (*b*).	Malt agar*.
Rhizopus nigricans (*c*); *Aspergillus niger* (c); *Penicillium notatum* (*c*); *Mucor erectus* (c); *M. hiemalis* (*c*).	Malt agar*.
Candida albicans (*a*); *Saccharomyces cerevisiae* (*c*).	Sabouraud Maltose Broth (Difco Ltd.).
Staphylococcus aureus (*d*); *Pseudomonas aeruginosa* (*d*); *Escherichia coli* (*d*); *Bacillus subtilis* (*d*).	Nutrient Broth No. 2 (Oxo Ltd.).
Streptococcus pyogenes (*d*); *Diplococcus pneumoniae* (*d*); *Corynebacterium diphtheriae* (*d*); *Actinomyces bovis* (*d*); *Pfeifferella mallei* (*d*).	Todd Hewitt Broth**.
Mycobacterium tuberculosis H37Rv (*d*); *M. phlei* (*d*).	Proskauer and Beck medium.

* Malt extract B.P. (Muntona Ltd.), 40 g.; Yeast extract (Oxo Ltd.), 5 g.; New Zealand Agar (Oxo Ltd.) 15 g.; Distilled water to 1 litre. The pH adjusted to 5·6–5·8 with lactic acid.

(*a*) Supplied through the courtesy of Dr. J. Walker, Director of Medical Mycology, London School of Tropical Medicine and Hygiene.

(*b*) Generously given by Col. R. Lewis, M.C., Director, 5280th Clinical Laboratory, U.S.A.F.

(*c*) Own isolate.

(*d*) National Collection of Type Cultures.

1 sq. cm. plaques, cut from a 10 day culture of *T. mentagrophytes* on an agar plate, were immersed in sterile horse serum for 15 seconds and then transferred to 5 ml. amounts of dilutions of the compounds in Dubos broth for periods of 1 hour, 24 hours and 5 days. After exposure the fungal plaques were immersed in 30 per cent v/v aqueous acetone for 10 minutes and transferred to 20 ml. of Sabouraud broth. Finally, the plaques were placed aseptically onto malt agar plates which were incubated for 14 days at 25 to 28°.

The results of these tests are shown in Table V.

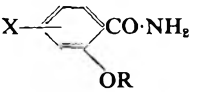
Results and Discussion

A few only of the salicylamide derivatives prepared exhibit high fungistatic activity (Table III). Consideration of the activities of 2-alkoxybenzamides indicates that there is a considerable degree of structural specificity in the activity of this group of compounds. Alkylation of the hydroxyl group of salicylamide by C_3–C_7 alkyl radicals confers activity against the dermatophytes. Maximal antifungal activity occurs with the

n-amyloxy and *n*-hexyloxy derivatives, the former proving significantly more active when the incubation period was extended to 14 days. This fungistatic effect is greatest when the alkyl substituent is unbranched and is no longer apparent with the *n*-octyloxy-derivative. The phenoxyacetic acid derivatives of this series although related to known herbicidal and fungicidal agents[7,8] do not inhibit growth of dermatophytes under the reported test conditions. Activity was not increased by the preparation of either nuclear substituted halogen derivatives or naphthamide

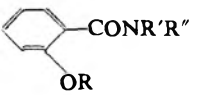
TABLE III

FUNGISTATIC ACTIVITIES OF SUBSTITUTED SALICYLAMIDES



X	R	Minimum inhibitory concentration μg./ml. †T.m.	T.t.	T.c.	T.r.	E.f.	M.a.	Anti-fungal index
H	H	>50	50	50	50	50	50	>50
H	Me^{19}	>50	—	—	50	>50	—	>50
H	Et^{12}	>50	—	—	50	>50	—	>50
H	Pr^{n1}	>50	50	25	25	25	6	>30
H	Pr^{i13}	>50	—	—	50	>50	—	>50
H	Bu^{n1}	12	25	12	12	12	6	13
H	Bu^{sec}*	>50	>50	>50	>50	>50	>50	>50
H	$C_5H_{11}{}^{n1}$	6	3	3	3	1	1·5	3
H	$C_5H_{11}{}^{i}$*	25	12	6	6	6	6	10
H	$C_6H_{13}{}^{n1}$	12	3	3	3	3	1·5	4
H	$C_7H_{15}{}^{n1}$	50	25	12	12	25	25	25
H	$C_8H_{17}{}^{n1}$; $-(CH_2)_3\cdot CH(CH_3)\cdot CH_2\cdot C(CH_3)_3$[1]; $C_{12}H_{25}{}^{n1}$; $C_{14}H_{29}{}^{n1}$; $-CH_2\cdot CO_2H^{14}$; $-(CH_2)_2\cdot CO_2H^{11}$; $-CH(CO_2H)\cdot CH_2$*; $-CH(CO_2H)\cdot C_6H_5$*; $-CH(CO_2H)\cdot C_6H_7{}^{n}$*; $-(CH_2)_2\cdot N(C_2H_5)_2\cdot HCl$.[21] All compounds have M.I.C. >50 against all fungi tested.							
5–Cl	H[16]	50	50	50	50	50	25	46
5–Cl	Bu^{n}*	50	25	50	50	50	25	41
5–Cl	$C_5H_{11}{}^{n}$*	>50	>50	>50	>50	>50	>12	>43
5–Cl	$C_6H_{13}{}^{n}$*	>50	>50	>50	>50	>50	25	>46
5–Br	$C_6H_{13}{}^{n}$*	>50	>50	>50	>50	>50	25	>46
3:5–Cl_2	H[16]	50	25	50	50	50	12	39
3:5–I_2	H*	50	25	50	25	25	6	30
3:5–I_2	$C_5H_{11}{}^{n}$*	>50	>50	>50	>50	>50	>50	>50
3:5–I_2	$C_6H_{13}{}^{n}$*	50	50	50	50	50	50	50
4:5–Cl_2	H*	50	25	25	25	25	6	26
4:5–Cl_2	Bu^{n}*	>50	50	50	50	50	50	>50
4:5–Cl_2	$C_5H_{11}{}^{n}$*	>50	>50	50	50	>50	50	>50
Benz·*d*	H[16]	>50	>50	>50	>50	>50	>50	>50
Benz·*d*	Bu^{n}*	50	50	50	50	50	50	50
Benz·*d*	$C_5H_{11}{}^{n}$*; $C_6H_{13}{}^{n}$*. M.I.C. >50 against all fungi tested.							

TABLE III—*continued*



R	R'	R"	Minimum inhibitory concentration µg./ml. T.m.†	T.t.	T.c.	T.r.	E.f.	M.a.	Anti-fungal index
H	H	Me^{17}	>50	—	—	>50	>50	—	>50
H	Me	Me^{18}	>50	>50	>50	>50	>50	>50	>50
H	Et	Et^{19}	>50	—	—	>50	>50	—	>50
H	H	Bu^{n20}	50	50	50	50	50	50	50
H	H	$C_6H_{13}{}^{n}$*	25	25	25	25	12	25	23
Bu^n	H	Bu^{n}*	25	12	12	12	12	12	14
$C_5H_{11}{}^{n}$	H	Bu^{n}*	25	12	12	12	12	12	14
$C_6H_{11}{}^{n}$	H	$C_6H_{13}{}^{n}$*	>50	>50	>50	>50	>50	>50	>50
$C_6H_{11}{}^{n}$	H	Ph*	>50	>50	>50	>50	>50	>50	>50
$C_6H_{13}{}^{n}$	H	Bu^{n}*	25	25	50	25	25	25	29
$C_6H_{13}{}^{n}$	H	Ph*	50	>50	>50	50	50	25	>46

* See Table I.
† T.m. = *T. mentagrophytes*, T.t. = *T. tonsurans*, T.c. = *T. concentricum*, T.r. = *T. rubrum*, E.f. = *E. floccosum*, M.a. = *M. audouini*.

analogues. All these latter derivatives were generally inactive at a concentration of 50 µg./ml., although a few halogenated compounds inhibited the growth of one organism, *M. audouini*, at low concentrations. This loss of potency on halogenation is in contrast to the increase in activity shown by the halogenated derivatives of phenol[9], but compares with the decrease in fungitoxicity of halogen substituted 2-phenylphenol derivatives[9]. The lack of fungistatic properties of the 3-alkoxy-2-naphthamides is parallelled by the recently reported[10] low antibacterial activities of naphthol derivatives.

TABLE IV

COMPARISON OF ANTIFUNGAL ACTIVITY OF 2-*n*-AMYLOXY BENZAMIDE WITH KNOWN FUNGISTATIC AGENTS

Compound	Minimum inhibitory concentration T.m.*	T.t.	T.c.	T.r.	E.f.	M.a.	Antifungal index
2-*n*-Amyloxybenzamide	6	3	3	3	1·5	1·5	3
N-*n*-Butyl 3-phenylsalicylamide	>50	25	25	6	>50	3	>26
Salicylanilide	12	6	6	3	6	1·5	6
8-Hydroxyquinoline	15	4	8	8	8	2	7
Undecylenic acid	>50	50	25	50	25	12	>35
Phenylmercuric acetate	25	1·5	25	6	6	6	12
Sodium ethylmercurithiosalicylate ..	12	1·5	12	6	1·5	1·5	6
Nystatin	15	4	8	15	4	4	8

* See footnote to Table III for definitions.

ANTIFUNGAL ACTIVITY OF SALICYLIC ACID DERIVATIVES

The preceding results prompted the preparation of a number of *N*-substituted and *ON*-disubstituted salicylamides, none of which showed activity comparable with that of 2-*n*-amyloxybenzamide.

The fungistatic activity of 2-*n*-amyloxybenzamide was compared with that of other compounds of known activity (Table IV). The 2-*n*-amyloxy derivative was found to be more active than *N*-*n*-butyl-3-phenylsalicylamide, undecylenic acid, salicylanilide, Nystatin, phenylmercuric acetate, sodium ethylmercurithiosalicylate, and 8-hydroxyquinoline.

TABLE V

COMPARATIVE FUNGICIDAL ACTIVITIES AGAINST *T. mentagrophytes*

Compound	Exposure period	Dilution μg./ml. 1000	100
N-Butyl-3-phenyl salicylamide ..	1 hr.	+	+
	24 hrs.	+	+
	5 days	−	+
2-*n*-Amyloxy benzamide	1 hr.	+	+
	24 hrs.	−	+
	5 days	−	+
Salicylanilide	1 hr.	+	+
	24 hrs.	+	+
	5 days	−	+

+, −, = Growth, or no growth on sub-culture at 14 days.

It seemed logical in considering the use of 2-*n*-amyloxybenzamide for the chemotherapy of dermatophyte infections to compare the fungicidal activity of this compound with other known antifungal agents. Preliminary results (Table V) indicate that it is a slow-acting fungicide comparable with salicylanilide and *N*-*n*-butyl-3-phenylsalicylamide.

Amyloxybenzamide inhibited our test range of fungi, at 25 to 28°, at the following concentrations in μg./ml. *Hormodrendron pedrosoi*, 25, *Sporotrichum schenkii*, *Allescheria boydii*, *Rhizopus nigricans*, *Aspergillus niger*, *Penicillium notatum* and *Mucor hiemalis*, 100, and *Mucor erectus* at >100. Of the yeasts, *Candida albicans* at 37° was inhibited at 62 and *Saccharomyces cerevisiae* at 25 to 28°, at 250. Of bacteria, at 37°, *Mycobacterium tuberculosis* H37Rv was inhibited at 31, *Mycobacterium phlei* at 125, *Corynebacterium diphtheriae*, *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Diplococcus pneumoniae* and *Pfeifferella mallei* at 250, *Pseudomonas aeruginosa* at 500, and *Actinomyces bovis* and *Escherichia coli* at >500.

2-*n*-Amyloxybenzamide is an antifungal agent with specificity for dermatophytes commonly responsible for mycotic infections in man. Animal tests have been commenced and these, together with further pharmacological investigations will form the subject of a later communication.

Acknowledgements. The authors wish to thank Mrs. S. Harvey, Mr. D. G. May and Mr. R. Shadbolt for experimental assistance.

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DISCUSSION

The paper was presented by MR. D. J. DRAIN.

The CHAIRMAN. The authors had not given any solubility data for the most active compound, and data should also be given about partition between non-aqueous and aqueous media.

DR. J. C. PARKINSON (Brighton). Did the fungistatic values alter with temperature? Had any tests been made at 37°?

MR. G. R. WILKINSON (London) asked about the toxicity of the compounds.

DR. G. E. FOSTER (Dartford) enquired about the mechanism of action of the substances.

MR. G. SYKES (Nottingham). It was common amongst the newer antifungal agents to be rather more specific and more active against pathogenic fungi than saprophytic types. What was the virtue of carrying out a fungicidal test at an interval of five days? Was fungistatic or fungicidal activity desirable for clinical use?

MR. T. D. WHITTET (London). There would be little difficulty in obtaining clinical material in man.

DR. B. K. MARTIN (Slough). Lack of success in the treatment of fungal infection was largely due to the use of fungistatic rather than fungicidal agents.

DR. J. B. STENLAKE (Glasgow). Had the solubilities been considered in relation to the size of the alkyl group, because as this was increased there was a steady drop in solubility and then a sudden drop.

MR. D. J. DRAIN replied. The compounds had not been tested at 37°, but the test method was rigorous. The acute LD50's for a number of

alkyl ethers of salicylamide both by oral and interperitoneal routes had been given by the authors in the *Journal of Pharmacy and Pharmacology* in 1952. No sensitising properties of the substances was detected when lotions were applied to animal or human skin. The test substances interfered with the metabolism of the organism. The pharmaceutical preparation of an antifungal substance was as important as the inherent activity of the substance itself, and they had concluded that clinical trials would be necessary. Bushby and Stewart in 1949 were unable to find any correlation between fungicidal activity and the activity in their animals. A marked difference was found in the *in vivo* effects depending on the base used. There was little correlation between relative fungistatic or fungicidal potencies, and there were no clinical results on their substances as yet. They considered that a five-day fungicidal test was different from a fungistatic test. The dissociation constants of the amides had not been determined, but it was certain that they would be very weak bases. The solubility of the amyloxybenzamides in aqueous solution had not yet been determined, but it would probably be about 50 to 100 μg./ml. In alcohol, chloroform and benzene the compounds were readily soluble, and the partition ratios between water and a fatty solvent would be in favour of the organic solvent. The problem of solubilities was being investigated. A sudden drop in solubility probably occurred between the butyl and amyl members of the series.

THE RESISTANCES OF VEGETATIVE BACTERIA TO MOIST HEAT

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Washed suspensions of *Escherichia coli* required much shorter exposure to heating at 57° for sterilisation than unwashed suspensions, the greater resistance of which was due to materials carried over from the culture medium. The heat resistances of washed suspensions resuspended in solutions of electrolytes differed from those in non-electrolytes. The mode of protection is discussed in terms of the charge and permeability of the bacterial surface. Varying methods of culture and periods of growth markedly affected the heat resistances of washed suspensions. Aeration, exclusion of air, presence or absence of carbon dioxide and the surface moisture of solid media all influenced the heat resistance. Washed suspensions of *E. coli*, on storage, retained heat resistance poorly, in contrast to retention of viability and resistance to bactericides and bacteriostats.

Killing of bacteria by moist heat causes leakage from the cells of substances which will support growth of unexposed *E. coli*, but which will not support growth of survivors. Growth is also supported by killed cells themselves as distinct from substances leaking from the cells.

The killing of vegetative bacteria by exposure to moist heat and the alteration of heat resistance have received much attention since the early investigations of Chick[1]. The investigations of death rates of *Escherichia coli* at elevated temperatures carried out by Jordan, Jacobs and Davies[2] used constant food supply and aeration[3] and give little information of the behaviour if these conditions are not met. This paper presents results with washed suspensions of *E. coli* approximating to a broth culture in the stationary phase of growth.

Experimental

Bacterial suspensions. The organism was *Escherichia coli* Type I, a 44° positive laboratory strain, formerly N.C.T.C.5933. Suspensions were prepared from slope cultures[4,5], washed by three times centrifuging at 3000 r.p.m. for 25 minutes, resuspended in standardised volumes of sterile water, and finally adjusted to a density of 2×10^9/ml.

Materials. All chemical substances used were of A.R. purity with the exception of calcium chloride hydrate and glucose of Pharmacopoeial purity, "Oxoid" peptone, and Davis agar and gelatin. Nutrient media contained 1 per cent peptone, 0·5 per cent sodium chloride and were adjusted to pH 7·0. Solid media contained in addition 2 per cent Davis agar.

Methods. The suspension in 5 ml. volumes was sealed in sterile 5 ml. ampoules, weighted with small lead weights and immersed in a waterbath maintained at the desired temperature within limits of $\pm 0{\cdot}1°$. After exposure, an ampoule was removed, cooled at room temperature for 15 minutes, thoroughly shaken, opened, and a sample diluted by a series of

tenfold dilutions with sterile water. Drops of these dilutions were placed with the Cook and Yousef[6] pipette on the surfaces of overdried plates to obtain viable counts of surviving organisms[7]. Plates were incubated at 37° for 12–15 hours and colonies counted from between 10 and 20 replicate platings. The following factors were found to be important:

The length of time the ampoules were allowed to stand at room temperature before dilution; a decrease in the numbers of surviving organisms was found if the ampoules were allowed to stand for more than 30 minutes. The count was approximately halved when suspensions heated at 57° for 1 hour were allowed to stand for 2½ hours.

TABLE I

NUMBERS OF SURVIVING VIABLE *E. coli* AFTER HEATING A WASHED SUSPENSION CONTAINING 2×10^9/ML. AT DIFFERENT TEMPERATURES AND TIMES

Time (minutes)	Viable count/ml. suspension			
	Temperature (° C.)			
	65	63·5	59	57
1	$4{\cdot}86 \times 10^7$			
3	$8{\cdot}11 \times 10^3$			
4	$7{\cdot}20 \times 10^1$	$1{\cdot}19 \times 10^6$		
5		$1{\cdot}04 \times 10^4$	$4{\cdot}91 \times 10^6$	
6		$2{\cdot}34 \times 10^3$		
10			$2{\cdot}38 \times 10^6$	
20			$7{\cdot}64 \times 10^5$	$1{\cdot}52 \times 10^9$
30			$2{\cdot}10 \times 10^2$	$6{\cdot}66 \times 10^8$
40				$1{\cdot}89 \times 10^8$
60				$1{\cdot}21 \times 10^6$
80				$9{\cdot}00 \times 10^1$

Delay in plating out the dilutions reduced the viable count.

Plates maintained at room temperature until the end of a series of experiments gave a lower count than those transferred to the incubator immediately after inoculation. Similar effects were not seen with unheated control suspensions. Organisms which survived the heat treatment may fail to reproduce unless rapidly transferred to an appropriate medium at a suitable growth temperature.

Statistical examination showed that the heterogeneity of colony counts among replicate platings generally increased as the mortality from heat treatment increased. Values of χ^2 corresponding to $P = 0{\cdot}05$ or over were obtained except where the number of colonies of survivors from the undiluted suspension was small.

To select a suitable temperature for future experiments, the numbers of viable organisms were counted after exposure of washed suspensions to varying temperatures for suitable times. Some results are listed in Table I. A temperature of 57° gave a substantial reduction of viability after 1 hour, and seemed convenient. Numbers of surviving organisms are shown plotted logarithmically against duration of heating for washed and unwashed suspensions at 57° in Figure 1. The curves are of similar sigmoid shape, the differences between the resistance of the suspensions increasing with exposure.

The lower resistance of the washed suspension may be due either to the removal of material derived from the medium (peptone, agar, salts) or to the removal of metabolites which exert a protective effect. When washed cells were resuspended in the condensate washed from several uninoculated agar slopes, the numbers of survivors approximated to those found with unwashed suspensions (Table II). Thus the first of the alternatives was likely to be true. Also increase in temperature during centrifugation and mechanical effects in precipitation and resuspension of the organisms were unlikely to affect heat resistance. Washed cells were next suspended in dilutions of a peptone water, 1 per cent peptone, 0·5 per cent sodium chloride, pH 7·0, when even 1 : 100 dilutions increased heat resistance. Finally, suspensions with varying concentrations of peptone were employed, without added electrolyte, in the suspending medium. The counts after heating at 57° for 1 hour are shown in Table III.

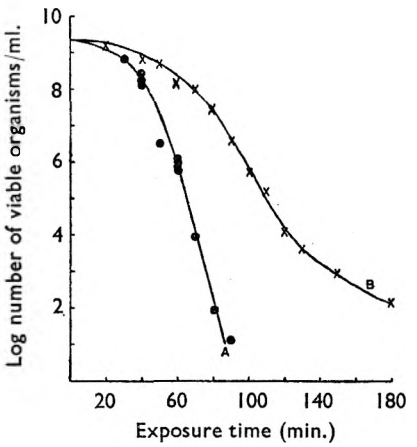

FIG. 1. The loss of viability of *Escherichia coli* when heated at 57°. A, washed suspensions; B, unwashed suspensions.

Since these peptone solutions were more acid than those previously used, washed cells were resuspended in 0·5 per cent peptone adjusted to 3 different hydrogen ion concentrations. The counts per ml. after heating for 1 hour were as follows: pH 5·5, $2{\cdot}44 \times 10^8$; pH 6·8, $2{\cdot}36 \times 10^8$; pH 8·6, $1{\cdot}28 \times 10^7$. It appears that small variations from neutrality influence the results only on the alkaline side.

TABLE II

EFFECT OF ADDITION OF SLOPE CONDENSATE TO WASHED SUSPENSION OF *E. coli* ON RESISTANCE TO HEATING AT 57°

Exposure time (min.)	Viable count/ml. suspension		
	Without slope condensate	With slope condensate	Unwashed suspension
50	$1{\cdot}77 \times 10^7$	$3{\cdot}56 \times 10^8$	$4{\cdot}81 \times 10^8$
80	$3{\cdot}00 \times 10^2$	$3{\cdot}62 \times 10^7$	$2{\cdot}26 \times 10^7$

In view of the common belief, cited by McCulloch[8] and Fay[9], that proteins and other colloidal materials are able to protect bacteria against killing by moist heat, solutions of agar and gelatin were examined. Both of these substances in low concentration were found to increase the heat resistance of washed cells (Table IV). If these substances and peptone exerted a protective action by virtue of their colloidal nature, a loss of protection should result from degradation by hydrolysis. Weighed

quantities of the three substances were dissolved in 4N sulphuric acid and autoclaved in glass-stoppered tubes for 8 hours at 121°. The cooled solutions were neutralised with chalk to avoid a high sulphate concentration, and were boiled and filtered. The cooled filtrates were adjusted to volume, sterilised by autoclaving and the washed organisms suspended in

TABLE III

EFFECT OF ADDITION OF VARYING CONCENTRATIONS OF PEPTONE ON HEAT RESISTANCE OF *E. coli* TO 57° FOR 60 MINUTES

Peptone concentration (per cent w/v)	Viable count per ml.	Peptone concentration (per cent w/v)	Viable count per ml.
0	$8{\cdot}32 \times 10^6$, $5{\cdot}22 \times 10^5$	0·10	$7{\cdot}82 \times 10^7$
0·005	$1{\cdot}40 \times 10^8$	0·15	$1{\cdot}08 \times 10^8$
0·025	$4{\cdot}86 \times 10^8$	0·20	$1{\cdot}39 \times 10^8$
0·050	$6\,12 \times 10^6$, $7{\cdot}48 \times 10^6$	0·30	$1{\cdot}44 \times 10^8$
0·075	$1{\cdot}60 \times 10^7$	0·50	$2{\cdot}18 \times 10^8$

these solutions. Results of survivor counts are shown in Table IV, together with the results from a "blank" solution prepared without the three substances. Increased resistance to the heat treatment was found with all three hydrolysates, but the "blank" solution and a separately prepared saturated aqueous solution of calcium sulphate had about an equal effect. The effects of the hydrolysis were apparently obscured by the protection afforded by salts present in the original substances and derived from the reagents.

TABLE IV

EFFECTS OF ADDITION OF PEPTONE, GELATIN, AGAR AND THEIR HYDROLYSATES ON RESISTANCE OF *E. coli* TO HEATING AT 57° FOR 60 MINUTES

Substance	Concentration (per cent w/v)	Viable count per ml.	
		Not hydrolysed	Hydrolysate
Peptone	0·5	$2{\cdot}12 \times 10^8$	$3{\cdot}40 \times 10^8$
Gelatin	0·05	$1{\cdot}04 \times 10^8$	$2{\cdot}10 \times 10^8$
Agar	0·05 0·005	$8{\cdot}28 \times 10^7$ $5{\cdot}22 \times 10^6$	$4{\cdot}44 \times 10^7$
Blank	—	$2{\cdot}42 \times 10^8$	
Calcium sulphate	Saturated (20°)	$4{\cdot}17 \times 10^8$	

Effects of Electrolytes on Heat Resistance

Washed cells were resuspended in sterilised solutions of sodium chloride, calcium chloride, magnesium sulphate and sodium sulphate. The numbers of survivors after heating for 1 hour at 57° are shown plotted logarithmically against molar salt concentration in Figure 2. Even dilute solutions of all the salts had a protective action, but solutions more dilute than 0·01M were not examined; attention was given to the stronger solutions.

At the salt concentrations associated with minimal resistance (0·2 to 0·4M sodium chloride and about 0·2M of the other salts), growth of

colonies of the surviving organisms was so much slower than at either lower or higher concentrations that incubation for 18–24 hours was required before colonies could be counted. Anomalous counts were obtained from heated suspensions of sodium chloride in excess of 1·5M: drops of the undiluted suspension gave an uncountable number of colonies, whereas the first tenfold dilution gave few colonies and the successive dilutions were sterile. This was explained by plasmoptysis of the cells on transference to a medium of lower osmotic pressure in the first dilution. When the sodium chloride concentration of dilution fluid was the same as the suspension medium, normal counts were obtained.

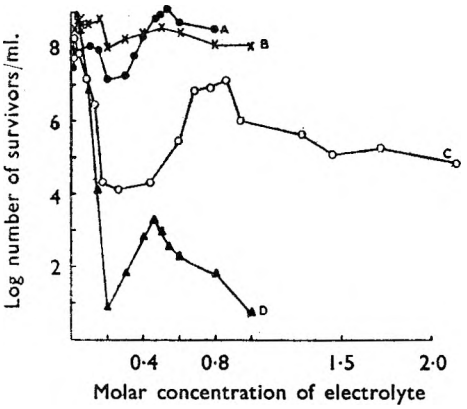

FIG. 2. The effects of electrolytes on the heat resistance of washed suspensions of *E. coli*. Plotted values are logarithms of numbers of organisms/ml. surviving exposure for one hour at 57°, at different molar salt concentrations. A, sodium sulphate; B, magnesium sulphate; C, sodium chloride; D, calcium chloride.

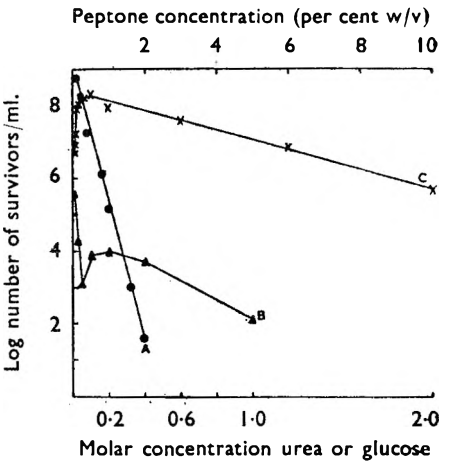

FIG. 3. The effects of organic compounds on the heat resistance of washed suspensions of *E. coli*. Logarithms of numbers of organisms/ml. surviving exposure for one hour at 57° in the presence of different concentrations of A, urea, B, glucose, and C, peptone.

The Effects of Other Substances

For comparison with the electrolytes used, the effects of non-ionisable substances were investigated. Results obtained when solutions of urea and glucose were used as suspension media are shown in Figure 3. Decreasing resistance with increasing urea concentration was very steep, very few surviving organisms remaining when 0·4M was used. In concentrations of 0·6, 1·0 and 2·0M urea no surviving bacteria were detected, even when the remainder of the contents of the ampoule was added to 200 ml. of a liquid medium, known to support growth of very small inocula of *E. coli* and incubated for a long period. The examination of peptone was extended to more concentrated solutions, and the survivor counts are plotted logarithmically against concentration per cent w/v in Figure 3.

Alterations of Heat Resistance with the Use of Different Cultural Conditions

Suspensions were prepared from 24 hour surface growth of *E. coli* on overdried and undried agar plates; overdried plates incubated in McKintosh and Fildes' jars with widely differing carbon dioxide contents, overdried plates filled to different depths with agar and sealed with cellulose tape, and sealed plates the lids of which were filled with soda lime. Survivor counts obtained after the heat treatment are recorded in Table V.

TABLE V

Numbers of *E. coli* surviving exposure to 57° for 60 minutes, the washed suspensions derived from different cultures

Culture	Cultural conditions	Age (hours)	Viable count per ml.	
			Before treatment	After treatment
Undried plate	20 ml., unsealed	24	$1{\cdot}99 \times 10^9$	$4{\cdot}90 \times 10^8$
Overdried plate	20 ml., unsealed	24	$1{\cdot}97 \times 10^9$	$5{\cdot}88 \times 10^8$
	10 ml., sealed	24	$2{\cdot}10 \times 10^9$	$1{\cdot}99 \times 10^3$
	40 ml., sealed	24	$2{\cdot}01 \times 10^9$	$2{\cdot}60 \times 10^4$
	20 ml., sealed, approx. $\frac{1}{4}$ CO_2	24	—	$1{\cdot}48 \times 10^4$
	20 ml., sealed, approx. $\frac{3}{4}$ CO_2	24	$1{\cdot}91 \times 10^9$	$3{\cdot}46 \times 10^2$
	20 ml., sealed, almost solely CO_2	24	$1{\cdot}69 \times 10^9$	$1{\cdot}68 \times 10^2$
	20 ml., sealed, over soda lime	24	$1{\cdot}74 \times 10^9$	$2{\cdot}33 \times 10^3$
	20 ml., sealed, over soda lime	48*	$1{\cdot}50 \times 10^9$	0
Peptone water	Not aerated, not agitated	24	$1{\cdot}73 \times 10^9$	$1{\cdot}10 \times 10^8$
		48	$1{\cdot}78 \times 10^9$	$8{\cdot}68 \times 10^4$

* Strong odour of ammonia on opening plate.

Washed suspensions were also obtained from the cells centrifuged from cultures in peptone water. The cultures were either aerated at a rate rapid enough to justify saturation with oxygen or were not aerated or agitated. All cultures were incubated at 37°. Figure 4 shows the logarithms of survivor counts after heating for 1 hour at 57° plotted against duration of incubation of aerated fluid cultures (curve B), the initial viability of each suspension expressed as a percentage of the viability of a suspension prepared from a 12 hour culture (curve C), and the viable counts of the fluid culture (curve A). Heat resistance of cells from the youngest culture (12 hours) is low and rapidly increases with age in accordance with the findings of Chick[1] and Elliker and Frazier[10], who used non-aerated cultures. The increase in resistance coincides with a sharp fall in the viability of the suspensions (curve C). A fall in suspension viability may mean a reduction in viability of the culture; but, since the suspensions were adjusted densiometrically by measuring the total bacterial substance present, it may also mean an increase in average cell size. Maximal resistance was achieved after growth for about 24 hours.

Longer incubation decreased heat resistance, the lowest resistance being met after about 35 hours, when a further increase was apparent, although subject to doubt in view of wider discrepancies between results from duplicate cultures than had hitherto been encountered. The sharp decline in heat resistance after reaching a maximal resistance does not appear so far to have been reported, and may be explained by the use of

washed suspensions and aerated culture. Aerated cultures achieve a stationary population much more rapidly than static cultures (curves A and D, Fig. 4), and suspensions from static cultures gave a more constant resistance with varying incubation time of the culture (Table V).

Heat Resistance of Stored Suspensions

In view of the findings of Cook, Steel and Wilis[11] on the maintenance of resistance of stored bacterial suspensions to bactericides and bacteriostats, washed suspensions of *E. coli* were stored at room temperature in glass-stoppered bottles and subjected to heat treatment after varying periods. Some results are listed in Table VI. Suspensions were adjusted to twice the usual density, i.e. 4×10^9/ml., and were diluted with an equal volume of either water or a 1 per cent w/v peptone solution before exposure to heat. Loss of heat resistance in stored suspensions is rapid, particularly within the first 48 hours, but suspension in 0·5 per cent peptone gives protection during the first few days only.

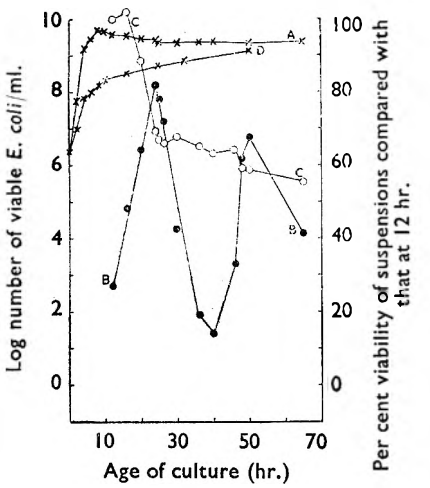

FIG. 4. The effects of age of culture of *E. coli* on the viability and heat resistance of washed suspensions. Suspensions were prepared from aerated broth cultures. A relates the viable count/ml. of broth culture to duration of incubation; C was obtained by plotting the per cent viability of washed suspensions compared with that of a suspension prepared from a 12 hour culture; B refers to the surviving organisms after heating the washed suspensions for one hour at 57°; D shows the viability of an unaerated, static broth culture after varying periods of incubation.

Growth-supporting Materials from Heat-killed Organisms

Previously[11] *E. coli* has been found to increase in viability when the cells from a suspension stored at 37° for 2 months were resuspended either in an aerated suspension supernatant, or in water. It was concluded that the organisms which had survived storage were able to grow on substances provided by dead cells. Since amino acids leak from vegetative bacteria when killed by moist heat[12], whether heat-killed cells could support bacterial growth was investigated.

A washed, living suspension of *E. coli* was prepared, a portion reserved for inoculation of the killed preparations, and the remainder filled into ampoules and heated at 65° for 45 minutes, which results in sterilisation. The cells were centrifuged, resuspended in water, and this washing repeated 5 times. The first and second washings were reserved and separately filtered through sintered glass bacterial filters. 5 ml. portions of each filtrate were inoculated with one drop of living *E. coli* suspension

so diluted that a count of about 1×10^5/ml. resulted. The determined initial viable count was $1{\cdot}51 \times 10^5$/ml. Counts per ml. after incubation for 24 hours were as follows:

In the first washings, $1{\cdot}63 \times 10^7$, and in the second, $1{\cdot}21 \times 10^6$.

In the first washings, after boiling 1 minute and cooling, $1{\cdot}71 \times 10^7$. Inoculation of 5 ml. sterile distilled water and incubation gave a count of $9{\cdot}00 \times 10^4$/ml. The first and second washings of the living suspension gave counts after inoculation and incubation of $1{\cdot}40 \times 10^8$ and $2{\cdot}57 \times 10^6$/ml. respectively, so that little growth would be expected from a third washing.

TABLE VI

NUMBERS OF VIABLE *E. coli* PER ML. SURVIVING EXPOSURE TO 57° FOR 60 MINUTES IN STORED WASHED SUSPENSIONS

Culture	Medium	Age of suspension (days)						
		0	1	2	5	7	14	28
Slope	Water 0·5 per cent peptone	$6{\cdot}90 \times 10^6$ $1{\cdot}21 \times 10^9$	$1{\cdot}51 \times 10^6$ $6{\cdot}72 \times 10^7$	$8{\cdot}28 \times 10^3$ $3{\cdot}57 \times 10^7$	$6{\cdot}12 \times 10^3$ $6{\cdot}66 \times 10^8$	$1{\cdot}17 \times 10^4$ $9{\cdot}02 \times 10^8$	$1{\cdot}11 \times 10^3$ $8{\cdot}40 \times 10^2$	$2{\cdot}04 \times 10$ $1{\cdot}44 \times 10^2$
Aerated broth (24 hr.)	Water	$1{\cdot}53 \times 10^8$	$2{\cdot}24 \times 10^2$	—	—	0	—	—
Unaerated broth (24 hr.)	Water	$1{\cdot}10 \times 10^6$	$2{\cdot}53 \times 10^6$	—	$4{\cdot}47 \times 10^3$	—	—	—
Overdried plate	Water	$5{\cdot}88 \times 10^6$	$6{\cdot}18 \times 10^5$	$8{\cdot}90 \times 10^3$	—	—	—	—

The results indicate that killing of suspensions of *E. coli* under the above conditions is accompanied by a loss of growth-promoting material to the suspension medium. Such materials are stable to boiling, and support growth, but not so well as the first washing in preparation of the living suspension, which contains nutrients removed from the agar slope.

The washed, killed cells from the above experiment were suspended in water, adjusted to a density of 2×10^9/ml., and placed in 10 ml. volumes in capped tubes which were incubated at 37°. After incubation for 27 days, half of the suspension was filtered to remove bacteria and the filtrate and the unfiltered suspension inoculated with freshly prepared suspension of *E. coli*. On incubation, the following viable counts per ml. were obtained.

Incubation time, hr.	0	16	48
Count in filtrate	$1{\cdot}96 \times 10^5$	$1{\cdot}61 \times 10^5$	$4{\cdot}74 \times 10^4$
Count in suspension ..	$2{\cdot}18 \times 10^5$	$1{\cdot}78 \times 10^7$	$2{\cdot}51 \times 10^7$

It appears that the prolonged storage of a thoroughly-washed suspension of heat-killed organisms in water results in the release of little more of the growth materials that occurred during the killing process. The system of cells + suspending fluid, however, is able to support growth, a finding which supports previous hypotheses[11,13] that bacterial cells

themselves, apart from products of metabolism and death, may provide nutrient for growth of living cells.

That a small proportion of organisms surviving a heat treatment might be able to grow using materials derived from the dead cells was investigated. Washed suspensions were heated for various times at 57°, 59° and 65° and viable counts of survivors determined. The suspensions were then incubated at 37° for 24 and 48 hours and the counts again estimated. There was no definite increase in viability after incubation. In fact, where the numbers of surviving organisms were initially small (less than 0·1 per cent survival), incubation usually showed a decrease in viability. Either the organisms surviving the heat treatment suffered damage which prevented their growth under conditions in which unheated cells can grow, or the milder conditions employed in these experiments caused insufficient damage to or leakage of contents from the killed cells to support growth.

Similar results have since been reported by Chambers, Tabak and Kabler[14], who mixed the treated suspensions of *E. coli* with equal volumes of 0·1M phosphate buffer solution or of solutions of Krebs cycle metabolites in phosphate buffer before incubation. The same workers substantiated the findings of Garvie[15] that organisms surviving treatment with chlorine, increased in viability after inactivation of the chlorine and transference to the above solutions. They considered that the increase in viability was due rather to multiplication of survivors than to a "reactivation" of presumed killed cells as postulated by Heinmets and others[16] Our evidence of growth of *E. coli* in stored suspensions[11] and of growth supported by killed cells suggests, contrary to Garvie's findings, that killed cells might easily be a source of nutrient in growth after chlorine treatment.

Discussion

Washed suspensions of *E. coli* require a considerably shorter time for virtual sterilisation than unwashed suspensions. The substances which confer resistance to unwashed suspensions are those carried over from the culture medium. This is suggested by the finding that peptone, agar and sodium chloride in low concentration are all able to confer similar resistance to washed cells. Peptone and agar may not act by the colloidal properties of their solutions but possibly because they contain ionisable compounds. Both substances contain inorganic salts.

The effects of added salts, in varying concentrations, on the heat resistance of washed suspensions show differences in the toxicity of the different ions. Calcium is much more toxic than magnesium or sodium; chloride is more toxic than sulphate. High toxicity of calcium salts to *E. coli* in suspensions at room temperature has been recorded by Winslow and Falk[17] and explained by the degree of dissociation of calcium salts by Mitchell[18]. Greater effect in preventing growth of several micro-organisms in liquid media by Ca^{++} than by Mg^{++} or Na^{+} has been described by several workers[17,19,20] and under similar conditions SO_4^{-} has been alleged[20] to be less toxic than Cl^{-}. Winslow and Falk found a minimal rate of

loss of viability of washed suspensions of *E. coli* at a sodium chloride concentration of about 0·25M at pH 7·0 and room temperature. This, however, is within the concentration range of maximal loss of viability at 57° (Fig. 2). Thus the salt appears to exert different effects at different temperatures. Winslow and Falk concluded that the salt acted mainly in an osmotic capacity because other univalent electrolytes behaved in the same way.

Effect of varying the concentration of electrolyte (Fig. 2) reveals a similar behaviour with all the salts examined. Variation in heat resistance at different concentrations of magnesium sulphate were ill-defined, but not with the other salts. Very low salt concentrations cause an increased resistance; increasing concentrations result in loss of resistance until a concentration of minimal resistance is reached (0·2–0·4M sodium chloride, 0·2M of the other salts). Further increases in concentration lead to increased resistance, which reaches a maximal value at a concentration of about 0·9M sodium chloride and 0·45–0·5M of the other salts. When the concentration is still further increased there is a drop in resistance, the rate of which depends upon the toxicity of the salt.

Previous reports of the effects of salts on heat resistance are vague. Von Angerer and Küster[21] found that solutions of calcium chloride offered no protection to *E. coli*; Robertson[22] reported a decrease in resistance with "hypotonic" salt solutions; Rahn[23] attributed the effects of salts to their osmotic pressures. My results offer no simple explanation in terms of osmotic effect. A tentative explanation is advanced on the following lines. Considerable evidence has led to the suggestion[18] that damage to the osmotic barrier of bacteria may result from thermal damage. The maintenance of the functions of the cell surface will depend *inter alia* on the surface charge, which has been shown[24,25] to be negative at physiological pH. In small concentrations, salts of univalent cations are held[26] to increase the surface potential; with increasing concentrations, cations decrease the potential, although univalent and divalent cations are considered incapable of neutralisation and reversal of the charge on the surface. In this case, only the initial increase and succeeding decrease in resistance can be explained by respective charging and discharging by electrolyte; and the second increase in resistance at higher concentrations might be explained as an osmotic effect whereby the loss of cellular constituents is retarded. The bacteria, at the salt concentration of minimal resistance, may have a nearly neutralised surface potential, making them more susceptible to heat and recovery of survivors more lengthy.

In contrast with electrolytes, increasing concentrations of urea caused a sharp decline in heat resistance (Fig. 3) without giving protection at any but very low concentrations. Suspension of washed cells in glucose solutions had decreased heat resistance at all concentrations, except for a slight increase at 0·05–0·1M. Differences in the behaviour of solutions of urea and glucose might be expected. Sterilised solutions of urea were alkaline (1M solution of pH 9·0), whereas those of glucose were faintly acid (1M solution of pH 6·2). Loss of resistance was found to be smaller when the glucose (0·02M) was dissolved in a phosphate buffer solution

(pH 7·0), but the resistance was then lower than with the use of phosphate buffer alone. Also, urea is a small molecule with high permeability of the lipoid cell membrane[27], and probably of lower osmotic effect than glucose; moreover, a high penetration of the cell by urea may occur, with resulting toxicity.

Previous workers have pointed to protection of bacteria against heat in the presence of sugars. Rahn[23] considered that increased resistance in the presence of strong sugar solutions was generally accepted and was explained by a partial dehydration of the protoplasm; Von Angerer and Küster[21] found that starch and urea gave no protection to *E. coli* heated at 56°, but inclusion of glycerol, sorbitol, monosaccharides and disaccharides retarded death. Previous investigations of the effects of added substances are subject to the general criticisms that the effects of change of concentration have been inadequately studied. Also attempts have rarely been made to remove substances carried over from the culture medium which greatly affect the response to heat.

In conclusion, it is found that a difference exists between the effects of electrolytes and non-electrolytes on the resistance of bacteria to moist heat and hence it is suggested between their modes of protection. In the protection of bacteria against heat, added substances act not in an osmotic capacity alone: presence of ions, permeability to the cell membrane and inherent toxicity of the substance are of importance.

The estimation of survival of washed bacteria when grown under different cultural conditions illustrates the very wide observed fluctuations in heat resistance. The following general conclusions are put forward. Aeration during growth of a culture leads to increased resistance when the culture is 20–25 hours old. Exclusion of air or the presence of high carbon dioxide contents in the atmosphere of growths results in lowered resistance. Complete absence of carbon dioxide and absorption of the gas liberated during growth causes reduced heat resistance, especially on long incubation. When grown on a solid medium, a moist surface appears to result in higher heat resistance.

Acknowledgements. The author gratefully acknowledges the helpful comments of Dr. A. M. Cook and the valuable technical assistance given by Mr. A. Edwards and Miss R. McDonald.

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DISCUSSION

The paper was presented by the AUTHOR.

The CHAIRMAN. The surprising and interesting point was that growth could be supported by the killed cells themselves and not by metabolites or materials which had leaked out of those cells.

MR. K. A. LEES (London). The title was not sufficiently specific in that the paper dealt with one strain of one species only. The last paragraph of the Discussion referred to general conclusions, but if the work were repeated with other organisms it was doubtful if they applied.

DR. J. C. PARKINSON (Brighton) asked whether with glucose and urea the effect on lowered heat resistance was the effect of those substances or one of pH.

MR. H. D. RAPSON (Dorking). If some of the ions in a solution were fixed on the surface of the cell and the pH were changed the charge on the cell was also changed. Was the author able to determine the point at which the surface appeared to be of the same potential as the solution surrounding it? What was meant by the term "osmotic effect"?

MISS A. E. ROBINSON (London). Were the conclusions based upon the results at 57° significant at other temperatures? Reversal of charge had been demonstrated with certain divalent cations. The values for the internal osmotic pressure of *E. coli* quoted in the literature varied between 2 and 15–20 atmospheres. Had the author information about his strain of organism? Damage to the osmotic barrier for low molecular weight substances was a consequence of thermal damage to the bacteria.

MR. G. SYKES (Nottingham). If, after heating, the suspension were allowed to stand for $2\frac{1}{2}$ hours instead of 1 hour, the result of the test was varied. The same applied if the plates were allowed to stand for a long interval. Was it a question of changes of temperature, and would it apply if a liquid culture had been used instead of a solid one? The work underlined the unreliability of any test which applied a so-called 100 per cent kill. The 90 per cent survival limit was no better from the point of view of reproducibility than the 100 per cent limit. Would the author comment on the work of Berry and his colleagues that a six-fold replicate of a 100 per cent endpoint was perfectly reproducible?

DR. WILLS replied he had intended to convey general conclusions based on his results rather than general conclusions for all organisms. In the use of urea the pH had a large bearing on the result. He had not made any measurements of surface neutrality. By osmotic effect he had in mind a greater concentration of solute in the external environment of the cells which would prevent leakage of constituents from the cell. He had done nothing to identify the substances which leaked from the cells. Behaviour at other temperatures had not been investigated. It was a fact that thermal death might be ascribed to damage to the osmotic barrier. By careful control of a number of factors like the medium and the conditions of growth, results could be obtained by Berry's method which were of very much closer reproducibility than would have been the case by any other bactericidal evaluation. The kill was not 100 per cent by this method but it was very close. He defended extinction methods because he had found such methods reproducible in his own hands, and because they more nearly approached the conditions in which one was interested in disinfection.

PHARMACOLOGICAL PROPERTIES OF HYDRALLAZINE, DIHYDRALLAZINE AND SOME RELATED COMPOUNDS

BY S. M. KIRPEKAR AND J. J. LEWIS

From the Department of Materia Medica and Therapeutics, University of Glasgow

Received June 3, 1957

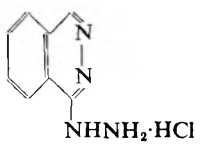
Hydrallazine and dihydrallazine cause adrenaline reversal and antagonise the pressor effects of noradrenaline on the cat blood pressure. Both hydrallazine and dihydrallazine antagonise vasoconstriction caused by adrenaline, noradrenaline, histamine, 5-hydroxytryptamine or barium chloride in isolated perfused preparations of the rat hind quarters or rabbit ear. In isolated aortic strips antagonism is shown to the constrictor action of adrenaline, noradrenaline, histamine and 5-hydroxytryptamine but not to barium chloride. Marked antagonism to 5-hydroxytryptamine is shown only on the perfused rat hind quarters or rabbit ear. Antagonism to pressor reflexes is of moderate potency or is absent. Applied locally to the carotid sinuses of the cat, hydrallazine causes a rise of blood pressure. Hydrallazine and dihydrallazine have mainly a peripheral site of action involving an effect upon the contractile elements of the blood vessel walls. 3-Phenyl-6-hydrazino-pyridazine HCl, 1-hydrazino*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate appear to have similar properties to hydrallazine and dihydrallazine.

HYDRALLAZINE and dihydrallazine have been used in the treatment of hypertension in the United States of America and Europe but they have not been widely used in Britain. Both compounds are potent hypotensive agents probably ranking next to the ganglion-blocking agents and veratrum preparations. They were synthesised by Druey and Ringier[1] in 1950 and early reports of their hypotensive activity were made by Gross, Druey and Meier in 1950[2] and by Craver and his colleagues (1950–1)[3,4]. The introduction of these compounds aroused interest since not only did they lower blood pressure, but they also increased renal blood flow in animals[2,5–7], and in man[8–12]. The mode of action of hydrallazine and dihydrallazine is not clearly understood. Various effects have been described, indicating a partly central action[4,5,14–17], a mainly peripheral point of attack[5,17,18], antagonism to 5-hydroxytryptamine[5,15], to rennin[5,19], to a cerebral vasopressor hormone[15], to adrenaline and noradrenaline[4,5,17,20,21] and inhibition of histaminase[22,23]. Antagonism to various pressor reflexes has been noted[5,14,17], especially in cats[5,17]. There is disagreement about some of the properties of hydrallazine. Erspamer[24] does not, for example, consider it to be a specific antagonist of 5-hydroxytryptamine, whilst, using dogs, Walker and his colleagues[20] could not show blockade of the carotid sinus pressor reflex; and Britton and his colleagues[25] could not inhibit the pressor response to hypoxia.

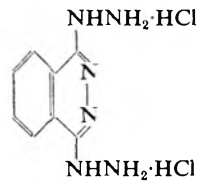
We have repeated some of the earlier investigations and extended them with the object of gaining some insight into the mode of action of these potentially valuable drugs.

Materials and Methods

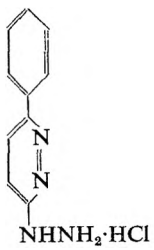
Perfusion fluids. The composition of these in g./litre was as follows. Frog Ringer's solution, NaCl 6·5, KCl 0·14, $CaCl_2$ 0·12, $NaHCO_3$ 0·2, glucose 1·0. Locke's solution, NaCl 9·0, KCl 0·42, $CaCl_2$ 0·24, $NaHCO_3$ 0·5, glucose 1·0. Tyrode's solution, NaCl 8·0, KCl 0·2, $CaCl_2$ 0·2, $MgCl_2$ 0·1, NaH_2PO_4 0·05, glucose 1·0, $NaHCO_3$ 1·0. De Jalon's solution, NaCl 9·0, KCl 4·2, $CaCl_2$ 0·06, $NaHCO_3$ 0·5, glucose 0·5.



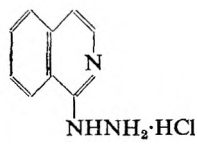
(I) Hydrallazine
(1-Hydrazinophthalazine-hydrochloride)



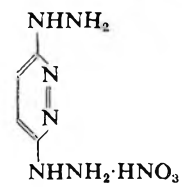
(II) Dihydrallazine
(1 : 4-Dihydrazinophthalazine-dihydrochloride)



(III) 3-Phenyl-6-hydrazino-pyridazine-hydrochloride



(IV) 1-Hydrazino-*iso*quinoline-hydrochloride



(V) 3 : 6-Dihydrazino-*pyridazine nitrate*

Drugs were dissolved in the appropriate saline solution. In studying drug antagonisms the following were used. Acetylcholine chloride (ACh), carbachol, histamine acid phosphate (Hm), (−)-adrenaline hydrochloride (Ad), (−)-noradrenaline bitartrate (NA), 5-hydroxytryptamine creatinine sulphate (5-HT), tubocurarine chloride (TC), decamethonium iodide (C 10), nicotine hydrogen tartrate (NHT), eserine sulphate (eserine), potassium chloride (KCl), barium chloride ($BaCl_2$) and atropine sulphate (atropine). The following drugs were investigated. Hydrallazine (I), dihydrallazine (II), 3-phenyl-6-hydrazino-pyridazine hydrochloride (III), 1-hydrazino-*iso*quinoline hydrochloride (IV) and 3 : 6-dihydrazino pyridazine nitrate (V). We are indebted to Dr. C. D. Falconer and Dr. F. Gross of Ciba Laboratories for supplies of these compounds.

The following preparations and techniques were used. The frog rectus abdominis muscle suspended in frog Ringer's solution at room temperature. The oestrous uterus of the rat suspended in oxygenated de Jalon's fluid at 29°. The rabbit duodenum in oxygenated Locke's solution at 37°. Isolated strips of the descending aorta of cats or rabbits cut spirally (Furchgott and Bhadrakom[26]) and perfused with Tyrode's solution with

O_2 and 5 per cent CO_2 at 37°. The isolated rabbit heart perfused by Langendorff's method[27] using oxygenated "double glucose" Locke's solution at 37°. The isolated guinea pig auricles in oxygenated "double glucose" Locke's solution at 29°. Strips of the terminal ileum of guinea pigs suspended in oxygenated Tyrode's solution at 34 to 35°. Segments of the diaphragm of the rat with the attached phrenic nerve, set up in "double glucose" Tyrode's solution with O_2 and 5 per cent CO_2 at 29°. The nerve was stimulated with a Dobbie McInnes stimulator, at a frequency of 6 or 8/minute at 6 volts; the pulse width was 1 to 3 msec. Finally the isolated rat hindquarters and rabbit's ear described by Burn[28] and perfused with oxygenated Locke's solution at room temperature.

In anaesthetised cats, constant pressor responses were obtained with (*a*) NA or Ad (1 to 2 μg./kg.), (*b*) clamping for 30 or 40 seconds with bulldog clips both common carotid arteries at a point immediately below the carotid sinuses, (*c*) stimulation of the cut central end of the vagus, (*d*) stimulation of the cut central end of the sciatic nerve, (*e*) stimulation of the splanchnic nerve, (*f*) compression with artery forceps for 15 to 40 seconds of the abdominal aorta at a point just above the coeliac artery, (*g*) anoxia and (*h*) hypoxia by inhalation of 95 per cent N_2 and 5 per cent O_2 for periods of 1 to 3 minutes. In stimulation of nerve square impulses were used at 5 to 125 volts and a frequency of 1200 to 1400/minute. The pulse width was 0·5 to 3 msec. Constant depressor responses were obtained to ACh (0·5 to 2 μg./kg.) or Hm (0·5 to 2 μg./kg.). In some cats a constant hypertension was maintained by intravenous infusion of a solution of 10 to 100 μg./ml. Ad or NA at 1·0 ml./minute. Contractions of the nictitating membrane were obtained in response to 5 to 10 μg./kg. Ad or NA, or to electrical stimulation of the cervical sympathetic. Drugs (1 mg./kg.) were administered once reproducible responses had been obtained.

In spinal cats, reproducible pressor responses were obtained to Ad or NA (1 to 3 μg./kg.) or to anoxia. Reproducible depressor responses were obtained to ACh or Hm (2 to 3 μg./kg.). Drugs (1 mg./kg.) were administered once reproducible responses had been obtained.

Results

Direct Effects

Smooth muscle. Hydrallazine and dihydrallazine (0·5 to 10 μg./ml.), increased the tone and the amplitude of peristaltic movements of isolated strips of rabbit duodenum (Fig. 1). Higher doses, 25 to 50 μg./ml., caused a contraction of the strip. The other compounds tested caused a slight increase in tone at doses of 2·5 to 12·5 μg./ml. Hydrallazine, (400 μg./ml.) and dihydrallazine (500 μg./ml.) caused a slight relaxation of aortic strips. All five compounds, 1 to 100 μg./ml., increased the outflow from the perfused rabbit's ear and rat's hindquarters. When injected into anaesthetised cats, 1 to 2 mg./kg. of hydrallazine, dihydrallazine and 3:6-dihydrazinopyridazine nitrate caused a delayed and gradual fall in the blood pressure with slight stimulation of respiration. One to 2 mg./kg. of 1-hydrazino-*iso*quinoline HCl or of 3-phenyl-6-hydrazino-pyridazine

HCl occasionally failed to produce a fall in the blood pressure, instead there was a gradual rise.

Cardiac muscle. Perfusion of the isolated rabbit heart with hydrallazine or dihydrallazine (1 to 10 μg./ml.) caused an irreversible decrease in amplitude but an increase in the rate, and in the outflow. 1-Hydrazino-*iso*quinoline HCl (10 μg./ml.) irreversibly decreased rate amplitude and

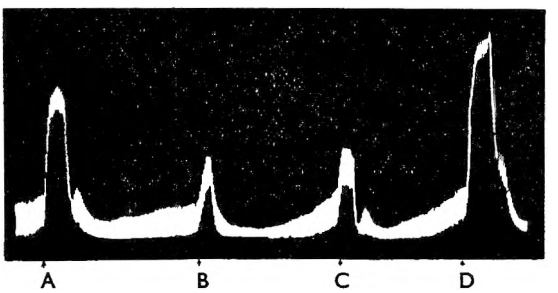


FIG. 1. Isolated rabbit duodenum.
At A, 30 μg./ml. of 1-4-dihydrazinophthalazine.
B, 12·5 ,, ,, 1-hydrazinophthalazine HCl.
C, 25 ,, ,, ,, ,,
D, 50 ,, ,, ,, ,,

outflow. 2·5 to 10 μg./ml. of hydrallazine or dihydrallazine initially increased the amplitude of beat of the isolated guinea pig auricles. This effect was followed by depression. The other compounds, 2·5 to 12·5 μg./ml., were ineffective or caused a fall in rate and amplitude.

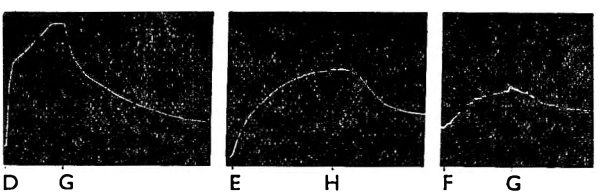


FIG. 2. Influence of hydrallazine on contractions of aortic strips induced by adrenaline, noradrenaline and 5-HT.
At D, 2 μg./ml. of (−)-adrenaline hydrochloride.
E, 5 ,, ,, (−)-noradrenaline bitartrate.
F, 2 ,, ,, 5-HT creatinine sulphate.
G, 400 ,, ,, hydrallazine.
H, 1 mg./ml. of hydrallazine.

Skeletal muscle. Hydrallazine or dihydrallazine (1 to 3 mg./ml.) reduced the twitch amplitude in the rat diaphragm preparation. Complete neuromuscular block was not seen and the muscle continued to respond to direct stimulation. No direct effects were observed on the frog rectus muscle or rat uterus at doses of 10 to 125 μg./ml. The guinea pig ileum showed no direct action unless spontaneous activity was high

when both hydrallazine and dihydrallazine 12·5 to 125 μg./ml. caused a contraction.

Antagonism to Adrenaline and Noradrenaline

Smooth muscle. Hydrallazine (400 μg./ml.) relaxed aortic strips caused to contract with Ad or NA (1 to 10 μg./ml.). Dihydrallazine (400 to 500 μg./ml.) was less effective (Fig. 2). Similar but much weaker effects followed the addition of the other three compounds (100 to 200 μg./ml.). When injected into anaesthetised cats, all five drugs, 1 mg./kg., altered the pressor response to Ad into a biphasic pressor-depressor response (Fig. 3), indicating an effect upon adrenaline vasoconstriction, but no effect upon the stimulant actions of Ad on the heart. When the blood

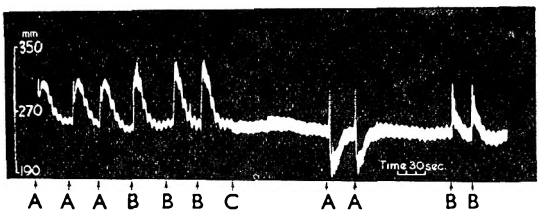


FIG. 3. Blood pressure record of a cat anaesthetised with ether-chloralose.
At A, 5 μg. of (−)-adrenaline i.v.
B, 5 μg. of (−)-noradrenaline i.v.
C, 1 mg./kg. of 1-hydrazinophthalazine HCl i.v.

pressure was raised by continuous perfusion of a solution of Ad or NA at constant rate, all five compounds antagonised the hypertension due to Ad more effectively than that due to NA (Fig. 3). Hydrallazine, dihydrallazine or 3:6-dihydrazinopyridazine nitrate, 1 mg./kg., partially antagonised the pressor response to NA. The other compounds had little or no effect. At doses of 1 to 10 μg./ml. hydrallazine and dihydrallazine antagonised Ad and NA (10 ng. to 1 μg.) vasoconstriction in the perfused rabbit ear or rat hindquarters. This effect was shared by 10 μg./ml. of 1-hydrazino-*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate, but not invariably by 10 μg./ml. of 3-phenyl-6-hydrazino pyridazine HCl. Hydrallazine and dihydrallazine (25 to 50 μg./ml.) showed no, or slight, antagonism to Ad (0·01 to 0·1 μg./ml.) or NA (0·25 to 1 μg./ml.) inhibition of ACh induced contractions of the rat uterus. The other three compounds themselves antagonised ACh and were not therefore tested. Only very slight antagonism to Ad or NA (0·013 to 0·1 μg./ml.) was shown on the rabbit duodenum. Contractions of the nictitating membrane induced by Ad were slightly antagonised by all compounds, excepting 3-phenyl-6-hydrazino-pyridazine HCl at doses of 1 mg./kg. Contractions due to NA or electrical stimulation were not affected.

Cardiac muscle. Hydrallazine and dihydrallazine (10 μg./ml.) caused slight potentiation of the effects of 0·5 to 2 μg. of Ad and NA on the isolated perfused rabbit's heart or of Ad (0·05 μg./ml.) and NA (0·025 μg./ml.) on the isolated guinea pig auricles, but 1-hydrazino-*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate showed no effect or caused slight

potentiation. 3-Phenyl-6-hydrazino-pyridazine HCl had no effect. These three compounds were tested at 2·5 to 12·5 μg./ml.

Vasopressor Reflexes

Pressor responses to compression of the common carotid arteries, stimulation of the central end of the cut vagus and hypoxia (Fig. 4) were either not antagonised or were slightly so by doses of 1 mg./kg. of all five drugs. At similar doses there was antagonism to the pressor response after stimulation of the cut central end of the sciatic nerve. Hydrallazine

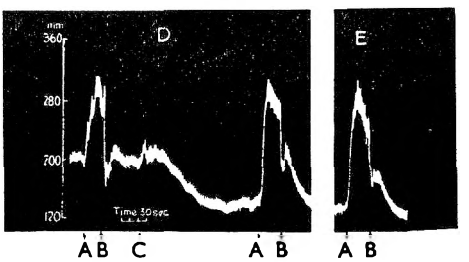


FIG. 4. Influence of hydrallazine on the pressor response to hypoxia. Record D, blood pressure of cat, wt. 2·5 kg., anaesthetised with ether-chloralose. Between A and B, inhalation of gas mixture of nitrogen 95 per cent and oxygen 5 per cent.

At C, 1 mg./kg. of hydrallazine i.v.
Record E, 30 min. after hydrallazine i.v.

and dihydrallazine (1 mg./kg.) antagonised the pressor responses after compression of the abdominal aorta (Fig. 5) or stimulation of the splanchnic nerve (Fig. 6). The other compounds at the same doses did not reliably block these responses. In some preparations no antagonism was shown.

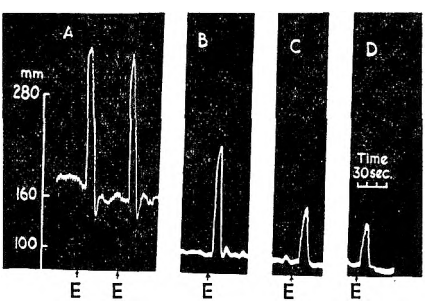


FIG. 5. Influence of hydrallazine on the pressor response to compression of the abdominal aorta. Blood pressure of cat, wt. 4 kg., anaesthetised with ether-chloralose.

A. Before hydrallazine.
B. 10 min. after 1 mg./kg. hydrallazine i.v.
C. 20 ,, ,, ,, ,, ,,
D. 30 ,, ,, ,, ,, ,,

At E, compression of the abdominal aorta for 15 sec.

Antagonism to 5-hydroxytryptamine. Marked antagonism was shown to the vasoconstrictor effects of 10 ng. to 1 μg. of 5-HT on the perfused rat's hindquarters or rabbit's ear by 10 μg./ml. of hydrallazine, dihydrallazine, 1-hydrazino-*iso*quinoline HCl or 3:6-dihydrazino-pyridazine nitrate. 10 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl was ineffective. On the other hand, 10 to 125 μg./ml. of hydrallazine or dihydrallazine potentiated the response of the guinea pig ileum to 5-HT (30 ng. to 3 μg./ml.).

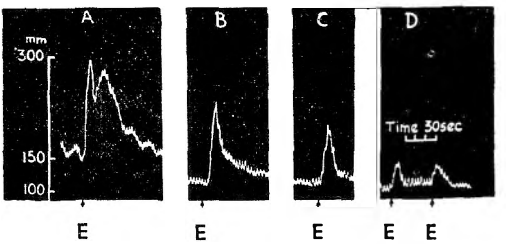


FIG. 6. Influence of hydrallazine on the pressor response to stimulation of splanchnic nerve. Blood pressure of cat, wt. 2·5 kg., anaesthetised with ether-chloralose.

A. Before hydrallazine.
B. 15 min. after 1 mg./kg. hydrallazine i.v.
C. 45 ,, ,, ,, ,, ,,
D. 75 ,, ,, ,, ,, ,,

At E, splanchnic nerve stimulated for 20 seconds (square impulses, 7·5 V., frequency 1400/min., width, 3 msecs.).

3-Phenyl-6-hydrazino-pyridazine HCl or 1-hydrazino-*iso*quinoline HCl (25 to 125 μg./ml.) antagonised 5-HT on this preparation, but 25 to 125 μg./ml. of 3:6-dihydrazino-pyridazine nitrate had no effect. Slight potentiation of 5-HT (1 to 3 μg./ml.) induced contractions of the rabbit duodenum was shown by 10 to 50 μg./ml. of hydrallazine or dihydrallazine. 2·5 to 12·5 μg./ml. of the other compounds had no effect. Hydrallazine and dihydrallazine (10 to 100 μg./ml.) showed slight antagonism to 5-HT (0·1 to 2 μg./ml.) contractions of the rat uterus. Similar effects were shown by 25 to 125 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl or 3:6-dihydrazino-pyridazine nitrate but 1-hydrazino-*iso*quinoline HCl (2·5 to 25 μg./ml.) was more potent. Hydrallazine and dihydrallazine (400 μg./ml.) relaxed aortic strips caused to contract by 5-HT 2 to 5 μg./ml. (Fig. 2).

Antagonism to Acetylcholine and Histamine

Smooth muscle. 12·5 to 125 μg./ml. of hydrallazine or dihydrallazine potentiated the response of the guinea pig ileum to ACh (0·1 to 1 μg./ml.). Hm contractions (0·1 to 0·5 μg./ml.) showed initial potentiation followed by antagonism. 25 to 125 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl or 1-hydrazino-*iso*quinoline HCl antagonised contractions due to both ACh and Hm but 3:6-dihydrazino-pyridazine nitrate at similar dose levels was ineffective. On the rat uterus 12·5 to 125 μg./ml. of hydrallazine or dihydrallazine caused a slight potentiation of the response

to ACh (0·1 to 0·25 μg./ml.), but 1-hydrazino-*iso*quinoline HCl (5 to 50 μg./ml.), 3-phenyl-6-hydrazino-pyridazine HCl (25 to 75 μg./ml.) and 3:6-dihydrazino-pyridazine nitrate (25 to 75 μg./ml.) antagonised it. 1-Hydrazino-*iso*quinoline HCl was the most potent. On the rabbit duodenum hydrallazine or dihydrallazine (12·5 to 50 μg./ml.) potentiated the contractions induced by ACh (0·02 to 0·1 μg./ml.) or Hm (2 to 20 μg./ml.). ACh induced contractions were slightly antagonised by 2·5 to 12·5 μg./ml. of the other three compounds. Hm or ACh induced contractions of aortic strips (Hm or ACh 1 to 10 μg./ml.) were relaxed by hydrallazine. Hm-induced vasoconstriction of the rat's hindquarters or rabbit's ear (Hm 1 to 10 μg.) was antagonised by 10 μg./ml. of hydrallazine or dihydrallazine. Slight antagonism was shown by the five compounds (1 mg./kg.) to the depressor effects of 0·5 to 2 μg./kg. of Hm and ACh on the blood pressure of anaesthetised or spinal cats. In some cats the depressor effects of Hm and ACh appeared to be slightly prolonged. When administration of Hm was followed by biphasic depressor-pressor response, hydrallazine or dihydrallazine or 1-hydrazino-*iso*quinoline HCl (1 mg./kg.) strongly antagonised the pressor component. A biphasic response to Hm cannot always be obtained. For this reason the effects upon it of the other two compounds have not yet been investigated.

Cardiac muscle. 10 to 25 μg./ml. of hydrallazine or dihydrallazine had no effect upon Hm acceleration of the guinea pig auricles (Hm 0·013 μg./ml.). The same compounds at doses of 10 μg./ml. had no effect upon ACh slowing of the isolated perfused rabbit heart caused by 0·5 to 1 μg. of ACh.

Skeletal muscle. 5 to 50 μg./ml. of hydrallazine or dihydrallazine and 2 to 5 μg./ml. of 1-hydrazino-*iso*quinoline HCl potentiated the response of the rectus muscle to ACh (0·1 to 0·5 μg./ml.). 5 to 25 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl or 3:6-hydrazino-pyridazine nitrate showed neither potentiation nor antagonism of ACh.

Other Drug Antagonisms

Skeletal muscle. 5 to 50 μg./ml. of hydrallazine or dihydrallazine potentiated the response of the frog rectus abdominis muscle to 0·25 to 0·75 μg./ml. KCl. 5 to 25 μg./ml. of the other three compounds had no effect or caused slight antagonism. C 10-induced contractions of the rectus (C 10, 2 to 3 μg./ml.) were antagonised by 5 to 25 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl, 1-hydrazino-*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate. Hydrallazine and dihydrallazine (50 to 100 μg./ml.) showed similar effects. On the other hand, 50 μg./ml. of hydrallazine or dihydrallazine potentiated the response to 1 to 3 μg./ml. NHT whilst the other three compounds at dose levels of 2·5 to 12·5 μg./ml. showed a slight antagonism. After C 10 had been washed out, 50 μg./ml. of hydrallazine or dihydrallazine and 25 to 50 μg./ml. of 1-hydrazino-*iso*quinoline HCl caused the rectus to contract even though they had no direct action on the tissue prior to C 10. After 5 to 25 μg./ml. of hydrallazine or dihydrallazine, atropine (0·1 to 2 μg./ml.) or TC (0·4 to 0·5 μg./ml.)

did not antagonise ACh induced contractions of the rectus. Eserine (2 to 4 μg./ml.) potentiation of ACh induced contractions was increased after hydrallazine or dihydrallazine (5 to 25 μg./ml.).

Smooth muscle. 12·5 to 125 μg./ml. of hydrallazine or dihydrallazine, 25 to 125 μg./ml. of 1-hydrazino-*iso*quinoline HCl or 3:6-dihydrazino-pyridazine nitrate did not modify contractions of the guinea pig ileum induced by 0·2 to 0·5 mg./ml. of $BaCl_2$. 25 to 125 μg./ml. 3-phenyl-6-hydrazino-pyridazine HCl showed slight antagonism. Marked antagonism to vasoconstriction of the rat hindquarters induced by 0·1 to 5 mg. $BaCl_2$ was shown by 1 to 10 μg./ml. of hydrallazine or dihydrallazine and 10 μg./ml. of 1-hydrazino-*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate but 10 μg./ml. of 3-phenyl-6-hydrazino-pyridazine HCl had no effect.

Skeletal muscle. Hydrallazine or dihydrallazine (200 to 500 μg./ml.) almost completely antagonised C 10 and NHT block of the rat diaphragm, but had no effect on the block caused by TC.

Discussion

Hydrallazine and dihydrallazine have certain properties in common: both antagonise the stimulant effects of Ad and NA. This is shown by their ability to cause adrenaline reversal and antagonism to NA on the blood pressure of the cat. They also antagonise the pressor component of the biphasic pressor-depressor response to Hm. Antagonism to Ad is also shown in the isolated perfused rat hindquarters and rabbit ear, on the nictitating membrane and on isolated aortic strips. There are wide variations in potency. It is greatest on the isolated perfused rat hindquarters and rabbit ear, moderate on aortic strips and the cat blood pressure, and low on the heart, auricles and nictitating membrane. Hypertension induced and maintained by constant rate infusion of Ad in cats is promptly reduced to normal levels but if NA is used hydrallazine and dihydrallazine are less effective.

Marked antagonism to 5-HT is shown only on the isolated perfused rat hindquarters or rabbit ear; in other preparations, it is weak or absent or there may even be potentiation of the response to 5-HT.

At the doses used none of the pressor reflexes was eliminated; but responses to stimulation of the cut central end of the sciatic nerve, to compression of the abdominal aorta and to stimulation of the splanchnic nerve, were reduced by both compounds. The responses to anoxia, hypoxia, carotid sinus occlusion and stimulation of the cut central end of the vagus were almost unaffected.

Neither hydrallazine nor dihydrallazine antagonises ACh. Potentiation is more usual. Responses to Hm may be potentiated but both drugs are potent antagonists of $BaCl_2$ or Hm vasoconstriction of the isolated perfused rat hindquarters and rabbit ear.

In our view, both hydrallazine and dihydrallazine have mainly a peripheral site of action. They appear to be able to abolish vasoconstriction in isolated vascular beds, irrespective of the nature of the chemical agent

constricting the blood vessels. This is shown in experiments on the perfused rat's hindquarters and rabbit ear when hydrallazine and dihydrallazine antagonise vasoconstriction due to Ad, NA, 5-HT, Hm or $BaCl_2$ and is also indicated by the increased outflow recorded from the isolated perfused heart, and by the direct vasodilator properties which both compounds possess. Hydrallazine and dihydrallazine seem to be adrenergic blocking agents but appear to be neither specific, selective nor potent. An effect upon the carotid body chemoreceptors does not seem likely since the pressor responses to hypoxia and anoxia are not affected. The carotid sinus pressor reflex is not abolished; this seems to rule out an effect upon the pressor receptors of this organ. We have carried out a few experiments similar to those described by Heymans[29] in which 6 mg. of hydrallazine dissolved in 1 ml. of saline were infiltered around both carotid sinuses of the cat. This caused a rise in the systemic blood pressure, an effect similar to that obtained by Heymans using adrenergic blocking agents including the ergot alkaloids. This points to a direct relaxant effect upon the muscle fibres, of the carotid sinus walls. When the walls are relaxed, intrasinusoidal pressure falls and this reflexly causes a rise in the blood pressure.

There is no specific antagonism to any one humoral agent. The effects observed may be due to a direct depressant action upon the contractile elements of the blood vessels. On the other hand they may react with the receptors involved in the process of vasoconstriction, making it impossible for compounds such as Ad, 5-HT, Hm, NA and $BaCl_2$ to exert their effects.

Hydrallazine and dihydrallazine potentiated the effects of KCl, ACh and NHT on the frog rectus. On the other hand, C 10 is antagonised and there is antagonism to TC block of ACh contractions. These effects are contradictory and at the moment we can offer no explanation of their mechanism.

3-Phenyl-6-hydrazino-pyridazine HCl, 1-hydrazino-*iso*quinoline HCl and 3:6-dihydrazino-pyridazine nitrate have some properties in common with hydrallazine and dihydrallazine, but in other respects they differ. All lower the cat blood pressure, 3:6-dihydrazino-pyridazine nitrate being the most potent. All antagonise vasoconstriction due to Ad, NA, $BaCl_2$, Hm and 5-HT in isolated vascular beds. Blockade of the pressor reflexes was weak or absent, but 3:6-dihydrazino-pyridazine nitrate antagonised the pressor response to stimulation of the cut central end of the sciatic nerve. Adrenergic blockage on other preparations was not seen. On the guinea pig ileum the three compounds non-specifically antagonise the contractions of ACh, Hm or 5-HT. They antagonise C 10 and NHT on the frog rectus, but do not antagonise contractions caused by ACh, but 1-hydrazino-*iso*quinoline HCl potentiated the response to ACh.

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DISCUSSION

The paper was presented by MR. S. M. KIRPEKAR.

DR. G. E. FOSTER (Dartford) asked about the toxicity of the compounds.

DR. G. BROWNLEE (London). What was the significance of the pressor component of adrenaline at AA in Figure 3? Did the authors prove it to be a cardiac effect? It was clearly different from the effect seen with noradrenaline. Was isoprenaline also used? If there were a peripheral site of action, then there seemed to be an effect in addition to the one being demonstrated. Since there was evidence that the pressor effects were being modified, the word "modified" and not "antagonise" might have been preferred.

MR. E. M. BAVIN (Ware). Were the effects tried in other hypotensive animals such as dogs and rats.

DR. B. K. MARTIN (Slough). The maximum concentration employed by the authors was roughly one thousand times that of the minimum concentration, but to consider the mode of action, it was necessary to operate in a much narrower concentration range.

MR. T. D. WHITTET (London). The compounds had not proved satisfactory by themselves, but when used with ganglion-blocking agents the total effect was increased.

MR. H. D. RAPSON (Dorking) said that the response curves looked like sigmoid curves. Was there any relationship between the significance

of the sigmoid curve and mode of action of pharmacologically active material?

The AUTHORS in reply said it had been shown that about 200 mg./70 kg. was not very toxic; and for dihydrallazine this could be increased to 500 mg./70 kg. LD50's in mice for hydrallazine were about 80 mg./kg. and for dihydrallazine about 200 mg./kg. Isoprenaline had not been used. The remaining pressor response was due to a cardiac effect and the evidence pointed to a direct action on the walls of the blood vessels rather than to some action in the brain or spinal cord. In Figure 5 it would be noticed that there was a fairly considerable fall in blood pressure over a period—in that case of thirty minutes. The word "antagonise" was used in the conventional sense. Other animals had not been used and in the experiments concentrations were maintained which were roughly equivalent to those found clinically in the blood. When hydrallazines were used with ganglion-blocking agents they were more effective. It was hazardous to deduce mathematical data from the response curves.

THE RHEOLOGY OF OIL-IN-WATER EMULSIONS

PART II. THE MICROSCOPICAL APPEARANCE OF EMULSIONS IN LAMINAR FLOW

BY ARNOLD AXON

From the Wellcome Chemical Works, Dartford, Kent

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A microscope cell has been made to examine semi-solid oil-in-water emulsions while flowing in a thin layer. Unautoclaved and autoclaved emulsions from cetyl alcohol, sodium lauryl sulphate, liquid paraffin with and without bentonite show differences in the dispersion of the globules. Those in the unautoclaved emulsions are mainly associated, "flocculated", into loose clusters in which each globule is free to move independently of its neighbours. Composite masses of disperse phase, called agglomerates, are formed in emulsions prepared from a preformed "emulsifying wax" which are absent from emulsions formed by adding sodium lauryl sulphate to the aqueous phase. A compact type of floccule occurs in the autoclaved emulsions containing bentonite. The globules are surrounded by a hydrated layer of bentonite which restricts their movement and provides a mechanical barrier against deformation. Rigidity is supplied by association of the hydrated bentonite particles in a network to form a gel. The globules in the autoclaved emulsions without bentonite are readily deformed and show complete freedom of movement with no association into clusters.

IN a previous paper[1] semi-solid emulsions were shown to have anomalous viscous behaviours which can be distinguished in a complete consistency curve determined on a rotational viscometer. Subsequently it was suggested[2] that the change from shear-rate thinning to uniform plastic flow, occurring when an emulsion containing bentonite was autoclaved, depended on the state of dispersion of the globules.

This paper records the differences in microscopical appearance of semi-solid oil-in-water emulsions prepared from liquid paraffin, sodium lauryl sulphate, cetyl alcohol with and without bentonite. The emulsions in motion are examined as a thin film and the observed microscopical differences are related qualitatively with the different anomalous viscous behaviours and the general macroscopic appearances. The presence of groups of globules (floccules or clusters) and of complex structures (agglomerates) which affect the appearance and consistency of the emulsions, and which are unrecognised in a static preparation, can be readily detected while the preparation is in motion. A microscope cell unit has proved valuable for the examination of emulsions of high disperse phase concentration. It is suitable for both direct and dark ground illumination.

The State of Dispersion of the Disperse Phase

The dispersion of fine particulate matter in a fluid may lead to the formation of small groups of particles. Pryce-Jones[3] found that the state of flocculation of the particles determined the macroscopical flow properties of paint and the ease of re-distribution of the sediment after storage.

The degree of dispersion of the globules in concentrated emulsions has received little study, although clustering of the globules has been postulated to explain variation in the gravity creaming of milk[4] and rubber latex[5,6], and the increase in viscosity of dairy cream which occurs in the re-bodying process[7], and also the increase in consistency caused by homogenisation of oil-in-water emulsions[8]. Clustering of globules is known to occur in fluid emulsions[9,10], and its extent assessed in a roughly quantitative way by measuring both the rate of creaming and the volume of the cream formed with time[11].

The Emulsion Globule Membrane

The existence of an emulsion membrane in milk was postulated by Ascherson in 1840 in the "Haptogen" membrane theory[12], and confirmed microscopically after staining[13,14] and also by electron photomicrographs[17,18]. This membrane has been isolated and shown to be a combination of protein and phospholipid[19,20]. Elkes and others[21] have demonstrated the orientation of protein and phospholipid at the oil : water interface. A similar membrane is postulated for rubber latex[22]. Emulsions with casein[23] and saponin[24] as emulsifying agents also show membranes. Many finely divided powders will orientate at the oil : water interface and act as emulsifying agents by forming a mechanical barrier which inhibits the coalescence of globules[25–27]. Levich[28] has suggested that the adsorbed stabiliser forms an envelope of structured liquid around each globule of the disperse phase. Bancroft in 1913 put forward the first definite theory of the role of the "soluble" emulsifying agent at the oil : water interface as a film of two surface tensions, one on the water side and one on the oil side[29].

The Microscopy of Disperse Systems

Green[30] has pointed out some of the difficulties, such as distortion of the globules and the apparently regular polyhedral appearance of stiff concentrated emulsions when examined under the microscope and has suggested a method for obtaining a satisfactory preparation. Emulsions and suspensions have been examined microscopically to obtain size distribution data[31,32] and to show the effect of freezing[33], electrical currents[34,35] and flocculating agents[36]. The flocculation of globules seem to have been confined to fluid preparations and dilutions of concentrated preparations in static mounts.

The rate of flow of disperse systems in capillary tubes has been used to determine the rheological properties of the dispersions[37,38]. Green and Haslam[39] have used a narrow capillary tube for the direct microscopical measurement of the plastic viscosity and yield value of paints, but it gives little indication of the finer details of interparticle relations.

Apparatus

A paper describing the use of an ultramicroscope for the detection of particles in smoke suggested a possible method for examining flowing emulsions[40].

The apparatus devised consists of a microscope cell which fits on the stage. A hypodermic syringe is attached to the perspex disc of the cell and provides a reservoir for the emulsion and a convenient means for propelling it. The cell unit (Fig. 1) consists of four parts: a pressure plate, a microscope cover glass, and a tunnelled perspex disc clamped together in a suitable metal fitment.

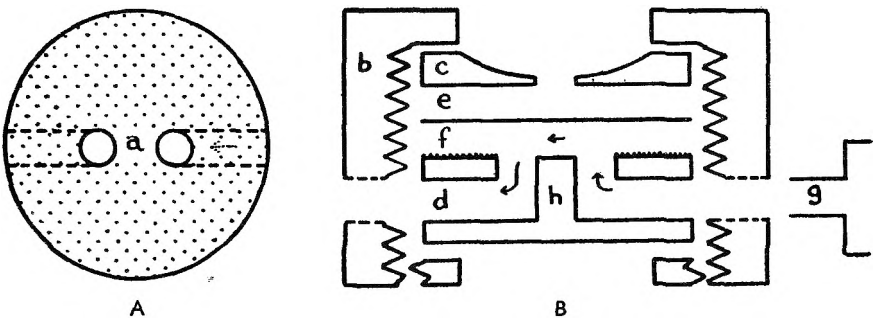

FIG. 1. Microscope cell unit. A. Plan of tunnelled gold-leafed perspex disc. B. Side elevation of cell unit.

a, Channel for laminar flow of the emulsion, from above, stippled area—gold leaf.
b, Brass union.
c, Pressure plate.
d, Channel.
e, Cover glass.
f, Gold leafed area.
g, Hypodermic syringe.
h, Perspex disc.

The tunnelled gold-leafed perspex disc. Two holes $\frac{1}{8}$ in. in diameter were bored diametrically opposite each other to within 1/16 in. of the centre of a circular piece of perspex $\frac{1}{4}$ in. thick. Two similar holes approximately $\frac{1}{8}$ in. apart were bored from the upper surface of the perspex disc to complete the tunnelling. Gold leaf was applied to the upper surface of the disc except for the area between the two orifices. A microscope cover glass was placed on top of the gold-leafed perspex disc to enclose a small channel for the passage of the emulsion.

A metal pressure plate was placed over the cover glass to prevent bowing under the pressure of the flowing emulsion, which was viewed through a small central hole $\frac{1}{8}$ in. in diameter. The cell was encased in a brass union provided with holes contiguous with those in the perspex disc. The apparatus is compact, easily made, assembled, used, dismantled and cleaned.

EXPERIMENTAL

Oil-in-water emulsions were prepared by two methods to the following formulae: 1, 2, 3, and 4 and to the same formulae: I, II, III, and IV with the addition of 2 per cent of bentonite. An 8 per cent w/w suspension of bentonite was prepared in distilled water before the preparation of the emulsions. The characteristics are shown in Table I.

	1	2	3	4
Sodium Lauryl Sulphate B.P. ..	0·2	0·4	0·6	0·8 g.
Cetyl alcohol	1·8	3·6	5·4	7·2 g.
Liquid Paraffin B.P.	25·0	25·0	25·0	25·0 g.
Distilled Water B.P. .. to	100·0	100·0	100·0	100·0 g.

Method A. The cetyl alcohol was heated to 115°, to reproduce the conditions used in method B for the preparation of "emulsifying wax". Liquid paraffin was added to the hot cetyl alcohol and re-heated to 70°. The sodium lauryl sulphate was dissolved in hot water at about 70° and made up to weight at 70°, with water or bentonite suspension. The aqueous phase was added to the oil phase and the mixture homogenised with an immersion type homogeniser for one minute. The emulsion was poured immediately into a previously warmed screw capped jar which was sealed with a white rubber closure and a metal screw cap. The jar was slowly rotated on rollers while cooling to 25°, when the emulsion was distributed in several jars and each was sealed as before.

Method B. An "emulsifying wax" was prepared as described for the B.P. preparation from cetyl alcohol and sodium lauryl sulphate. The sodium lauryl sulphate was dispersed in the melted cetyl alcohol at 95° and 4 g. of water added for each 100 g. of "emulsifying wax". The mixture was heated to 115°, with vigorous agitation until a translucent mass resulted. Liquid paraffin was added and the mixture heated to 70°. Sufficient distilled water or bentonite suspension at 70° was added to the mixture. The two phases were mixed and the emulsions prepared and packed as in Method A.

Subsequent Treatment of Emulsions

Heating to 115°. The jars of emulsion were autoclaved at 115° for 30 minutes and gently rotated on rollers while cooling to 25°. They were kept in a room maintained at 25° ± 1°.

Microscopical Appearance of Emulsions

Unautoclaved emulsions prepared without bentonite. The unautoclaved emulsions prepared by methods A and B flowed steadily through the microscope cell on application of slight pressure. The main body of globules moved in several streams or lamellae in which they have freedom of movement. The emulsion continued to flow after withdrawal of the applied pressure and gradually slowed and ceased. While most of the globules were stationary, some movement of the smaller globules in minute channels was often then seen. This system is described as being "loosely flocculated".

Emulsions prepared by methods A and B were readily distinguished. Those from an "emulsifying wax" (method B) showed numerous large masses (Figs. 2*b*, 3*a*) which were absent in emulsions prepared by method A (Fig. 2*a*). Each mass moved as a unit and was sufficiently compact to resist disruption, although its form was modified by pressures from the

TABLE I

THE CHARACTERISTICS OF SEMI-SOLID OIL-IN-WATER EMULSIONS

Without bentonite (white coloured)

	Macroscopical characters				Rotational viscometer consistency			Microscopical characters	
	General appearance		Feel	Consistency		U_{200}*	f_{200}†	Flow behaviour	Globules
Unautoclaved									
4.A	Granular	Glossy Wet	Wet and cold Glovelike Easily absorbed	Soft	Shear-rate thinning	4·20	853	Smooth lamellae Loosely flocculated	Readily distorted
4.B	Granular Grainy	Semi-matt Greasy	Warm and greasy Not readily absorbed	Soft	Shear-rate thinning	4·20	1110	Smooth lamellae Loosely flocculated Presence of agglomerates	Readily distorted
Autoclaved									
4.A	Smooth	Glossy Wet	Wet and cold Glovelike Easily absorbed	Soft	Shear-rate thinning	4·13	744	Globules completely free Deflocculated	Readily distorted
4.B	Smooth Slightly grainy	Semi-matt	Warm and greasy Not readily absorbed	Soft	Shear-rate thinning	4·79	866	Globules completely free Deflocculated	Readily distorted

With bentonite (cream coloured)

Unautoclaved									
IV.A ..	Granular	Glossy Wet	Wet and cold Glovelike Easily absorbed	Soft	Shear-rate thinning	5·09	1205	Smooth lamellae Loosely flocculated	Readily distorted
IV.B ..	Granular Grainy	Semi-matt Greasy	Warm and greasy Not readily absorbed	Firm	Shear-rate thinning	4·27	1706	Smooth lamellae Loosely flocculated Presence of agglomerates	Readily distorted
Autoclaved									
IV.A ..	Smooth	Glossy Wet	Wet and cold Glovelike Easily absorbed	Medium-firm	Plastic flow	5·75	1462	Smooth lamellae Flocculated	Sheathed in a hydrated layer
IV.B ..	Smooth Slightly grainy	Glossy Wet	Wet Not readily absorbed	Medium-firm	Plastic flow	5·60	1421	Smooth lamellae Flocculated	Sheathed in a hydrated layer

* U_{200}: The "plastic viscosity" for emulsions showing plastic flow, and the limiting viscosity for emulsions showing shear-rate thinning, determined at 200 r.p.m. ($91{\cdot}4^{-1}$ sec.).

† f_{200}: The yield value in dynes per sq. cm. for emulsions showing plastic flow, and the torque intercept for emulsions showing shear-rate thinning determined at 200 r.p.m. ($91{\cdot}4^{-1}$ sec.).

flowing globules. This mass did not appear to correspond to that of a cluster or of a multiple globule described by Seifriz[41]. It is described as "an agglomerate" and consisted of an apparently unorganised granular matrix in which individual globules and small groups of globules were present (Fig. 3*b*).

Unautoclaved emulsions containing bentonite. The unautoclaved emulsions containing bentonite were indistinguishable from those without bentonite. The bentonite particles presented no distinguishing characteristics.

Autoclaved emulsions without bentonite. The autoclaved emulsions prepared by methods A and B flowed readily through the microscope cell on application of a slight pressure. The globules moved with complete freedom without forming the distinct streams seen in the unautoclaved emulsions. The globules are fairly easily deformed. The flow continued after withdrawal of the applied pressure and gradually slowed and ceased. No agglomerates were present in either of the autoclaved emulsions. This system is described as "deflocculated" (Figs. 2*c*, 3*c*).

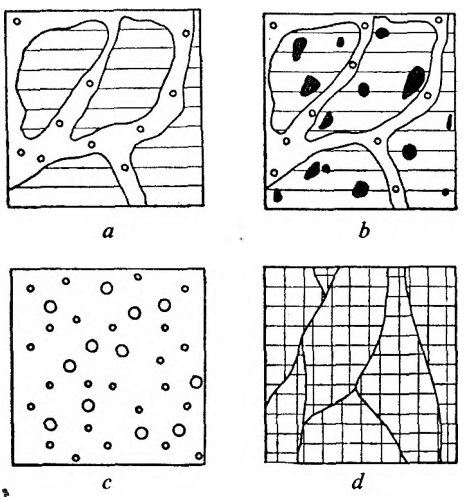

FIG. 2. Diagrammatic representation of the disperse phase in autoclaved and unautoclaved oil-in-water emulsions.

a, Unautoclaved emulsion prepared either with or without bentonite by Method A.

b, Unautoclaved emulsion prepared either with or without bentonite by Method B.

c, Autoclaved emulsion prepared without bentonite by both methods.

d, Autoclaved emulsion prepared with bentonite by both methods.

Horizontal lines, loose floccule of many hundred globules; squares, compact floccule of many hundred globules; open circles, globule of disperse phase; black areas, agglomerate.

Autoclaved emulsions containing bentonite. The autoclaved emulsions containing 2 per cent bentonite prepared either by methods A or B presented a marked resistance to flow and pressure was needed to begin flow. The flow ceased immediately on withdrawal of the applied pressure and a pulsating flow followed the intermittent application of pressure. A controllable rate of flow was satisfactorily achieved by using an Agla micrometer syringe. When in flow, the globules moved in several streams or lamellae, and in each stream moved with the same relative velocity. The globules appeared to have little individual freedom of movement, the larger globules showing a readily observable boundary around them which prevented distortion and seemed to link the globules together. This system is described as "flocculated" (Figs. 2*d*, 3*d*).

DISCUSSION

Some of the macroscopical differences seen in the appearance and consistency of emulsions can be related qualitatively to the observed microscopical differences. The flow of oil-in-water emulsions in a thin

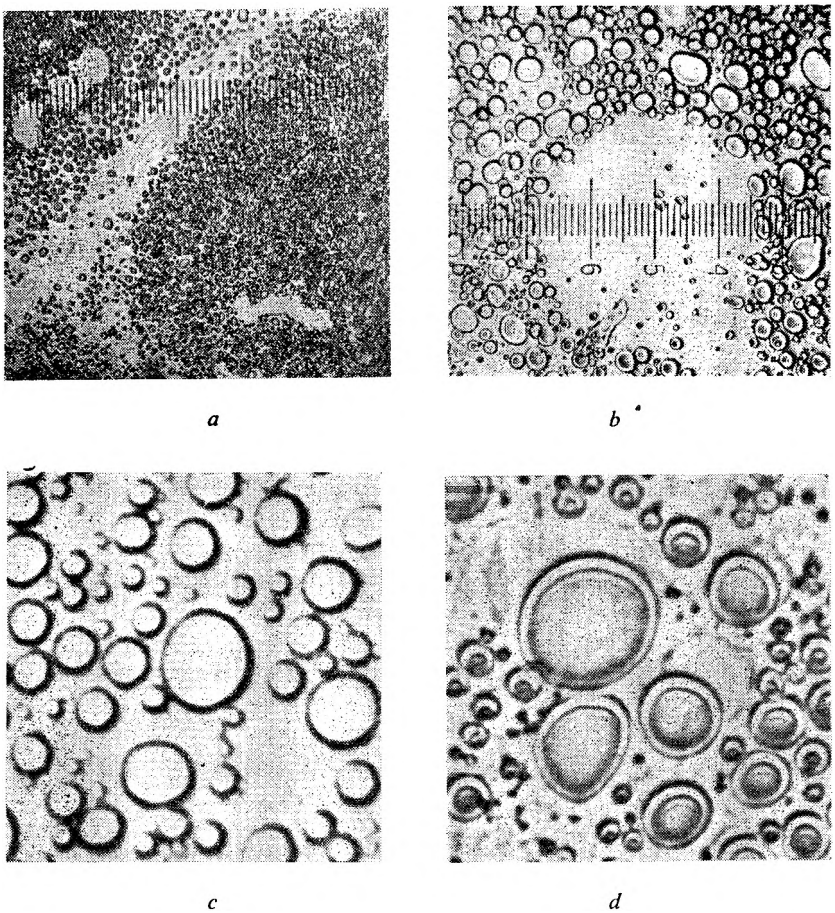

FIG. 3. Photomicrographs of:—

a. An unautoclaved emulsion (Method B). The presence of channels between the clusters in which isolated globules are freely moving and the presence of agglomerates within the clusters illustrate the characteristics typical of an unautoclaved emulsion prepared from an "emulsifying wax." (Compare Fig. 2*b*.) Magnification × 75.

b. An agglomerate (Method B). Magnification × 300.

c. An autoclaved emulsion prepared without bentonite (Compare Fig. 2*c*.) Magnification × 1000.

d. An autoclaved emulsion prepared containing bentonite. Three of the globules show the hydrated layer of bentonite typical of autoclaved emulsions. Magnification × 1000.

film shows differences in the disperse phase. The manner of movement of the globules and the relative pressure necessary to produce flow readily distinguishes different forms of globule association. Globules are associated into clusters in two different ways; the globules are free to move within the cluster (unautoclaved emulsions prepared with and without bentonite); the globules have little or no freedom within the cluster

(autoclaved emulsions prepared containing bentonite). The disperse phase can be obtained as globules which have complete freedom of movement (autoclaved emulsions prepared without bentonite); or dispersed as a composite mass, an agglomerate (unautoclaved emulsions prepared from an "emulsifying wax").

The "flocculation" of the globules in the unautoclaved emulsions is loose. Each globule is readily deformed and is free to move independently of its neighbours. Most of the globules are flocculated in this way, but other globules in the emulsion are completely free to move. This emulsion appears therefore as a heterogeneous dispersion of fairly dense floccules separated by a more fluid region in which the globules are free to move. On visual examination, these differences are recognised and described as "granularity".

A more compact type of flocculation of globules occurs in the autoclaved emulsions containing bentonite. The globules are restricted in their movement by a distinct zone which provides a mechanical buffer against deformation and a barrier to the approach of other globules. The floccules move as distinct streams or lamellae which are not separated by a more fluid region. This homogeneous dispersion produces an even scattering of light and is seen as a smooth and glossy emulsion.

The globules in the autoclaved emulsions prepared without bentonite are completely dispersed. All the globules show complete freedom of movement and are readily deformed. Since the globules are too small to be recognised by the eye as discrete units, the dispersion appears homogeneous and this produces an even scattering of light; it is a smooth and glossy emulsion.

The use of an "emulsifying wax" gives emulsions which show a striking microscopical appearance readily distinguished from unautoclaved emulsions prepared by method A. Numerous masses described as agglomerates are present. In the freshly prepared emulsions the matrix of the agglomerate appears to be of an unorganised granular nature, which in the aged becomes crystalline. The agglomerates are thought to be mainly composed of higher fatty alcohol. The presence of a large number of agglomerates increases the heterogeneity of the emulsion and gives a fine "grainy" appearance and a matt surface to the emulsion.

The consistency of an emulsion is a measure of the internal friction or its resistance to flow. Three factors concerned are, the resistance offered by the individual globules, the resistance offered by the clusters of globules, and the resistance offered by the agglomerates. The unautoclaved emulsions show an easy deformation of both the globules and floccules during flow; this would be expected therefore to produce a decrease in consistency with increasing rate of shear, namely shear-rate thinning. The presence of agglomerates and of the fine granular or colloidal dispersion of the cetyl alcohol in the emulsions prepared from an "emulsifying wax", would be expected to produce a noticeably thicker consistency. The emulsions would be expected to be slightly stiffer when prepared containing bentonite.

From the microscopical appearance of the autoclaved emulsions, those containing bentonite would be expected to offer the greatest resistance to flow. Deformation of the individual globules is prevented by a mechanical barrier and the free flow of the clusters is hindered by their compactness. The flow behaviour will show a distinct break when sufficient pressure has been applied to cause the rigidly held clusters to flow. Determination of a flow curve with the rotational viscometer, previously reported[1], does show a distinct yield value, before true flow begins. The bentonite particles appear to have become orientated at the oil : water interface during autoclaving to form a hydrated matrix around the oil globules. This matrix behaves as a bentonite gel and therefore imparts rigidity to the emulsion, changing the flow properties from shear-rate thinning to plastic flow.

The autoclaved emulsions prepared without bentonite would be expected to show the least resistance to flow because all the globules are readily deformed and completely free to move.

Acknowledgement. The author thanks Mr. M. H. Bennie of The Wellcome Chemical Works for his craftsmanship.

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DISCUSSION

The paper was presented by the AUTHOR.

MR. N. J. VAN ABBÉ (Loughborough). Modification due to reheating of emulsions not containing bentonite was often seen; this he thought was a matter of orientation of the hydrophilic-hydrophobic relation of the emulsifying agent. Was this taking part in the reported results? How much of the effect reported was due to the method of homogenisation or to ageing?

MR. HOBBS (Bristol). Had the behaviour of the emulsions in relation to changes in pH likely to be due to high temperature and time of heating been investigated?

MR. S. G. E. STEVENS (London). Were the effects the same for all kinds of bentonite?

MR. C. L. J. COLES (Greenford). Was not the granularity associated with solubility of cetostearyl alcohol in liquid paraffin at room temperature?

MR. A. E. DAVIS (Nottingham). Was there some special technique of applying gold leaf?

MR. H. D. RAPSON (Dorking). If a non-polar solvent were present, such as paraffin, there was inside the paraffin globule a system analogous to grease. This showed three or four phases which were temperature dependent and had hysteresis effect. If the solid phase was at equilibrium at the storage temperature, then it was probably a stable emulsion. If, however, it had been prepared at higher temperature, changes might take place at lower temperatures and the emulsion was probably unstable.

MR. N. J. VAN ABBÉ (Loughborough). Clarity might be obtained on some of the points by comparing the effect of sodium laurylsulphate emulsifying wax with non-ionic waxes.

MR. A. W. BULL (Nottingham) thought temperature was most important, particularly in relation to the lyophilic barriers. Had the jars been rotated on rollers at 45°, and then the emulsions allowed to cool without further agitation probably material differences would have been found.

MR. AXON replied he had not listed all changes in consistency. The point of interest was the concentration of the agglomerates which would

appear to increase with the concentration of the emulsifying wax. A change did take place in emulsions not containing bentonite, but it was not known whether this was due to change of orientation in the emulsifying agent at the interface. He had used only one homogeniser and had passed homogenised emulsions through a colloid mill where globule size had been reduced from 10 μ to 1 or 2 μ and the agglomerates had also been reduced in size. He had prepared emulsions by homogenisation only at 70°. The only difference after ageing of the emulsions was an observed increase in the crystalline nature of some of the disperse phase. He agreed that this was an important feature in maintaining stability. With a cetyl alcohol content of 10 per cent, globules with a marked crystalline nature were present but they were not observed in concentrations lower than 8 per cent. He did not know what happened to cetyl alcohol on homogenisation. There was little change in pH of emulsions containing bentonite which had been autoclaved. Only one bentonite had been used throughout and Mr. Coles was probably correct about the granularity. In making the cell units the Perspex was rubbed on paper and then applied to the gold leaf, which adhered for one operation. If gold size was applied to the Perspex the gold leaf adhered satisfactorily and could be used twenty times. The main point of the work was to be able to investigate a semi-solid emulsion a little better than was possible at the present time.

THE SPECTROPHOTOMETRIC DETERMINATION OF $\alpha\beta$-UNSATURATED ALDEHYDES AND KETONES WITH GIRARD-T REAGENT

PART I. ESSENTIAL OILS

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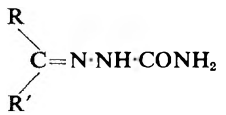
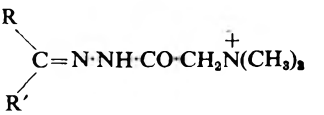
Spectrophotometric methods for the determination of citral, cinnamaldehyde and carvone in essential oils using Girard-T reagent are described. The results are compared with those obtained by the hydroxylamine hydrochloride method.

REVIEWS of the determination of aldehydes and ketones in volatile oils[1–5] and other substances[6] are frequent and indicate the difficulties inherent in the assays. Unless a reagent can be made almost specific for one type of carbonyl compound, for example *m*-phenylenediamine dihydrochloride for the $\alpha\beta$-unsaturated group[7], chemical methods do not distinguish between different types and the result is necessarily expressed in terms of the major component such as citral in lemon oils. This account is concerned with the development of a rapid spectrophotometric method for the determination of the $\alpha\beta$-unsaturated carbonyl compounds citral, cinnamaldehyde and carvone in volatile oils. The above mentioned *m*-phenylenediamine colour reaction[7] has been used for the determination of $\alpha\beta$-unsaturated aldehydes in oils, but it does not react with carvone, and, moreover, suffers from the disadvantage that the colours fade rapidly. Measurement of the general ultra-violet absorption of Californian and Italian lemon oils has been used[8,9] as a criterion of purity, but the high probability of irrelevant absorption in the appropriate wavelength region renders the method of doubtful value for quantitative work. Carvone has, however, been determined by means of the high intensity $\alpha\beta$-unsaturated carbonyl absorption[10] a carvone-free oil being used to compensate for the absorption by terpenes and other constituents. Interference due to irrelevant absorption can be overcome by conversion of the carbonyl compounds to the corresponding semicarbazones and 2:4-dinitrophenylhydrazones which exhibit characteristic high intensity absorption at longer wavelengths than those associated with the parent compounds. Such derivatives however still require organic solvents for solution, and the volatile oils would of necessity be retained in the solution undergoing measurement. This latter disadvantage can, however, be overcome by the use of Girard-T reagent, the derivatives of which are water-soluble and readily separated from non-carbonyl constituents of the oil. Like the semicarbazones Girard-T hydrazones of $\alpha\beta$-unsaturated carbonyl compounds show considerably greater absorption than the parent substances. This, together with the longer wavelength of the maxima, not only tends to reduce errors caused by irrelevant absorption, but also increases the sensitivity of the method by a factor of about 2·5.

Girard reagents[11] have been used extensively for the isolation of aldehydes and ketones from natural products and reviews appear occasionally on this subject[12,13]. For the quantitative determination of the hydrazones polarography[14–19] has proved a useful tool, but ultra-violet absorption has received little attention. Höyer[20] noted the similarity of the chromophore in semicarbazones (I) and Girard-T hydrazones (II).



(I)



(II)

and made use of the ultra-violet absorption to determine αβ-unsaturated ketosteroids in urine. Spectrophotometry was also used by Young[21] to confirm the formation of Girard-T derivatives of some saturated ketones. For quantitative work, however, the absorption of saturated derivatives, although intense at 230 to 240 mμ is of less value because of the increase in the absorption by the reagent in this region. Ideally, the maximum absorption should occur at a wavelength greater than about 265 mμ above which there is little interference. The Girard-T hydrazones of αβ-unsaturated carbonyl compounds comply with this condition.

Development of Method

Standards

Cinnamaldehyde yielded a crystalline Girard-T derivate m.p. 175° *E* (1 per cent, 1 cm.) at 313 mμ = 1492, but the poor analytical figures and its hygroscopic nature confirmed its unsuitability as an analytical standard. We therefore used pure cinnamaldehyde as standard, which *when treated by the given method*, gave an *E* (1 per cent, 1 cm.) value equivalent to that obtained with the crystalline Girard-T hydrazone. Thus despite our inability to isolate the corresponding crystalline Girard-T derivatives from citral and carvone we consider that *pure citral* and *pure carvone* provide satisfactory standards.

Pure citral was prepared by Tiemann's method as modified by Hibbert and Cannon[22]. The product is a mixture of citral-*a* and citral-*b*. As the ultra-violet absorption spectra of the corresponding semicarbazones are identical, it is assumed that those of the Girard-T hydrazones are also identical.

Carvone of satisfactory physical constants was prepared from dill oil through the semicarbazone which was isolated in the form m.p. 140 to 141°.

Typical absorption curves for reagent and the Girard-T hydrazones of citral, cinnamaldehyde and carvone are given in Figure 1. The molecular extinction coefficients of the hydrazones and those published for the corresponding semicarbazones (Table I) are similar. The bathochromic shift of the absorption maximum is attributed to solvent effect. This comparison provides support for accepting the curves obtained with citral, carvone and cinnamaldehyde (Fig. 1) as being due to the hydrazones although pure crystals of these hydrazones were not isolated. Derivatives

prepared from natural oils showed no variation in the wavelengths of the maxima (Table II).

We have shown (Fig. 2) that the absorption of all three compounds obeys the Beer-Lambert Law, and this eliminates the need for calibration curves in the methods described.

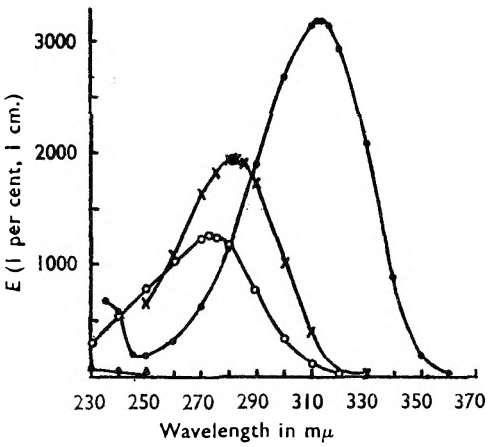


FIG. 1. Absorption spectra of citral (×), cinnamaldehyde (●) and carvone (○) after the treatment described under the appropriate method of assay. Girard-T reagent (▲).

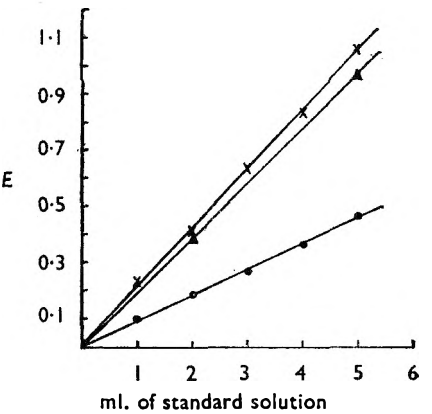


FIG. 2. Calibration curves for citral (×) 0·559 per cent w/v in ethanol; cinnamaldehyde (▲) 0·366 per cent w/v in ethanol; carvone (●) 0·770 per cent w/v in ethanol.

Reaction Solvents

Owing to the different rates at which aldehydes and ketones react with Girard-T reagent, different solvents were found to be necessary. Citral and cinnamaldehyde reacted quantitatively in ethanol, in agreement with the observations of Lederer and Nachmias[26]. Carvone required the solvent proposed by Girard and Sandulesco[11] which is a 10 per cent solution of glacial acetic acid in ethanol. Lemon oils, which contain only small quantities of carbonyl compounds, modify the solvent system appreciably and appear to slow the reaction. The decrease in the *E* (1 per cent, 1 cm.) value was seen in preliminary control experiments with solution of citral in oil of turpentine. The difficulty can be overcome by increasing the quantity of reagent and limiting the amount of essential oil. On the other hand the concentration of carbonyl compounds in cinnamon, dill and caraway oils is very much higher and in consequence the oil sample is much smaller so that the effect of terpenes in such materials was neglected. *E* (1 per cent, 1 cm.) values found in this series are lower than the standard figure for citral even when turpentine was absent. Satisfactory results were obtained only when the usual 30 seconds drainage time for pipetting ethanolic solutions was extended to 80 seconds for the citral solution at which time the pipette delivered exactly one tenth of the quantity of solution in a 50 ml. standard flask. Complete concordance was then found between results obtained by direct weighing of samples and those in

TABLE I

MOLECULAR EXTINCTION COEFFICIENTS OF GIRARD-T HYDRAZONES AND SEMICARBAZONES OF CITRAL, CINNAMALDEHYDE AND CARBONE

Compound	Girard-T hydrazone		Semicarbazone		Ref.
	ε	λ max. (mμ)	ε	λ max. (mμ)	
Citral	29,500	281	31,350	272	23
Cinnamaldehyde ..	42,000	313	40,200	310	24
Carvone	19,000	272·5	21,200	265	25

which a weighed quantity had been diluted and an aliquot portion taken by volume.

Reaction Time

Using a twofold excess of the reagent, reaction with citral and cinnamaldehyde in ethanol was complete in 10 minutes, and a standard time of 12 minutes was therefore chosen. As expected carvone was slow to react even in acetic acid-ethanol. Evaporation of the ether remaining after the extraction of the carvone Girard-T hydrazone yielded traces of oil when natural oils were examined. These residues gave positive reactions with 2:4-dinitrophenylhydrazine when less than 60 minutes were given for the reactions, confirming that the reactions were incomplete. We have confirmed spectrophotometrically that the reaction was complete in 60 minutes (Fig. 3) and a standard time of 70 minutes was adopted for carvone.

TABLE II

Oil	Quantity (mg.)	Dilution (ml.)	λ max. (mμ)	E (1 per cent, 1 cm.)
Citral	10–15	10 to 100	281	1937
Lemon	300	10 „ 100	281	
Lemon grass	15–20	10 „ 100	281	
Cinnamaldehyde ..	10–15	10 „ 250	313	3180
Cinnamon bark ..	20–30	10 „ 250	313	
Cinnamon leaf ..	200	10 „ 250	313	
Carvone	15–20	10 „ 100	272·5	1267
Dill	20	10 „ 100	272·5	
Caraway	20	10 „ 100	272·5	

Stability of Derivatives

Girard-T hydrazones regenerate the parent carbonyl compounds under acid-aqueous conditions, and as the final solutions in some of the assays were of pH 5·1 to 5·3, the optical density was checked at intervals to detect possible dissociation of the complex. No change in the optical density was observed over many hours.

Calculation of Results

Since the observed absorption is strictly proportional to concentration, results can be calculated by using the E(1 per cent, 1 cm.) values given in Table II. These values are referred to the parent carbonyl compound *when treated by the given method* and are not the E(1 per cent, 1 cm.) values for the corresponding Girard-T hydrazones.

Recovery Experiments with Citral

The quantitative nature of the method finally adopted for the determination of citral in essential oils was demonstrated by a series of recovery experiments on mixtures of pure citral and oil of turpentine. There was prepared 4·93, 4·11, 3·30 and 3·55 per cent w/w; found respectively, 4·95 (100·4), 4·11 (100), 3·34 (101·2) and 3·53 (99·5). Per cent recoveries are given in parentheses.

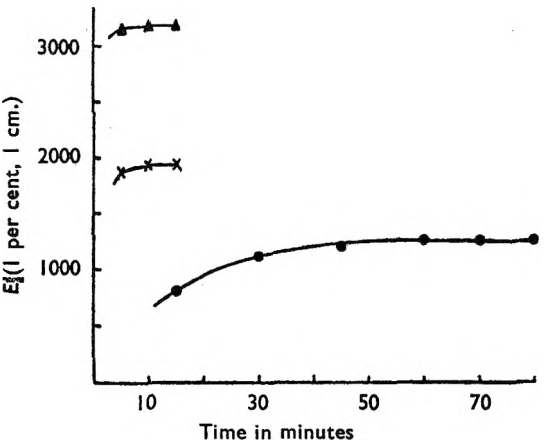


FIG. 3. Rates of reaction of citral (×), cinnamaldehyde (▲) and carvone (●) with Girard-T reagent in an appropriate solvent.

EXPERIMENTAL

Melting points are uncorrected. Ultraviolet absorption spectra were measured in 1 cm. cells using a Hilger Uvispek spectrophotometer, model H700/303.

Preparation of Standards. Citral. Commercial citral (49 g.) was added to a cooled solution of sodium sulphite (175 g.) and sodium bicarbonate (62·5 g.) in water (500 ml.), and the mixture was shaken vigorously for one hour. The oily phase was extracted with ether (2 × 300 ml.) and rejected. The aqueous phase was treated with 10 per cent sodium hydroxide solution until the solution became turbid and was extracted immediately with ether (2 × 300 ml.). The bulked ether layers were washed with tartaric acid solution until the washings were acid to litmus, dried with sodium sulphate and evaporated. The residue was fractionally distilled and the fraction (25·2 g.) b.p. 118°/16 mm. reserved as standard. The first runnings (4·5 g.) and residue (5·1 g.) were rejected. The standard was an almost colourless oil $n_D^{16\cdot5°}$ 1·4907, d_{20}^{20} 0·8895, E(1 per cent, 1 cm.) at 281 mμ = 1937 when treated by the method given below.

Cinnamaldehyde Girard-T hydrazone. Cinnamon oil (4 g.) was treated with Girard-T reagent (3 g.) in ethanol (10 ml.) and heated on a boiling water bath for 30 minutes. The crystalline product which separated on slow cooling was filtered off and recrystallised from ethanol as pale yellow needles, m.p. 175° (decomp.) E(1 per cent, 1 cm.) at 313 mμ = 1492.

Cinnamaldehyde. Cinnamaldehyde of reagent grade was distilled under reduced pressure and the pale yellow fraction b.p. 134°/22 mm. $d_4^{17°}$ 1·049, $n_D^{17°}$ 1·6215, used as standard.

Carvone. Oil of dill (22 g.) was heated for 1½ hours with semicarbazide hydrochloride (15 g.), sodium acetate (anhydrous, 12 g.) and ethanol (90 per cent, 200 ml.). The mixture was cooled, diluted with water (100 ml.) and set aside until the turbid liquid deposited a bulky mass of

crystals. The semicarbazone was washed free from oil, and recrystallised from ethanol as colourless flat needles (9 g.) m.p. 140 to 141°. The crystals were decomposed by addition of phthalic acid (3 g.), and steam-distillation until 1200 ml. of distillate had been collected. The carvone was isolated from the distillate by ether extraction, drying with sodium sulphate and evaporation of the ether. Distillation of the residue under reduced pressure yielded a colourless oil (3·5 g.) b.p. (bath temp.) 120°/18 mm. $n_D^{20°}$ 1,5000, $d_{18°}^{18°}$ 0·9625, $[\alpha]_{18°}$ +61·7° (C = 2·2 in ethanol), E(1 per cent, 1 cm.) 272·5 mμ = 1267 when treated by the method given below.

Reagents

Girard-T reagent recrystallised twice from ethanol and stored over sulphuric acid; ethanol (absolute); solvent ether; glacial acetic acid (A.R.); N sodium hydroxide.

Method for the Determination of Citral and Cinnamaldehyde in Essential Oils

Boil an accurately weighed quantity of the oil (see Table II for the appropriate quantity)* for 12 minutes under reflux with ethanol (5 ml.) and Girard-T reagent (0·2 g.). Cool the mixture and transfer to a separator with the aid of *ether* (50 ml.). Wash out the reaction flask with water (25 ml.) and transfer the washings to the separator. Shake the mixture gently, allow to separate and run the lower aqueous layer into a 500 ml. graduated flask. Wash the reaction flask and ether layer with two further quantities of water, each of 10 ml., and add the washings to the flask. Adjust to 500 ml. with water, mix well and dilute an aliquot portion so as to obtain a suitable optical density for measurement in 1 cm. cells at the wavelength of peak absorption (see Table II for the appropriate dilution). Carry out a blank determination without the oil and use as reference solution in matched cells. Calculate the percentage of carbonyl compound by means of the given E(1 per cent, 1 cm.) constants (Table II).

Preliminary Experiments with Carvone

(i) Carvone, in ethanol, treated with Girard-T reagent as described under the Method for the Determination of Citral in Essential Oils did not react.

(ii) Addition of glacial acetic acid (10 per cent v/v) to the reaction mixture promoted reaction which was complete in 60 minutes (Fig. 3).

Method for the Determination of Carvone in Essential Oils

Boil an accurately weighed quantity of oil (see Table II for the appropriate quantity) for 70 minutes under reflux with ethanol (5 ml.), glacial acetic acid (0·5 ml.), and Girard-T reagent (0·2 g.). Cool the mixture and transfer, with the aid of ether (50 ml.), to a separator which contains

* For small quantities the most convenient method is to dilute the oil with ethanol so that 5 ml. of the dilution contains the required amount.

sufficient N sodium hydroxide to neutralise 9/10 of the acetic acid. Complete the assay as described under the Method for the Determination of Citral in Essential Oils.

Results and Discussion

The well-known hydroxylamine hydrochloride method[27] determines total aldehydes or ketones in volatile oils and it appeared of interest to compare the results with those obtained by the spectrophotometric method (Table III). The latter method is specific for $\alpha\beta$-unsaturated compounds so that citronellal and methylheptenone in lemon oils would not be estimated.

TABLE III

Results of spectrophotometric and chemical methods of examination of essential oils

Oil	Found (per cent w/w)	
	Citral	
	Girard-T	Chemical
Standard citral (re-examination)	99·3*	98·7*
Commercial citral A	90·1	—
,, ,, B	93·2	—
,, ,, B*	92·2*	92·7*
Lemon C	3·70 : 3·72	4·1†
	3·74 : 3·73	
	3·73	
,, D	3·55	4·3†
,, E	3·77	4·5†
Lemon grass F	66·9	69·8
,, ,, G	67·3	71·3
	Cinnamaldehyde	
Cinnamaldehyde, reagent grade	97·7	97
Cinnamaldehyde (from bisulphite comp.)	99·8	—
Cinnamon bark H	63·4	64·5†
,, ,, I	62·5	61·9†
,, ,, J	64·1	64·0†
,, ,, K	58·3 : 58·9	59·8
,, leaf L	1·67 : 1·66	2 (approx.)
	Carvone	
Carvone (synthetic), reagent grade	95·0	97·5
Carvone (from semicarb.)	100·0	101
Caraway M	54·3 : 54·3	54·8
,, N	49·4 : 49·1	55 approx.)
Dill	45·7	53 (,,)

* Assays after six week interval in which visible deterioration had occurred in the standard citral.
† Chemical analysis by Stafford Allen and Sons Ltd.

The differences found were so large (Table III) and raised such doubts in our results that the standard and the commercial citral (B) were re-examined by both methods. Satisfactory agreement was obtained so that differences in the figures for lemon oils themselves by the two methods can only be attributed to the presence of other carbonyl compounds. The slightly lower figures obtained for citral on re-examination were probably caused by deterioration, since the standard had acquired a distinct yellow colour during the interval of six weeks between assays. Before a final conclusion can be reached on natural lemon oils a larger number of samples must be examined.

Cinnamon oils may contain *o*-methoxycinnamaldehyde which would be calculated as cinnamaldehyde by the spectrophotometric method. Closer agreement between the results by both methods was expected and actually found (Table III); cinnamon leaf oil (L) proved very difficult to assay

chemically because of the small cinnamaldehyde content so that an approximate result only was obtained. No difficulty was experienced when using Girard-T reagent.

Dihydrocarvone is likely to occur with carvone in caraway and dill oils but agreement was obtained between both methods for a genuine sample of English caraway oil (Table III M). The samples of caraway (N) and dill oils are of interest. Both were discoloured and of considerable age but examination by the official method indicated a satisfactory carvone content. The endpoint in both assays however was doubtful as a pink tint persisted and increased in intensity on the addition of a large excess of alkali. As the spectrophotometric method on the caraway oil gave results which did not comply with the British Pharmacopoeia requirements, the physical constants of the oil were determined. These were satisfactory except for the optical rotation ($+64°$) which is outside the official limits. It therefore affords a measure of support for the spectrophotometric result.

The results so far obtained indicate the utility of the Girard-T reagent, particularly where only small quantities of aldehyde occur for example in cinnamon leaf oils. The method provides a closer approximation to the true citral content of lemon oils though this may not necessarily be a guide to the organoleptic properties of these oils.

We wish to thank Dr. W. Mitchell of Stafford Allen and Sons Ltd. for gifts of essential oils and allowing us to include their results of several chemical assays. Thanks are also due to Miss E. Heggarty and Mr. W. Gardiner for their help.

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THE SPECTROPHOTOMETRIC DETERMINATION OF $\alpha\beta$-UNSATURATED ALDEHYDES AND KETONES WITH GIRARD-T REAGENT

PART II. KETOSTEROIDS

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A rapid spectrophotometric method is suggested for the determination of ethisterone and of methyltestosterone in tablets by reaction with Girard-T reagent.

THE principle of the method described by Stenlake and Williams[1] is now applied to the determination of $\alpha\beta$-unsaturated ketosteroids, in particular, ethisterone and methyltestosterone in tablets. Assays for these tablets are not given in the British Pharmacopoeia and the methods described in the United States Pharmacopeia XV involve long extraction procedures. Hot glacial acetic acid which has been used as a solvent in the preparation of Girard-T hydrazones[2,3] readily extracted the ketosteroids from tablet bases, to give a solution suitable for treatment with Girard-T reagent. As expected[2–4], reaction was complete within three minutes at 100° with both ethisterone and methyltestosterone, and this was adopted as the standard reaction time. The stability of the final solutions and agreement with the Beer-Lambert law was satisfactory.

Preliminary observations showed that sucrose and glucose interfered to a marked extent when present as solids in the reaction mixture. This interference was reduced when solutions which were saturated at room temperature with respect to the sugars were used. Two methods are therefore described, a general method applicable to tablets of unknown composition, and a direct one which can be used when the tablet basis is known, and does not contain glucose or sucrose.

EXPERIMENTAL

Standards

Ethisterone was recrystallised from ethanol. After drying at 105° for 2 hours the crystals had the following constants: m.p. 271–274°, $[\alpha]_D^{17°}$ + 33° (*c*, 1·1 pyridine), *E* (1 per cent, 1 cm.) 766 at 282 mμ when *determined by the method described.* The corresponding *E* (1 per cent, 1 cm.) values for the anhydrous manufacturing samples of ethisterone were: Batch 1: (i) 762, (ii) 762; Batch 2: 759.

Methyltestosterone was recrystallised from aqueous ethanol. After drying at 105° the crystals had the following constants: m.p. 165·5–167°, $[\alpha]_D^{17°}$ + 84·5° (*c*, 1·02 in ethanol), *E* (1 per cent, 1 cm.) 771 at 283 mμ when *determined by the method described.* The anhydrous manufacturing sample gave *E* (1 per cent, 1 cm.) 768 at 283 mμ by the same method.

Method for the Determination of E (1 *per cent*, 1 *cm.*) *Constants*

Dissolve the ketosteroid (about 75 mg. accurately weighed) in glacial acetic acid and adjust to 50 ml. To the solution (1 ml.) in a dry test-tube add Girard-T reagent (20 mg.). Plug the tube loosely with cotton wool and place in a boiling water bath for 3 minutes, swirling the tube gently at first to ensure solution of the reagent. Cool, dilute with water and transfer immediately to a 200 ml. standard flask which contains water (100 ml.) and sufficient N sodium hydroxide to neutralise 9/10 of the acetic acid. Wash out the tube and add the washings to the flask. Adjust to volume with more water, mix well and measure the optical density of the solution in 1 cm. cells at the appropriate wavelength of maximum absorption using water as reference solution. Repeat the operation, omitting the ketosteroid. Calculate the *E* (1 per cent, 1 cm.) constant from the difference between the optical densities.

General Method for Tablets

Take a sample of 20 tablets and determine the average weight. Powder the tablets and transfer an accurately weighed quantity, equivalent to about 10 mg. of ketosteroid, to a dry test-tube. Add about 7 ml. glacial acetic acid, and heat in a boiling water bath for 2 minutes, stirring the mixture thoroughly. Cool the mixture and filter through a small plug of cotton wool directly into a 10 ml. graduated flask using slight suction. Treat the residue with three successive quantities of glacial acetic acid each of 1 ml. as before, cool and pass the extract through the filter. Finally extract the residue with glacial acetic acid, 3 ml., cool and filter into a separate container. Use this filtrate to adjust the contents of the standard flask to volume and reserve the remainder for use in the preparation of a blank solution. Continue as described under Method for Determination of *E* (1 per cent, 1 cm.) Constants from "To the solution (1 ml.) . . ." but using as a blank 1 ml. of the reserved portion of glacial acetic acid treated in the same way. Calculate the concentration of ketosteroid in the tablet using the appropriate constant.

Direct Method for Tablets

Take a sample of 20 tablets and determine the average weight. Powder the tablets and transfer an accurately weighed quantity, equivalent to about 1 mg. of ketosteroid, to a dry test-tube. Add 1 ml. of a freshly prepared 2 per cent w/v solution of Girard-T reagent in glacial acetic acid and proceed as described in the Method for the Determination of *E* (1 per cent, 1 cm.) Constants from "Plug the tube loosely . . .", using as a blank the appropriate quantity of tablet basis treated in the same way. Calculate the concentration of ketosteroid in the tablets using the appropriate constant.

Efficiency of Extraction

A mixture of ethisterone (17·7 mg.), acacia (15 mg.) and lactose (0·4 g.) was treated as described under General Method for Tablets. The recovery of ethisterone was 100 per cent.

The extracted tablet residues from two determinations on Batch P (Table II) were bulked, boiled with glacial acetic acid (4 ml.) for 1 minute and digested at 100° for 5 minutes. The mixture was cooled, filtered and the filtrate (1 ml.) treated as described under Method for Determination of *E* (1 per cent, 1 cm.) Constants. A normal blank value only was obtained.

The extracted tablet residue from one of the assays was dissolved in water and the insoluble portion, after separation, was treated as described under Direct Method for Tablets from "Add 1 ml. . . .". No absorption corresponding to $\alpha\beta$-unsaturated ketosteroid was obtained.

Effect of Tablet Bases

The substances (see Table I for names and quantities) were treated as described under General Method for Tablets. The optical densities obtained using distilled water as reference solution are recorded in Table I.

TABLE I

EFFECT OF TABLET BASES

Substance	Quantity (g.)	Optical density
Reagents	—	0·020
Lactose	0·3	0·025
Mannitol	0·3	0·020
Dextrin	0·3	0·015
Tragacanth	0·05	0·020
Sucrose	0·3	0·070, 0·074
Glucose..	0·3	0·058

Lactose (0·05 g.) was treated as described under the Direct Method for Tablets. The optical density obtained was 0·04 using distilled water as reference solution. Sucrose (0·05 g.) was treated as for lactose above. The optical density found was 0·18.

Examination of Ethisterone Tablets Batch AA

The powdered tablets (4·475 g.) were extracted continuously for 4 hours with light petroleum, the extract evaporated and the residue (A, 8·1 mg.) reserved. Extraction was continued for a further 4 hours with chloroform, the extract was evaporated and the residue (B) dried to constant weight at 105° (yield 0·1225 g.). From residue (B) ethisterone in a tablet of average weight = 5·53 mg. or 110·5 per cent of labelled strength.

Examination of Residues A and B by the Girard-T Spectroscopic Method

Residue A was dissolved in 5 ml. of glacial acetic acid, and 1 ml. treated as described under Determination of *E* (1 per cent, 1 cm.) constants. Ethisterone found: 43·5 per cent (equivalent to 3·5 mg.). Residue B was dissolved in glacial acetic acid and made up to 100 ml. with more glacial acetic acid. The solution (1 ml.) was treated as described under Determination of *E* (1 per cent, 1 cm.) Constants. Ethisterone found: 86·3 per cent (equivalent to 105·8 mg.). Total ethisterone: 109·3 mg. equivalent to 4·93 mg./tablet, or 98·6 per cent of labelled strength.

Results and Discussion

The results obtained by the Girard-T spectroscopic assay of ethisterone and methyltestosterone in tablets are recorded in Table II. The speed with which the method can be applied represents a considerable advance on the U.S.P. XV method for ethisterone, for which the extraction procedures alone require 8 hours.

TABLE II

Girard-T spectroscopic assay of ethisterone and methyltestosterone

Tablets	Batch	Strength (mg.)	Found (mg.)	Label strength per cent	Label* strength per cent
Ethisterone	P	5	4·72	94·4	104
			4·79	95·8	
			4·74	94·8	
,, ...	Q	5	4·83	96·6	96·5
,, ...	R	10	9·1	91·0	91
,, ...	S	10	9·35	93·5	102
,, ...	T	10	9·12	91·2	95·7
			9·14	91·4	
,, ...	U	10	9·58	95·8	100
,, ...	V	10	9·66	96·6	102
,, ...	W	25	23·6	94·4	97
,, ...	X	25	23·7	94·8	101
			23·5	94·0	
,, ...	AA	5	4·91	98·2	100·4
			4·93	98·6	
Methyltestosterone	Y	3·6	3·50	97·2	100
,, ...	Z	3·6	3·54	98·3	97

* Results supplied by Organon Laboratories Ltd.

Although the results (column 4) appear to show low recoveries, control experiments on extracted tablet basis showed no unextracted ketosteroid. Recovery was also quantitative from a mixture of known composition. Two factors contribute to the apparently low figures, one being the fact that our constants were determined on recrystallised anhydrous samples of ethisterone and methyltestosterone. These give slightly higher E (1 per cent, 1 cm.) values than the manufacturing samples examined. The actual manufacturing samples used in the production of the various batches of tablets were not available to us, so that correction of the results was not possible Secondly some differences were found between the average weight of the tablets and the theoretical manufacturing weight, and corrections for these are incorporated in the Organon Laboratories results which are quoted in column 6 by kind permission of Dr R. P. Edkins. These results, obtained by a different method, show reasonable agreement with our own in several instances but notable discrepancies exist.

The results obtained on the batch AA of ethisterone tablets provide an interesting comparison of the Girard-T spectroscopic and the U.S.P. XV extraction methods. The latter, which is based on a gravimetric determination of chloroform-soluble matter after a preliminary extraction with light petroleum, gave a high result equivalent to 110·5 per cent of labelled strength. Application of the Girard-T spectroscopic method to the chloroform-soluble residue showed that only 86·3 per cent of this extract, equivalent to 95·4 per cent of the labelled strength, was ethisterone. It is significant that a similar examination of the light-petroleum-soluble

extractive showed that 43·5 per cent, equivalent to 3·16 per cent of labelled strength, was ethisterone, giving a total yield by this method equivalent to 98·6 per cent of the labelled strength.

Acknowledgements. We wish to thank Dr. R. P. Eckins of Organon Laboratories Ltd. for samples of ethisterone, methyltestosterone and tablets of these substances.

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DISCUSSION

The papers were presented by DR. W. D. WILLIAMS.

The CHAIRMAN said that it was necessary not only to determine how much $\alpha\beta$-unsaturated ketone was in the tablet, but also to identify it. The authors had emphasised that the extinctions were almost identical for the two compounds and, therefore, the active ingredient must be isolated.

MR. H. B. HEATH (Sudbury). Had the authors any comment on the application of the method to determinations of the citral content of solutions in 40 per cent ethanol or *iso*propyl alcohol?

MR. A. R. ROGERS (Brighton). Had the authors checked that extinction was proportional to path length?

MR. S. G. E. STEVENS (London) referring to Table II of Part I asked how a deviation from the quantities quoted would affect accuracy of the answer? Were Stafford Allen's samples tested at the same time, or were they oils which had been stored and had possibly undergone some degree of resinification?

Under "Efficiency of Extraction", in Part II, the authors had used a mixture containing acacia and stated that the recovery was 100 per cent. One would have thought a number of saccharides would probably have been produced, which may have caused the ill effects reported in the case of glucose and lactose, and it would be difficult to get a 100 per cent recovery.

DR. W. MITCHELL (London). The method took considerably longer to perform than the method of the British Pharmacopoeia and the results with lemon oil were consistently much lower. It was desirable to test more samples before drawing any final conclusions. There might be a slight error in the citral standard, because it was difficult to get it completely free from methyl heptenones. The fall in results of so-called pure citral might be explained by the production of methyl heptenone on subsequent storage.

DR. G. E. FOSTER (Dartford). Was it necessary to make a calibration curve with each batch of reagent? Had any work been done on the injections of the hormones?

The AUTHORS replied that the only alcoholic solutions used had been in 95 per cent ethanol, and it would seem that the method could not be applied to 50 or 60 per cent ethanol solutions, because on heating there might be hydrolysis. There was a possibility of testing without heating since the reagent reacted readily with citral. A 1 cm. cell was used throughout and the quantities were such that one obtained suitable extinction reading on the spectrophotometer. They had no information on the age of the samples which Stafford Allen had sent, but the analyses were made about three weeks after they were made by the manufacturer. The amount of acacia involved was only 15 mg., heated for three minutes, so it would probably not interfere to any great extent. Reasonably anhydrous crystalline hydrazone gave values which compared favourably with those obtained from citral itself. The reagents had been recrystallised twice from ethanol and the constants obtained were identical. There was interference with the reaction when it was applied to injections such as progesterone in oil and with ethyl oleate the blanks were large. Ten variations had not produced a method which was successful in the presence of ethyl oleate.

THE DETERMINATION OF MORPHINE IN OPIUM AND SOME OF ITS GALENICAL PREPARATIONS

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A method of assay for morphine in opium depending upon precipitation of the dinitrophenylether with 1-fluoro-2:4-dinitrobenzene has been critically examined. The morphine is first separated from other alkaloids and extraneous matter by elution from an alumina column with an organic solvent and subsequent extraction with alkali. The method, which is rapid, has been applied to many galenical preparations and, in cases where the pharmacopoeial method based on the colorimetric nitroso reactions may give high results, figures in accordance with the expected values have been obtained.

In 1935 Mannich described a method of determination of mophine in which the dinitrophenylether was precipitated with 1-chloro-2:4-dinitrobenzene[1], and the application of such a method to the determination of morphine in opium was attempted. The procedure was critically assessed in 1937 by Nicholls[2], who concluded that it was unsatisfactory for opium since he obtained very discoloured residues which contained an appreciable proportion of methoxyl group indicating that other phenolic alkaloids such as laudanine had also been precipitated. In 1951 Dann and Wippern[3] investigated and recommended the use of 1-fluoro-2:4-dinitrobenzene for the precipitation of morphine since they found it more rapid than the chloro-reagent. This material was used for the determination of morphine in opium by Svendsen and Aarnes in 1955[4], but in view of the principle shortcoming of this method, namely the possible co-precipitation of other phenolic alkaloids, the validity of the procedure does not appear to have received much investigation. Moreover the results quoted for a number of samples of opium of unspecified origin were erratic, a spread of over 5 per cent being obtained with 10 determinations on a single sample. The method appeared to be relatively simple to operate and worth further investigation in an attempt to obtain a suitable assay for opium also applicable to its galenical preparations. The method described by Svendsen and Aarnes depends upon the precipitation of morphine with fluorodinitrobenzene after separation from other alkaloids and much extraneous matter by elution from an alumina column with an organic solvent and subsequent extraction with alkali. To assess the method the precipitation stage was first examined.

The Precipitation of Morphine with Fluorodinitrobenzene

In the published method an alkaline solution of the morphine is just neutralised with hydrochloric acid and adjusted to a weight of 30 g. with water; a solution containing 250 mg. of fluorodinitrobenzene in 30 ml. of acetone is added, followed by 5 ml. of 25 per cent ammonia. After

standing for four hours at room temperature the precipitate is filtered, washed twice with 2 ml. quantities of acetone followed by two 2 ml. quantities of water, dried for 1 hour at 80° and weighed.

Recovery experiments on known weights of a recrystallised sample of morphine alkaloid gave a series of erratic results, all being in excess of the expected value, some by as much as 10 per cent. It was thought that the washing and drying conditions might contribute to these results since they seemed inadequate. We therefore examined the effect of washing the residue with acetone and water. Aliquot portions of a solution of morphine were precipitated and washed with varying quantities of acetone, or with acetone followed by water, and the weights of residues after drying for 1 hour at 80° were recorded. To assess the purity of each residue, as anhydrous morphine dinitrophenylether, the methods described by Mannich[1,5] which are based on aqueous titration procedures, were applied but found to be unsatisfactory. Non-aqueous titration with perchloric acid in glacial acetic acid proved to be satisfactory, giving a readily detectable end point with crystal violet as an indicator; a typical titration curve is shown in Figure 1. Table I shows the results of the washing tests, the recovery of morphine being calculated both from the weight of residue and from the titration figure.

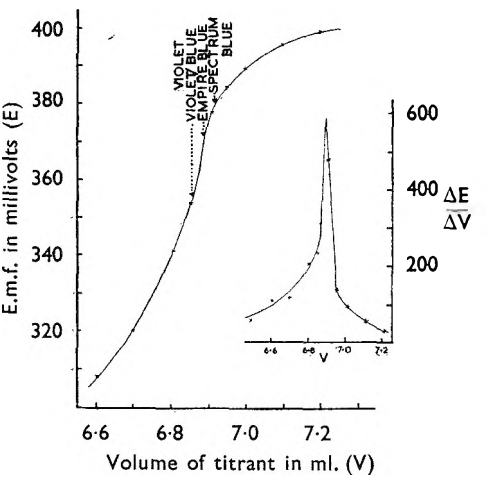

FIG. 1. Titration curves of morphine dinitrophenylether in glacial acetic acid with 0·05N perchloric acid.

TABLE I

EXAMINATION OF WASHING CONDITIONS FOR MORPHINE DINITROPHENYLETHER

Washing employed	Wt. of residue* (g.)	Apparent recovery, per cent	
		Volumetric	Gravimetric
None	0·2375	119·8	160·3
2 ml. acetone	0·2200	105·1	148·4
2 × 2 ml. acetone	0·1967	101·5	132·8
2 × 2 ml. acetone and 1 × 2 ml. water	0·1603	99·7	108·1
†2 × 2 ml. acetone and 2 × 2 ml. water	0·1630	99·7	110·0
3 × 2 ml. acetone	0·1611	100·1	108·7
4 × 2 ml. acetone	0·1481	99·9	100·0
5 × 2 ml. acetone	0·1480	99·9	99·9
6 × 2 ml. acetone	0·1479	99·7	99·8
7 × 2 ml. acetone	0·1479	99·7	99·8
8 × 2 ml. acetone	0·1478	99·6	99·7
9 × 2 ml. acetone	0·1475	99·4	99·5
10 × 2 ml. acetone	0·1473	99·4	99·4

* The quantity of morphine precipitated in each test should yield 0·1481 g. of dinitrophenylether.
† The conditions prescribed by Svendsen and Aarnes.

Some titratable impurity is present in the precipitate but is removed by washing with acetone; the high titration values for unwashed material might be accounted for by a trace presence of ammonia or of ammonium chloride but this would not account for the high gravimetric values. Washing with water increases the gravimetric values, presumably due to inadequate drying, whilst continued washing with acetone causes a gradual loss due to solubility. Little is published on the solubility of morphine dinitrophenylether, Mannich[1] stating it to be soluble with difficulty in acetone; a solubility-time test was therefore made. Finely powdered morphine dinitrophenylether was shaken vigorously with acetone at 17° for periods up to one hour. The solubility at 17° in 100 ml. of acetone was: 10 minutes 8 mg., at 20 minutes 12 mg., at 30 minutes 15 mg., at 40 minutes 19 mg., at 50 minutes 22·5 mg., at 60 minutes 26 mg., showing a slow progressive solubility with time.

TABLE II

COMPARATIVE REACTIVITY OF FLUORO- AND CHLORODINITROBENZENE

Per cent recovery of anhydrous morphine

	Precipitation time, hours					
	2	3	4	6	18	24
Fluorodinitrobenzene A ..	100·0	100·1	100·1	—	100·2	—
,, B ..	—	—	100·9	—	—	—
			100·2			
,, C ..	99·9	100·2	100·0	—	100·5	—
,, D ..	99·9	100·0	100·0	—	100·5	—
Chlorodinitrobenzene ..	—	—	92·4	96·0	97·7	100·1

(A) Commercial liquid I. (B) Crystalline material. (C) Commercial liquid II. (D) Laboratory prepared liquid.

The time of standing necessary before filtration of the precipitate was next examined, and a comparison was made between the use of the fluoro- and the chloro- reagents. The results recorded in Table II illustrate that, as reported by Dann and Wippern, the fluoro- compound is much more reactive than the chloro- compound. Several different samples of 1-fluoro-2:4-dinitrobenzene were used. A note on the reagent is included as an appendix.

In an examination of conditions for drying the precipitate aliquot portions of a solution of morphine were precipitated, the residue obtained being dried at different temperatures for 1 hour. Drying at 80° gave a pale yellow residue of weight closest to theoretical (0·1599 g. found; 0·1598 g. theoretical). Increasing the temperature by stages to 160° resulted in progressive increase in discolouration and weight of residue to a dark brown colour and 0·1612 g. at 160°. These results suggests that some oxidation occurs at higher temperatures. To confirm this a weight of morphine dinitrophenylether was heated for periods, each of $1\frac{1}{2}$ hours, at temperature intervals of 10° between 80° and the melting point at about 250°. Darkening commenced at 110° becoming more marked until, after 160° the precipitate had become brown; further periods of heating caused a fall in weight, and an uncertain melting point in the region of

250°. Professor Clement Duval, of the Sorbonne, kindly made a thermogravimetric analysis of morphine dinitrophenylether prepared by the proposed method and his results support the view that a gradual oxidation occurs as the temperature is raised and that there is a sudden fall in weight at a temperature of about 250°. For these reasons the drying conditions of 80° for 1 hour were considered satisfactory.

The nature of the acetone-soluble impurity in morphine dinitrophenylether precipitated by the fluoro- reagent was next examined. Blank determinations in which 30 ml. of water replaced the morphine solution showed that a precipitate was rapidly formed when the fluoro- reagent was used, but that no precipitate resulted from use of the chloro- reagent. The precipitate was found to consist of 2:4-dinitroaniline, a further demonstration of the remarkable reactivity of the fluorine-substituted nitrobenzene.

An investigation was now made of the extraction procedure of Svendsen and Aarnes, the principles of which had been earlier suggested by Graf[6]. In this procedure the morphine-containing material (1 g. of opium) is triturated with 3 ml. of methanol and 1 ml. of 25 per cent ammonia until a homogeneous mass is formed. It is then triturated with 15 g. of aluminium oxide and the powder mixture so obtained is run into a glass tube; extraction is by eluting the column with 240 ml. of a mixture in the proportion 3 parts of chloroform to one of *iso*propyl alcohol. The morphine is extracted from the eluate by shaking with successive quantities of 0·1N sodium hydroxide and the bulked extracts, after neutralisation with hydrochloric acid, are evaporated to 30 ml. After cooling, the reagent solution is added and the determination made as previously described. Known quantities of morphine were subjected to this procedure and recoveries between 99·5 and 100 per cent were obtained in all cases. All of the morphine was found to be eluted from the column with less solvent than the 240 ml. prescribed. In every experiment 100 ml. of eluting solvent was found to be more than sufficient.

The Determination of Morphine in Opium

The determination of morphine in a sample of Turkish opium was then examined.

Reagents

Acetone B.P.C.; alcohol 95 per cent B.P.; aluminium oxide, Brockmann chromatographic grade; dilute solution of ammonia B.P.; chloroform—*iso*propyl alcohol mixture (chloroform B.P. 3 parts, *iso*propyl alcohol B.P.C., 1 part); solution of 1-fluoro-2:4-dinitrobenzene (a 0·8 per cent w/v solution in acetone); hydrochloric acid solution, 1N; sodium bicarbonate solution (a saturated solution of sodium bicarbonate B.P. in water); sodium hydroxide solution 0·1N.

Method

Powder a representative sample of the opium and accurately weigh about 1 g. into a small porcelain dish. Triturate with 4 ml. of a 3:1

mixture of 95 per cent ethanol and dilute solution of ammonia to an homogeneous cream. Add aluminium oxide gradually and continue triturating until a free-flowing powder is obtained. Transfer the powder to a dry chromatographic tube of about 1·5 cm. diameter and 40 cm. long, previously plugged lightly above the tap with cotton wool. Remove any adhering powder from dish and pestle with cotton wool moistened with alcohol, and add to the tube. Insert the lower end of the tube through a bung fitting into the neck of a 250 ml. separator and elute with 100 ml. of chloroform-*iso*propyl alcohol mixture, adjusting the rate of elution to about 1·5 ml./minute using slight positive pressure if necessary.

Extract the chloroform-alcohol solution in a separator by shaking gently with 20 ml. of 0·1N solution of sodium hydroxide. Allow to separate. Run off the organic phase into a second separator. Filter the aqueous phase through a cotton wool plug into a 150 ml. beaker.

TABLE III

ANALYSIS OF TURKISH OPIUM

	Per cent anhydrous morphine, calculated to the dried opium			
Batch	Proposed method		B.P. method	U.N. method[7]
5402	15·78	15·75	15·55	15·68
	15·51	15·84	15·60	—
6227	15·70*		15·40	15·70
			15·45	—

* Mean of 15 determinations, spread −0·13 to +0·14.

Extract the organic phase with two further quantities of 0·1N sodium hydroxide solution, each of 15 ml., filtering the extract into the same beaker. Add N HCl to the bulked aqueous solutions until just acid to litmus paper and concentrate on a steam bath to 30 ml. Cool, add 30 ml. of solution of 1-fluoro-2:4-dinitrobenzene, followed by 5 ml. of dilute solution of ammonia. Stir gently to mix, cover the beaker and set aside at 15 to 20° for 4 hours, after which decant the supernatant through a tared sintered-glass filter (porosity No. 3). Use the filtrate in portions of 2 to 3 ml. to quantitatively transfer the dinitrophenyl ether to the filter. Rinse the beaker with 2 ml. of acetone and transfer to the filter. After several seconds contact with the crystals remove the acetone by applying gentle suction. Repeat this washing procedure with three further portions of acetone, each of 2 ml. Dry the residue for 1 hour at a temperature of 80°; each g. of residue is equivalent to 0·6319 g. $C_{17}H_{19}O_3N$.

Total working time is about $1\frac{1}{2}$ to $1\frac{3}{4}$ hours and the whole determination may be made in less than a day. Results in Table III show the reproducibility of the method and a comparison of results with those obtained by two other methods.

Difficulties were encountered when the method was applied to opium of Indian origin. The eluates were much darker than those from Turkish opium and in the subsequent extraction with sodium hydroxide emulsions tended to form which were sometimes difficult to break. Moreover the precipitate was much darker than that from morphine or Turkish opium

and appeared to be contaminated with a resin-like material. Morphine figures calculated from the weight of the precipitate were erratic and, in general, were substantially higher than those by the B.P. method. When titrated by the non-aqueous procedure previously described, the residues from Indian opium gave figures for purity ranging between 88 and 95 per cent compared with 99 per cent from Turkish opium. Because the interfering material was readily extracted from the organic layer by sodium hydroxide it was assumed to be either acidic or phenolic in nature.

TABLE IV

EFFECT OF WASHING ELUATES FROM INDIAN OPIUM WITH SODIUM BICARBONATE SOLUTION

Vol. of saturated $NaHCO_3$ solution used for washing	Per cent anhydrous morphine		
	Proposed method		
	Gravimetric	Volumetric	B.P. method
2 × 25 ml.	13·32	12·88	12·75
2 × 25 ml.	13·47	12·96	12·78
3 × 20 ml.	12·86	12·85	—
4 × 20 ml.	12·98	12·98	—
4 × 20 ml.	12·92	12·91	—

Attempts were therefore made to separate it from the morphine by extracting the organic eluate with sodium bicarbonate solution before removal of the morphine with sodium hydroxide. The bicarbonate washings, while not removing morphine, extracted some material which, on subsequent treatment with fluorodinitrobenzene, gave an amorphous precipitate, soluble in glacial acetic acid, but the solution did not titrate with perchloric acid. A number of estimations were made, varying the volume of bicarbonate solution used in washing. In each, washings were bulked, shaken with two 10 ml.-portions of chloroform-*iso*propyl alcohol mixture, and the three organic phases combined before extraction of the morphine with sodium hydroxide. On shaking the washed organic phases with sodium hydroxide there was little or no emulsification; morphine dinitrophenylether was precipitated in the usual way and the purity of the residues determined by titration with perchloric acid. Results in terms of anhydrous morphine are given in Table IV.

Results obtained by different methods on two samples of Indian opium are shown in Table V.

Titration of the impure residue is not satisfactory as the end point is sometimes difficult to detect because of the colour; furthermore the volume of 0·05N perchloric acid used is only about 7 or 8 ml. The method of choice is the gravimetric procedure after washing with a standard solution of sodium bicarbonate and is as follows:

Proceed by the method described for Turkish opium to the words ". . . elute with 100 ml. of chloroform-*iso*propyl alcohol mixture." Wash the eluate in the separator by shaking gently with four 20 ml.-portions of saturated sodium bicarbonate solution. Combine the four washings and extract with two 10 ml.-volumes of chloroform-*iso*propyl alcohol mixture. Reject the aqueous phase, combine the two washings

with the original washed eluate and continue by the method for Turkish opium from the words "Extract the chloroform-*iso*propyl alcohol solution in the separator. . . ."

As with Turkish opium the results are a little higher than those obtained by the B.P. method but are in close agreement with those obtained by the United Nations method.[7]

TABLE V

ANALYSIS OF INDIAN OPIUM

	Per cent anhydrous morphine					
	Precipitation of morphine as dinitrophenylether					
	No washing with $NaHCO_3$		Washing with $NaHCO_3$			
Batch	Gravimetric	Volumetric	Gravimetric	Volumetric	B.P.	U.N.[7]
4749	13·32	12·66	12·67	12·66	12·43	12·58
	13·21	12·69	12·69	12·67	12·31	—
	—	—	12·58	12·57	—	—
	—	—	12·72	12·70	—	—
	—	—	12·58	12·54	—	—
	—	—	12·74	12·70	—	—
2458	13·64	13·00	12·98	12·98	12·75	12·99
	13·60	12·91	12·92	12·92	12·78	13·01
	13·70	—	—	—	—	—

COMPARISON OF THE RESIDUES OBTAINED FROM OPIUM WITH PURE MORPHINE DINITROPHENYLETHER

Samples of morphine dinitrophenylether prepared from pure morphine, Turkish opium and Indian opium using 1-fluoro-2:4-dinitrobenzene and from pure morphine using the chloro- reagent were compared.

Assuming the empirical formula $C_{17}H_{18}O_3N{\cdot}C_6H_3(NO_2)_2$ with a molecular weight of 451·42 theoretical values are C, 61·2 per cent, H, 4·69 per cent, N, 9·30 per cent, methoxyl content nil. Residues contaminated with other phenolic alkaloids of opium would be expected to give a significant methoxyl value. Values found are, per cent:

	C	H	N	From non-aqueous titration	Methoxyl
From morphine with F cpd	60·9	5·0	9·2	99·9	less than 0·1
From morphine with Cl cpd	60·7	4·8	9·0	99·6	—
From Turkish Opium	61·2	4·8	9·3	99·8	less than 0·1
From Indian Opium	61·0	5·0	9·5	99·7	less than 0·1

Infra-red spectra suggested that samples precipitated from opium are identical with those precipitated from morphine. The presence of dintroaniline in insufficiently washed samples prepared with fluorodinitrobenzene influences the spectrum of morphine dinitrophenylether, causing modifications at 9·40 μ, 10·85 μ, 11·97 μ and 13·05 μ. Spectra of samples prepared by the proposed assay process confirmed that all the dinitroaniline had been removed. The spectrum of pure morphine dinitrophenylether over the range 5 μ to 15 μ is shown for reference in Figure 2.

Thus the possibility that the precipitates from the opium samples might be contaminated with other phenolic alkaloids is neither supported by the infra-red spectra, nor by the very low methoxyl contents found.

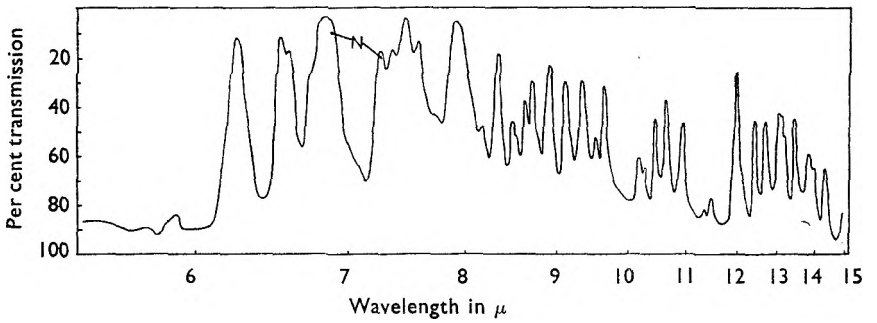

FIG. 2. Infra-red spectrum of morphine dinitrophenylether in Nujol mull. N = Nujol bands.

THE INFLUENCE OF OTHER ALKALOIDS OF OPIUM AND RELATED MATERIALS ON THE PRECIPITATION OF MORPHINE DINITROPHENYLETHER

Codeine, thebaine, papaverine and narcotine. 0·1 g. of each was subjected to the precipitation procedure already described and since no phenolic groups are present, no acetone-insoluble precipitate was obtained. Recoveries of anhydrous morphine in the presence of these alkaloids are shown in Table VI.

TABLE VI

RECOVERY EXPERIMENTS ON ANHYDROUS MORPHINE IN THE PRESENCE OF OTHER ALKALOIDS OF OPIUM AND RELATED MATERIALS

Alkaloid or group of alkaloids	Amount present (g.)	Anhydrous morphine		Per cent recovery of morphine
		Added (g.)	Recovered (g.)	
Codeine	0·1	0·1206	0·1205	99·9
Papaverine	0·1	0·1215	0·1216	100·1
Narcotine	0·1	0·1324	0·1324	100·0
Thebaine	0·1	0·0895	0·0896	100·1
*"Porphyroxine-meconidine"	c 0·015	0·1190	0·1189	99·9
*Laudanine, etc.	c 0·015	0·1074	0·1077	100·2
*"Unknown Base"	c 0·005	0·1014	0·1015	100·1
*Codeine and cryptopine	c 0·05	0·1076	0·1075	99·9
Pseudomorphine	0·1	0·1071	0·1072	100·1
Apomorphine	0·1	0·1011	0·1014	100·3
Ethylmorphine	0·1	0·1154	0·1158	100·3
Diamorphine	0·1	0·1029	0·1030	100·1

* Residues obtained in the U.N. assay.

Minor phenolic alkaloids, principally laudanine, codamine and narcotoline. No supply of these was available. In the United Nations "Unified Analysis of Opium for alkaloids"[7], however, a method is given whereby various groups of alkaloids are separated and estimated. Two groups of phenolic alkaloids are separated, one, the so-called "porphyroxine-meconidine" group, the other laudanine and related alkaloids. The complete assays were made on two samples of opium of Indian origin and one of Turkish and the residues from each separation were precipitated. No acetone-insoluble residues were obtained from any of the fractions other than the morphine one. Further supplies of these groups of alkaloids were then similarly obtained from one of the Indian opiums

and recovery experiments on anhydrous morphine were made in their presence. The results are included in Table VI. Appreciable colours were obtained when small quantities of the minor phenolic residues were tested by the nitroso-morphine reaction.

Pseudomorphine. This is 2-2′-dimorphine[8] and although it was obtained from opium by Pelletier[9] it is not known whether it is actually present in opium or formed during extraction[10]. A sample was prepared by the method described by Polstorff[11] and subjected to the proposed precipitation procedure, after identification by the micro-technique of Clarke and Williams[12]. From the crystalline nature of the material obtained it appeared that something other than 2:4-dinitroaniline had precipitated but the residue was entirely soluble in the stipulated quantity of acetone used for washing. A recovery experiment on morphine made in the presence of pseudomorphine is included in Table VI.

Apomorphine, ethylmorphine and diamorphine. The precipitation procedure described was carried out on samples of these alkaloids. Whereas ethylmorphine and diamorphine gave no precipitates other than of 2:4-dinitroaniline, apomorphine gave a resinous residue but this was completely soluble in the volume of acetone used for washing. Recoveries of anhydrous morphine in the presence of these alkaloids are shown in Table VI.

Application of the Method to Galenical Preparations

Having obtained satisfactory results on both Turkish and Indian opiums the method was extended to the determination of morphine in galenical preparations; most preparations to which the method has been applied are official and with a few exceptions were compounded from Turkish opium.

Preparations of the British Pharmacopoeia

Tincture of Opium. Take 10 ml. of sample in a porcelain dish and evaporate to dryness on a steam bath. Continue by the appropriate method for raw opium.

The results per cent w/v anhydrous morphine in tincture of opium from Turkish and Indian sources, obtained on routine batches are:

Turkish,	proposed method,	1·04, 1·04;	B.P.	method,	1·03, 1·03
,,	,,	,,	1·00, 1·00;	,,	,,	1·00, 0·99
,,	,,	,,	1·06, 1·07;	,,	,,	1·07, 1·04
Indian (unadjusted)	,,	2·13,	;	,,	,,	2·12

Camphorated Tincture of Opium. With this preparation we find that a high result may be obtained by the official colorimetric method and this has also been observed by others[13]. A number of samples were prepared from Tinctures of Opium already assayed and the results obtained by the proposed method and the B.P. method are shown in Table VII. The theoretical morphine contents quoted are based upon results by the B.P.

method of assay on the original tinctures. The recommended method is as follows:—

Take 100 ml.* in a porcelain dish and evaporate to about 10 ml. on a steam bath. Add about 5 g. of aluminium oxide and continue the evaporation to dryness. Continue by the appropriate method for raw opium.

TABLE VII

PER CENT W/V ANHYDROUS MORPHINE IN CAMPHORATED TINCTURE OF OPIUM

Tincture of Opium used	Sample No.	Per cent anhydrous morphine w/v		
		Theoretical	Proposed method	B.P. method
8243	1	0·049	0·049	0·058
	2	0·053	0·053	0·055
			0·053	0·054
8303	3	0·053	0·054	0·062
8597	4	0·050	0·051	0·060
	5	0·050	0·051	0·056
				0·058

Powdered Ipecacuanha and Opium. The official assay is based on the measurement of colour by the nitroso reaction first reported by Radulescu[14]. Being a general reaction for most phenols[15,16] a colour may be given by the minor phenolic alkaloids of opium or by the phenolic alkaloids of ipecacuanha. Despite the use of a compensating technique described in the B.P., routine analyses may yield high figures (Table VIII). Another source of error in the official method arises from a difference of tint in the final matching between the standard and the sample.

TABLE VIII

PER CENT W/W ANHYDROUS MORPHINE IN POWDERED IPECACUANHA AND OPIUM

Batch No.	Proposed method	B.P. method
24338	0·97	1·17
	0·97	1·16
4564	0·99	1·11
	0·98	—
301	0·98	1·31
26647*	1·01	1·26
	1·01	—
25595*	1·01	1·25
	1·02	—
26645	0·97	1·31
2467	0·97	1·14
23559†	Operator 1 1·01	1·21

* B.P. 1914 formula.
† For batch 23559, Operators 1–7 had the following further results by the proposed method: (Op. 1) 1·01, 1·00; (Op. 2‡) 0·99, 1·01; (Op. 3‡) 1·00; (Op. 4‡) 0·97; (Op. 5‡) 0·99; (Op. 6) 1·01; (Op. 7‡) 0·98.
‡ These operators were carrying out the assay for the first time.

The possibility of interference with the proposed gravimetric method by alkaloids of ipecacuanha was considered and emetine, cephaëline and psychotrine were extracted from powdered ipecacuanha and subjected to precipitation tests. No acetone insoluble precipitate was obtained from any of the alkaloids under consideration and results of recovery experiments on anhydrous morphine in the presence of these alkaloids are:

		Morphine, g.		Per cent recovery
	g.	Added	Recovered	
Emetine	0·1	0·1143	0·1141	99·8
Cephaeline	0·1	0·1129	0·1134	100·4
Psychotrine	0·05	0·1146	0·1148	100·1

* For manufacturing control purposes this volume presents no difficulty, but with a suitable semi-micro technique it could be reduced to 25 ml.

The recommended method of assay is as follows:—

Take about 5 g. accurately weighed and continue by the appropriate method for raw opium.

Aromatic Powder of Chalk and Opium. Satisfactory results cannot be obtained on this material. The trituration and column procedure is far too cumbersome because of the large bulk of material and extraction and precipitation after lime treatment yielded results which were only about 90 per cent of theory.

Preparations of the British Pharmaceutical Codex

Dry Extract of Opium. This can be assayed directly as for raw opium using about 0·5 g. accurately weighed. A sample which gave a figure of just under 20·2 by the B.P.C. method gave results of 20·29 and 20·27 per cent w/v anhydrous morphine.

Concentrated Camphorated Tincture of Opium. The recommended method of assay is as follows:—

Take 25 ml. of sample in a porcelain dish and evaporate to about 10 ml. on a steam bath; continue by the method for Camphorated Tincture of Opium from the words "Add about 5 g. of aluminium oxide. . . ."

The results, per cent anhydrous morphine content, from tincture prepared from two batches of Tinctures of Opium are: (1) proposed method 0·414, 0·414; B.P.C. method 0·462. (2) (manufacturing batch) proposed method 0·431, 0·433; B.P.C. 0·483. A theoretical figure of 0·413 for batch (1) is derived from the B.P. assay of the Tincture of Opium used.

Liniment of Opium B.P.C. 1949. There is no interference from Liniment of Soap; the recommended method of assay is as follows:—

Take 10 ml. of sample in a porcelain dish and evaporate to dryness on a steam bath; continue by the method for raw opium. A sample which gave a result of 0·54 per cent w/v by the B.P.C. method gave a figure of 0·55 per cent by the proposed method.

Papaveretum. Officially, papaveretum may be prepared either ". . . from opium by converting the total alkaloids into the hydrochlorides . . ." or by ". . . mixing suitable proportions of the hydrochlorides of morphine, codeine, narcotine and papaverine". Since codeine, narcotine and papaverine have been shown to cause no interference in the precipitation of morphine as the dinitrophenylether, it is unnecessary to adopt the column separation where papaveretum is a mixture of pure alkaloids.

Recommended assays are as follows:—

For material prepared from the total alkaloids of opium take 0·2 g. accurately weighed in a porcelain dish and proceed by the method for raw opium.

For material prepared by mixing the hydrochlorides of morphine, codeine, narcotine and papaverine—take 0·2 g. accurately weighed in a 150 ml. beaker, add 30 ml. of water and stir to dissolve. Continue by the method for raw opium commencing with the words "To the cooled solution add 30 ml. of solution of 1-fluoro-2:4-dinitrobenzene. . . ."

The results, per cent anhydrous morphine, in 2 batches of papaveretum, are: (1) column treatment, 49·9, 49·9; B.P.C. method 49·8; (2) column treatment, 48·5; direct precipitation, 48·6; B.P.C., 48·5.

Injection of Papaveretum. Phenylmercuric nitrate does not interfere with the method. Even in the presence of 20 mg. (hundredfold excess) quantitative recoveries of anhydrous morphine were obtained. Morphine may be determined, using 10 ml. with or without column treatment. A number of samples of the injection was prepared and the recovery of anhydrous morphine in g. calculated. The theoretical amount calculated from the estimate made on the papaveretum used is given in parentheses. With column treatment, 0·1041 (0·1043); 0·1028 (0·1029); 0·1046 (0·1044); and without column treatment, 0·1128 (0·1128); 0·1068 (0·1067).

Tablets of Papaveretum. Take a quantity of powdered tablets, accurately weighed, equivalent to about 0·2 g. of papaveretum and proceed by the method for raw opium.

The content of anhydrous morphine in grains/tablet of two batches was: (1) proposed method 0·089, B.P.C. method 0·099; proposed method 0·090, B.P.C. 0·097; (2) proposed method 0·085, B.P.C. 0·103; proposed method 0·087, B.P.C. no figure. The nominal content of papaveretum in each tablet was $\frac{1}{6}$ grain.

Liquid Extract of Poppy, B.P.C. 1949. On evaporating the sample to dryness, it becomes resinous and cannot be completely homogenised with the aluminium oxide. If the extract is treated by the method of Bennett and Garratt[17] the dinitrophenylether procedure may be applied satisfactorily. The recommended method of assay is as follows:—

Take 50 ml. of sample in a 250 ml. stoppered flask, add 20 ml. of water and 150 ml. of *iso*propyl alcohol and shake vigorously for 2 to 3 minutes. Allow to stand for 5 minutes and pour off the alcohol into a 500 ml. separator. Redissolve the residue by shaking with a further 10 to 20 ml. of water, then add 50 ml. of *iso*propyl alcohol, shake again and pour off the alcohol into the separator. Repeat the process of dissolving the residue in 10 ml. of water and shaking with 20 ml. of *iso*propyl alcohol twice more, bulking the alcoholic extracts in the separator. Shake the separator and filter the alcoholic extracts through a plug of cotton-wool into an evaporating dish. Evaporate the solvent on a steam bath, triturate the residue with 3 ml. of 95 per cent alcohol and sufficient dilute solution of ammonia to ensure alkalinity to litmus paper. Continue by the general method for Turkish opium.

On a batch of Liquid Extract of Poppy B.P.C. 1949 results of 0·172 per cent and 0·169 per cent w/v of anhydrous morphine were obtained by the B.P.C. method and 0·160 per cent and 0·159 per cent w/v by the proposed method.

The method has not been applied to a number of products such as Liquid Extract of Opium B.P.C., Sedative solution of Opium B.P.C. 1949 and Ammoniated Tincture of Opium B.P.C. 1949 which would obviously present no difficulty. One preparation has not been assayed satisfactorily: Ointment of Gall with Opium B.P.C.

APPENDIX

A Note on 1-*Fluoro*-2:4-*dinitrobenzene*

In the initial experiments on the determination of morphine in opium a pale yellow crystalline solid (m.p. 26·5°) of commercial origin was used. Further supplies of reagent from this source were unobtainable, and a supply was obtained from a second commercial source. This was a yellow liquid, b.p. 167° at 11 mm. pressure, wt./ml. at 20°, 1·5640, refractive index at 20° 1·5686. Doubt arose whether this liquid was the same material as the crystalline solid and a search of the literature was made.

A synthesis of fluorodinitrobenzene is described by Finger and Finnerty[18] which, when carried out, yielded a pale yellow viscous oil. Material prepared in this way has been used for the majority of the determinations described in the present work. The method of preparation was as follows:

Prepare a mixture of 900 ml. of concentrated sulphuric acid and 300 ml. of concentrated nitric acid, heat to 50 to 60° and add, with constant stirring, 192 g. of fluorobenzene at such a rate as to maintain the temperature between 70 and 75°. When the addition is complete maintain the reaction mixture at a temperature of 85° for 30 minutes, cool and pour into 4 litres of ice-cold water. Leave overnight and distil the oil under reduced pressure on the following day. A yield of about 250 g. was obtained, b.p. 166° at 11 mm. pressure, wt./ml. at 20° 1·5429, refractive index at 20° 1·5682. A nitrogen determination by Dumas' method gave 15·30 per cent (theory 15·06 per cent). Two derivatives were prepared. (i) anilide, m.p. 157·4 to 157·5° (156°)[19]; (ii) *p*-chlorophenylmercaptan, m.p. 124·8 to 125·3° (123°)[19]. Zahn and Würz[20], however, described a synthesis (not attempted) which yielded a product with b.p. 108° and m.p. 25·8°. Heilbron and Bunbury[21] quote the *p*-fluoromononitrobenzene as existing in two forms, the stable one of which has m.p. 26·5 to 27°. As it seemed improbable that the crystalline material could have been the mono-nitro compound since morphine recoveries would then have been about 11 per cent higher than theory, the possibility of polymorphism in the dinitro compound was considered. Rheinboldt and Perrier[22,23] quote the 1-fluorodinitrobenzene as having m.p. of 26° and 28° and state[24] that polymorphism in this compound, as in many others where 2:4-dinitrobenzene is substituted in the 1- position, is very pronounced.

The manufacturers of the crystalline sample later supplied a further quantity of 1-fluoro-2:4-dinitrobenzene. This was a yellow liquid, b.p. 167° at 11 mm. pressure and refractive index at 20° = 1·5684. The various samples gave essentially identical infra-red spectra and since the spectrum does not appear to have been recorded in the literature, this is reproduced in Figure 3 over the range 5 μ to 15 μ. Furthermore, quantitative recoveries of morphine were obtained with the various samples, and the solid and liquids would thus appear to be polymorphic forms of 1-fluoro-2:4-dinitrobenzene.

During the precipitation of morphine with fluoro-dinitrobenzene the solution gradually changes colour from yellow to dark reddish orange.

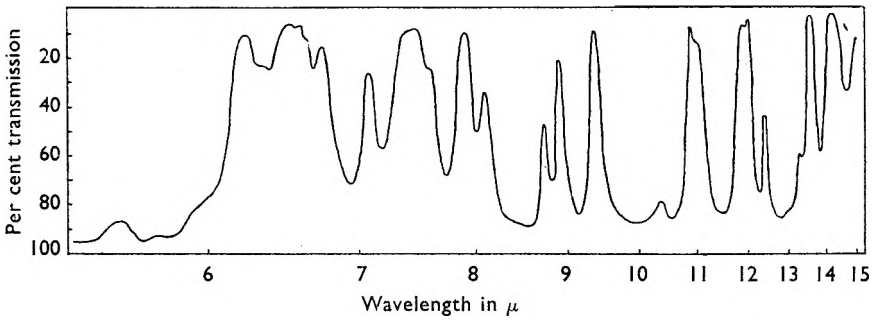

FIG. 3. Infra-red spectrum of 1-fluoro-2:4-dinitrobenzene (capillary film).

After about two hours a bright yellow material of different crystalline form and readily distinguishable from morphine dinitrophenylether begins to deposit slowly. These changes are not observed when chlorodinitrobenzene is used. In this case the solution becomes pinkish-purple after only a few minutes standing, gradually increasing in intensity to a reddish-purple which remains for about 30 hours before fading to an orange-yellow. No co-precipitate appears within about 36 hours.

Tests were made with the fluoro reagent in the absence of morphine; the colour changes and deposition of a bright yellow precipitate as described above were again noticed. This precipitate was insoluble in water, soluble with difficulty in cold alcohol but more readily soluble in hot alcohol and very readily soluble in acetone. After filtering and washing several times the material was recrystallised from aqueous alcohol and shown to be 2:4-dinitroaniline as follows:

(i) M.p. 179·6 to 180·2° (180°)[19]. (ii) Mixed m.p. with pure 2:4-dinitroaniline 179·8 to 180·2°. (iii) Nitrogen content by the method of McCutchan and Roth[25] 22·80 per cent (theory 22·94 per cent). (iv) Acetyl derivitive m.p. 121·8 to 122·3° (120°). (v) Identity of infra-red spectrum with that of pure 2:4-dinitroaniline. The ease with which this material may be prepared using fluorodinitrobenzene as opposed to chlorodinitrobenzene[26] again illustrates the remarkable reactivity of the fluorine substituted compound.

It should be noted that 1-fluoro-2:4-dinitrobenzene is a vesicant and care should be taken to avoid contact with the skin.

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DISCUSSION

The paper was presented by MR. C. A. JOHNSON.

MR. H. B. HEATH (Sudbury). Why was the opium and ammonia first mixed with the alumina? How much alumina was used?

DR. G. E. FOSTER (Dartford). A similar method was published in the Australian Chemical Institute proceedings in 1946 by Dr. Trautner. What was the minimum quantity of morphine which could be estimated by their method?

DR. L. SAUNDERS (London). About three years ago there appeared in *Analytical Chemistry* a method describing the use of ion exchange resins for extracting morphine. Had this method been tried?

MR. R. L. STEPHENS (Portsmouth). Had the authors tried to estimate morphine in Tincture of chloroform and morphine?

MR. W. SMITH (Ware). Was the vesicant nature of fluorodinitrobenzene any disadvantage to the method.

MR. C. A. JOHNSON replied. The quantity of alumina varied a little with the product being assayed, but was about 10 to 15 g. Alumina was added until a free flowing powder was obtained. The method could be operated successfully with about 25 ml. of camphorated tincture of opium. The method with ion exchange resins published in *Analytical Chemistry* had been tried, but they had not obtained quantitative results. Attempts had been made to apply the method to Tincture of chloroform and morphine, but the trouble involved in setting up the column was such as to make it not worth while. The quantity of water required cancelled out any variation in the alumina used. With reasonable care the vesicant nature of fluorodinitrobenzene was no disadvantage.

THE PREPARATION AND PROGESTATIONAL ACTIVITY OF SOME ALKYLATED ETHISTERONES

By A. David, F. Hartley, D. R. Millson and V. Petrow

From The Research Laboratories, The British Drug Houses, Ltd., London, N.1, and Godalming, Surrey

Received June 17, 1957

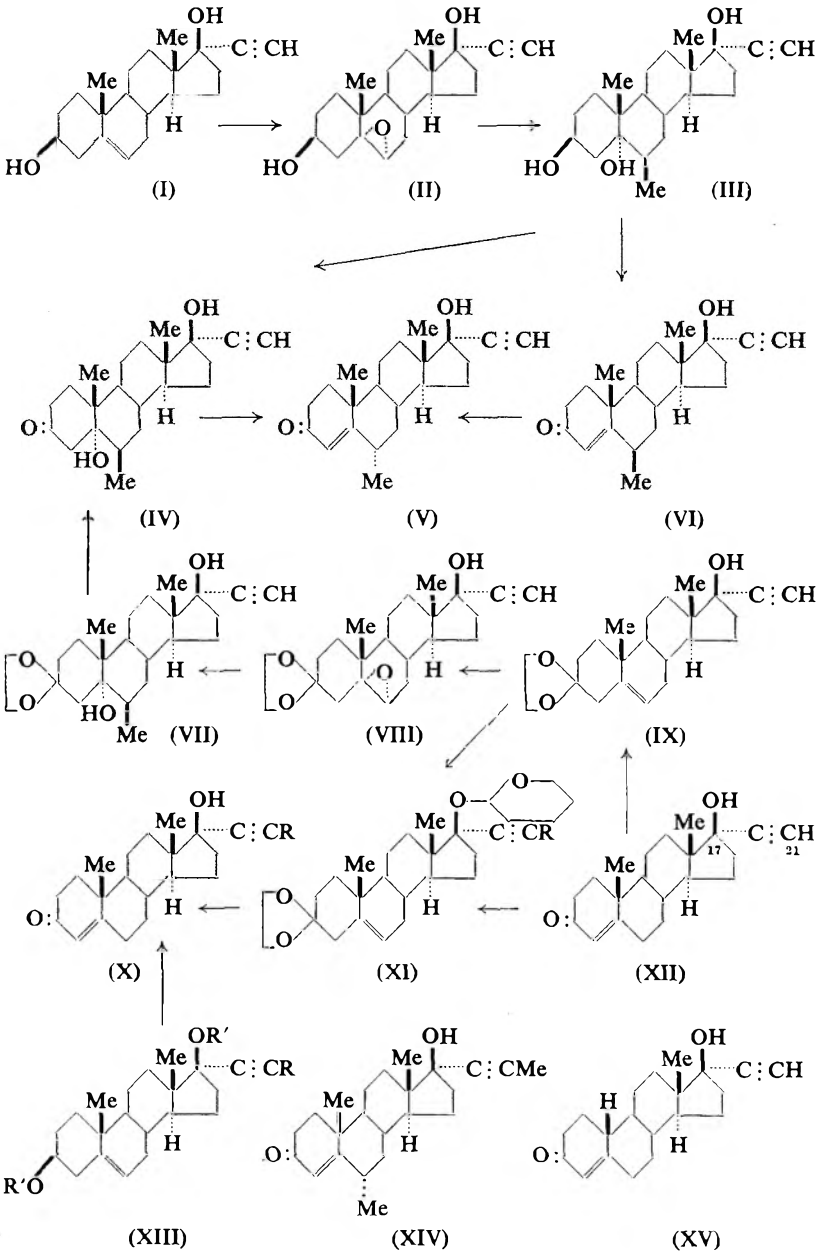
The preparation and progestational activity of some 6-alkyl, 21-alkyl and 6:21-dialkyl derivatives of ethisterone are described. 6α:21-Dimethylethisterone (XIV), the most potent compound of the series, given by mouth proved to have approximately twelve times the activity of ethisterone in the Clauberg test.

The present studies were initiated in 1954 and had as their object the preparation of an orally-acting progestational agent more potent than ethisterone (XII). At that time the literature contained several references to unsuccessful attempts to achieve the same objective. In addition, the paper by Djerassi, Miramontes, Rosenkranz and Sondheimer[1] had just appeared describing the partial synthesis of 19-norethisterone (XV), which had several times the activity of ethisterone in the Clauberg test. The 19-nor steroid type (XV), however, appeared unattractive for development as its preparation involved Birch reduction of oestradiol methyl ether, a raw material which could hardly be regarded as abundant and cheap.

At that time the interests of our research group had been focussed on speculations concerning the possible role of methylated steroids, and in particular 4:4-dimethyl steroids, as biogenetic precursors of the steroid hormones[2]. It seemed desirable therefore to consider the methylated derivatives of ethisterone. Selection of 6-methylethisterone for prior study stemmed largely from biogenetic considerations. The liver was known to deactivate steroid hormones in several ways including β-hydroxylation at C(6)[3]. By blocking this centre with a methyl group we hoped to prevent such oxidation and thereby enhance biological activity by the oral route.

Preparation of the isomeric 6α- and 6β-methylethisterones was achieved in the following way[4]. Ethynylandrostenediol (I) was converted into the 5α:6α-epoxide (II), which passed smoothly into 17α-ethynyl-6β-methylandrostane-3β:5α:17β-triol (III) on reaction with methyl magnesium iodide. Oppenauer oxidation of the latter compound afforded 6β-methylethisterone (VI), which was readily isomerised to 6α-methylethisterone (V) by alkaline or acidic reagents. The same compound (V) was additionally obtained by oxidising the triol (III) to the 3-oxo derivative (IV), which was converted into the desired product by dehydration and epimerisation.

An alternative route to 6α-methylethisterone (V) employing ethisterone (XII) as starting material was also developed[5]. In this process ethisterone (XII) was converted into the 3:3-ethylenedioxy-derivative (ethylene ketal)



(IX), a transformation accompanied by migration of the 4:5-ethylenic linkage into the 5:6-position. Treatment of this compound with monoperphthalic acid gave the 5α:6α-epoxide (VIII), which passed into the 6β-methyl-5α-hydroxy steroid (VII) on reaction with methylmagnesium iodide. This product (VII) readily lost the ketal group on contact with

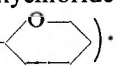
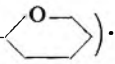
very dilute acid to give the intermediate (IV), which was transformed into 6α-methylethisterone (V) as before. 6β-Ethylethisterone was prepared in essentially the same way (see exptl.).

Biological study of 6β-methylethisterone (VI) proved disappointing, the compound being only about one-third as potent as ethisterone. The 6β-ethyl-derivative was virtually devoid of activity. 6α-Methylethisterone (V), in contrast, was found to be approximately seven times as active as ethisterone in the Clauberg test.

The marked superiority of 6α- over 6β-methylethisterone as a progestational agent is difficult to interpret in terms of increased resistance to 6β-hydroxylation by the liver[3], and it seems likely that other factors are involved. This view is strengthened by the observation that similar quantitative differences obtain with other 6-methylated steroid hormone pairs prepared in our laboratories. The role played by the alkyl group in modifying biological activity, however, remains to be elucidated.

We next turned our attention to the alkylation of ethisterone at C(21). The reasons which prompted this choice stemmed from the structural analogy between ethisterone and methyltestosterone, which could both be regarded as 17-substituted testosterone derivatives.

Now stepwise increase in the alkyl chain attached to C(17) in 17α-methyltestosterone changes markedly the biological properties of this orally active androgen. In analogy stepwise increase in the alkynyl chain attached to C(17) in 17α-ethynyltestosterone (ethisterone) to give 21-alkyl derivatives was thought worthy of study.

Preparation of the 21-alkyl derivatives of ethisterone (X) was accomplished by the following route[6]. Ethynylandrostenediol (XIII; R = R′ = H) was treated with 2:3-dihydropyran and phosphorus oxychloride to give the bis-tetrahydropyranyl ether (XIII; R = H, R′ = ). This operation proved necessary to avoid alkylation of the hydroxyl groups during the subsequent operation. The bis-tetrahydropyranyl ether was then converted into the C(21)-lithium derivative by treatment with lithamide in liquid ammonia, which was then alkylated with an alkyl halide to give the derivative (XIII; R = alkyl, R′ = ). The product obtained by removing the protecting groups was submitted to Oppenauer oxidation to give the ethisterone homologue (X; R = alkyl). Alternatively, the 3:3-ethylenedioxy-derivative of ethisterone (IX) was treated with 2:3-dihydropyran to give the product (XI; R = H), which was alkylated as before yielding the intermediate (XI; R = alkyl). Treatment of this compound with dilute acid furnished the 21-alkyl-ethisterone (X; R = alkyl).

Bioassay of the ethisterone homologues (X; where R = Me, Et, *n*-Pr and *n*-Bu, respectively) revealed an interesting gradation of properties.

21-Methylethisterone was found to be about three times as active as ethisterone. Increase in the size of the alkyl substituent led successively to a decrease of activity, the *n*-butyl derivative being less active than ethisterone itself.

Having established that the biological activity of ethisterone was enhanced by (i) a 6α-methyl substituent and (ii) an alkyl group (Me > Et > Pr) at C(21), it was clearly essential to determine whether these effects would prove additive. We therefore synthesised the 21-methyl and 21-ethyl derivatives[6] of 6α-methyl-ethisterone by procedures based on those described above. Biological study of these compounds revealed that the effects were, broadly speaking, additive. Thus 6α:21-dimethyl-ethisterone (XIV) proved to be approximately twelve times and 21-ethyl-6α-methylethisterone nine times more active than ethisterone in the Clauberg test. 6α:21-Dimethylethisterone (XIV) is thus the most potent orally active progestational agent based upon 10:13-dimethylperhydro-*cyclo*pentenophenanthrene that has yet been reported. Clinical studies are in progress.

Experimental

Melting points are uncorrected.

Preparation of 6β-ethylethisterone (with Miss D. Wedlake, B.Sc.).—3β-Acetoxy-5α:6α-epoxy-17α-ethynylandrostan-17β-ol[1] (13·1 g.) in benzene (500 ml.) was added rapidly to a stirred Grignard reagent prepared from magnesium (7·2 g.), ethyl iodide (42 g.) and ether (320 ml.). The mixture was distilled until the vapour temperature reached 78° when more benzene (300 ml.) was added. After heating under reflux for 5 hours the mixture was cooled, treated with a slight excess of dilute sulphuric acid, and the organic layer washed with water and dried. The residue obtained by removal of the solvent was crystallised from aqueous methanol to give *6β-ethyl-17α-ethynylandrostane-3β:5α:17β-triol*, needles, m.p. 143° to 145° $[\alpha]_D^{20}$ − 45° (*c*, 1·0 in pyridine). Found: C, 73·6; H, 10·3. $C_{23}H_{36}O_3$ requires C, 73·0; H, 10·2 per cent.

The foregoing triol (4·5 g.) in toluene (250 ml.) and *cyclo*hexanone (110 ml.) was treated with aluminium *iso*propoxide (2·5 g.) in toluene (12 ml.). The mixture was heated under reflux for 40 minutes, cooled, washed with dilute sulphuric acid, then with water, and the solvents removed by steam distillation. The product was purified from methanol to give *6β-ethylethisterone*, rods, m.p. 260° to 264°, $[\alpha]_D^{20}$ + 9° (*c*, 0·7 in pyridine). Found: C, 80·9; H, 9·4. $C_{23}H_{32}O_2$ requires C, 81·1; H, 9·5 per cent.

Estimation of Progestational Activity

McPhail's modification[7] of the Clauberg test was employed. Immature female rabbits weighing between 800 to 1,200 g. were sensitised with a total dose of 15 μg. of oestrone in 0·6 ml. of a mixture of ethyl oleate (20 per cent) with arachis oil, given intramuscularly, in three equal amounts, on days one, three and five of the test.

Ethisterone and the test compounds were given orally in four equal parts on days seven, eight, nine and ten. Doses were given suspended in 5 ml. of a mucilage of acacia (5 per cent). The animals were killed on day eleven and frozen sections of uteri of 20μ thickness were prepared, stained with haematoxylin and examined for progestational response. Projection

drawings of the uteri were prepared and the responses estimated by measuring the fraction of endometrium occupied by glandular tissue. In addition, McPhail's method of scoring was used and good agreement between the methods was found. Potencies were however calculated using the first method which being susceptible of repeated check measurements was considered to be less liable to error from bias.

The relative activities of four 21-alkyl derivatives of ethisterone were estimated in a multiple four point assay, using fifty rabbits. Table I records the results.

TABLE I

MEAN PROGESTATIONAL RESPONSES IN FIVE RABBITS PER DOSE AFTER ORAL ADMINISTRATION OF FOUR 21-ALKYL DERIVATIVES OF ETHISTERONE

Compound	Response per cent glandular tissue		Approximate relative potency
	Dose 2·5 mg./kg.	Dose 7·5 mg./kg.	
Ethisterone	54·4	67·0	1·0
21-Methylethisterone	66·4	70·8	3·0
21-Ethylethisterone	58·2	69·3	1·6
21-Propylethisterone	56·0	64·6	1·0
21-Butylethisterone	50·3	58·6	0·5

The 6α-methyl-, 6α:21-dimethyl-, and 6α-methyl-21-ethyl-derivatives were also compared in a multiple six point assay, using sixty rabbits. Table II records the results.

TABLE II

MEAN PROGESTATIONAL RESPONSES IN FIVE RABBITS PER DOSE AFTER ORAL ADMINISTRATION OF VARYING AMOUNTS OF 6α-METHYL, 6α:21-DIMETHYL AND 6α-METHYL-21-ETHYL DERIVATIVES OF ETHISTERONE

Compound	Dose mg./kg.	Response		Relative potency (P = 0·95)
		McPhail	Glandular tissue per cent	
Ethisterone	2·5	1·16	45·8	
	5·0	1·82	54·8	1·0
	10·0	2·54	68·4	
6α-Methylethisterone	0·3	0·76	38·2	
	0·6	1·60	50·8	6·54
	1·2	2·76	66·8	(4·84–8·70)
6α:21-Dimethylethisterone ..	0·3	1·62	53·4	
	0·6	2·36	59·6	11·5
	1·2	3·20	73·4	(8·67–15·5)
6α-Methyl-21-ethyl ethisterone	0·3	1·12	43·8	
	0·6	1·86	57·6	9·24
	1·2	3·26	73·2	(6·93–12·4)

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1. Djerassi, Miramontes, Rosenkranz and Sondheimer, *J. Amer. chem. Soc.*, 1954, 76, 4092.
2. Cf. Cooley, Ellis and Petrow, *J. chem. Soc.*, 1955, 2998; Adams, Patel, Petrow, Stuart-Webb and Sturgeon, *ibid.*, 1956, 4490.
3. See, for example, Miller and Axelrod, *Metabolism*, 1954, 3, 438.

4. B.P. Provisional Specifications Nos. 15889/55, 16645/55, 17799/55, 18118/55. Burn, Ellis, Petrow, Stuart-Webb and Williamson, in the press; Ackroyd, Adams, Ellis, Petrow and Stuart-Webb, in the press.
5. B.P. Provisional Specification No. 9378/56. Cooley, Ellis, Kirk and Petrow, in the press.
6. B.P. Provisional Specification No. 2820/57.
7. McPhail, *J. Physiol.*, 1935, **83**, 145.

DISCUSSION

The paper was presented by DR. V. PETROW.

THE CHAIRMAN. Preliminary clinical tests confirmed that 6α-methyl ethisterone was at least three times as active as ethisterone. Clinical confirmation about the 6α:21-dimethyl compound was not yet available.

DR. L. M. ATHERDEN (Sunderland) noted that ethisterone possessed a double bond in the 4:5 position which would be hydrogenated in the liver. Would the 6α-methyl compound be more active because the hydrogenation would be hindered?

DR. J. B. STENLAKE (Glasgow). Had the study of the 6α-alkyl substances been carried any further?

DR. G. BROWNLEE (London). Had the substances any androgenic activity?

DR. V. PETROW replied that he knew of no published work describing the fate of ethisterone in the body. The study of the 6α-alkyl substances had not been continued because of the drop in activity.

DR. DAVID added that the substances possessed no androgenic properties; at very high and unphysiological doses the 6α-methyl compound showed a very slight anabolic effect.

SOME EFFECTS OF INCREASING STILLHEAD SURFACE AREA ON LIQUID ENTRAINMENT DURING DISTILLATION

By David Train and Bayardo Velasquez-Guerrero

Department of Pharmaceutics, School of Pharmacy, University of London, Brunswick Square, W.C.1

Received July 1, 1957

The effect on the amount of entrainment passing during distillation when the surface area of a stillhead is increased has been investigated. The conditions suitable for supporting a liquid film on such a surface and which favour flooding at high flow rates have been observed.

Shotton and Habeeb[1] investigated the entrainment of liquid during distillation in straight vertical stillheads, and we have studied the effect of increasing the wall surface area of such a stillhead whilst maintaining constant its effective area of cross-section.

Using essentially the same apparatus and techniques as those already described[1,2], this increase in wall surface area was achieved by introducing into the stillhead a *thin* diametrical septum of phosphor-bronze (Fig. 1) which had been dull chromium plated to give it wetting characteristics similar to those of glass. The diameters of the stillheads were 1 in. and $1\frac{1}{2}$ in. respectively and the length of each was maintained at 15 in. to limit one of the variables. The maximum distillation rate was increased from 18 l. to 33 l. per hour by using the main steam supply. A dry sample of this steam was passed into the reboiler containing a fluorescein solution. The quantity of liquid entrained was assessed as before, by estimating fluorimetrically the amount of fluorescein passing from the boiler to the separator and the condenser.

Results

Figure 2 summarises the results of some 450 individual fluorescein estimations. The curves are derived from points which represent the means of 2 to 4 replicate determinations. Curve A represents 31 points, B, 26 points, C, 47 points, and D, 42 points. The average increment was 500 ml. The quantity of fluorescein collected beyond the stillhead was expressed as μg./l. of distillate and represented the degree of contamination of distillate by entrainment, each μg. of fluorescein being equivalent to 0·001 ml. of original solution. Extension of the distillation rate gave new data on the behaviour of the system in the upper range.

Discussion

It has been assumed that for a given rate of distillation the amount of entrainment will be consistently the same. It was shown by Shotton and Habeeb that the diameter of the stillhead was an important factor in governing the amount of entrainment. It would be expected therefore that a reduction in a radial path by introducing a septum, whilst leaving the total cross-sectional area unchanged, would reduce the amount of entrainment passing through the stillhead. This has been demonstrated in the present study (cf. curve A with B and curve C with D in Fig. 2).

These curves have the same general form as those previously reported and may be divided into three distinct stages.

The first stage was attributed by Shotton and Habeeb to be a region where streamline flow is predominant and it is to be expected that any droplets, being carried up in the cores of vapour flowing in a streamline manner between the wall surfaces, will pass into the separator without loss. Those droplets near a wall surface will be arrested and the presence of the septum reduces the amount of entrainment for any given distillation rate. The slope of the curve is at a constant angle for a given stillhead whether with or without the septum.

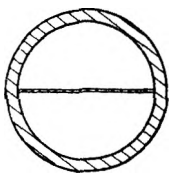


Fig. 1. Thin diametrical septum of phosphor-bronze.

The second portion of the curves shows how the additional wall area reduces the amount of entrainment passing. Since this region is in the turbulent range local eddies with radial components in the vapour stream induce droplets to move in some direction not truly along the axis of flow, so that there is a greater possibility that a droplet caught in such an eddy will be arrested on the wall. As the rate increases the degree of turbulence increases and this favours greater catchment so that the amount of entrainment passing per unit volume of distillate stays substantially the same. The presence of a septum reduces the entrainment passing, but since measurement of the *total* amount of liquid entrained in the vapour stream was not possible, we have been unable to assess whether the increase in entrainment is in proportion to the increase in perimeter caused by the insertion of the septum.

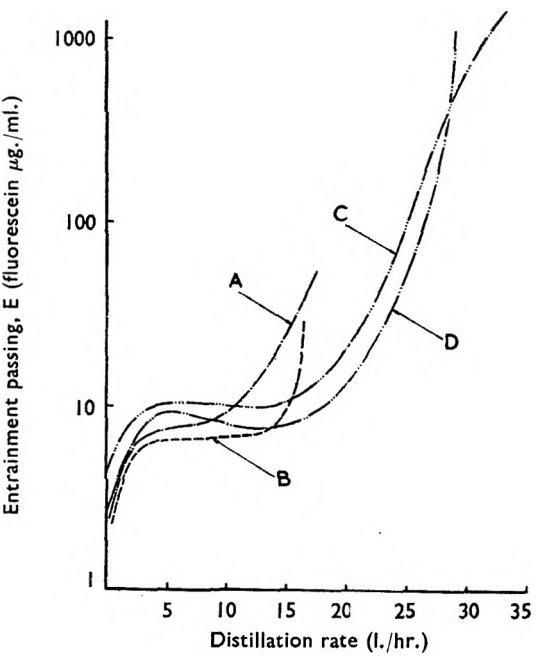


FIG. 2. Results of 450 estimations using stillheads with and without a septum.

A. Stillhead 1 inch diameter without septum.
B. ,, ,, ,, with ,,
C. ,, 1½ inches ,, without ,,
D. ,, ,, ,, with ,,

The third stage was given the name of "gross carry over" by Shotton

and Habeeb. In the present work careful observations of the conditions obtaining in the stillhead at the onset of this stage showed that the frictional drag forces of the rising vapour were sufficient to maintain a thin film of liquid on the walls of the stillhead in spite of the gravitational force tending to drain it downwards. As the distillation rate increased the wall film became thicker and finally, at extremely high rates, the film was dragged as a whole in an upward direction; it even coalesced across the head to form slugs of liquid which continued to be driven upwards by the rapidly ascending vapour. This final condition is similar to that found in the movement of two phases in wetted wall columns[3] in the condition known as "flooding".

The insertion of a septum delays the onset of gross carry over since with increased surface area in the stillhead, there is a greater area of liquid film to be supported. This requires a greater drag force which can be achieved only under the experimental conditions by increasing the rate of distillation. However, when there is sufficient liquid being held so that coalescence into liquid slugs is facilitated, the shorter radial path produced by the presence of the septum is advantageous and so flooding, as defined above, occurs at a lower distillation rate than it would have done in the same stillhead without a septum.

There is close agreement with the earlier work[1] when an analysis of the results from plain stillheads is made to relate the entrainment passing (E) with Reynolds Number ($\rho ud/\eta$). No acceptable correlation has been established which allows the incorporation of those results when a septum is used. Various possibilities for finding a relation between the area of cross-section, wetted perimeter, or mean radial path and the quantity of entrainment passing were tested without success. A possible explanation is that the particular shape of septum used gives conditions which makes the interpretation of results a difficult procedure.

Acknowledgement. We should like to thank Professor E. Shotton for his advice and interest throughout this work.

REFERENCES

1. Shotton and Habeeb, *J. Pharm. Pharmacol.*, 1954, **6**, 1023.
2. Shotton and Habeeb, *ibid.*, 1955, **7**, 456.
3. Perry, *Handbook of Chemical Engineering*, 3rd Ed., McGraw Hill, London, 1950, p. 686.

DISCUSSION

The paper was presented by MR. B. VELASQUEZ-GUERRERO.

THE CHAIRMAN. Had the authors considered the possible value of dropwise promotors in reducing entrainment?

MR. J. H. OAKLEY (London). What was the effect of increasing the number of septa, and would a septum in conjunction with a conventional baffle further reduce the entrainment?

PROFESSOR K. BULLOCK (Manchester). Was there much difference between the replicate determinations?

MR. H. B. HEATH (Sudbury). Did lagging the stillhead make any difference?

MR. J. H. OAKLEY (London) referred to the two pairs of graphs from which it appeared that an increase in the diameter of the stillhead reduced the effect of the septum.

MR. R. L. STEPHENS (Portsmouth) pointed out that if a second septum were placed in the distillation column and a voltage applied across the septa the effect might be that of electrostatic precipitation.

THE AUTHORS replied. An increase in number of septa would reduce the amount of entrainment, but flooding would occur earlier. The use of a baffle above the septum would probably reduce entrainment. The results were the average of five or six distillations. The difficulty was to keep the distillation rate constant. Lagging the stillhead made a difference because it affected the amount of condensation. It might be conjectured that the greater the scale the less the entrainment caught, but it was the difficult problem of comparing the effect of increase in surface area with that of increase in area of cross-section. There was no evidence that the particles were charged sufficiently to be affected electrostatically.

ALPHA- AND BETA-PRODINE TYPE COMPOUNDS: CONFIGURATIONAL STUDIES

By A. H. Beckett, A. F. Casy, G. Kirk and J. Walker

From the Chelsea School of Pharmacy, Chelsea Polytechnic, London, S.W.3

Received June 27, 1957

The addition of various lithium aryl compounds to 1:3-dimethyl-4-piperidone is investigated. Those stereoisomers present as the major proportion of the respective stereoisomeric mixtures are shown to have similar infra-red absorption characteristics which are different from those exhibited by those isomers formed in minor amount. The stereochemistry of addition to 6-membered alicyclic and heterocyclic ketones is discussed; on the basis of these discussions, and the rates of hydrolysis of appropriate esters, the above isomers formed in major amount are allocated the *trans* (Me/Ar) configuration. The analgesic activities in mice of various stereoisomeric amino-alcohols and esters are given.

The addition of phenyl lithium to 1:3-dimethyl-4-piperidone yields two diastereoisomeric alcohols from which two analgesically active propionoxy esters, alphaprodine and betaprodine have been prepared[1]. In animals, betaprodine is much more active than alphaprodine[2] although the differences in observed potencies are less in man[3]. Results of hydrolysis studies which indicate that betaprodine should be allocated the *cis* (Me/Ph) and alphaprodine the *trans* (Me/Ph) configuration have previously been presented[4].

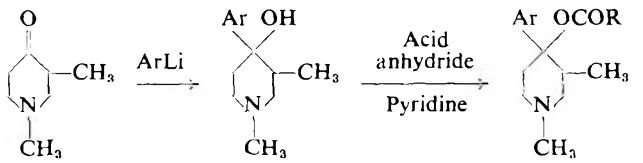
The stereochemical compositions of the products of addition of certain lithium aryl compounds to 1:3-dimethyl-4-piperidone are now considered. Information of isomeric composition is of value in assigning configurations to various stereoisomers and also supplements information from hydrolysis experiments with stereoisomeric esters. There is the additional object of producing compounds of known configuration for analgesic tests to provide further information about the stereochemical requirements of analgesics[5–7] and the analgesic receptor site[8,9].

Preparation of Compounds and Separation of Isomers

The key intermediate, 1:3-dimethyl-4-piperidone (I), was prepared by cyclisation of β-carbomethoxyethyl-β-carbomethoxypropylmethylamine and subsequent decarboxylation[10]. Treatment of the ketone with a lithium aryl gave a mixture of tertiary alcohols (II) which was esterified by refluxing with an acid anhydride in the presence of pyridine. Attempts to separate the diastereoisomers involved fractional crystallisation of the free alcohols from hydrocarbon solvents, or the ester hydrochlorides from ether-ethanol.

Separation of the alcohols derived from phenyl lithium was achieved by crystallisation of the propionoxy ester hydrochlorides; alphaprodine was obtained from the first four, and betaprodine from the fifth crop.

Adsorption chromatographic separations were unsuccessful. The infra-red absorption spectra of the derived isomeric alcohols (alpha- and beta-prodine alcohols) showed distinct differences in three regions (see Table II).

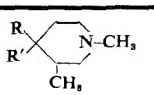


(I) (II) (III)

The *m*-tolyl alcohols (II, Ar = *m*-$CH_3 \cdot C_6H_4$) were separated by crystallisation from *n*-hexane; 15 recrystallisations were necessary to separate the pure isomers. The infra-red absorption spectrum of one isomer (A) resembled that of alphaprodine alcohol; that of the other isomer (B) resembled that of betaprodine alcohol.

TABLE I

CHARACTERISTICS AND PROPORTIONS OF ISOMERS OBTAINED BY THE ADDITION OF LITHIUM ARYLS TO 1:3-DIMETHYL-4-PIPERIDONE



		Reaction product		Isomer A		Isomer B		
R	R'	Wt. in g.	m.p. ° C.	Wt. in g.	m.p. ° C.	Wt. in g.	m.p. ° C.	Ratio A : B
C_6H_5	$OCOC_2H_5$	11·0	181–197	7·6	218–220	2·6	181–185	3 : 1
o-$CH_3 \cdot C_6H_4$	OH	6·5	83–85	4·96	86–87	0	—	mainly one isomer (A)
m-$CH_3 \cdot C_6H_4$	OH	14·7	104–108	10·58	88·5–89·5	1·28	114–115	9 : 1
p-$CH_3 \cdot C_6H_4$	OH	6·3	softened 85 m.p. 122–133	4·21	135–136	0·04*	103–104	A > B
o-$OCH_3 \cdot C_6H_4$	OH	7·3	109	7·18	111·5	0	—	mainly one isomer (A)

* Isolated by hand picking from a weight of 1·8 g., m.p. 81–91°; the latter was examined spectroscopically for infra-red absorption and exhibited characteristics of both isomers.

The first three crops obtained by crystallisation of the *p*-tolyl alcohols (II, Ar=*p*-$CH_3 \cdot C_6H_4$) from *n*-hexane gave one pure isomer (A) with a sharp melting point, little changed upon recrystallisation, and an infra-red absorption spectrum resembling that of alphaprodine alcohol. Further crops gave material melting over a wide range 40° or so below the melting point of the initial crops. Crystallisation of the corresponding propionoxy ester hydrochlorides from ether-ethanol again gave sharp melting point initial crops; further crops melted at similar temperatures but showed softening 40° or so below the final melting point. A small quantity of a second isomer (B) was obtained by hand-picking crystals from the tenth recrystallisation of the free alcohols; the infra-red absorption spectrum of this isomer resembled that of betaprodine alcohol, while that of a powdered sample of the tenth fraction showed characteristics of both isomers.

Treatment of the piperidone (I) with *o*-tolyl lithium gave a product which, upon recrystallisation, was recovered in high yield with little change in melting point; the infra-red absorption spectrum of this alcohol resembled that of alphaprodine alcohol. A similar result was found upon treatment of the piperidone (I) with *o*-methoxyphenyl lithium. These last two additions result in virtually single products and isomeric forms, if they occur, represent only a small proportion of the reaction products.

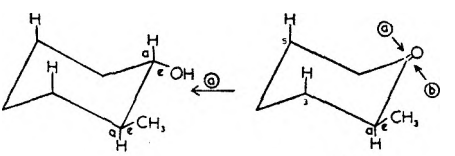
An estimate of the ratio of isomer weights is summarised in Table I. The weights of the isolated isomers accounted for the major portion of the reaction product, and their relative proportions may therefore be used as a basis for a discussion of the isomeric composition of the products of addition of lithium aryl compounds to 1 : 3-dimethyl-4-piperidone.

Discussion

Stereochemistry of Addition to Ketones

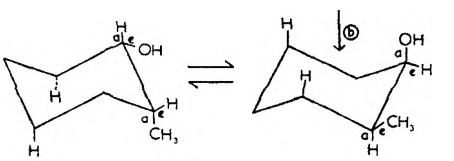
The stereochemical composition of the products of addition to the carbonyl group of cyclic ketones will be mainly dependent upon two factors. The first is the difference in the steric environment about the two directions of approach to the C atom of the carbonyl group and the second, the relative thermodynamic stability of the isomers. This will influence the rate of formation of the isomers from the transition complexes. The importance of these factors may be shown by the following examples.

In the reduction, other than hydrogenation, of 2-methyl*cyclo*hexanone, which exists mainly in the conformation IV, attack from side (*a*) to yield the *trans* isomer (V) will be more hindered, mainly from the 3 and 5



(V)
trans isomer

(IV)



(VI)
cis isomer

axial H-atoms, than that from side (*b*) to yield the *cis* isomer (VI). But the *trans* isomer (V) is thermodynamically more stable than the *cis* isomer. For example, equilibration of the alcohols yields 99 per cent of the *trans* isomer[11]. The two effects are in opposition, and the yields of 58 and 31 per cent of the *cis* isomer, upon reduction of the ketone by

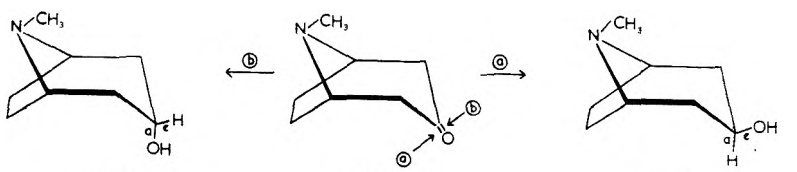
aluminium isopropoxide and sodium borohydride in methanol[11] respectively, are explicable in terms of the larger reducing species of the former reagent. [Dauben and others[11] explain these results by assuming that attack from side (*a*) and (*b*) in IV are equally favoured; the high proportion of *cis* isomers in the product is explained by these authors as resulting from the contributions of the less stable conformation of 2-methyl*cyclo*hexanone in which the axial methyl group hinders attack from its side of the carbonyl group.]

The equatorial 2-methyl group of IV constitutes a small steric factor operating on the same side as the 3 and 5 axial H-atoms. The following predictions may therefore be made about the isomeric composition of the products of reduction of 2 (or 4) monoalkyl*cyclo*hexanones.

The product will contain more of the *cis* isomer than the product of equilibration; the proportion of this isomer will increase with increasing size of reducing reagent species. Increase in bulk of the 2-alkyl substituent will increase the relative proportion of the *cis* isomer. Reduction of the 4-alkyl derivatives will give relatively less, compared with equilibrated mixtures, of the *cis* isomer than reduction of the corresponding 2-alkyl derivatives.

The recorded values[11–13] for the stereochemical composition of the reduction product of various alkyl*cyclo*hexanones supports these predictions, although some values are only of a semi-quantitative character.

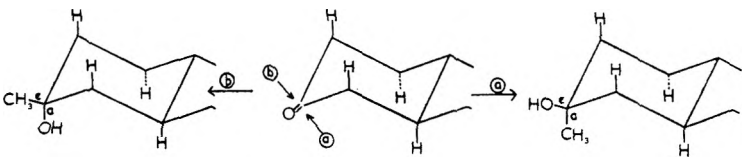
Reduction of heterocyclic ketones may be considered similarly. Beckett and others[14] have shown that reduction of tropinone (VII), by various reducing agents, yielded much more of the thermodynamically less stable tropine (VIII) than was present in "equilibrated" tropine or



ψ-tropine (IX). This was expected, since approach from side (*a*) is less favoured sterically than that from side (*b*); changes in the reducing species yielded larger proportions of tropine under conditions in which larger species would be expected.

The formation of isomers by addition of Grignard reagents, or lithium derivatives, to alicyclic or heterocyclic ketones will be also influenced by the factors governing the stereochemistry of addition to ketones. Cyclic transition states will be involved but the rate-controlling step of such reactions involves addition of the R of the reagent to the C atom of the carbonyl group. Equilibration of these tertiary alcohols to give a measure of the relative thermodynamic stabilities of the isomers is usually precluded. However, examination of isomeric compositions (the isomer formed in larger amounts will be derived from attack from the least hindered side of the molecule, if the energy contents of the isomers are not

too dissimilar) and the effect of an increase in the size of the hydrocarbon portion of the addendum (increase will favour more the attack from the least hindered side) may be used for tentative configurational assignment to the isomers. Few examples of such additions are available in which quantitative results of isomeric composition have been obtained. Barton and others[15] reported that the addition of methylmagnesium bromide to 5·2 g. cholestanone (X) gave 2·9 g. of the α-OH isomer (XI) and 2·2 g. of the β-OH isomer (XII); thus the isomer resulting from attack from the

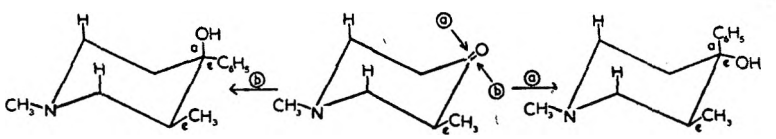


(XI) (X) (XII)

least hindered side of the carbonyl group is formed in major amount. We find that the addition of phenyl lithium to tropinone gives a tertiary alcohol in 97 per cent yield with the OH in the same configuration as that of tropine, showing predominant attack from the least hindered side.

Configurational Assignments to Prodine-type Compounds

Beckett and Walker[4], from hydrolysis experiments, have allocated the *trans* (Me/Ph) configuration to alphaprodine (propionoxy ester of XIV) and the *cis* (Me/Ph) configuration to betaprodine (propionoxy ester of



(XIV) (XIII) (XV)

XV). The preceding arguments indicate that the *trans* (Me/Ph) isomer should be formed in larger amount upon addition of phenyl lithium to 1:3-dimethyl-4-piperidone (XIII) since attack from the least hindered side (*b*) would be favoured. Careful fractional crystallisation of the propionoxy ester hydrochlorides derived from the reaction product yielded the ratio of 3:1 of alphaprodine to betaprodine, thus supporting the previous assignments.

Addition of *m*-tolyl and *p*-tolyl lithium gave isomeric pairs of compounds; the isomers were not present in equal amounts (see Table I). The predominant isomer would be expected to have the same configuration as the alcohol (XIV) from alphaprodine. Even higher proportions of the isomers configurationally related to XIV would be expected by using an *o*-methyl or *o*-methoxy group substituted in phenyl lithium. These substitutions will increase the bulk of the addendum in the vicinity of the centre which itself will form a bond with the carbonyl group on addition. The results (Table I) indicated that increases in the size of the

addendum led to the formation of one isomer almost exclusively. Evidence was therefore sought for the configurational identity of the isomers (Type A, see Table II) formed in major amount on the addition of phenyl lithium and *m*- and *p*-tolyl lithium to 1:3-dimethyl-4-piperidone (XIII) and the single isomers (Type A, see Table II) formed upon addition of

TABLE II

CHARACTERISTICS OF INFRA-RED ABSORPTION SPECTRA OF 1:3-DIMETHYL-4-ARYL-4-SUBSTITUTED PIPERIDINES

R	R′	Isomer	Absorption peaks of characteristic frequency (cm.$^{-1}$)						
			Region A (990–1020 cm.$^{-1}$)	Region B (1350–1385 cm.$^{-1}$)			Region C (2670–2780 cm.$^{-1}$)		
C_6H_5	OH	A*	1000	1355		1383	2670 (w)	2735 (s.sh.)	2760
o-OCH_3·C_6H_4	OH	A	1001	1357		1380	2675 (w)	2730 (w)	2760
o-OCH_3·C_6H_4	$OCOCH_3$	A	1001	1365		1380	2675	2725 (w)	2760
o-CH_3·C_6H_4	OH	A	1001	1352		1376	2680 (s.sh.)	2740 (w)	2775
m-CH_3·C_6H_4	OH	A	1000	1355		1383	2680 (w)	2745 (s.sh.)	2770
p-CH_3·C_6H_4	OH	A	1002	1354		1382	2675 (w)	2730 (s.sh.)	2755
C_6H_5	OH	B†	no peak		1372	1380		2740	2775
m-CH_3·C_6H_4	OH	B	no peak		1376	1383		2740	2780
p-CH_3·C_6H_4	OH	B	no peak		1372	1383		2725	2760

w = Weak peak; s.sh. = Slight shoulder; * Alcohol from alphaprodine
A = Isomer in major amount; B = Isomer in minor amount; † Alcohol from betaprodine

o-tolyl and *o*-methoxyphenyl lithium to this ketone. Infra-red absorption measurements (see Table II) revealed a consistent pattern for these isomers in the regions, 990 to 1020 cm.$^{-1}$, 1350 to 1385 cm.$^{-1}$ and 2670 to 2780 cm.$^{-1}$, which was completely different from that shown by the isomers (Type B, see Table II) formed in lesser amount upon the addition of phenyl lithium and *m*- and *p*-tolyl lithium to the piperidone (XIII). Our own hydrolysis experiments show that esters of the isomers exhibiting the infra-red pattern of Type B (betaprodine type) hydrolyse more readily than the corresponding esters of their stereoisomeric alcohols with the Type A infra-red pattern.

It is therefore concluded that Type A isomers (Table II) have the *trans* (Me/Ar) configuration (configurationally related to XIV) whereas Type B isomers have the *cis* (Me/Ar) configuration as shown in XV.

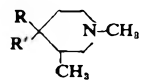
ANALGESIC COMPARISONS IN MICE

The analgesic activities of certain of the compounds reported in this paper are summarised in Table III. Compounds 1, 3–9, and 12–14 all have been assigned a *trans* configuration and must therefore be compared with alphaprodine, *trans* Me/Ph configuration, in any assessment of structure-activity relationships. The results are summarised as follows:— Replacement of the 4-phenyl group of alphaprodine by a 4-tolyl group results in a fall in activity; replacement by *p*-tolyl, *o*-tolyl and *m*-tolyl gives progressively less active compounds.

In the 4-tolyl series, replacement of the 4-propionoxy by a 4-acetoxy group results in a fall in activity; this fall is greatest in the *p*- and *m*-tolyl compounds and least in the *o*-tolyl compound. The activity in the acetoxy

TABLE III

ANALGESIC ACTIVITIES* OF 1:3-DIMETHYL-4-ARYL-4-SUBSTITUTED PIPERIDINES



	R	R′	Configuration (Me/Ar)	Salt	Analgesic activity (morphine = 100)
1	OH	*o*-CH_3·C_6H_4	*trans*	base	20
2	,,	*m*-CH_3·C_6H_4	*cis*	,,	<20
3	,,	*m*-CH_3·C_6H_4	*trans*	,,	<20
4	,,	*p*-CH_3·C_6H_4	,,	,,	<15
5	,,	*o*-OCH_3·C_6H_4	,,	,,	<20
6	$OCOCH_3$	*o*-CH_3·C_6H_4	,,	HCl	70
7	,,	*m*-CH_3·C_6H_4	,,	,,	<20
8	,,	*p*-CH_3·C_6H_4	,,	,,	30
9	,,	*o*-OCH_3·C_6H_4	,,	base	30
10	$OCOC_6H_5$†	C_6H_5	*cis*	HCl	870
11	,, ‡	C_6H_5	*trans*	,,	200
12	,,	*o*-CH_3·C_6H_4	,,	,,	85
13	,,	*m*-CH_3·C_6H_4	,,	,,	50
14	,,	*p*-CH_3·C_6H_4	,,	,,	150
15	Pethidine				40

* Determined in mice by subcutaneous injection, using adaptation of the "hot plate" method as described by Janssen and Jageneau [17].
† Betaprodine.
‡ Alphaprodine.

esters is *o*-tolyl > *p*-tolyl > *m*-tolyl. The free alcohols (Compounds 1–5) are less than one fifth as active as morphine.

EXPERIMENTAL

All m.ps. are uncorrected. Microanalyses were by Mr. G. S. Crouch, School of Pharmacy, University of London.

Equivalent weights of the bases were determined by titration with 0·02N perchloric acid in glacial acetic acid using Oracet Blue B as indicator. Titration of the hydrochloride salts in non-aqueous media in the presence of mercuric acetate was by the method of Pifer and Wollish[16].

1:3-*Dimethyl-4-piperidone.* This was prepared by the method described by Howton[10]; equiv. wt. 124, theory 127, hydrochloride, needles from ether-ethanol, m.p. 195°; Howton[10] gives m.p. 194·9° to 195·3°.

1:3-*Dimethyl-4-phenyl-4-propionoxypiperidine hydrochloride isomers* (*alpha- and beta-prodine*). The piperidone (I) (15 g.) was added dropwise with stirring to a cooled, (ice-salt bath) solution of phenyl lithium in ether (200 ml.) prepared from lithium (3·05 g.) and bromobenzene (34 g.). The mixture was stirred for 2 hours at room temperature and then added to crushed ice and glacial acetic acid (26 ml.). The solid which separated was washed with ether, the base liberated with strong aqueous ammonia and extracted with ether. After drying with Na_2SO_4, the solvent was removed to give a mixture of the crude alcohols (21 g.). The crude alcohols (12 g.) were refluxed for 4 hours with propionic anhydride (20 g.) and pyridine (20 ml.), the solvents removed under reduced pressure, the residue made alkaline with strong aqueous ammonia and the free base extracted with ether. After drying with Na_2SO_4, the solvent was removed to give a mixture of the crude propionoxy esters (13·7 g.). A solution of the crude esters (11·7 g.) in ether (160 ml.) was saturated with dry hydrogen chloride, the solid which separated collected, washed

with ether and dried in a vacuum desiccator to give the crude ester hydrochlorides (11·9 g.). Fractional crystallisation of the latter mixture (11 g.) from ether-ethanol gave alphaprodine (7·6 g.), m.p. 218° to 220° and betaprodine (2·6 g.) m.p. 181° to 185°. Further crystallisation of alphaprodine from acetone raised its m.p. to 220° to 221°; crystallisation of betaprodine from methyl ethyl ketone gave material m.p. 195° to 196°. Ziering and Lee[1] give alphaprodine m.p. 212° to 214° and betaprodine 190° to 192°. A mixture of alphaprodine (2 g.), ethanol (5 ml.) and 5 per cent aqueous sodium hydroxide (20 ml.) was refluxed for 3 hours, saturated with K_2CO_3 and the free base extracted with ether. After drying with $MgSO_4$, the solvent was removed to give α-1 : 3-dimethyl-4-phenyl-4-piperidinol, m.p. 101° to 102° after recrystallisation from *n*-hexane. Ziering and Lee[1] give m.p. 103°. β-1 : 3-Dimethyl-4-phenyl-4-piperidinol was prepared in a similar way from betaprodine and had m.p. 118° to 119° after recrystallisation from *n*-hexane. Lee informs us that the m.p. is 116° to 117°.

1 : 3-*Dimethyl-4-o-methoxyphenyl-4-piperidinol and its acetoxy ester.* The piperidone (I) (6·35 g.) was added dropwise with stirring to a cooled (ice-salt bath) solution of *o*-methoxyphenyl lithium in ether (50 ml.) prepared from lithium (1·36 g.) and *o*-methoxybromobenzene (18·7 g.). The reaction mixture was treated as above, the free base liberated with strong aqueous ammonia and extracted with chloroform. After drying with Na_2SO_4 the solvent was removed to give a solid (7·3 g.) m.p. 109°. Fractional crystallisation of this solid from light petroleum (b.p. 60° to 80°) gave needles of 1 : 3-*dimethyl-4-o-methoxyphenyl-4-piperidinol* (7·18 g.) m.p. 111·5°. Found: C, 71·2; H, 8·6; N, 6·0 per cent; equiv. wt. 235. $C_{14}H_{21}O_2N$ requires C, 71·4; H, 8·9; N, 5·95 per cent; equiv. wt. 235. A mixture of the alcohol (2 g.), acetic anhydride (2 ml.) and pyridine (2 ml.) was refluxed for 3 hours, the solvents removed under reduced pressure, the residue made alkaline with strong aqueous ammonia and the free base extracted with ether. After drying with Na_2SO_4, the solvent was removed to give 1 : 3-*dimethyl-4-o-methoxyphenyl-4-acetoxypiperidine* (2 g.) m.p. 112° to 113° after recrystallisation from light petroleum (b.p. 60° to 80°). Found: C, 69·3; H, 8·4; N, 5·0 per cent; equiv. wt. 280. $C_{16}H_{23}O_3N$ requires C, 69·3; H, 8·4; N, 5·05 per cent; equiv. wt. 277.

1 : 3-*Dimethyl-4-o-tolyl-4-piperidinol and its esters.* The piperidone (I) (5·08 g.) was added dropwise with stirring to a cooled solution of *o*-tolyl lithium in ether (50 ml.) prepared from lithium (0·7 g.) and *o*-bromotoluene (8·55 g.). The reaction mixture was treated as above, the free base liberated with strong aqueous ammonia and extracted with ether. After drying with Na_2SO_4, the solvent was removed to give a pale yellow oil (6·5 g.) which solidified on storing in a vacuum desiccator. Fractional crystallisation of the solid m.p. 83° to 85° from *n*-hexane gave the *piperidinol* (II, Ar = *o*–$CH_3 \cdot C_6H_4$) (4·96 g.) needles m.p. 86° to 87°. Found: C, 76·7; H, 9·4; N, 6·35 per cent; equiv. wt. 223. $C_{14}H_{21}ON$ require C, 76·65; H, 9·7; N, 6·4 per cent; equiv. wt. 219. The alcohol was esterified with acetic anhydride as above; the *acetoxy ester* (III,

Ar = *o*-$CH_3 \cdot C_6H_4$; R = CH_3) gave a *hydrochloride*, needles from ether-ethanol, m.p. 222° to 223°. Found: C, 64·5; H, 8·1; N, 4·65 per cent; equiv. wt. 301. $C_{16}H_{24}O_2NCl$ requires C, 64·5; H, 8·1; N, 4·7 per cent; equiv. wt. 298. The *propionoxy ester* (III, Ar = *o*-$CH_3 \cdot C_6H_4$; R = Et), prepared in an analogous manner using propionic anhydride, gave a *hydrochloride*, needles, m.p. 198° to 199°. Found: C, 65·5; H, 8·6; N, 4·3 per cent; equiv. wt. 314. $C_{17}H_{26}O_2NCl$ requires C, 65·5; H, 8·4; N, 4·5 per cent; equiv. wt. 312.

1:3-*Dimethyl*-4-*m-tolyl*-4-*piperidinol isomers and esters.* The piperidone (I) (12·7 g.) was added dropwise with stirring to a solution of *m*-tolyl lithium in ether (200 ml.) prepared from lithium (1·53 g.) and *m*-bromotoluene (18·8 g.). The reaction mixture was treated as above, the free base liberated with strong aqueous ammonia and extracted with ether. After drying with Na_2SO_4, the solvent was removed to give a solid (14·7 g.) m.p. 104° to 108° after softening at 80°. Fractional crystallisation of this solid from *n*-hexane gave the piperidinol (II, Ar = *m*-$CH_3 \cdot C_6H_4$) in two isomeric forms: *Isomer A*, needles (10·58 g.) m.p. 88·5° to 89·5°. Found: C, 76·4; H, 9·3; N, 6·3 per cent; equiv. wt. 221. $C_{14}H_{21}ON$ requires C, 76·65; H, 9·65; N, 6·4 per cent; equiv. wt. 220; *Isomer B*, prisms (1·28 g.) m.p. 114° to 115°. Found: C, 76·85; H, 9·5; N, 6·1 per cent; equiv. wt. 221. $C_{14}H_{21}ON$ requires C, 76·65; H, 9·65; N, 6·4 per cent; equiv. wt. 220. The *acetoxy ester* of Isomer A, prepared as above, gave a *hydrochloride*, platelets from ether-ethanol, m.p. 238·5°. Found: C, 64·1; H, 8·4; N, 4·7 per cent; equiv. wt. 300. $C_{16}H_{24}O_2NCl$ requires C, 64·5; H, 8·1; N, 4·7 per cent; equiv. wt. 298. The *propionoxy ester* of Isomer A, prepared as above, gave a *hydrochloride*, needles from ether-ethanol, m.p. 233°. Found: C, 65·5; H, 7·8; N, 4·5 per cent; equiv. wt. 314. $C_{17}H_{26}O_2NCl$ requires C, 65·5; H, 8·4; N, 4·5 per cent; equiv. wt. 312.

1:3-*Dimethyl*-4-p-*tolyl*-4-*piperidinol and esters.* The piperidone (I) (5·08 g.) was added dropwise with stirring to a cooled solution of *p*-tolyl lithium in ether (50 ml.) prepared from lithium (0·7 g.) and *p*-bromotoluene (8·55 g.). The reaction mixture was treated as above, the free base liberated with strong aqueous ammonia, and extracted with chloroform. After drying with Na_2SO_4, the solvent was removed to give a solid (6·3 g.) m.p. 128° to 133° after softening at 85°. Fractional crystallisation of this solid from *n*-hexane gave the *piperidinol* (II, Ar = *p*-$CH_3 \cdot C_6H_4$) (4·21 g.), needles, m.p. 135° to 136° (*Isomer A*). Found: C, 76·6; H, 9·3; N, 6·4 per cent; equiv. wt. 221·5. $C_{14}H_{21}ON$ requires C, 76·65; H, 9·7; N, 6·4 per cent; equiv. wt. 220. A final crop of crystals (1·8 g.) m.p. 81° to 91° equiv. wt. 222, theory 220, was obtained which could not be further purified by fractional crystallisation; these crystals were hand-picked to give a small quantity of *Isomer B*, m.p. 103° to 104°. *Isomer A* gave an *acetoxy ester hydrochloride*, prepared as above, plates from ether-ethanol, m.p. 218° to 219°. Found: C, 60·8; H, 8·2; N, 4·2 per cent; equiv. wt. 311. $C_{16}H_{24}O_2NCl H_2O$ requires C, 60·8; H, 8·3; N, 4·4 per cent; equiv. wt. 316. The *propionoxy ester hydrochloride*, prepared as above, gave needles from ether-ethanol, m.p. 221° to 222°. Found: C, 65·1; H, 8·1;

N, 4·4 per cent; equiv. wt. 314. $C_{17}H_{26}O_2NCl$ requires C, 65·5; H, 8·4; N, 4·5 per cent; equiv. wt. 312.

Infra-red Absorption Measurements

Determinations were carried out in carbon disulphide solution, concentration range 0·3 to 0·9 per cent w/v. Calibration was accurate to ± 3 cm.$^{-1}$ over the region 650 to 2000 cm.$^{-1}$ and ± 5 cm.$^{-1}$ over the region 2000 to 5000 cm.$^{-1}$. Infra-red spectra were measured on a Hilger H.800 double-beam automatic recording spectrophotometer fitted with sodium chloride optics, run in cells of path length 0·75 mm. and compensated with carbon disulphide.

Acknowledgements. The authors thank Smith, Kline and French Laboratories for making available an infra-red spectrophotometer, and Mr. T. H. E. Watts for carrying out the measurements. They also thank Dr. P. Janssen of Eupharma, Beerse, Belgium, for the analgesic results.

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DISCUSSION

The paper was presented by DR. A. F. CASY.

THE CHAIRMAN. It seemed that analgesic activity had been measured only by the hotplate method. The results required further consideration before one attempted to draw too close a deduction about the relative effects of ortho, meta and para substituents.

PROFESSOR K. BULLOCK (Manchester) also suggested that certain of the biological results were not sufficiently different to warrant conclusions being drawn from them.

DR. G. E. FOSTER (Dartford). It would seem that there were asymmetric carbon atoms present in the compounds. Had consideration been given to resolving them and testing the isomers?

DR. L. SAUNDERS (London). Did the active compounds confirm the authors' receptor site theory?

DR. J. B. STENLAKE (Glasgow). In the discussion of a previous paper on the receptor site theory he had put forward the view which was contrary to that held by the authors that the Randall and Lehman figure should be reversed. With regard to the infra-red results, particularly the region B (1380 to 1385 cm.$^{-1}$) was it to be understood that absorption in that region was C-methyl absorption? If so, he took it that the more or less constant band about 1380 was C-methyl. Doublets in that region were well known, and duality of those bands seemed to be correlated with configuration.

DR. A. F. CASY, in reply, agreed that not too much significance should be put on the biological results. The replacement of the phenyl by an aryl group brought about a reduction in activity. Resolution had been carried out in the case of the betaprodine, and one optical isomer was considerably more active than the other. The work described in the paper was confined to configuration, and at this stage he would prefer not to comment on the receptor site theory. No attempt had been made to relate the characteristics of infra-red absorption spectra to structural features.

DR. N. J. HARPER added that Dr. Janssen had shown the results which he obtained could be duplicated in a different laboratory. The original structures of alpha and betaprodine were assigned by Lee, but Beckett and Walker developed their structures as a result of hydrolysis studies, and it was significant that the results agreed with the idea of a receptor site.

THE INACTIVATION OF PYROGENS BY GAMMA RADIATION

By T. D. Whittet and W. P. Hutchinson

From The Pharmaceutical Department, University College Hospital, the Medical Unit, University College Hospital Medical School, London, and the Isotope Division, A.E.R.E., Harwell, Berks.

Received July 3, 1957

Gamma irradiation in a dose of $5{\cdot}0 \times 10^6$ rad reduced the pyrogenicity of London tap water but $10{\cdot}0 \times 10^6$ rad was required for it to pass the B.P. test, 20×10^6 rad reduced the pyrogenicity of a pyrogen from *S. abortus equi* but 25×10^6 rad was required before it passed the B.P. test.

The fact that micro-organisms can be inactivated by exposure to ionising radiations has been known for many years. The possibility of using such radiation for the treatment of food has been the subject of a review by Hannan[1]. Ionising radiations have also been used for the sterilisation of numerous pharmaceutical and surgical products. Their use in these fields has been summarised by Horne[2].

The late D. E. Lea's book "Actions of radiations on living cells"[3] describes radiation effects in great detail. It has been suggested that doses about 2×10^6 rad or more are required to inactivate all micro-organisms. Vegetative organisms are killed with $0{\cdot}5 \times 10^6$ rad and moulds and yeasts with $1{\cdot}0$ or $1{\cdot}5 \times 10^6$ rad. Spores are more resistant and need $2{\cdot}0 \times 10^6$ rad. Virus particles are inactivated only at still higher amounts of $2{\cdot}0$–$5{\cdot}0 \times 10^6$ rad. The inactivation amount depends upon the number of organisms and their environment. Exceptionally large amounts are needed with massive numbers or special conditions, for example in anoxia.

Davis, Dole, Izzo and Young[4] examined the haemolytic effects of radiation and Huber[5] investigated the inactivation of the virus of homologous serum jaundice.

Because of the possible use of radiation methods to sterilise plasma we have examined the effect of gamma radiation on pyrogens, since these are a possible source of contamination in plasma.

Experimental

The sources of pyrogens used were London unsterilised tap water, which has been found to be pyrogenic[6,7], and lipopolysaccharide Wander, Pyrexal, a potent pyrogen from *Salmonella abortus equi* supplied in ampoules containing 1 μg. in 2 ml.

Samples of each were irradiated with various doses of gamma radiation and were injected intravenously into the ear veins of sensitised rabbits of 2 to 2·5 kg. The doses used were 10 ml./kg. of tap water and 1 μg. per rabbit of Pyrexal. A dose of 10 ml./kg. of unsterilised tap water could be expected to give a response of about 1·5°. The dose of Pyrexal represents about 250 times the dose which could normally be expected to give a response of 0·6°. The rabbits were kept in restraining boxes during

the experiment and their temperatures were measured by means of a thermistor electrical thermometer for at least one hour before and three hours after injection. The results are shown in Tables I and II.

TABLE I

THE EFFECT OF GAMMA RADIATION ON PYROGENICITY OF LONDON TAP WATER

Irradiation dose (10^6 rad)	Number of rabbits	Mean rise ° C.	Limits
0·5	3	1·38	Limits 1·65° and 0·88°, i.e. + 19·6 − 36·2 per cent
1·0	2	1·36	,, 1·43° and 1·30°, ,, + 5·1 − 4·4 ,, ,,
2·0	3	1·04	,, 1·30° and 0·85°, ,, + 25·0 − 18·27 ,, ,,
3·0	2	1·23	,, 1·53° and 0·93°, ,, + 24·1 − 24·1 ,, ,,
5·0	12	0·59	S.D. 0·47° Coefficient of variation 80·1 per cent
5·0	7	0·78	,, 0·52° ,, ,, ,, 66·4 ,, ,,
5·0	12 + 7	0·66	,, 0·48° ,, ,, ,, 72·1 ,, ,,
7·5	12	0·85	,, 0·32° ,, ,, ,, 37·9 ,, ,,
10·0	12	0·35	,, 0·18 ,, ,, ,, 50·3 ,, ,,
20·0	3	0·28	Limits 0·45° and 0·10°, i.e. + 60·71 − 64·28 per cent

TABLE II

EFFECT OF GAMMA RADIATION ON PYREXAL

Irradiation dose (10^6 rad)	Number of rabbits	Mean rise ° C.	Limits
2·0	3	1·71	Limits 1·90° and 1·50°, i.e. + 11·11 − 12·3 per cent
4·0	3	1·02	,, 1·50° and 0·43°, ,, + 47·05 − 57·8 ,, ,,
20·0	9	0·64	S.D. 0·148° Coefficient of variation 21·9 ,, ,,
22·5	12	0·27	,, 0·35° ,, ,, ,, 129·6 ,, ,,
25·0	12	0·38	,, 0·61° ,, ,, ,, 160·0 ,, ,,
45·0	12	0·15	,, 0·14° ,, ,, ,, 93·3 ,, ,,
30·0	3	0·47	Limits 0·55° and 0·30°, i.e. + 17·0 − 36·2 per cent
35·0	3	0·22	,, 0·43° and 0·10°, ,, + 95·5 − 54·5 ,, ,,
40·0	3	0·28	,, 0·63° and 0·03°, ,, + 125·0 − 89·3 ,, ,,

RESULTS

The irradiation of London tap water with doses of ionising radiations of from 0·5 to 3·0 × 10^6 rad, had little effect on its pyrogenicity (Table I). With a dose of 5 × 10^6 rad the results were variable. Two groups would have passed the old B.P. test and one would have passed the new test. The results of the experiments on twelve rabbits just failed to pass the new test but on the mean value it would have passed the old test by 0·01°. This appears to be a dose just below the inactivating one. Because of this doubt another seven tests were made on samples given this treatment and the mean result of these was 0·77°. Samples of London tap water treated with 10 × 10^6 rad and 20 × 10^6 rad easily passed the B.P. test.

The irradiation of Pyrexal solution (1 μg. in 2 ml.) with 2 × 10^6 rad and with 4 × 10^6 rad (Table II) did not appreciably reduce its pyrogenicity to rabbits in a dose of 0·5 μg./kg. The irradiation with 20 × 10^6 rad reduced its activity to about one third and 25 × 10^6 rad practically destroyed the activity. This is a severe test since the amount of pyrogen injected is approximately 250 times that necessary to produce a pyrogenic response.

DISCUSSION

There appear to be few references to the effects of ionising radiations on pyrogens. Bellamy and Lawton[8] report that human plasma irradiated

with $2{\cdot}0 \times 10^6$ rad or $5{\cdot}0 \times 10^6$ rad passed the standard pyrogen test in rabbits but we have now learned from them that the unirradiated plasma was not pyrogenic.

Pyrogens can be inactivated by ionising radiations but the dose is greater than that required to kill micro-organisms. The method might be of practical use in removing the pyrogenicity of intravenous preparations such as plasma or protein hydrolysates if it could be shown that the products were not damaged by radiation.

Appendix

The samples were treated with gamma radiation from a cobalt 60 source. This was in the form of a hollow cylinder and the samples were placed in the centre, thus ensuring a uniform reproducible dose.

The dose rate was measured using a standard chemical method; the oxidation of a solution of ferrous ion (ferrous ammonium sulphate). The production ferric ions during irradiation is, within limits, independant of the initial concentration of ferrous ion and is proportional to the dose. The total dose which can be measured is limited by the oxygen available in the solution. The ferric ion concentration was measured at 304 mμ and 25° as soon as possible after irradiation, with a Unicam S.P.500 spectrophotometer.

At the time of measurement the dose rate was 7200 I rad/minute. One rad denotes an energy deposition of 100 ergs per g. of material irradiated.

Acknowledgements. We wish to thank Dr. W. Wild of the Chemistry Division, A.E.R.E., for permission to use the cobalt 60 source, and Dr. O. Westphal of Dr. A. Wander, Forschungsinstitut, Baden, for generous supplies of Pyrexal.

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6. Whittet, *J. Pharm. Pharmacol.*, 1954, **6**, 304.
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DISCUSSION

The paper was presented by MR. T. D. WHITTET.

DR. G. E. FOSTER (Dartford). Sterilisation by gamma radiation often caused decomposition. Had the water been tested for peroxides, for example?

DR. J. C. PARKINSON (Brighton). Was plasma fit for use after being irradiated? What was the effect of irradiation at higher than room temperature?

MR. D. H. O. GEMMELL (Glasgow). Had the authors irradiated Pyrexal as a solid or in solution?

MR. J. W. HADGRAFT (London). After autoclaving London tap water the author had found it to comply with the B.P. test for absence of pyrogens. The water had been used without filtration, and it might well be that the pyrogens had been adsorbed on to the finely precipitated salts. There was an alternative explanation that the water was not pyrogenic in the first place. The paper seemed to indicate that the substance present in London tap water which caused an increase in body temperature was more sensitive to gamma radiation than known pyrogens or *Salmonella abortus equi.*

MR. K. L. SMITH (Nottingham). Irradiation caused heparin to lose potency: heparin was a sulphonated polysaccharide and it had been suggested the pyrogens were polysaccharides. Was the reason that London tap water when autoclaved was rendered non-pyrogenic because it was heavily chlorinated?

DR. H. DAVIS (London). At University College Hospital some years ago a famous surgeon insisted that every injection of 5 per cent dextrose should be made with London tap water which was filtered and then boiled. In ten years hundreds of those injections were given to patients, without a rigor occurring. He was not convinced that the evidence which had been put forward as to the pyrogenicity of London tap water was sufficient. Had correlation of the rise in temperature of the rabbit and the production of rigor in a human been attempted?

MR. G. SYKES (Nottingham). One assumed that the mechanism of sterilisation by irradiation was by denaturation of proteins in the organisms. Presumably the proteins in plasma were closely allied to those in bacteria, and it was difficult to see why irradiation should attack organism protein and not plasma protein.

DR. L. SAUNDERS (London). Was the method feasible for treating water continuously?

PROFESSOR J. P. TODD (Glasgow). There was a general assumption that all substances producing a rise in temperature were polysaccharides. The production of a rise in temperature was only one of many phenomena associated with physiological stress. A great many substances could do that, not necessarily a bacterial polysaccharide, which was reasonably thermostable. Possibly there was another type of substance in London tap water, protein-like in nature.

DR. T. WALLIS (London) understood that if a precipitate were produced in the liquid the pyrogen would be adsorbed on the precipitate. Had the authors determined the hardness of the London water, because if London water were heated a precipitate was obtained which might be unobserved by anybody who had either boiled or autoclaved the water, because it might adhere to the side of the vessel in which the heating was done.

DR. J. G. DARE (Leeds) commented that there was evidence, for certain specified pyrogens, correlating the effects of injection into rabbits and man.

DR. J. W. FAIRBAIRN (London) was surprised at the very high coefficients of variation in Tables I and II.

MR. WHITTET replied. Radiations damaged a number of compounds, and some peroxide was formed. The dose necessary to destroy pyrogens would render plasma unfit for use. Pyrexal solution containing 1 μg./2 ml. ready for injection was irradiated. Thames water was strongly pyrogenic, and on autoclaving its pyrogenicity was not destroyed. Primary filtered London tap water was strongly pyrogenic after autoclaving, but Thames mains water after secondary filtration was not pyrogenic after autoclaving. The response from London tap water gave the same shape of curve as pure pyrogens. Primary filtered London tap water was pyrogenic both before and after autoclaving, but if it were left standing it lost its pyrogenicity after a month or two. A large number of samples of different origins had been examined at all seasons of the year, with both mains water and water from cisterns, and no differences had been found in any of the samples. Glass ampoules became brown when subjected to irradiation, and there was slight effervescence when an ampoule was opened which had been subjected to a high dosage. There was no apparent effect on the rabbits at all. He did not consider chlorination was the reason for the pyrogenic activity, as water before chlorination behaved in the same manner as it did after chlorination. He doubted whether it would be a feasible method for continuous water treatment. What convinced him that pyrogens were the cause was the fact that raw and primary filtered water behaved exactly as bacterial pyrogens. The material was extremely thermostable, but in secondary filtered water there was thermal instability. With unsterilised water the time: temperature curve was that of a typical bacterial pyrogen. There was no appreciable precipitate visible after autoclaving, but it was possible that one had formed and adhered to the glass. The autoclaved samples were injected unfiltered. The results shown in the Tables were not intended to give a quantitative measure of pyrogenicity.

THE PHARMACOLOGY OF SOME HYDROXYBENZYL*ISO*QUINOLINE DERIVATIVES

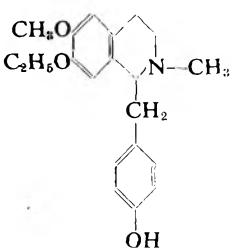
By J. J. Lewis and M. S. Zoha*

From the Department of Materia Medica and Therapeutics, Glasgow University

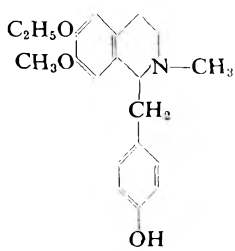
Received June 3, 1957

(±)-1-(4′-Hydroxybenzyl(-2-methyl-6-methoxy-7-ethoxy-1:2:3:4-tetrahydro-*iso*quinoline and its dextrorotatory and laevorotatory isomers and methochloride, (±)-1-(4′-hydroxybenzyl)-2-methyl-7-methoxy-6-ethoxy-1:2:3:4-tetrahydro-*iso*quinoline, its laevorotatory isomer and methochloride and a berberine-like compound related to tetrahydroworenine have been tested for curare-like activity. Little activity was noted, but on the rat diaphragm and frog rectus muscle quaternisation of the racemic compounds increased this. The tertiary bases possessed potent convulsant properties.

The compounds I and II were synthesised by Jeffreys[1] during studies on the constitution of the di-tertiary alkaloid *iso*chondrodendrine (III). These compounds are related chemically to armepavine[2], coclaurine[3], *iso*coclaurine[3] and magnocurarine[4] (IV-VII). *iso*Coclaurine and magnocurarine have been reported to possess some curare-like activity. Coclaurine is inactive. Compounds I and II are possible degradation products of *iso*chondrodendrine which is chemically somewhat similar to tubocurarine (a di-quaternary base). We thought it worth-while to test



(I)



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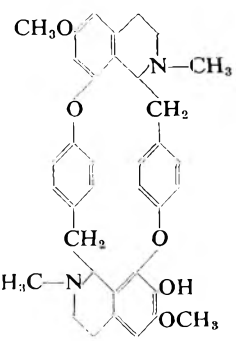

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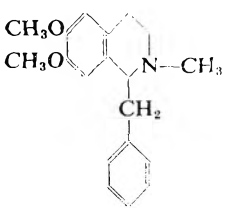

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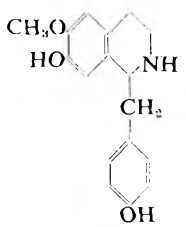

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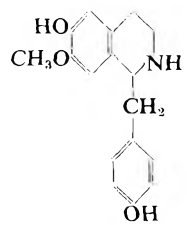

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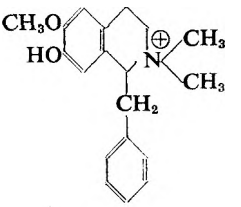
* Pakistan Government Scholar.



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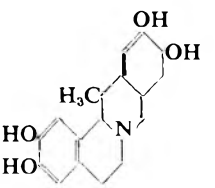

(VIII)

these compounds for curare-like activity and also to test the corresponding quaternary salts, especially since magnocurarine, which is a quaternary base, has been reported to possess some curare-like activity[4]. We have made some comparisons with tubocurarine and with a berberine-like compound (VIII) which contains an *iso*quinoline ring system[5].

Materials, Methods and Results

The composition of the perfusion fluids used in this investigation was as follows:—

Frog Ringer's solution: NaCl 6·5 g., KCl 0·138 g., $CaCl_2$ (anhydrous) 0·12 g., $NaHCO_3$ 0·2 g., dextrose 1·0 g., distilled water to 1000 ml. Locke's solution: NaCl 9·0 g., KCl 0·42 g., $CaCl_2$ (anhydrous) 0·24 g., $NaHCO_3$ 0·5 g., dextrose 1·0 g., distilled water to 1000 ml. Tyrode's solution: NaCl 8·0 g., KCl 0·198 g., $CaCl_2$ (anhydrous) 0·2 g., $NaHCO_3$ 1·0 g., $MgCl_2$ 0·1 g., NaH_2PO_4 0·005 g., dextrose 1·0 g., distilled water to 1000 ml.

Drugs used were as follows: acetylcholine chloride (ACh), (—)-adrenaline hydrochloride (Ad), (—)-noradrenaline hydrochloride (NA), tubocurarine chloride (TC), decamethonium iodide (C10), histamine acid phosphate (Hm), 5-hydroxytryptamine creatinine sulphate (5-HT), phentolamine, dibenamine and hydergine.

The compounds investigated were: 1, (±)-1-(4′-hydroxybenzyl)-2-methyl-6-methoxy-7-ethoxy-1:2:3:4-tetrahydro*iso*quinoline (I) and its dextrorotatory (I*d*) and laevorotatory isomers (I*l*) and the quaternary derivative (I*q*) (methochloride); 2, (±)-1-(4′-hydroxybenzyl)-2-methyl-7-methoxy-6-ethoxy-1:2:3:4-tetrahydro-*iso*quinoline (II) and its laevorotatory (II*l*) isomer and the quaternary derivative (II*q*) (methochloride); 3, a compound related to tetrahydroworenine[5], 5:6:13:13a-tetrahydro-2:3:10:11-tetrahydroxy-8H-dibenzo-(a, g)-pyridocholine hydrochloride, described as (VIII).

The tertiary compounds are insoluble in water but can be dissolved in dilute hydrochloric acid at about pH 5. The quaternary compounds are freely soluble in water.

Frog Rectus Abdominis Muscle

The rectus muscle was dissected and suspended in a 20 ml. bath containing oxygenated frog Ringer's solution at room temperature. Reproducible submaximal contractions were obtained to ACh (0·1 to 0·2 μg./ml.) and C10 (2·0 to 2·5 μg./ml.) added at 5 minute intervals and left in contact

with the tissue for 1·5 minutes. Drugs in doses of 12·5 to 250 μg./ml. were added ½ minute before the next addition of ACh or C10 and left in contact with the tissue for 2 minutes.

There was antagonism by all compounds to ACh-induced contractions and a graded effect was seen. Comparing the potency against TC, I was one fiftieth, II about one four hundredth, VIII about one hundredth, and I*l*, I*d* and II*l* had a potency of one four hundredth, one sixtieth and one four hundred and fiftieth respectively. I*q* and II*q* had respectively about one thirtieth and one fiftieth of the potency of TC. None of these compounds had any direct effects on the rectus. All antagonised contractions by 2 to 2·5 μg./ml. C10, but high doses were needed. The effects of these compounds were additive to those of TC.

Frog Sartorius Muscle Ischiad Nerve Preparation

The sartorius muscle with the ischiad nerve was dissected. The nerve was drawn through the membrane of a fluid electrode and the muscle nerve preparation suspended in an 80 ml. bath containing oxygenated frog Ringer's solution at room temperature. The muscle was stimulated indirectly by using square pulses at a frequency of 12/minute at 10 V., pulse width 0·5 msec. Drugs at doses of 25 to 200 μg./ml. were added to the bath and kept in contact with the muscle for 3 minutes.

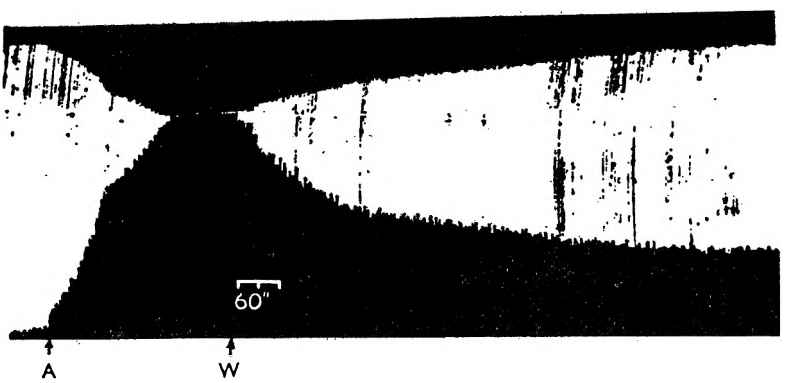

FIG. 1. Frog sartorius muscle-ischiad nerve preparation. Contractions due to indirect square pulses, 12/min. 12 V., 0·5 msec. width.
At A, 62·5 μg./ml. compound II. At W, wash out.

Only I, II and VIII were tested. The doses given of these three compounds inhibited the response to indirect stimulation. They were much less potent than TC. After washing there was complete recovery of the contraction (Fig. 1).

Rat Phrenic Nerve Diaphragm Preparation

The dissection was made as described by Bülbring[6] and the muscle-nerve preparation suspended in an 80 ml. bath containing Tyrode's solution at 29° and gassed with 95 per cent O_2 and 5 per cent CO_2. A Collison's electrode was used and the muscle stimulated indirectly using square

pulses at a frequency of 6/minute at 12 V.; pulse width 1·0 msec. Drugs were added to the bath and kept in contact with the muscle for 3 minutes.

On this preparation these compounds had only very weak curare-like activity. I*q* and II*q*, the quaternary salts of I and II were about five times more potent than the tertiary bases from which they were prepared. Complete neuromuscular block could be produced but high doses of about 0·25 mg./ml. had to be used. After the response to indirect

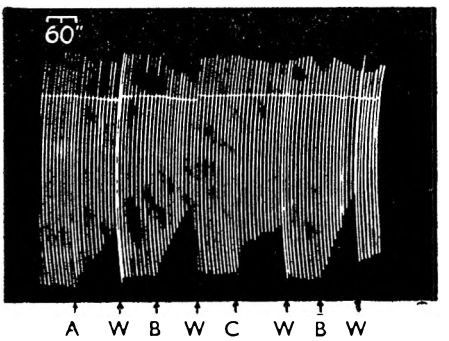

FIG. 2*a*. Rat phrenic nerve-diaphragm preparation. Contractions due to indirect square pulses, 6/min., 12 V. 1 msec. width.

At A, 2·5 μg./ml. tubocurarine chloride.
At B, 3·0 μg./ml. tubocurarine chloride.
At C, 0·6 mg./ml. compound I.
At W, wash out.

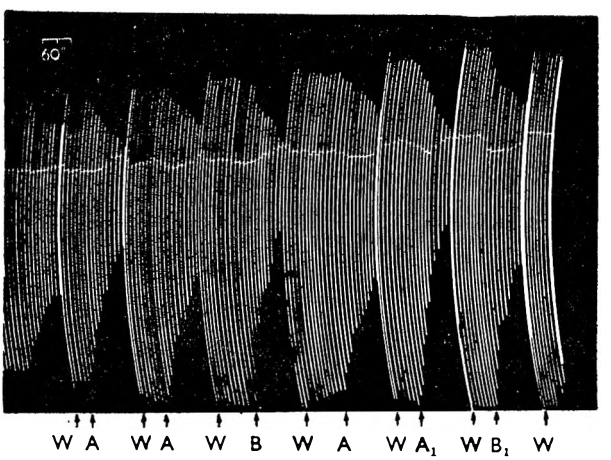

FIG. 2*b*. Rat phrenic nerve-diaphragm preparation as Fig. 2*a*.

At A, 5 μg./ml. tubocurarine chloride.
At A_1, 10 μg./ml. tubocurarine chloride.
At B, 300 μg./ml. compound II.
At B_1, 250 μg./ml. compound II.
At W, wash out.

stimulation had failed, the muscle still responded to direct stimulation. The tertiary bases were about 250 times less potent (Fig. 2*a*) and compound (VIII) had about one fourtieth of the potency of TC (Fig. 2*b*).

Isolated Kitten Heart

Kitten hearts were perfused by Langendorff's method[7] using oxygenated Locke's solution at 37°. The outflow was measured by collecting the perfusate for periods of 5 minutes into a measuring cylinder. Drugs were administered by injection into the aortic cannula.

Ten to 20 μg. of II*l* increased the rate and amplitude of the heart; 20 μg. of I*l* had a similar effect. These effects were reversible. II and VIII had no effect.

Guinea Pig Ileum

About 3 cm. of the terminal ileum was suspended in a 4 ml. bath containing oxygenated Tyrode's solution at 31°. Reproducible submaximal contractions were obtained by adding ACh (0·05 to 0·2 μg./ml.) or Hm (0·05 to 0·2 μg./ml.) at 3 minute intervals and leaving in contact for $\frac{1}{2}$ minute at doses of 25 to 250 μg. Drugs were added $\frac{1}{2}$ or 1 minute before the next addition of ACh or Hm. Contact of drug with tissue was for 1 or 1·5 minutes.

All compounds inhibited contractions by ACh or Hm. There was a graded inhibition according to dose. No direct effects were seen.

Cat Gastrocnemius Muscle Sciatic Nerve Preparation

In cats weighing 2·0 to 3·5 kg. anaesthesia was induced by ether and maintained by intravenous chloralose (80 mg./kg.). The gastrocnemius muscle was partially freed from the surrounding tissues and the achilles tendon severed just above its insertion into the calcaneus. The tendon was attached by means of a strong linen thread to a myograph lever. The sciatic nerve was partly dissected on the lateral aspect of the thigh and stimulated by means of platinum electrodes which were placed on the nerve just above the emergence of the anterior tibial nerve. Stimulation was by square pulses at a frequency of 6/minute at 12 V.; pulse width 2·5 msec. A Dobbie McInnes square wave generator was used. Drugs were administered by the cannulated external jugular vein.

The quaternary bases at doses of 1 to 2 mg./kg. did not depress the response of the muscle to indirect stimulation. Similarly the tertiary bases at 1 to 4 mg./kg. had no neuromuscular blocking activity. There was some spontaneous muscular twitching.

Cat Blood Pressure

In cats weighing 2·0 to 3·5 kg. anaesthesia was induced by ether and maintained by intravenous chloralose (80 mg./kg.). Blood pressure was recorded from the common carotid artery. Drugs were administered by the cannulated external jugular vein. In some experiments the spinal cat was used.

Results showed that I (1·0 to 2·0 mg./kg.) always caused a biphasic depressor-pressor response. The fall in blood pressure was marked but the ensuing rise was small (Fig. 3). I*d* and I*l* showed qualitatively similar effects but both depressor and pressor components were less. When I was given after bilateral mid-cervical vagotomy, the depressor component was lost and only a rise in blood pressure was seen (Fig. 3). In a few

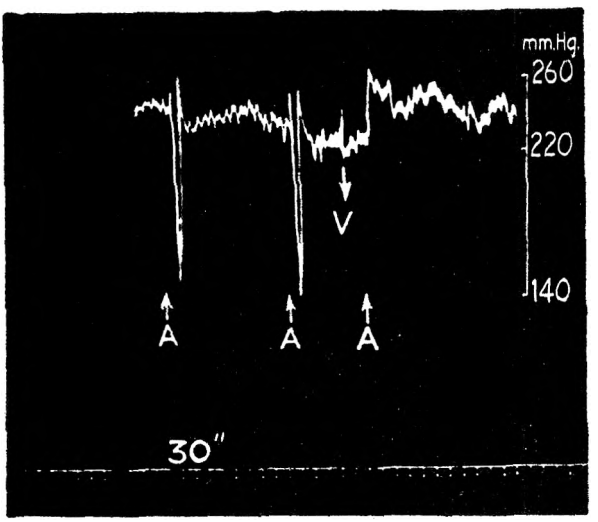

FIG. 3. Cat 2·5 kg. anaesthetised with ether-chloralose. Record of blood pressure from common carotid artery.
At A, 0·8 mg./kg. compound I intravenously.
At V, bilateral cervical vagotomy.

experiments I*l* caused a rise only in blood pressure. I*q* (0·5 to 1·0 mg./kg.) caused a rise in blood pressure (Fig. 4). II and II*l* at dose levels of 1·0 to 2·0 mg./kg. caused respectively a slight rise in blood pressure and a biphasic depressor-pressor effect. II*q* (0·5 to 1·0 mg./kg.) also caused a rise in blood pressure (Fig. 4). VIII (1·0 to 2·0 mg./kg.) had no effects on the blood pressure. The pressor responses were not blocked by phentolamine (up to 5 mg./kg.), dibenamine (up to 25 mg./kg.) or hydergine (up to 0·4 mg./kg.). These compounds did not antagonise the characteristic effects on the blood pressure of ACh (0·5 to 1·0 μg./kg.), Hm (0·5 to 1·0 μg./kg.), Ad (1·0 to 2·0 μg./kg.) or 5-HT (1·0 to 2·0 μg./kg.).

Higher doses when given repeatedly in anaesthetised cats caused respiration to stop and artificial respiration had to be given.

When doses of 1 mg./kg. or more were given at intervals of $\frac{1}{4}$ to $\frac{1}{2}$ hour in anaesthetised or spinal cats, convulsions were caused. Convulsant activity was not shown following the first dose but on repeated administration of each drug convulsant activity became apparent. This appeared to be a cumulative effect. Convulsions were preceded by twitching movements which became clonic convulsions. There were periods of quiet between convulsions.

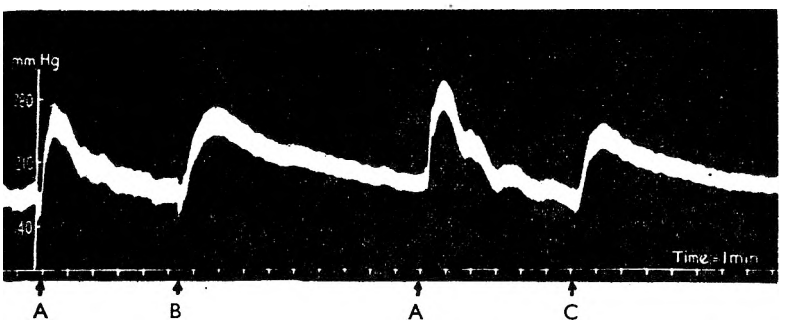

FIG. 4. Cat female, 4·0 kg. anaesthetised by intraperitoneal chloralose. Record of blood pressure from common carotid artery.

At A, 0·2 μg./kg. noradrenaline hydrochloride intravenously.

At B, 0·5 mg./kg. compound I*q* intravenously.

At C, 0·5 mg./kg. compound II*q* intravenously.

Toxicity

Drugs were given by intraperitoneal injection into groups of mice weighing 40 to 50 g.

In mice I, II and VIII showed potent convulsant activity. These compounds were roughly equipotent with leptazol. The mice showed tonic-clonic convulsions with maximal hind leg extensor spasm.

DISCUSSION

The compounds which we have tested possess little or no skeletal neuromuscular blocking activity but the preparation of the quaternary salts of I and II increased their potency on the rat diaphragm and frog rectus muscle. Compound II is less potent than compound I which would indicate that transposition of the ethoxy and methoxy groups had altered activity.

All of the tertiary bases were convulsants but we have had insufficient supplies of the quaternary compounds to test them for convulsant activity.

Acknowledgment. We are indebted to Dr. J. A. D. Jeffreys for samples of the compounds tested.

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DISCUSSION

The paper was presented by DR. M. S. ZOHA.

DR. J. B. STENLAKE (Glasgow). What was the activity of the quaternary salts of isochondrodendrine which would be structurally related to tubocurarine?

DR. M. S. ZOHA replied. The quaternary salts of isochondrodendrine were not examined.

THE OXIDATION OF SOLUBILISED AND EMULSIFIED OILS

I. Oxidation of Benzaldehyde in Potassium Laurate and Cetomacrogol Dispersions

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The solubility of benzaldehyde in solutions of potassium laurate and cetomacrogol, has been measured at 20°. Oxidation rates of emulsified and solubilised benzaldehyde have been observed manometrically at 25°. The effect of concentration of surface-active agent on the oxidation rate of benzaldehyde has been studied. Emulsified benzaldehyde oxidised at a greater rate than solubilised benzaldehyde. Emulsified benzaldehyde was more susceptible to the action of copper catalysts than was solubilised benzaldehyde. Possible reaction mechanisms and loci of reaction are discussed.

The solubility of water insoluble organic substances in aqueous solutions of soap has been known and used since 1874[1]. The structure and properties of these solutions have been reviewed by Winsor[2] and Klevens[3].

Synthetic surface-active agents, and in particular the non-ionic group, have recently become of increasing importance in pharmacy. The preparation of aromatic waters[4], aqueous solutions of phenobarbitone[5], 2-methyl-1 : 4-naphthoquinone[6], steroid hormones[7], vitamin preparations[8–13], nonsteroidal oestrogen[14], solubilised tolu in Syrup of Tolu[15], water miscible flavouring oils for use in antacid preparations[16], and solubilised volatile oils for use in perfumery[17], have all been suggested uses.

The oxidation of solubilised oil-soluble vitamins has been the subject of several studies. Coles[9] and Kern[10] considered that the aqueous dispersions were more stable than the oily ones, whilst Patel, Kumptu and Radhakrishna[11] found the reverse. The latter authors also found differences in the stability of vitamin A palmitate and vitamin A alcohol in aqueous media.

Recently the Pharmaceutical Society's Scientific Publications Department in their preliminary work on the deterioration of solubilised volatile oils, have compared the rate of oxidation of benzaldehyde in Cetomacrogol and ethanol[18].

The present work undertakes the study of the oxidation of oils in the presence of surface-active substances. Benzaldehyde, methyl linoleate, and methyl oleate were chosen as simple reference compounds because of the complex oxidation reactions of natural products. All these compounds oxidise by a chain reaction involving the intermediate formation of a peroxide. The effect of water soluble and oil soluble catalysts were also observed on the oxidation, as these may be expected to yield different results in systems possessing an oil : water interface. Copper sulphate and copper laurate were the catalysts chosen. Potassium laurate which can

be prepared in a high state of purity, and Cetomacrogol B.P.C., are the two surface-active substances studied.

EXPERIMENTAL

Materials

Potassium Laurate. Because aqueous solutions of potassium laurate hydrolyse on storage the solid was prepared from Eastman Kodak lauric acid and carbonate-free ethanolic potassium hydroxide. Molar solutions were prepared from the crystalline soap and diluted as required.

Cetomacrogol B.P.C. 'Texofor A1P' was used. It is a creamy white solid with a C:H:O ratio of 57·9:9·7:32·4. This corresponds to an average chain length of 34 to 36 polyethylene oxide units which is longer than that quoted in the B.P.C. specification. The material had a melting point of 45·5° and an acetyl value of 41·3 using the methods described in the B.P.C. Measurements using a Du Nouy tensiometer indicated a critical micelle concentration of 1×10^{-6} to 1×10^{-7} M.

A stock solution of 0·1 M (Molecular weight taken as 1300) was prepared and diluted as required.

Benzaldehyde. Analar benzaldehyde was further purified by two distillations under nitrogen at reduced pressure (b.p. 69°/15 mm.).

Catalysts. Copper sulphate (Analar) and copper laurate, prepared from copper sulphate and potassium laurate solutions by double decomposition, were used as catalysts.

Sequestering Agent. An 0·01M solution of the di-sodium salt of tetra-acetic acid Laboratory Reagent Grade was used.

Freshly glass-distilled water was used for all experiments.

Experimental Methods

Measurement of solubilisation. The solubility of benzaldehyde in solutions of potassium laurate and cetomacrogol was estimated by weighing a series of known quantities of benzaldehyde into ampoules containing the detergent and rotating the ampoules in a water bath at 20° ± 0·05°. The end point was estimated visually; the average between an undersaturated and an oversaturated ampoule differing by 1 mg. or less of benzaldehyde was taken as the solubility. As the concentration of detergent decreased, the volume of solution was increased from 5 to 250 ml. Glass stoppered flasks were used for volumes over 20 ml.

Measurement of oxidation. Because of the small quantities of benzaldehyde involved, it was necessary to have a micro method of following the oxidation, and a Warburg apparatus was used to measure oxygen uptake. The apparatus consisted of a series of manometers one arm of which was connected to the reaction flask, and the other was open to the atmosphere. A bath temperature of 25° ± 0·005° and a shaking rate of 100 strokes a minute was employed. Solutions or emulsions were prepared under standard conditions and 2 ml. samples used in the reaction flasks. An equilibration period of five minutes was allowed between closing the taps and taking the readings. All burette readings were converted to μl. of oxygen taken up. The experiments were duplicated

and averaged. Experiments were also made to show that the rate of diffusion of oxygen was not a limiting factor in the experiments.

Cetomacrogol, being an ether, is liable to peroxidise, but the rate was slow, even in the presence of copper catalysts. However, the oxidation of cetomacrogol proceeds at a greater rate in the presence of benzaldehyde, as a result of the oxidation of the latter.

The addition of benzoic acid to the oxidising systems did not alter the rate of oxidation, which indicates that the benzoic acid produced did not have any catalytic effect on the reaction.

TABLE I

THE SOLUBILITY OF BENZALDEHYDE IN SOLUTIONS OF CETOMACROGOL AT 20°

Molar concentration of cetomacrogol	Wt. solubilised g. benzaldehyde/l.*	Mol. benzaldehyde/ mol. cetomacrogol
0·000051	0·53	98
0·000076	0·76	95
0·000103	0·82	76
0·000507	1·84	34
0·00103	2·72	25
0·00253	3·83	14
0·00507	4·72	8·8
0·0126	6·69	5·0
0·0219	8·40	3·6
0·0439	12·77	2·7
0·0507	13·40	2·5
0·1013	24·86	2·3

* The solubility of benzaldehyde in water was deducted from the weight solubilised and was used as a correction in calculating the molar ratio.

TABLE II

THE SOLUBILITY OF BENZALDEHYDE IN SOLUTIONS OF POTASSIUM LAURATE AT 20°

Molar concentration of potassium laurate	Wt. solubilised g. benz./l.*	Mol. benzaldehyde/ mol. potassium laurate
0·01	0·040	0·038
0·02	0·157	0·074
0·03	1·56	0·49
0·04	2·01	0·47
0·05	5·32	1·00
0·06	7·18	1·13
0·07	8·98	1·21
0·08	10·18	1·20
0·10	14·44	1·36
0·225	36·76	1·54
0·45	90·02	1·89

* The solubility of benzaldehyde in water was deducted from the weight solubilised and was used as a correction in calculating the molar ratio.

Titration of oxidised samples and pH readings. All titrations and pH readings were made with a glass:calomel electrode system. The "apparent" pH of potassium laurate was read with an "alkacid" glass electrode. A modified Agla micrometer syringe was used as a micro burette. Nitrogen was passed over the titration solution throughout and a stream of nitrogen used to stir the solution and prevent further oxidation. The 0·1N NaOH was prepared from 10 N carbonate free NaOH immediately before use.

The iodometric titration of the peroxide formed during the oxidation. The iodometric method of Boehm and Williams[19] was used with the following modifications; the flask was left in the dark for 15 minutes, as the

release of iodine in the presence of cetomacrogol was slow. Water was not added at the end of the reaction as this prevented the formation of a colour with the starch indicator. To prevent any further oxidation nitrogen was passed into the flask during the course of the titration.

RESULTS

Solubility of Benzaldehyde in Aqueous Solutions of Cetomacrogol and Potassium Laurate

The solubility of benzaldehyde in solutions of cetomacrogol and potassium laurate is shown in Tables I and II respectively.

The Effect of Concentration of Surface-active Agent on the Oxidation of Benzaldehyde

The effect of adding increasing amounts of surface-active agent to benzaldehyde, was to bring about the progressive change from suspensions, through emulsions, to the solubilised state. The oxidation of these three systems (calculated as ml. oxygen uptake per g. of benzaldehyde) is shown in Figures 1 and 2. In both, the rate fell as the solubilised state was approached. This is better shown in Figures 3 and 4 where the rate of oxidation, ml. of oxygen taken up per g. of material per hour, is plotted. Further addition of surface active-agent to the solubilised benzaldehyde did not alter the rate of oxidation. With benzaldehyde solubilised with potassium laurate there is a marked delay in onset of oxidation, but not with cetomacrogol.

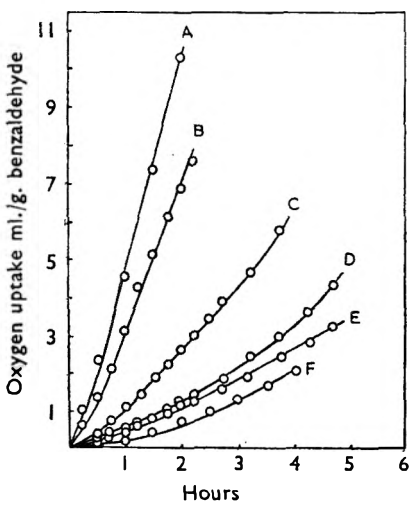

FIG. 1. The uptake of oxygen (in ml. O_2/g. of benzaldehyde) by benzaldehyde dispersed in varying concentrations of cetomacrogol. Temperature 25°. Benzaldehyde concentration 15 mg./ml.

A, suspended benzaldehyde in water
B, emulsified ,, ,, 0·01M cetomacrogol
C, ,, ,, ,, 0·02M ,,
D, solubilised ,, ,, 0·028M ,,
E, ,, ,, ,, 0·036M ,,
F, ,, ,, ,, 0·06M ,,

Variation in Rate of Oxidation with Changes in the Concentration of Benzaldehyde

Cetomacrogol. The effect of increasing the concentration of benzaldehyde in 0·06 M cetomacrogol solutions was investigated. Up to 3 mg.

benzaldehyde/ml. was soluble in water. From 3 to 15 mg. of benzaldehyde/ml. a solubilised system existed, and above this concentration emulsion droplets separated.

Figure 5 shows that the oxidation rate of benzaldehyde dispersed in cetomacrogol is similar, in the range 0 to 15 mg./ml. for 1×10^{-5} M

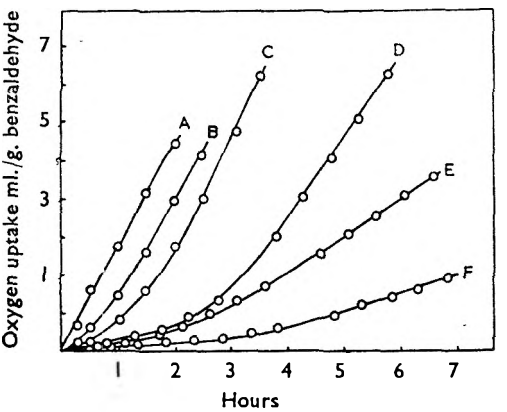

FIG. 2. The uptake of oxygen (in ml. O_2/g. of benzaldehyde) by benzaldehyde dispersed in varying concentrations of potassium laurate. Temperature 25°. Benzaldehyde concentration 15 mg./ml.

A, emulsified benzaldehyde in 0·04M potassium laurate
B, ,, ,, ,, 0·06M ,, ,,
C, ,, ,, ,, 0·064M ,, ,,
D, ,, ,, ,, 0·072M ,, ,,
E, solubilised ,, ,, 0·08M ,, ,,
F, ,, ,, ,, 0·12M ,, ,,

copper sulphate, 1×10^{-5} M copper laurate, and without copper and with the addition of 0·01M di-sodium tetra-acetic acid. In the range 15 to 40 mg./ml. the rate of oxidation is greatest with copper sulphate, is next with copper laurate and is least without copper and containing the sequestering agent.

Systems containing potassium laurate. Figure 6 shows that in the solubilised range, 0 to 20 mg./ml. oxidation is at the same rate in the catalysed and uncatalysed preparation; in the range 20 to 55 mg./ml. emulsions are oxidised faster with copper.

Oxidation in light petroleum. The effect of variation of benzaldehyde concentration on the uncatalysed oxidation of benzaldehyde in redistilled light petroleum (b.p. 100 to 120°) was studied. With increasing concentration the rate of oxidation per g. of benzaldehyde showed a simple proportionate increase as expected from the Law of Mass Action.

Effect of Catalyst Concentration on the Oxidation

Increasing the concentration of copper from 1×10^{-6} to 1×10^{-4} M increased proportionately the rate of oxidation in solubilised and emulsified states, the latter being more susceptible.

Effect of Addition of Cetomacrogol to a Suspension which is Already Oxidising

Five suspensions of benzaldehyde in distilled water, 15 mg./ml. were allowed to reach a constant rate of oxidation and at this time cetomacrogol solutions contained in the side-arms of the reaction flasks were added, and the readings continued. In Figure 7 is shown the rate of oxidation of the benzaldehyde suspension A, and the rate of oxidation of emulsified benzaldehyde, B and C. Curve B is based upon benzaldehyde emulsified with the addition of 0·01M cetomacrogol and C with 0·02M. Also shown are curves D and E containing 0·03 and 0·04M cetomacrogol, amounts sufficient to solubilise the benzaldehyde. After the characteristic delay period of about 30 minutes the rates of oxidation of both the emulsified and the solubilised benzaldehyde are similar, and nearly as great as the benzaldehyde suspension.

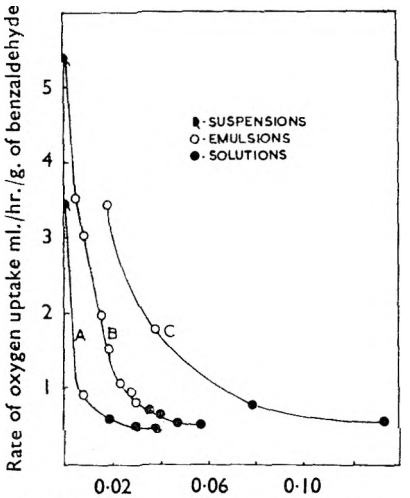

FIG. 3. The influence of cetomacrogol concentration on the rate of oxidation of benzaldehyde at 25°. Catalyst concentration 1×10^{-5}M copper sulphate. (Each point represents the average of four determinations.)

A, Benzaldehyde 10 mg./ml.
B, ,, 15 ,,
C, ,, 25 ,,

Results of the Titration of Benzaldehyde in Cetomacrogol

The apparent pKa of the acid produced was in the range 4·4–4·6. This agreed with the apparent pKa of benzoic acid in cetomacrogol solution. Perbenzoic acid has a pKa of 7·9[20] and the absence of a break in the titration curve other than that at pKa 4·4, suggests that perbenzoic acid is not produced. The amount of benzoic acid produced accounts for only half the oxygen taken up. It seems likely that the remainder of the oxygen is used in the peroxidation of the cetomacrogol.

DISCUSSION

In passing from a suspension, through an emulsion, to a solution, the rate of oxidation of benzaldehyde was reduced with both the anionic and non-ionic surface-active agents.

Oxidation of benzaldehyde is known to proceed by a chain reaction involving at least three distinct steps; initiation, propagation, and termination. The oxidation may be initiated photochemically[21] by suitable free radical sources such as easily dissociable peroxides[22], or by electron transfer between the aldehyde on the peracid formed during oxidation

with the metal catalyst[23]. The reaction scheme proposed by Bawn for oxidation of the latter type in solution was as follows:

$$Co^{+++} + C_6H_5CHO \rightarrow Co^{++} + C_6H_5CO^{\bullet} + H^{+} \quad .. \quad .. \quad (1)$$

$$C_6H_5CO^{\bullet} + O_2 \rightarrow C_6H_5COOO^{\bullet} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

$$C_6H_5COOO^{\bullet} + C_6H_5CHO \rightarrow C_6H_5COOH + C_6H_5CO^{\bullet} \quad .. \quad (3)$$

$$2C_6H_5COOO^{\bullet} \rightarrow \text{inert products} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

$$\frac{dO_2}{dt} = \frac{k_1^{\frac{1}{2}}k_3}{k_4^{\frac{1}{2}}} [Co^{+++}]^{\frac{1}{2}}[PhCHO]^{3/2}$$

The rate determining reaction was (1). The relationship between [benzaldehyde]$^{3}/^{2}$ and oxygen uptake was found to be linear and the slope of the regression line changed sharply between the solubilised and emulsified phase. Since the rate of oxidation was dependent on [benzaldehyde]$^{3}/^{2}$ a reaction similar to that suggested by Bawn is likely to occur. However, in the present work it was found that the rate of oxidation was directly proportional to catalyst concentration within the range 1×10^{-6} to 1×10^{-4} molar and that the catalyst coefficient was greater in the emulsions than in the solubilised preparations.

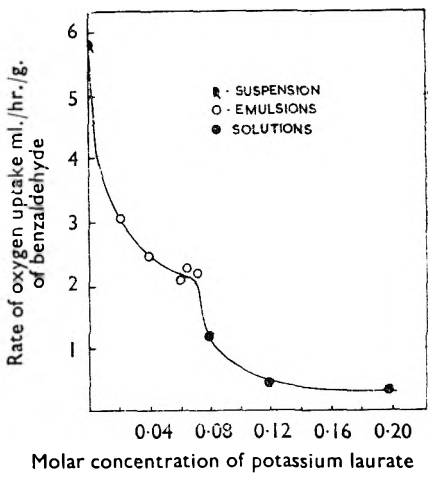

FIG. 4. The influence of potassium laurate concentration on the oxidation rate of benzaldehyde at 25°. Benzaldehyde concentration 25 mg./ml. Catalyst concentration 1×10^{-5}M copper sulphate.

The study of the oxidation of benzaldehyde in aqueous dispersions was complicated by the possible different sites of reaction. In an emulsion the benzaldehyde is present in two phases in equilibrium; one composed of discrete globules of emulsified benzaldehyde each of which possesses an oriented layer of surface-active agent, and a continuous phase consisting of benzaldehyde solubilised in micelles, and also in true solution in the water. The three possible loci of reaction in the system are:

(*a*) In the benzaldehyde phase, in emulsion droplets or micelles. (*b*) At the benzaldehyde:water interface. (*c*) In the water phase (excluding micelles).

From Figure 5 it is seen that the rate of oxidation of solubilised benzaldehyde in the presence of catalysts is only slightly higher than for the uncatalysed reaction. A different result was obtained in the emulsified system, where catalysts have a marked effect. The catalyst copper sulphate was insoluble in benzaldehyde, and it was thus highly improbable that the proposed chain reaction would occur within the benzaldehyde

globule. However, copper sulphate was a better catalyst than the oil soluble copper laurate, and it is therefore probable that the initiation reaction took place at the benzaldehyde: water interface more readily than in the globule itself. The poorer catalytic effect of the copper laurate may also be due to hydrolysis, which would reduce the effective concentration of copper ions. In potassium laurate systems any copper sulphate introduced undergoes double decomposition to copper laurate and

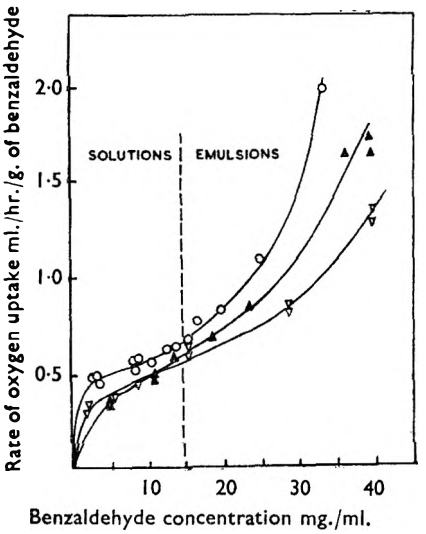

FIG. 5. Oxidation rate of benzaldehyde dispersed in cetomacrogol solutions showing the effect of variation of benzaldehyde concentration. Cetomacrogol concentration 0·06M, temperature 25°.

⊙ 1×10^{-5}M copper sulphate
▲ 1×10^{-5}M copper laurate
▽ uncatalysed

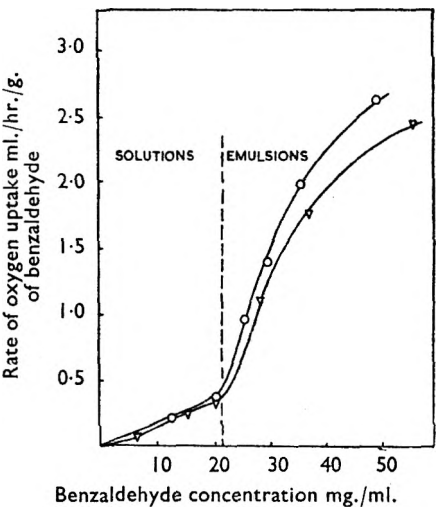

FIG. 6. Oxidation rate of benzaldehyde dispersed in potassium laurate solutions showing the effect of variation of benzaldehyde concentration. Potassium laurate concentration 0·12M, temperature 25°.

⊙ 1×10^{-5}M copper sulphate
▽ uncatalysed

thus reduces the effectiveness of the catalyst. It is likely that the copper laurate would be oriented at the benzaldehyde: water interface, so that it is probable that a similar initiation of the chain reaction most probably takes place there.

However, on increasing the interface, by incorporating the benzaldehyde in micelles, the rate decreased until in the solubilised state it was apparently independent of concentration of surface-active agent. This is the reverse of what would be expected if the reaction took place solely at the benzaldehyde: water interface. The suggestion of Kern[10] that the micelle acted as a barrier to the diffusion of oxygen appears to be doubtful as the surface of a spherical micelle, which consists of loosely packed polar heads, is unlikely to act as a barrier to the diffusion of oxygen into the non polar centre. Also, benzaldehyde is more probably contained between the palisade of the soap molecules, or bonded at or near the surface of the micelle.

The evidence so far gives some indication that most oxidation occurred within the emulsion globules of benzaldehyde. The reduction in oxidation rate as the globule size was reduced could be accounted for by the terminal reaction occurring at the benzaldehyde : water interface. From an examination of Figure 7 this possible mechanism does not appear to be the principle one, since additions of cetomacrogol to already oxidising suspensions of benzaldehyde produce emulsions and "solutions" which oxidised at about the same rate. This indicated that once oxidation had been initiated in the micelle or emulsion droplet, it proceeded rapidly. The stability of the solubilised systems shown in Figure 3 could be due to the chain reaction being retarded in some way, or due to the mutual destruction of free radicals once they are oriented at an interface.

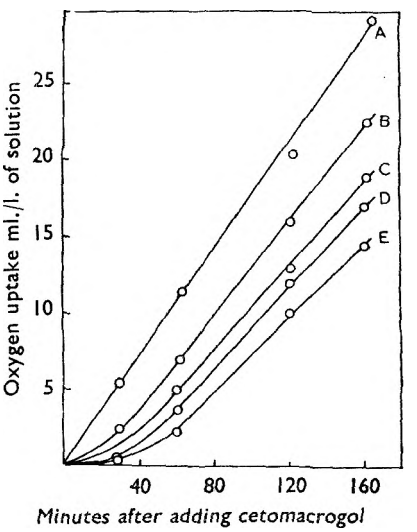

FIG. 7. The effect of adding cetomacrogol to an oxidising suspension of benzaldehyde. Concentration of benzaldehyde 15 mg./ml. Uncatalysed reaction at 25°.

A, suspended benzaldehyde in water
B, emulsified ,, ,, 0·01M cetomacrogol
C, ,, ,, ,, 0·02M ,,
D, solubilised ,, ,, 0·03M ,,
E, ,, ,, ,, 0·04M ,,

In the solubilised state oxidation was apparently confined to reaction in true solution in the water. If the oxidation took place only in the water then the rate should be independent of the interfacial area, provided that the diffusion of benzaldehyde from the "reservoir" of the micelle into solution was not a limiting factor. In support of this was the fact that once the solubilised state was reached, there was very little reduction in the rate of oxidation. It was of interest to note that the oxidation rate of a saturated solution of benzaldehyde in water was 750 and 2200 μl./g./hour at 25° and 35° respectively. This was only slightly above the oxidation rate for benzaldehyde solubilised by cetomacrogol or potassium laurate. It was thus probable that in the solubilised state oxidation took place almost exclusively in true solution in the water.

The oxidation of benzaldehyde was further complicated by the reaction of the surface-active agent with intermediates in the oxidation process. A study of the products of the reaction shows that accumulation of perbenzoic acid in the presence of cetomacrogol was unlikely, but that the cetomacrogol itself was peroxidised, and benzoic acid formed. Usually perbenzoic acid has been found to be the main oxidation product when

there is as much as 20 per cent decomposition of benzaldehyde. This is one probable reason for the low oxidation rate in solubilised systems, as the chain propagating perbenzoate radical would be reduced to the inactive benzoic acid.

Another possible complication is polymerisation of the benzaldehyde. Normally benzaldehyde does not polymerise easily, but it has been shown that solubilisation in soap micelles offers conditions favourable to polymerisation. According to Harkins[24], the predominant factor in the early stages is solubilisation of the monomer. The possibility that polymerisation is a complicating factor in these reactions is being investigated. If, in fact, polymerisation of benzaldehyde does take place, then the effective concentration available for oxidation would be lowered, and thus the rate of oxidation reduced. From the relation between oxygen uptake and the disappearance of benzaldehyde from the systems studied, we have evidence that decomposition of benzaldehyde proceeded other than by oxidation, and it is probable that polymerisation was taking place.

Acknowledgements. The authors wish to thank the Pharmaceutical Society of Great Britain for an educational grant to one of them (J.R.N.), and to Glovers Chemicals, Ltd., for samples of cetomacrogol. We are indebted to Dr. H. S. Bean for many helpful discussions.

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DISCUSSION

The paper was presented by MR. J. R. NIXON.

DR. K. R. CAPPER (London). In related work using benzaldehyde, he had obtained results which were difficult to explain. More oxygen

disappeared than expected. The authors commented, "It seems likely that the remainder of the oxygen is used in the peroxidation of the cetomacrogol." It appeared that in a solution of benzaldehyde in alcohol most of the oxygen was not used in oxidation of the benzaldehyde, but of the alcohol. It would be of interest to study the effect of antoxidants.

MR. N. J. VAN ABBÉ (Loughborough). What was the chronic toxicity of the non-ionic surface-active agents?

DR. L. SAUNDERS (London). It was stated that the rate of oxidation was dependent on [benzaldehyde]$^{3/2}$ using a simple solution of benzaldehyde in water. Later the statement was made that the poorer catalytic effect of copper laurate might be due to incomplete ionisation, but it might be due to hydrolysis. It would seem that incorporating the benzaldehyde in micelles protected the benzaldehyde from oxidation. Interaction between the benzaldehyde and the soap was being obtained.

MR. A. AXON (Dartford). Were the end products of the oxidation of benzaldehyde observed as emulsions, solutions or crystals?

THE AUTHORS replied. They hoped to study the effects of added antoxidants. The low toxicity of the polyoxyethylene non-ionics was well known. The potentiometric measurements were their own. The poorer catalytic effect of copper laurate seemed to be a matter of hydrolysis. Benzaldehyde is most stable in micelles, but the mechanism is unknown. They did not consider interaction between the peroxide and surface-active agent to be entirely responsible for the low oxidation rate. If oxidation were allowed to proceed further, crystals of benzoic acid were obtained; in solubilised preparations crystals had never been observed.

THE USE OF OXIDISED CELLULOSE FOR THE DETERMINATION OF STRYCHNINE IN PHARMACEUTICAL PREPARATIONS

By D. A. Elvidge and K. A. Proctor

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With oxidised cellulose as a carboxylic cation exchange medium strychnine can be separated from extraneous interfering materials in a pure form for spectrophotometric assay. When brucine is also present a 2-point spectrophotometric procedure is adopted. Results compare well with chemical assays with a coefficient of variation of about 1 per cent. The oxidation procedure used in the official assay of nux vomica completely destroys brucine with no loss of strychnine.

In 1955 Brealey and Proctor[1] reported spectrophotometric methods for the determination of the therapeutically active ingredient in injection solutions containing chlorocresol. It was realised that these methods had only a limited application and investigations were therefore continued with the aim of finding a general method by which any injection solution capable of spectrophotometric assay could be so determined in the presence of any interfering bacteriostatic agent. Early in 1956 Freeman[2] described the use of oxidised cellulose for alkaloidal analysis and we have applied it[3] to the separation and determination of the active principle and bacteriostatic agent in seventeen different injection solutions, almost all official preparations. In these the active ingredient is assayed spectrophotometrically. Phenol, chlorocresol and benzyl alcohol all interfere with the direct spectrophotometric determination of injections but may be separated by oxidised cellulose.

Many of the preparations examined could not be purified sufficiently for spectrophotometric determination by oxidised cellulose alone, because of the very high proportion of interfering materials which also occasionally prevented the repeated use of the column. Some preliminary purification, usually by treatment with alumina, was therefore necessary. Where the sample contained either coloured materials which were not retained on alumina, or salts which had been shown by Freeman[2] to hinder the retention of alkaloids on oxidised cellulose, it was necessary to extract with solvent.

The methods are divided into those involving (*a*) strychnine and (*b*) nux vomica. The preliminary purification and chromatographic stages were identical for both.

Preparations Containing Strychnine Alone

Tablets and pills, and a liquid containing nicotinamide, caffeine and riboflavine were examined.

Preliminary Purification

(*a*) Treatment with Brockman alumina.

Apparatus, Pyrex tube (2·5 × 30 cm.) with tap, plugged with glass wool.

Preparation of column. Slurry 20 g. of alumina with 70 per cent ethanol, transfer to the tube and allow to settle under gentle air pressure.

Preparation of sample. Weigh a suitable amount of the powdered sample into a 50 ml. conical flask, heat on a steam bath with 25 ml. of 70 per cent ethanol and filter through No. 30 paper onto the alumina column. Extract the residue in the flask with two further portions of 15 ml. of 70 per cent ethanol and transfer to the column. After these extracts have passed through the column wash with small quantities of 70 per cent ethanol and collect the total eluate in a 100 ml. graduated flask. After dilution to volume with the same solvent transfer a suitable aliquot to an oxidised cellulose column.

(*b*) Solvent extraction with chloroform B.P.

Preparation of sample. Pipette a suitable aliquot into a 100 ml. separating funnel, make alkaline with a few drops of dilute ammonia and extract with 20 ml. of chloroform for two minutes. Transfer the chloroform layer into a second separating funnel and wash with 10 ml. of water. Run off the washed extract into a flask, and extract the original sample in a similar manner with three further portions of 20 ml. of chloroform. Wash these further extracts with the same 10 ml. of water and add to the original extract. Transfer the bulked chloroform extracts to an oxidised cellulose column.

Chromatography

Prepare a 1 g. column of oxidised cellulose, Eastman Kodak (16 to 22 per cent carboxylic content) as described in our previous communication[3]. If preliminary purification is unnecessary make an extract or dilution of the sample with 70 per cent ethanol and transfer a suitable aliquot to the column. When preliminary purification has been made by solvent extraction wash the oxidised cellulose column with absolute ethanol before adding the chloroform solution. A suitable amount of strychnine for chromatography is about 0·5 mg.

After the alkaloidal extract has passed through the column under gentle air pressure, adjusted to give a flow rate of about 3 ml. per minute, wash the column with successive amounts of water until the eluate shows an extinction of less than 0·005 at 250 mμ. Where purification by solvent extraction has been used pass 10 ml. of absolute ethanol through the column before washing with water. After washing, elute the alkaloid from the column with N sulphuric acid and collect 50 ml. of eluate in a graduated flask.

Spectrophotometric Determination

Measure the extinction of the eluate from the oxidised cellulose column in a suitable spectrophotometer at 254 mμ in a 1 cm. cuvette, with N

sulphuric acid in the compensation cuvette. Calculate the amount of strychnine from the formula,

$$\text{per cent strychnine} = \frac{E\,(1\text{ per cent, }1\text{ cm.}) \times 100.}{375}$$

Table I gives the results with tablets, pills and a tonic. Replicates were within 1 per cent of the mean. These results were not compared with chemical assays the accuracy of which is poor in comparison. The quantitative nature of the method may, however, be seen from the following recovery experiments.

TABLE I

SAMPLES CONTAINING STRYCHNINE ONLY

Sample	Strychnine content	
	Expected	Found
Compound tablets of ferrous carbonate	0·033 grains/tab.	0·033 grains/tab.
Tablets of ferrous carbonate with arsenic and strychnine	0·0100 ,,	0·0098 ,,
		0·0108 ,,
Tablets of reduced iron with arsenic and strychnine	0·0166 ,,	0·0161 ,,
		0·0167 ,,
Compound Pills of Phenolphthalein B.P.C.	0·0125 ,,	0·0125 ,,
Compound tablets of glycerophosphates	0·0109 ,,	0·0119 ,,
Tonic	0·084 mg./ml.	0·087 mg./ml.

1. A sample of strychnine pills was dissolved in 70 per cent ethanol and made up to a suitable volume. One aliquot was examined directly after dilution in N sulphuric acid; a further aliquot was passed through an oxidised cellulose column, washed and eluted with N sulphuric acid. Comparison of the two solutions showed that 98·6 per cent of the strychnine was recovered from the column.

2. To a sample of compound tablets of ferrous carbonate, containing an expected amount of 0·033 grains of strychnine per tablet, a weight of strychnine equivalent to 0·0300 grains/tablet was added and the whole subjected to alumina treatment followed by chromatography.

The recovered amount of strychnine was 0·0297 grains/tablet, or 99·0 per cent recovery.

3. To a sample of tablets of ferrous carbonate with arsenic and strychnine, containing an expected amount of 0·0100 grains strychnine per tablet, a weight of strychnine equivalent to 0·00982 grains/tablet was added and the whole subjected to treatment with alumina followed by chromatography.

The recovered amount of strychnine was 0·0100 grains/tablet, or 102 per cent recovery.

The purity of the strychnine obtained may be judged by the absorption spectrum of a final N sulphuric acid eluate from the tonic, which coincides with the absorption spectrum of pure strychnine between 240 mμ and 295 mμ.

PREPARATIONS CONTAINING NUX VOMICA

A number of pharmaceutical preparations contain nux vomica. The official chemical method of assaying strychnine in nux vomica also

extracts brucine, which is removed by chemical destruction under controlled conditions. This is followed by re-extraction and titration of the strychnine.

Various workers[4–6] have shown that strychnine can be determined spectrophotometrically in the presence of brucine. We have found these two alkaloids to be isolated in a pure state from preparations containing nux vomica by the use of oxidised cellulose and that as a result it is possible to estimate strychnine with an accuracy of about ± 1 per cent. Furthermore, these determinations can generally be carried out within two hours with only 500 μg. of strychnine. A chemical assay requires at least 20 mg.

Details of the 2-point spectrophotometric procedure are given below. In N sulphuric acid strychnine exhibits a characteristic spectrum with a maximum at 254 mμ, whilst brucine has two maxima at 264 mμ and 300 mμ respectively. In a mixture of approximately equal quantities of the two alkaloids, as in nux vomica, a maximum is observed at about 262 mμ with an inflexion at 300 mμ, so that these two wavelengths are most suitable for the application of a 2-point method.

The E (1 per cent 1 cm.) values of strychnine and brucine at 262 mμ and 300 mμ are as follows:—

	Strychnine	*Brucine*
262 mμ ..	322	312
300 mμ ..	5·16	216

It can be shown that, for a solution containing only strychnine and brucine the observed extinctions are A and B at 262 mμ and 300 mμ respectively, it follows that $x = 0{\cdot}318A - 0{\cdot}460B$ where x is the concentration of strychnine per cent. Nine different preparations containing nux vomica were examined.

Tincture of Nux Vomica B.P.

(*a*) 5 ml. of sample was diluted to 100 ml. in ethanol, 10 ml. was placed on a 1 g. column of oxidised cellulose, washed with 20 ml. of alcohol, then with 50 ml. of water, and the alkaloids finally eluted with N sulphuric acid and diluted to 50 ml.

(*b*) To 5 ml. of sample was added 5 ml. of a 0·125 per cent solution of strychnine in ethanol, the mixture diluted to 100 ml. and 5 ml. chromatographed as above.

The tincture was found to contain 0·118 and 0·120 per cent of strychnine, and the tincture to which 0·125 per cent of strychnine was added contained 0·244 and 0·244 per cent, so that 0·125 per cent strychnine was recovered from each of the latter two samples.

These results agree with the result of the official chemical assay (0·119 per cent of strychnine).

Mixture of Potassium Bromide and Nux Vomica B.P.C.

This preparation contains 4·17 per cent of tincture of nux vomica and 4·57 per cent of potassium bromide in addition to a small amount of amaranth. The presence of potassium bromide and the dye-stuff

prevented the direct application of the mixture to an oxidised cellulose column, and preliminary purification by solvent extraction was necessary.

Some initial experiments were done on two mixtures. The first was prepared by dissolving 0·0520 g. of strychnine in 20 ml. of ethanol, adding 0·104 g. of amaranth, 4·57 g. of potassium bromide and 2·5 ml. of chloroform and diluting to 1 litre with water. The second solution was prepared with the same quantities of ingredients, together with 0·0508 g. of brucine.

10 ml. of each solution was made alkaline with dilute ammonia and extracted with chloroform as described in the preliminary purification method.

The first mixture gave duplicate assays of 0·00524 and 0·00520 per cent of strychnine (theory = 0·00520 per cent). The second mixture gave an average result of 0·00514 per cent (theory = 0·00520 per cent), with a coefficient of variation of $\pm 1{\cdot}22$ per cent (four determinations).

A further mixture containing potassium bromide, amaranth, strychnine, 0·0052 per cent, and brucine was prepared and assayed in duplicate by three methods:—

(i) Solvent extraction and chromatography as described above. (ii) Extraction as above, destruction of the brucine by the official method, and re-extraction of the strychnine followed by spectrophotometric determination. (iii) The official method. Found by methods (i) 0·00525 and 0·00525, (ii) 0·00506 and 0·00517, and (iii) 0·0052 and 0·0053 per cent of strychnine.

The agreement is good and confirms the accuracy of the spectrophotometric method which also saves time.

Prepared Nux Vomica B.P.

The official method requires a continuous extraction procedure with boiling chloroform for at least four hours. Preliminary experiments showed that the alkaloids were extracted easily by boiling with 70 per cent ethanol. When an aliquot of this extract was chromatographed on oxidised cellulose and washed with water only, high results were obtained and the ultra-violet spectrum suggested that this was due to extraneous absorption. By washing with chloroform the interfering material was eliminated and close agreement with chemical assays was obtained. From the calculated strychnine (and brucine) concentrations a composite absorption curve was obtained using spectrophotometric data for the pure alkaloids and corresponded closely with that of the extract, indicating the high state of purity obtained by the chromatographic procedure.

An improvement was made by extracting the sample in a Soxhlet apparatus with 70 per cent ethanol for two hours. Longer periods of extraction of four hours yielded no increase of strychnine.

To test the reproducibility of the method a total of six assays in duplicate were carried out on one sample. The mean was 1·22 per cent strychnine with a coefficient of variation of $\pm 1{\cdot}4$ per cent. Duplicate chemical assay gave results of 1·22, 1·23 per cent of strychnine.

The method finally adopted therefore for this material is as follows:—

Weigh 1 g. of finely powdered sample into a Soxhlet thimble, add 80 ml. of 70 per cent ethanol and reflux for two hours. After cooling, transfer the extract to a 100 ml. graduated flask and make up to volume with 70 per cent ethanol. Place 5 ml. of this solution on an oxidised cellulose column, prepared as previously described. Wash the column with 10 ml. of absolute ethanol, 50 ml. of chloroform, 10 ml. of ethanol and 50 ml. of water in succession. Elute the alkaloids with 50 ml. of N sulphuric acid, collect the eluate in a graduated flask and measure the extinctions at 262 mμ and 300 mμ. Calculate the strychine content from the equation.

Miscellaneous Preparations

Six further preparations examined gave no difficulties and need not be discussed individually. The details of method of purification, quantities, etc., are given in Table II, and the results in Table III. Determinations were carried out in duplicate, and replicates were within 1 per cent of the mean.

TABLE II

METHODS FOR SAMPLES CONTAINING NUX VOMICA

Sample	Amount taken	Purification method	Dilution	Aliquot taken for chromatography
Dry Extract of Nux Vomica B.P. ..	0·10 g.	None	→100	10 ml.
Liquid Extract of Nux Vomica B.P. ..	5 ml.	,,	→100 : 10 → 100	10 ,,
Compound Pills of Aloin and Podophyllin B.P.C. 1934	3 pills	(*a*)	None	Whole
Compound Pills of Aloin B.P.C. 1934 ..	3 ,,	,,	→200	30 ml.
Pills of Aloes and Nux Vomica B.P.C. ..	5 ,,	,,	→200	20 ,,
Compound Bismuth Mixture with Pepsin B.P.C. 1934	10 ml.	(*b*)	None	Whole

TABLE III

RESULTS ON SAMPLES CONTAINING NUX VOMICA

Sample	Strychnine content		
	Expected	Found	Chemical assay
Dry Extract of Nux Vomica B.P.	5·0 per cent	5·00 per cent	5·14 per cent
Liquid Extract of Nux Vomica B.P. ..	1·50 ,, ,,	1·52 ,, ,,	1·54 ,, ,,
Compound Pills of Aloin and Podophyllin B.P.C. 1934	0·0025 grains/pill	0·00209 grains/pill	
Compound Pills of Aloin B.P.C. 1934 ..	0·025 ,,	0·0217 ,,	
Pills of Aloes and Nux Vomica B.P.C. ..	0·0125 ,,	0·0120 ,,	
Compound Bismuth Mixture with Pepsin B.P.C. 1934	0·0105 per cent	0·0117 per cent	0·0114 per cent

THE EFFECT OF OXIDATION ON STRYCHNINE-BRUCINE MIXTURES

The described method was suitable for examining one aspect of the validity of the correction factor (1·02) applied to compensate for loss of strychnine in the official method of assay of preparations containing nux vomica.

Preliminary experiments were done by mixing suitable aliquots of strychnine sulphate solution with an aqueous solution of potassium nitrate and potassium sulphate of a concentration equivalent to that

obtained by neutralisation of the official oxidation mixture. The recovery of strychnine with the recommended 1 g. of oxidised cellulose was low and variable, but quantitative recoveries were obtained with a 2·5 g. column. For six determinations the average recovery was 100·3 per cent with standard deviation $\pm$0·2 per cent.

An aqueous solution of strychnine sulphate equivalent to 1·00 per cent of strychnine was prepared and the acidity adjusted by mixing 10 ml. of this solution with 5 ml. of 9 per cent sulphuric acid immediately before adding 2 ml. of nitric acid and a few crystals of sodium nitrite. In this way possible errors due to incomplete dissolution of the strychnine were avoided. After exactly 30 minutes at 20° the solution was adjusted to about pH 7 with N potassium hydroxide, transferred to a 250 ml. graduated flask with water and diluted to volume. Immediately 5 ml. was pipetted on a column of 2·5 g. of oxidised cellulose, washed with 100 ml. of water and the alkaloid eluted with N sulphuric acid. The strychnine was determined by spectrophotometric measurement at 254 mμ and the results of five assays gave an average recovery of 100·0 per cent, with standard deviation of $\pm$0·3 per cent. There is thus no evidence for any destruction of strychnine.

To complete the investigation the effect of the official oxidation mixture on brucine was examined. After oxidation of 0·1 g. quantities and chromatographic treatment it was impossible to measure spectrophotometrically any residual brucine. There was a small amount of general extraneous absorption which, if calculated as brucine, would give a maximum of 0·05 per cent of brucine, implying a minimum of 99·95 per cent destruction. Thus, there are no grounds for the application of the correction factor for loss of strychnine by oxidation.

We thank Mr. C. A. Johnson, Mr. W. E. Drinkwater and Mr. K. Rogers for chemical determinations.

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DISCUSSION

The paper was presented by MR. D. A. ELVIDGE.

The CHAIRMAN. The reason for the factor of 1·02 in the B.P. assay of nux vomica became less obvious as the result of this work.

DR. D. C. GARRATT (Nottingham). It was Corfield who introduced the factor of 1·02, after showing that the weight of strychnine was less than the figure obtained by titration. Foster's work may now have solved the question.

MR. H. B. HEATH (Sudbury) had made a number of spectrophotometric determinations of strychnine and brucine mixtures and found that the

method was inaccurate and he asked how it followed that $x = 0{\cdot}318A - 0{\cdot}460B$. He had found that sodium nitrite had a very marked absorption at 300 mμ. What was the number of the Brockman grade of alumina used?

DR. G. E. FOSTER (Dartford). How was the theoretical amount of strychnine reported in Table III determined?

DR. W. MITCHELL (London). Could coarsely crushed nux vomica be readily extracted by boiling with ethanol? The liquid reaching the extraction thimble would be stronger than 70 per cent.

MR. S. G. E. STEVENS (London). Did all batches of oxidised cellulose give the same type of absorption characteristics and was there any experience when other permitted dyes were present?

DR. L. SAUNDERS (London). It was not clear that oxidised cellulose was comparatively unstable at room temperature and had to be kept refrigerated. Had it been found necessary to apply blanks for absorbing material derived from the oxidised cellulose and had the exchange capacities been determined in order to standardise the material?

MR. H. B. HEATH (Sudbury). Was it necessary to wash the column after eluting with sulphuric acid?

DR. G. E. FOSTER said it was not clear whether the authors had carried out assays of nux vomica using the B.P. chloroform extraction method as well as the ethanol method recommended. His experience of spectrophotometric methods had been that although one worker with his own spectrophotometer might obtain results accurate within 0·1 per cent, when assays were carried out in independent laboratories the agreement was not so good.

DR. G. BROWNLEE (London) raised the question of the use of the term "error" by chemists to record the observed deviations of replicates and by other scientists to indicate the error of the estimate. Had not the time arrived for analysts to record the latter? The analyst would lose nothing by stating the intrinsic error of his estimation, and he would strengthen his own hand by recording the variations from day to day or from laboratory to laboratory.

DR. GARRATT agreed with Dr. Brownlee. The precision of many determinations was known, but not the accuracy.

MR. H. D. RAPSON (Dorking). Spectrophotometers might be subject to error owing to diurnal temperature variations, although the more recent instruments did not show such an error. He would use a set of standard solutions.

MR. C. A. JOHNSON (Nottingham). Despite the instability of the oxidised cellulose, could it not be used over a long period for a number of determinations?

In reply MR. ELVIDGE said that there were two simultaneous equations for the additive absorption spectrum, but these were not included in the paper. For pure strychnine the standard deviation was calculated as $\pm 0{\cdot}3$

per cent, but with strychnine and brucine there would be a slightly larger error. They had found that all the salts came through the column, only strychnine being left and, therefore, there was no interference from sodium nitrite. The alumina was Brockman Grade I. He had had no experience with coarsely crushed nux vomica. The temperature of the alcohol vapour recorded by a thermometer in the Soxhlet thimble indicated a strength of 70 to 72 per cent. After saying that the figure of 0·033 grains of strychnine was determined on one sample of tablets, and it was assumed that others had the same amount. There were different grades of oxidised cellulose. For strychnine assay a content of 16 to 22 per cent of free carboxyl was satisfactory, but for some other alkaloids the 10 to 12 per cent grade must be used. Amaranth and dyes of a similar type were used in the preparations. Oxidised cellulose was unstable, particularly in the dry state, but appeared more stable under water, and the powdered material was always kept in a refrigerator. In all the batches examined there was virtually no blank, and even at 200 mμ it was found to be only 0·002. Provided simple solutions practically free from suspension were used, up to 10 ot 12 consecutive determinations could be carried out successfully. It was advisable after, say, a month to discard the column. The variation of wavelength in one of his calibrated spectrophotometers had been no greater than $\frac{1}{2}$ mμ during one year. It was doubtful whether that shift would cause any appreciable error.

OBSERVATIONS ON THE INTRAVENOUS TOXICITY AND CHELATING ACTIVITY OF SODIUM STIBOGLUCONATE

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The optical activity, calcium chelating power and intravenous toxicity, but not the leishmanicidal activity, of a sodium stibogluconate solution varies with pH and age. As the optical rotatory power increases, the calcium chelating property decreases, and the solution becomes less toxic. Solutions of sodium stibogluconate injected intravenously into mice produce hypocalcaemia, which may be the cause of death. Production of hypocalcaemia may be a phenomenon common to many substances with chelating powers, a factor to be considered in the design of comparative toxicity tests.

The lack of relation between the analytical and toxicity specifications in the B.P.C. monograph for sodium stibogluconate led to this investigation. Goodwin[1-4] has demonstrated that the acute toxicity of a sodium stibogluconate solution decreased with pH, whereas its leishmanicidal activity was unaffected. We had observed that traces of excess chelating agent increased the intravenous toxicities of metal chelates in mice. We therefore investigated the techniques for assessing the toxicity of sodium stibogluconate, and also certain of its physical and chemical properties with the object of relating the observations.

Experimental Methods

Solutions for toxicity studies, unless otherwise stated, were prepared at room temperature by grinding the solid to a paste with water and then diluting to the required volume. Concentrations were chosen to give the LD50 dose in approximately 0·2 ml. of solution. The doses were injected intravenously into GFF male mice, each of between 16 to 22 g., in groups of 5. A constant rate of injection of 1 ml./30 seconds was used. Mortalities occurring after 1 hour were not included.

Blood samples of 1 ml. in paraffin waxed glass tubes, 3 in. long, 0·375 in. internal diameter, were slowly inverted at 30 second intervals and their clotting times observed. Leishmanicidal activity was tested by Goodwin's[1] method as follows.

Hamsters which had been infected four weeks previously with *L. donovani* were grouped in fives. The animals were anaesthetised and spleen biopsies made. Smears were made from the tissue removed. The following day the animals received a single subcutaneous injection of sodium stibogluconate. A week later a second biopsy was made and spleen smears again made.

The effectiveness of the sample was estimated by the comparison of the number of parasites per 100 spleen cell nuclei before and after treatment. Counts of approximately 1000 spleen cell nuclei were made in each case.

The power of the solution to chelate calcium was determined by the technique of Mehltretter and others[5]. Sodium oxalate, 2 ml. of a 2 per cent solution, was added to a suitable quantity of sodium stibogluconate, 20 to 50 ml. of 10–30 per cent solution. The mixed solution was titrated with calcium acetate, 10 per cent, until a permanent white cloudiness appeared within 2 minutes.

Infra-red absorption spectra of Nujol mulls were recorded on a Perkin-Elmer, Model 21 double-beam spectrophotometer with a sodium chloride prism.

The rotatory powers of 10–30 per cent solutions in 2 dcm. tubes were measured with sodium light. pH values of similar solutions were measured potentiometrically.

Results

The LD50 value calculated by a log probit method for a solution of sodium stibogluconate freshly prepared at room temperature increased with time. The results are shown in Table I. The decrease in toxicity with increasing age of solution was confirmed by the figures in Table II.

TABLE I

Reduction of intravenous toxicity of a freshly prepared 10 per cent solution of sodium stibogluconate with time

Dose mg./kg.	Age of solution									
	1 min.	15 mins.	1 hr.	2 hrs.	3 hrs.	5¾ hrs.	8 hrs.	1 day	2 days	13 days
900	1/5									
1000		0/5								
1100	1/1									
1200		1/5								
1300	1/1	0/4								
1400			1/5							
1500		5/5	3/5	0/5						
1700				0/5						
1850				2/5						
2000			1/1	2/5						
2150				4/5	2/5					
2300					2/5					
2500					3/5	2/5	1/5	0/1		
2600							6/10	1/5		
2700						4/5	7/10	5/10		2/5
2850							5/5	5/11	0/2	
3000						3/3		6/7	2/5	4/5
3200								7/1	3/5	
3400									2/3	
3600									1/1	
Graphical LD50 (common slope)	1000	1300	1500	2000	2300	2500	2600	2800	3100	2800

Entries in the Table: no. of deaths/no. of mice injected. The correlation coefficient for LD50 and age of solution in the period from 5 minutes to 8 hours is $r = 0{\cdot}897$ ($n' = 5$), a value which is improbable by chance. ($0{\cdot}01 > P > 0{\cdot}001$.)

The toxicities of samples of different pH values are given in Table III. They show that toxicity decreases with increase in acidity and also that the solutions became less toxic with time.

Since the toxicity studies suggested that some change occurred in sodium stibogluconate solutions, measurements of optical activities against time, pH value and temperature were made. The results are summarised in Figure 1.

The curves show mutarotation positively to an equilibrium value that increases with fall of pH. The figures for rotation of the freshly prepared solutions are of little value because of the difficulty in deciding zero time. Equilibrium was rapidly reached in warmed solutions.

Infra-red spectra of solids, that in solution had pH values 6·3, 6·0 and 5·5, showed that the carbonyl band of the COONa group (*c.* 1595 cm.$^{-1}$) progressively diminished and the COOH band (*c.* 1670 cm.$^{-1}$) increased.

TABLE II

ACUTE TOXICITY TO MICE OF 15 PER CENT SODIUM STIBOGLUCONATE SOLUTIONS 1 AND 24 HOURS OLD

Dose mg./kg.	Solution 1 hour old							Solution 24 hours old							
	Lab. std.	399/3A	415A/2	416A/2	424/B	425/B	S.W.B.	Lab. std.	399/3A	415A/2	416A/2	424/B	425/B	S.W.B.*
750	0/1	0/3	0/5		0/5	0/1								
900		0/2	3/5		5/10	0/5								
1050		0/5	5/5		6/6	5/5								
1125				3/10										
1200					2/2	1/1						1/10		
1275	0/5	2/5	2/2	7/10										
1350					2/2	1/1		0/1		0/5	2/10	5/10		
1500	5/5	5/5	2/2	2/2	2/2	1/1		0/1		9/10	5/10	7/10	0/5	
1650										5/5	8/10	4/5	6/10	
1800									0/1	1/1	1/1	4/5	5/5	
1950									4/10		1/1	2/2		
2100									6/10		1/1	1/1		
2250	2/2							3/10	6/10	1/1	1/1	1/1		
2400								6/10	6/10					
2550								7/10						
2880	2/2						0/1	1/1						
3000														
3840							0/3							0/3
4130							2/5							0/5
4420							3/5							4/5
4800							4/5							3/3
Graphical LD50	1390	1310	880	1200	900	975	4280	2400	2210	1580	1650	1420	1610	4230

Entries in the table are deaths out of number of mice injected.
*Solution was at pH 5·4 and was warmed during preparation.
$F = 62{\cdot}95$, $n_1 = 1$, $n_2 = 5$ (P 0·001)
The toxicity at 24 hrs. is less than that at 1 hr.

For a solid precipitated from a solution of pH 3·5 there was a strong COOH band, but none for lactone nor COONa group, although the sodium content of the dry solid was 70 per cent of that present in a material of pH 6·3.

After thus establishing that the toxicities and rotatory powers of freshly prepared sodium stibogluconate solutions change with time and pH, their powers to chelate calcium were investigated. The results summarised in Figure 2 show chelating power to decrease with increasing rotatory power.

TABLE III

DECREASE IN TOXICITY OF SODIUM STIBOGLUCONATE WITH DECREASE IN pH VALUE AND AGE OF SOLUTION

Material* injected	Concentration of injected solution per cent	pH	LD50 mg./kg. at hours after dissolution of solid	
			1 hr.	24 hrs.
399/O	15	6·3	1455	2360
399/P	30	5·4	2775	3375
424 B/O	15	6·57	1130	2360
424 B/P_1	30	5·67	2200	3500
424 B/P_2	30	3·48	4800	5930
415/O	15	6·45	880	1760
415/P	30	5·4	2250	2580
416/O	15	6·35	—	1840
416/P	30	5·6	—	2550
425/O	15	6·35	—	1950
425/P	30	5·6	—	2775
M.R.C. standard	30	5·35	—	4125
	30	6·0	—	2440
	15	6·6	—	1610

* O = Original; P = pH adjusted and reprecipitated. The probability of finding this correlation between pH and toxicity by chance is approximately 1 in 100.

The calcium chelating power of freshly prepared solutions *in vivo* were then assessed. A 15 per cent solution (w/v) of sodium stibogluconate was more toxic than the same solution with the addition of 1 per cent of calcium chloride. The two solutions were compared with the standard preparation by the B.P.C. test for undue toxicity. The solution with no calcium killed 90 per cent of the animals injected, that with calcium only 10 per cent. A 2·2 kg. rabbit injected rapidly with 4·4 ml. of a freshly prepared 30 per cent solution of sodium stibogluconate convulsed violently but quickly recovered. A blood sample, taken from the ear immediately after injection, took 60 minutes to clot; one taken before the injection took 8 minutes.

Another rabbit given four times the dose received by the first, but in doses of 1·1 ml. at 8 minute intervals, showed no distress. Its blood clotting time doubled after the first injection, increased to fourfold with further injections and then began to fall.

No difference in leishmanicidal activity was observed for solutions between pH limits 6·25 and 5·5.

Discussion

Since mutarotation is a manifestation of molecular change, solutions exhibiting this phenomenon may change their biological properties with age. These properties will depend on the relative proportions of epimers present at the time of measurement.

The mutarotation curves show (Fig. 1) that the modification occurring in a freshly prepared solution of sodium stibogluconate is not of the first order[6]. They suggest the possible existence of at least three components in the equilibrium mixture, but insufficient is known about the binding of the antimony in the material to make it possible to determine the mechanism of the changes. However, from the infra-red spectra and sodium contents of the dried material it can be deduced that unlike that in sodium gluconate most of the sodium is not attached to the carboxylic group.

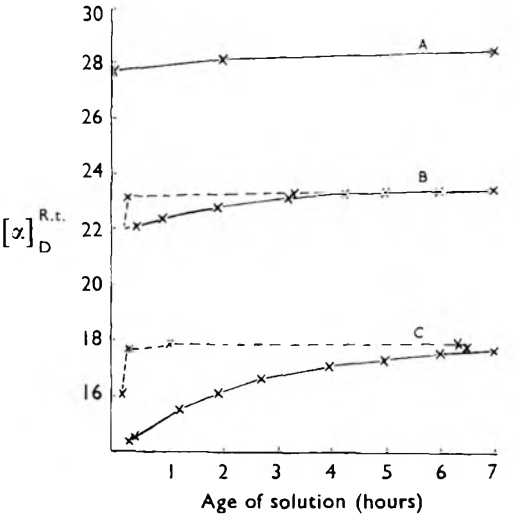

FIG. 1. Changes in molecular rotation of sodium stibogluconate with pH and age of solution: A, at pH 3·5, B, at pH 5·5 and C, at pH 6·4.
x———x Solution prepared and kept at room temperature (R.t.)
x– – –x Solution prepared at 50° and kept at room temperature (R.t.)

The decreases in acute toxicities of sodium stibogluconate solutions on standing or on acidification (Tables I, II and III) indicate the formation of a less toxic epimer. Moreover, since the calcium chelating powers of the solutions decrease *pari passu* with the toxicities to mammals, it is suggested that the phenomena are related. Therefore the molecular species present in a freshly prepared solution of pH say, 6·5, can chelate blood calcium more strongly than can the epimer produced on standing or by acidifying the solution.

The convulsions and seven-fold increase in blood-clotting time of a rabbit injected with freshly prepared sodium stibogluconate indicate that the blood calcium has been removed by the injected drug. But the rapid recovery by the first rabbit, and the tolerance shown by the second, which received repeated injections, suggest either that the removal of blood calcium is reversible or that calcium reserves may be mobilised rapidly. The rate of injection will affect toxicity if the rate of the reverse action is similar to the rate of chelation.

The mutarotation curves (Fig. 1) show that equilibrium is rapidly reached in warmed solutions, so that the toxicities of solutions warmed or

autoclaved before injection will not decrease with time. (Sample S.W.B. in Table II.)

Maffii and others[7] have shown that the intravenous toxicities of tetracyclines in mice are reduced if saline instead of water is used as the solvent. Nicolle and Weisbuch[8] demonstrated that potassium chloride decreases the rates of mutarotation of certain sugars. These phenomena may be relevant, for a single experiment has shown that potassium chloride modifies the rotation of a sodium stibogluconate solution. The phenomena also indicate that apparently minor changes in experimental conditions may affect biological activity and allow wrong conclusions to be drawn about an injected drug.

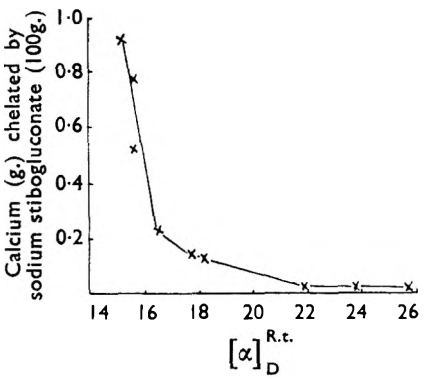

FIG. 2. Calcium chelating activity of sodium stibogluconate solutions plotted against optical activity.

It is therefore suggested that when drugs with chelating properties are compared for toxicity the conditions of the test should be such that the chelating activities are similar. Sodium stibogluconate is an example. At present its solutions are tested for undue toxicity according to the Pharmacopoeial test for neoarsphenamine. This seems hardly logical, as Neoarsphenamine solutions become more toxic with age, while sodium stibogluconate solutions become less so. It is suggested that test solutions should be equilibrated before injection and compared with a standard solution at the same pH and concentration : also the solid material should be dissolved under identical conditions and the solutions injected at the same rate.

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DISCUSSION

The paper was presented by DR. J. GREEN.

The CHAIRMAN. It might have been better to have stated specifically a desirable method for achieving equilibrium before testing for toxicity and to have given the appropriate pH value. It was not possible from the results quoted to discover why the leishmanicidal activity did not vary with age and pH.

DR. G. E. FOSTER (Dartford) was able to confirm that the addition of calcium reduced toxicity, but not that toxicity decreased on keeping. Official monographs included a test for the presence of trivalent antimony since it was thought that toxicity was due to this, but it now seemed that a stringent test was not necessary. Tests to exclude calcium had been tried but failed probably owing to the chelating effect of the stibogluconate.

DR. J. GREEN replied that the point to which the Chairman had referred was being rectified. Specific recommendations about pH were not made because the matter was about to be discussed by the British Pharmacopoeia Commission. The present B.P.C. monograph stated that tests should be made on an autoclaved solution, when many of the phenomena observed were encountered. There was much smaller change in toxicity at the lower pH and it was at about pH 6 and above where chelating activity was the greatest. As the pH dropped the chelating activity became less and changes in toxicity also became less. All the samples in question were tested for trivalent antimony, but it was not responsible for the toxicity.

THE NON-AQUEOUS TITRATION OF PHENOLIC COMPOUNDS

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The published methods for the titration of very weak acids are reviewed and the choice of a suitable solvent, electrode system and titrant discussed. Ethylenediamine as a solvent is rejected for routine purposes because it is toxic and unpleasant in use. Dimethylformamide is a suitable solvent for phenolic substances and gives a titration curve with a step at the end point of at least 75 mV. This is nearly the bottom limit of practicability, using tetrabutylammonium hydroxide in benzene and methanol as titrant. The electrode system preferred for its reliability is a glass indicator electrode and a saturated calomel half cell, slightly modified, as reference electrode.

The volumetric determination of very weak acids by titration in non-aqueous solvents has been investigated by a number of workers, the earliest successful method being that of Moss, Elliott and Hall[1] in 1948 using ethylenediamine as solvent and a solution of sodium aminoethoxide in ethylenediamine as titrant. Since that time, a number of different solvents and titrants have been proposed and the use of various electrode systems has been reported.

The purpose of this paper is to present a procedure for the potentiometric determination of very weak acids that is simple, convenient and sufficiently reliable for routine use. No attempt has been made to use visual indicators because the change in e.m.f. at the end point of the average titration is considered to be too low to give a sharp colour change.

Electrodes

The choice of a suitable electrode system presents difficulties when ethylenediamine is used. Moss, Elliott and Hall[1] used an antimony indicator electrode with a second antimony electrode immersed in the titrant stream, electrical connection being maintained by allowing the tip of the burette jet to dip below the surface of the solution being titrated. A hydrogen indicating electrode was also found to be satisfactory with the antimony reference electrode, while a calomel reference electrode was considered to be less convenient. The glass electrode does not respond to changes in acidity in ethylenediamine in the presence of sodium ions and in fact, Katz and Glenn[2] have used it as a reference electrode under these conditions, with antimony or platinum as indicating electrode. Sprengling[3] found difficulty in obtaining reproducible curves with the antimony indicating electrode using ethylenediamine and benzene with *iso*propyl alcohol as solvents, while Gran and Althin[4] reported that they had been unable to obtained good indication of end points with either the antimony or the platinum electrodes systems used by Moss, Elliott and Hall.

We have found the antimony:antimony system unreliable and the observed variability does not appear to be dependent on the solvent or titrant chosen, replicates of the same titration often giving different titration curves.

In a suitable solvent system, the glass electrode as indicating electrode is the most stable and reproducible of those examined and, in our hands, the saturated calomel electrode is the most convenient and reliable reference electrode. Gran and Althin[4] devised a non-aqueous "calomel electrode" for use as a reference in titrations in ethylenediamine with bright platinum as indicating electrode. This employed ethylenediamine saturated with mercurous chloride and lithium chloride in place of the usual aqueous potassium chloride. We have used an electrode prepared on similar lines, but found in a variety of titrations that the curves showed shallower steps than with a conventional sleeve-type calomel electrode. This latter type of reference electrode has been used throughout the work described in this paper and for routine application it is useful to separate the ground sleeve from the main bulk of potassium chloride in the body of the electrode vessel with a plug of Gooch asbestos drawn into the capillary by suction.

Solvent

Ethylenediamine is the solvent of choice for the titration of very weak acids but for routine use, its highly unpleasant nature makes it desirable to find a satisfactory alternative. *n*-Butylamine and dimethylformamide are less basic than ethylenediamine, but dimethylformamide in particular seems suitable for the titration of all but the most weakly acidic phenols. In addition, it appears to be a better solvent than either ethylenediamine or *n*-butylamine for phenolates; many of the phenols examined give a precipitate during titration in ethylenediamine, even with an ammonium type base as titrant. Purification of ethylenediamine by distillation before use is essential, and should be repeated weekly. Dimethylformamide is more stable and can be treated as later described.

Titrant

Alkali methoxides have been widely used as basic titrants in non-aqueous solvents, the sodium, potassium or lithium methoxides dissolved in the minimum of methanol and diluted with a neutral solvent such as benzene, being usually chosen. Cundiff and Markunas[5] have discussed the disadvantages of these titrants, one of which is the formation of gelatinous precipitates during a titration which obscure the end point. Sodium aminoethoxide was recommended by Moss Elliott and Hall[1] but this is troublesome to prepare and uses ethylenediamine as solvent. Deal and Wyld[6] recommended the use of tetrabutylammonium hydroxide to overcome the problem of precipitation, but this use was not strictly non-aqueous, since an *iso*propyl alcohol and water mixture was used as solvent. Harlow, Noble and Wyld[7] extended the work of Deal and Wyld, but still retained *iso*propyl alcohol as solvent. Cundiff and Markunas[5]

investigated the potentialities of this titrant quite extensively, using benzene as solvent with the addition of sufficient methanol to keep the quaternary base in solution. Recently van der Heijde and Dahmen[8] used tetrabutylammonium hydroxide in pyridine, and found it necessary to store it at $-20°$ to avoid decomposition of the solution due to the Hoffmann degradation. We find that dimethylformamide is a good solvent for the base, but, after an induction period of about 2 hours decomposition takes place at room temperature. Any other conditions of storage are out of the question for a titrant for general use and, therefore, we have employed the solution of tetrabutylammonium hydroxide in benzene and methanol described by Cundiff and Markunas[5]; this is stable if stored so as to minimise the absorption of carbon dioxide.

Apparatus

The titration vessel comprises a 50 or 100 ml. tallform beaker closed with a cork shive; rubber is attacked by dimethylformamide. Three holes in the shive take respectively the two electrodes and the tip of the burette. Magnetic stirring is employed. When working on N/25 scale or weaker it is advisable to flush the vessel and contents with nitrogen; no elaborate precautions to exclude moisture or carbon dioxide during the course of the titration are necessary when using solutions stronger than N/50. The burette is conveniently a 10-ml. micro burette graduated to 0·05 ml. In our experiments, the cell e.m.f. was measured with a Pye direct reading pH meter with a glass and calomel electrode system. The calomel half cell used is described above and the conventional glass electrode was immersed in dimethylformamide for 24 hours before use. One electrode should be kept for this type of work exclusively and stored in dimethylformamide.

Reagents

Dimethylformamide. The pure grade of this solvent should be neutralised to the blue colour of azo violet and it is then suitable for immediate use if the titration is carried out on the deci-normal scale. The blank titration on an average sample should not exceed 0·8–1·0 ml. 0·1N base per 100 ml. For use in smaller scale titrations, the dimethylformamide should be shaken occasionally during 24 hours with barium oxide and redistilled; after this treatment the solvent can be stored in a well filled glass-stoppered bottle for some weeks without development of more than a trace of acidity.

Benzene Analar grade used without further treatment.

Methanol B.P.C. is distilled from sodium metal.

Tetrabutylammonium hydroxide is prepared from the iodide by the method of Cundiff and Markunas[5]. A solution so prepared is approximately 0·1N and can be standardised by titrating a known weight of pure dry benzoic acid dissolved in dimethylformamide using a glass: calomel electrode system, the end point being indicated by a large increase in e.m.f. (about 500 mV. on the addition of about 0·1 ml. of tetrabutylammonium hydroxide solution).

Harlow, Noble and Wyld[7] have stated that the quaternary hydroxide may be prepared from the iodide by passing a methanol solution of the latter through a strongly basic exchange resin in the form of its free base. We have experienced difficulty in obtaining reasonable yields by this method; also the resin could not be regenerated.

General Method

Dissolve an accurately weighed quantity of the compound in neutralised dimethylformamide in a volumetric flask and dilute to volume. Transfer a suitable aliquot, equivalent to about 0·5 mg. equivalent of the phenol, to the titration vessel and dilute to about 25 ml. with neutralised dimethylformamide. Titrate potentiometrically with 0·1N tetrabutylammonium hydroxide in benzene and methanol, stirring constantly and plot the

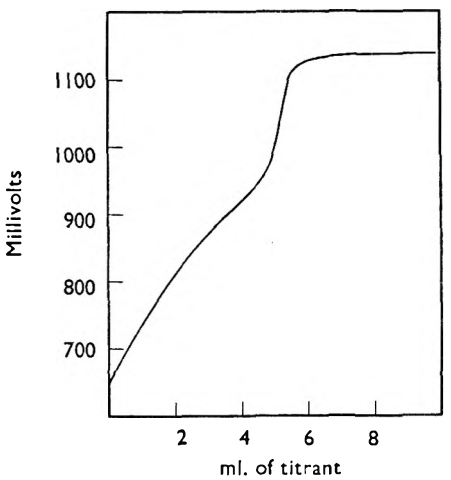

FIG. 1. Titration curve of phenol.

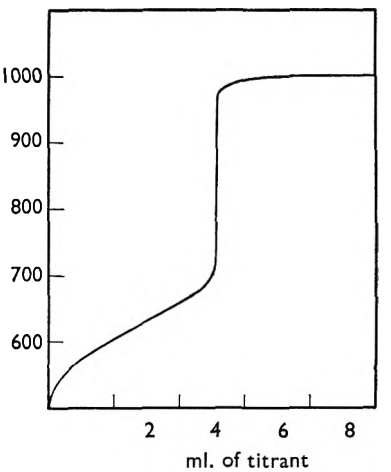

FIG. 2. Titration curve of vanillin.

titration curve. Determine the end point either by geometrical construction or by inspection. For routine purposes, the end point can be selected by calculating $\Delta E/\Delta V.$, or by plotting the differential curve.

Discussion

Figures 1–2 show typical titration curves. The rather steep slope of that part of the curves preceding the end point is characteristic of this solvent-electrode system. The height of the step in the e.m.f. to volume of titrant curve is about 100 to 150 mV. for most of the phenolic compounds examined. Some, like resorcinol give a step of about 75 mV., which is approaching the lower limit of practicability while for others the step height might reach 300 to 400 mV. Picric acid, a relatively strong acid, gives a potential change at end point over practically the whole range of the solvent, producing a step of nearly 1·5 V.

The superiority of tetrabutylammonium hydroxide as a titrant for phenolic compounds in dimethylformamide with the glass and calomel

electrode system is shown by the increased step height obtained compared with the more usual alkali methoxide solution. Thus, a number of compounds have been titrated with tetrabutylammonium hydroxide in benzene and methanol and with lithium methoxide in the same solvent with the results shown in Table I.

TABLE I

TITRATION OF PHENOLIC COMPOUNDS WITH TETRABUTYLAMMONIUM HYDROXIDE IN BENZENE AND METHANOL AND WITH LITHIUM METHOXIDE IN THE SAME SOLVENT

Compound	Step height, mV.	
	Tetrabutylammonium hydroxide	Lithium methoxide
Phenol	125	25
Resorcinol	75	10
n-Propyl *p*-hydroxybenzoate	300	50
Picric acid	1400	1200
Eugenol	100	15

Table II gives the step heights obtained with the phenolic oestrogens. Each of the three synthetic hormones, although dihydroxy compounds, give only one step, corresponding to the titration of a single phenolic group.

TABLE II

STEP HEIGHTS OBTAINED WITH PHENOLIC OESTROGENS

Compound	Step height, mV.
Stilboestrol	140
Hexoestrol	125
Dienoestrol	120
Oestradiol	100
Oestrone	160
Ethinyloestradiol	100

TABLE III

STEP HEIGHTS OF PHENOLIC COMPOUNDS SUCCESSFULLY TITRATED WITH TETRABUTYLAMMONIUM HYDROXIDE

Compound	Step height, mV.
Phenol	125
Cresol, mixed isomers	150
4-Chloro-3-cresol	180
4-Chloro-3 : 5-xylenol	200
Eugenol (4-allyl-2-methoxy-phenol)	110
Thymol	120
Vanillin	300
Resorcinol	75
Hexyl-resorcinol	80
Dithranol	450
Propyl gallate	280
n-Propyl *p*-hydroxy benzoate	300
Picric acid	1400
Dichlorophene	150

With oestradiol and ethinyloestradiol, the curve shapes are anomalous; the rather steep slope before the end point which is characteristic of the solvent and electrode system is repeated after the end point. Thus it is advisable to plot the differential curve when titrating these substances.

Other Phenolic Substances

Table III gives the step heights of a number of phenolic compounds of pharmaceutical interest which have been successfully titrated with tetrabutylammonium hydroxide.

The polyhydric phenols resorcinol, hexyl-resorcinol, dithranol, propyl gallate and dichlorophene, give only one inflection point on the titration curve that is sufficiently well-marked to be used as an indication of the end point; thus, in each case, the equivalent is equal to the molecular weight.

Acknowledgement. The authors wish to express their thanks to Dr. R. E. Stuckey for his interest and encouragement.

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DISCUSSION

The paper was presented by MR. J. ALLEN.

The CHAIRMAN wondered to what extent the position of the step was a diagnostic character of a particular molecule; had one to depend upon the step height itself or were these only characteristic of a group of related substances?

MR. C. A. JOHNSON (Nottingham). Fritz and Yanamura had used triethylbutylammonium hydroxide which they regarded as more basic than tetrabutylammonium hydroxide. By their method, using acetone as a solvent he had not been able to observe any definite step comparable with the one now recorded. Would the authors comment on the basicity of the two materials and had they any experience with sulphonamides which had been titrated successfully by Fritz and Yanamura. Why did the authors use the calomel electrode in the way described?

MR. H. D. RAPSON (Dorking). Had the authors tried conductometric methods as these had certain advantages over potentiometric methods?

MR. S. G. E. STEVENS (London). Had the bubbling of nitrogen as a means of flushing and stirring been considered, and what was the source of the acidity which developed in the dimethylformamide previously treated with barium oxide, and presumably stored under nitrogen?

MR. H. B. HEATH (Sudbury). Had the authors any experience of the determination of eugenol in clove oil or phenolic bodies in ointment bases.

In reply, MR. J. ALLEN said that the basicity of quaternary ammonium hydroxides increased as the weight of the substituting radicals increased to a maximum at tetrabutyl, which was one of the reasons for choosing

this substance. A step height was obtained with phenol which it was not possible to obtain with other solvents. Acetone in certain electrode systems would depress the step and height. The solvent must not only possess intrinsic basic strength, but must also have a long basic range so that potential change at any particular addition was a steep one. Ethylenediamine was basic but it had a short range, pyridine which was less basic had a slightly longer range, and dimethylformamide was less basic but had a very long range. Since submitting the paper it had been possible to obtain with slight modification, two steps with dihydric phenols but there were no means of differentiating between two similar phenols having only one reactive group. They had had no great success in determining phenolic materials extracted from galenical preparations when there were also phenolic impurities in the extract. With dimethylformamide the junction potential between the electrode and the solvent was high, and even with a ground sleeve it was found that there was leakage and therefore Gooch asbestos had been used as a filler as it eliminated all leakage. High frequency conductivity methods had been tried but using the solvent system decided upon, the change of slope in the conductivity curve at the endpoint was small, and did not appear to give a sufficiently definite indication of the position of the endpoint for general routine purposes. The diagnostic properties of conductivity curves depend on the solvent. When water was used the curves could be useful, but in the case of non-aqueous solvents there was suppression of diagnostic characters of the conductivity curve. A nitrogen bubbler as stirrer had been used, but flushing with nitrogen was carried out only if using a weaker solution than N/25. The solution of tetrabutylammonium hydroxide was stored under nitrogen, but not the dimethylformamide. With reasonably pure dimethylformamide if using N/10 titrant it was only necessary to neutralise the acidity, but if N/50 were used it was necessary to resort to purification. It then probably remained stable under nitrogen, but if kept in a bottle a slight amount of acidity developed. However, it could then be used even with N/50 reagent if the acidity were first neutralised.

MR. STEVENS said it was surprising to hear that dimethylformamide did not seem to affect glass electrodes.

MR. ALLEN replied that glass electrodes could not be used with ethylenediamine because in that solvent the sodium ion gave a potential to the glass electrode, but in dimethylformamide that did not happen and an electrode stored in it for twelve months was still satisfactory. It was necessary first to separate the phenolic material to be determined from all other phenols; oils and fats would also interfere.

A COMPARATIVE EXAMINATION OF SOME SAMPLES OF DIGITOXIN

By E. H. B. Sellwood*

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Received June 17, 1957

Twelve samples of digitoxin have been examined. Little difference was found between them by physical constants or by colorimetric assays using sodium picrate or xanthydrol. Three samples were more potent biologically than the others but the difference is not a simple function of the gitoxin content which ranged from a few per cent to more than 20 per cent.

The British Pharmaceutical Codex, which describes Digitoxin as "the crystalline glycoside digitoxin associated with traces of more soluble glycosides and usually a small proportion of a sparingly soluble glycoside gitoxin", recognises the difficulty in preparing the pure glycoside. On the other hand the French Codex 1949, United States Pharmacopeia XV and International Pharmacopoeia, all describe single substances.

Further differences are shown in the standardisation for, while digitoxin B.P.C. is required to have a potency, biologically, of at least 900 units per gram and digitoxin U.S.P. to contain at least 90 per cent of $C_{41}H_{64}O_{13}$ determined by the Baljet reaction applied to a chromatographic eluate, digitoxin of the French Codex and of the International Pharmacopoeia is standardised by physical constants alone. These and other physico-chemical constants recorded in the literature show wide variations and Demoen and Janssen[1] suggested that the physico-chemical properties of pure digitoxin are still largely unknown; however with the rapid growth of chromatographic methods and improved chemical assays for digitoxin and gitoxin their comments do not describe the present position. Nevertheless a number of problems remain to be solved including the feasibility of eliminating the biological assay, and estimation of the gitoxin impurity.

In this communication the results of some physical measurements, and chemical and biological assays, of a number of samples of digitoxin are reported and their significance discussed.

Experimental Methods

Colorimetric assays with sodium picrate[2]. About 5 mg. of "digitoxin", accurately weighed, was dissolved in 100 ml. *iso*propanol and allowed to stand overnight to ensure complete solution. 5 ml. portions were then mixed with 5 ml. of sodium picrate reagent (1 per cent trinitrophenol 95 ml., 5 per cent sodium hydroxide, 5 ml., freshly mixed) and the maximum optical density measured using the EEL photoelectric colorimeter (Ilford filter 623, maximum transmission at 495 mμ), the instrument being balanced to zero with a blank consisting of 5 ml. *iso*propanol and 5 ml. reagent. Results were expressed as the extinction coefficient, E (1 per

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cent, 1 cm.), or as percentage purity, taking the Canadian standard as 100 per cent.

Colorimetric assays with xanthydrol reagent. About 10 mg. of digitoxin, accurately weighed, was dissolved in 100 ml. of glacial acetic acid (A.R.) and 10 ml. of the solution diluted to 100 ml. with more acetic acid. 5 ml. portions of the diluted solution were placed in colorimeter tubes and 5 ml. of 0·25 per cent solution of xanthydrol in glacial acetic acid and 0·1 ml. of hydrochloric acid were added to each. A reagent blank containing no digitoxin was prepared in the same way. The contents of the tubes were stirred, the tubes corked and allowed to stand protected from light. Readings of optical density were taken at intervals from 2 hours onwards using Ilford filter 624 (maximum transmission at about 520 mμ) until the maximum density was reached. The results were calculated from the extinction coefficient E (1 per cent, 1 cm.) = 1520 obtained using digitoxose[2], on the basis of 3 molecules of digitoxose ($C_6H_{12}O_4$) ≡ 1 molecule of digitoxin ($C_{41}H_{64}O_{13}$).

Biological assays were carried out against standard digitalis, using guinea pigs, by the method of B.P. 1953 (page 830).

Melting points were measured by the B.P. capillary tube method.

Specific rotations were determined at 20° using the sodium D line on 1 per cent solutions in chloroform in a 2 decimetre tube.

Ultra-violet absorption. Determination were made using the Beckman Spectrophotometer Model DU fitted with photo-multiplier attachment. Absolute ethanol (A.R.) was refluxed with zinc dust and potassium hydroxide and redistilled rejecting the first and last 10 per cent. Sixteen to 18 mg. of the sample, accurately weighed, was dissolved in 50 ml. of the purified ethanol, 5 ml. of the solution was diluted to 50 ml. with more ethanol and the optical density measured at 217 mμ.

Gitoxin content. Paper chromatograms were run using the system carbon tetrachloride : ethanol : water and the glycosides eluted in xanthydrol reagent by the method described previously[2].

Results

Five commercial and six laboratory-prepared samples of digitoxin were examined. U.S.P. reference standard digitoxin was not available but a sample of the Canadian standard was tested. The results are summarised in Table I.

Discussion

The results of the colorimetric assays and determinations of gitoxin content and ultra-violet absorption are sufficiently close to justify considering all the samples as a single group. Only samples 6, 7 and 12 meet the requirements of the British Pharmaceutical Codex, although these differ but little from the others in their chemical and physical properties. Thus only half would pass the official test for melting point (at least 240°) although it is well known that lower values are frequently obtained. Similarly because of the small angular rotation of a 1 per cent solution of

TABLE I

ASSAYS AND PHYSICAL CONSTANTS FOR 12 SAMPLES OF DIGITOXIN

		Melting point (B.P.C. 240°)	$[\alpha]_D$ (B.P.C. about + 18°)	Xanthydrol method digitoxin (Calc. from assay of digitoxose) per cent	Picrate method calculated as		Biological assay		Gitoxin content per cent	Ultra-violet absorption Log ϵ
					E (1 per cent, 1 cm.)	Purity per cent (Sample 6 = 100)	Units/g.	Fiducial limits		
1	Commercial 1 ..	236°	+20°	99†	172	89‡	774	563–1064	14	4·13
2	" 2 ..	232°	+21°	97	172	89	655	440–976	23	4·15
3	" 3 ..	228°	+17°	97	185	96	788	—	7	4·19
4	" 4 ..	249°	—	100	177	92	760	—	12	—
5	" 5 ..	225°	+21·4°	98	190	98	1050	—	10	4·18
6	Canadian Standard	245°	—	98	193	100	1368	876–2136	11	4·17
7	Laboratory 1 ..	254°	+18°	95	193	100	1368*	876–2136	8	4·18
8	" 2 ..	245°	+20°	100	170	88	723	602–869	17	4·18
9	" 3 ..	255°	+15°	103	181	94	881	743–1045	20	4·18
10	" 4 ..	229°	+19·7°	92	192	99	894	641–1246	17	4·16
11	" 5 ..	230°	+18·9°	94	189	98	761	602–963	13	4·15
12	" 6 ..	251°	+17°	95	174	90	1401	865–2271	9	4·19

* Original assay 10 years ago, 1298 units/g.

† Standard deviation of 5 determinations ± 2·23.

‡ Standard deviation of 5 determinations ± 2·45.

digitoxin (approximately 0·4°), the specific rotation serves only as a confirmatory test of identity and is used in the B.P.C. as such.

Petit and colleagues[3] suggested that, although the low solubility of gitoxin does not permit the actual determination, its specific rotation in chloroform would be higher than that of digitoxin. However in the present work there is no indication that the values obtained are even a guide to the gitoxin content. Determinations of specific rotation may nevertheless be of value in detecting isomerisation as a sample of digitoxin which had become partially isomerised through accidental contact with alkali had a specific rotation of − 49·5° instead of the normal + 18°.

Colorimetric assays gave results of the expected order although the xanthydrol method showed three samples to be slightly low in digitoxose content. Values of *E* (1 per cent, 1 cm.) in the picrate method are 10 to 20 per cent lower than some recorded in the literature but this is attributed to the use of a filter type instrument which gives lower results than the prism type[4] and to the use of *iso*propanol in which optical densities are a few per cent lower than those obtained when using ethanol. The lowest extinction coefficients were about 10 per cent less than the highest but all the samples are probably normal in respect of the unsaturated lactone ring. This is confirmed by the ultra-violet absorption which in all samples showed a maximum at 217 mμ with log ε of the expected order. The partially isomerised digitoxin gave in the picrate method *E* (1 per cent, 1 cm.) = 61 and ultra-violet absorption, log ε = 3·72, thus showing a serious loss of the lactone function.

Whereas the colorimetric assay results varied from the mean by up to 6·5 per cent, the lowest biological assay was 31 per cent below the mean, and the highest 45 per cent above. Furthermore, there is no indication that the results of the biological assays reflect the equivalent of digitoxin as measured either by the picrate method or by the sugar content determined by the xanthydrol method, but neither the chemical nor the biological assays measure digitoxin alone.

Three samples by biological assay possess a distinctly higher potency than the others and, although these have a lower gitoxin content, the reduction in gitoxin does not account fully for the increase in potency. Apart from the lower gitoxin content these samples do not differ markedly physically or chemically from the others.

Assays of sample 7, initially and after storage for ten years, show that digitoxin in the solid state can be expected to maintain its potency indefinitely.

Chromatographic separation followed by colorimetric assay indicated that most of the samples contained a considerable proportion of gitoxin but, while with some a high gitoxin content was associated with a poor biological assay, this was not so with all. Samples 7, 8, 9, 10, and 12 were examined chromatographically by Tschesche who in a personal communication reported all to contain gitoxin while samples 8, 9, and 10 contained gitaloxin also.

From our chromatograms also gitoxin was found to be the main impurity. On some of our paper strips, however, between the spots of

digitoxin and gitoxin, there was a trace of a substance giving a blue fluorescence when dipped into phosphoric acid and its R_F value suggested it to be gitoxigenin. Tschesche's results indicate that this was not gitoxigenin itself, but the gitoxigenin glycoside gitaloxin (16-formyl gitoxin). This would, in contrast to gitoxigenin, react with xanthydrol and so might cause errors in quantitative determinations of the digitoxin and gitoxin zones but as it appears to be present only in traces such errors should be small.

While the potency of digitoxin containing an unknown proportion of gitoxin may readily be determined by biological methods, it is very desirable from the analytical viewpoint that a pure substance should be employed. The problem is essentially one of reducing the gitoxin content to an acceptable level. As gitoxin produces in the colorimetric assays optical densities comparable to those given by digitoxin and has similar ultra-violet absorption and optical rotation, a specific quantitative test for gitoxin is required. Fluorimetric[5–7] and spectroscopic methods[4] have been proposed which will detect the presence of less than 1 per cent of gitoxin in digitoxin. It is desirable, therefore, that a more sensitive test than the Keller-Kiliani should be introduced in the Official Standards in order to limit the gitoxin content, as digitoxin of commerce must contain at least 10 per cent before any appreciable red tinge is produced in this test.

The physico-chemical constants for the samples examined are, in general, consistent and the colorimetric assays of most are concordant both for a method based on the sugar side chain and a second on the lactone ring. Provided therefore that a suitable limit test for gitoxin could be applied, or the digitoxin freed from gitoxin chromatographically before a colorimetric assay is performed (as in U.S.P. XV), there seems to be no reason why digitoxin should not be standardised by physico-chemical methods alone, so rendering unnecessary the costly and time consuming biological methods.

Acknowledgements. The author thanks Dr. H. O. J. Collier for the biological assays and for his comments on the results and also Professor R. Tschesche of Hamburg for chromatographic examinations and Mr. R. V. Swann, Chemist in charge of the Physical Laboratory, for ultra-violet measurements.

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DISCUSSION

The paper was presented by the AUTHOR.

In a written communication DR. J. M. ROWSON said the results in the paper did not suggest that the application of a limit test for gitoxin would permit the evaluation of digitoxin by chemical and physical methods alone. The penultimate paragraph should be modified to read "freed from gitoxin and other glycosides or aglycones chromatographically". Why were samples 6, 7 and 12 of higher potency if gitoxin content is not related to potency, were these three samples free from gitaloxin, and what was believed to be the biological potency of pure digitoxin? For several digitoxin samples he had found "spots" on paper chromatograms in the region of gitaloxin and hoped to publish these results soon since it seems possible that this impurity (of high potency) may account for the occasional digitoxin of high potency.

DR. G. E. FOSTER (Dartford). Had the author any figures for digitoxin when the Keller-Kiliani and 3:5-dinitrobenzoic acid methods were used. In the B.P.C. a fundamental rule of biological assay was being broken in that digitoxin was compared with digitalis which was not a comparison of like with like and that might be the cause of some of the errors in the assay. If digitoxin were made too pure it was less soluble and as soon as injections were made up, it crystallised out.

MR. J. C. HANBURY (Ware). The activity of digitoxin appeared to depend on the botanical origin, and the very pure samples were usually either of French or Danubian origin, but no one knew what the glycosides were which contributed to the very high biological activities. Pharmacologists stated that they had examined samples which physically and chemically were nearly pure digitoxin, with biological activity around 1800 or 2000 units.

MR. W. SMITH (Ware). What was the potency the Canadians claimed for their standard, and how was this standardised?

MR. S. G. E. STEVENS (London) suggested that infra-red spectrophotometry might help in this investigation.

In reply MR. SELLWOOD said that gitoxin was the main impurity in digitoxin, others were present only in small proportions. He was unable to explain why the three samples 6, 7 and 12 were so different on the biological assay as it was not related to the gitoxin content. If Dr. Rowson had found a trace of another active substance it might help to solve the problem. He had used the 3:5-dinitrobenzoic acid and Keller-Kiliani methods, but had relied on the picrate method as being the most sensitive method based on aglycones. Xanthydrol was very satisfactory and sensitive and there was adequate coverage of aglycone and sugar content by the two colorimetric methods. English leaf was capable of yielding a very good sample of digitoxin and he had found a high proportion in the leaf grown by Dr. Rowson. There seemed to be traces of impurities which affected biological potency to a considerable extent, and they must be

extremely potent substances. It was difficult to purify digitoxin beyond a certain limit without great loss of material, and that was why it was necessary to accept a product which would contain only 90 per cent digitoxin as suggested by U.S.P. He had no further information about the biological potency of the Canadian standard. It was taken as a standard, but not as 100 per cent digitoxin, in Table I, because in common with sample 7 it gave the highest picrate assay, and also had the greatest biological potency. The only difference between digitoxin and gitoxin was a single hydroxyl grouping, which altered chemical properties and biological activity, though there was confusion about the biological potency of gitoxin.

SOME *NSN*-TRIS-QUATERNARY NEUROMUSCULAR BLOCKING AGENTS*

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The tris-quaternary compounds 7-ethyl-7-thioniatridecylenebis(triethylammonium) triiodide (Dihexasulphonium triethiodide; DHSE), 6-ethyl-6-thioniaundecylenebis(triethylammonium) triiodide (Dipentasulphonium triethiodide; DPSE), and 7-methyl-7-thioniatridecylenebis(trimethylammonium) triiodide (Dihexasulphonium trimethiodide; DHSM) have been synthesised. DPSE and DHSE have been shown to be neuromuscular blocking agents, which act by a mechanism resembling that of tubocurarine and gallamine rather than decamethonium. DPSE and DHSE are about equipotent with tubocurarine on the cat gastrocnemius muscle, but less potent on the frog rectus abdominis muscle and rat diaphragm, and the head drop doses are higher. Neuromuscular block can be reversed by neostigmine, eserine, and edrophonium. DHSE and DPSE do not cause ganglion blockade or histamine release, do not increase heart rate and are less potent than tubocurarine in causing respiratory paralysis.

The properties and modes of action of neuromuscular blocking agents which act by competition, by depolarisation or by an intermediate type of action have been carefully investigated[1-9]. These studies have led to the synthesis of suxamethonium, benzoquinonium and gallamine. Gallamine, a tris-ethonium compound, possesses properties similar to tubocurarine, and lacks some of the undesirable properties of the latter but it is less potent. On the other hand it sometimes causes tachycardia[10,11]. Thioalkane-$\alpha\omega$-bis-quaternary salts have been shown to possess neuromuscular and ganglion blocking activity[12,13]. 3-Thiapentane-1:5-bis(trimethylammonium iodide) (I, $n = m = 2$, R = Me) was described by Marxer and Miescher[14], whilst Andrews, Bergel and Morrison[12] have prepared a series of thioalkane-bis(quaternary ammonium) salts (I, $n = m = 2$, R = Me; $n = 2$, $m = 3$, R = Me; $n = m = 3$, R = Et; $n = m = 4$, R = Me; $n = 4$, $m = 6$, R = Me) and dithioalkane-bis(quaternary ammonium) salts (II, $n = m = 2$, R = Me; $n = m = 3$, R = Et; $n = m = 4$, R = Me). As with the corresponding polymethylene compounds, those compounds with more than seven atoms between the quaternary nitrogen atoms exhibit predominantly neuromuscular blocking action, whilst the lower homologues exhibit a depressor action on cat blood pressure and block transmission at the cat superior cervical sympathetic ganglion.

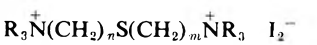
Chemical

The availability of a series of long chain bisdiethylaminoalkyl sulphones[15] (III) prompted us to investigate the corresponding dioxothiaalkane-$\alpha\omega$-bistriethylammonium salts for neuromuscular blocking activity.

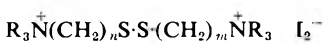
* Patent rights pending. † Pakistan Government Scholar.

For this purpose, 4-dioxothiaheptane-1 : 7-bis(triethylammonium iodide) (IV, $n = 3$) and 4-dioxothiahepta-2 : 5-diene-1 : 7-bis(triethylammonium iodide) (V) were readily obtained by direct quaternisation of the corresponding bisdialkylaminoalkyl sulphones[15] with ethyl iodide. Bisdiethylaminohexyl sulphone (III, $n = 6$) and bisdiethylaminodecyl sulphone (III, $n = 10$) however, yielded oily products with ethyl iodide, which crystallised only on long standing *in vacuo*. Fakstopp[16] has recently reported side reactions in the preparation of quaternary salts from amino sulphones.

A chance observation showed that bis-6-diethylaminohexyl sulphide[15] yielded 7-ethyl-7-thioniatridecylenebis(triethylammonium) triiodide) (VI, $n = 6$, R = Et; dihexasulphonium triethiodide; DHSE) when treated with excess ethyl iodide under reflux. The use of limited amounts of ethyl iodide (2 mol.) gave the same product, though in reduced yield and failed to yield the expected 7-thiatridecan-1 : 13-bis(triethylammonium iodide) (I, $n = m = 6$, R = Et).



(I)



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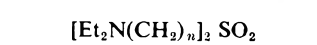

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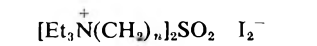

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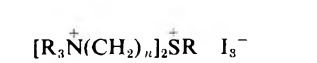

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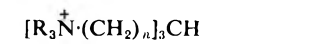

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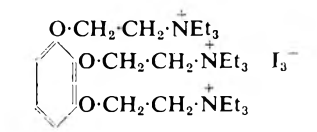

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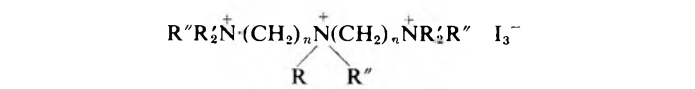

(IX)

Compounds of this type have not previously been examined for neuromuscular blocking activity, though a number of tris-quaternary nitrogen compounds have been described. Kensler, Zirkle, Matallana and Conduris[17] prepared a series of compounds, VII, where $n = 2$, 3, or 4 and R = Me or Et, of which the most active, VII ($n = 4$, R = Et), showed about 40 per cent of the activity of gallamine (VIII) in the rabbit head drop assay. DHSE and DPSE resemble gallamine in possessing three quaternary centres although one is now sulphur. The steric pattern of the three quaternary centres, however, is non-linear in the compounds VII and in gallamine, whilst DHSE and DPSE resemble decamethonium in that the molecules are linear, and of approximately equivalent chain length. On

the other hand DHSE and DPSE resemble gallamine in that all three are ethonium compounds, whilst decamethonium is a methonium compound. The short-chain linear tris-quaternary nitrogen compounds, IX, $n = 2$ or 3, described by Marxer and Miesher[14] and by Delaby, Damiens and Marquist[18], as expected, exhibit ganglion-blocking activity. Longer-chain linear tris-quaternary nitrogen compounds have not been examined for neuromuscular blocking activity. We therefore attempted the preparation of further compounds of the *NSN*-trisquaternary type VI. Bis-dimethylaminohexyl sulphide, prepared by condensation of 6-chloro-hexyldimethylamine[12] with sodium sulphide, unlike bisdiethylaminohexyl sulphide, gave two products with methyl iodide, depending upon the reaction conditions. With a limited amount of methyl iodide in the cold 7-thiadecan-1 : 13-bis(triethylammonium iodide) (I, $n = m = 6$, R = Me), was obtained, whilst excess reagent under reflux gave 7-methyl-7-thioniatridecylenebis(trimethylammonium) triiodide, (VI, $n = 6$, R = Me; dihexasulphonium trimethiodide, DHSM).

Bisdiethylaminopentyl sulphide was prepared by the following reaction sequence:

$$Et_2N{\cdot}(CH_2)_3Cl \xrightarrow[NaOEt]{CH_2(COOEt)_2} Et_2N(CH_2)_3CH(COOEt)_2 \xrightarrow[2{\cdot}EtOH-H_2SO_4]{1{\cdot}H^+}$$

$$Et_2N(CH_2)_4COOEt \xrightarrow{LiAlH_4} Et_2N{\cdot}(CH_2)_5OH \xrightarrow{SOCl_2} Et_2N{\cdot}(CH_2)_5Cl \xrightarrow{Na_2S}$$

$$[Et_2N{\cdot}(CH_2)_5]_2\ S$$

It underwent a stepwise reaction with ethyl iodide, so that even when the latter is present in excess, 6-thiaundecan-1 : 11-bis(triethylammonium iodide) (I, $n = m = 5$, R = Et), was always deposited as the primary product of the reaction. More prolonged treatment with ethyl iodide gave 6-ethyl-6-thioniaundecylene bis(triethylammonium) triiodide (VI, $n = 5$, R = Et; dipentasulphonium triethiodide; DPSE).

5-Thianonane-1 : 9-bis(triethylammonium iodide) (I, $n = m = 4$, R = Et) obtained from 1-chloro-4-bromobutane by the action of first triethylamine and then sodium sulphide, failed to yield the corresponding tris-quaternary compound with excess ethyl iodide even under forcing conditions.

Experimental

Melting points are uncorrected. We are indebted to Dr. A. C. Syme, Mr. W. McCorkindale and Mr. W. Gardiner for the microanalyses.

4-Dioxothiaheptane-1 : 7-bis(triethylammonium iodide). Bis-3-diethylaminopropyl sulphone[15] (3·87 g.) was refluxed with ethyl iodide (5 ml.) for 30 min. Excess reagent was removed by distillation under reduced pressure and the residue crystallised from ethanol (90 per cent) to yield pale yellow crystals (4·15 g. 51·9 per cent) of *4-dioxothiaheptane-1 : 7-bis(triethylammonium iodide)*, m.p. 220–222°. Found: C, 35·7; H, 7·0; N, 4·7; I, 42·7 per cent; $C_{18}H_{42}O_2N_2SI_2$ requires: C, 35·8; H, 7·0; N, 4·6; I, 42·0 per cent.

4-*Dioxothiahepta*-2:5-*diene*-1:7-*bis*(*triethylammonium iodide*). Bis-3-diethylaminoprop-1-enyl sulphone dihydrochloride[15] (0·94 g.) in water (2 ml.) was basified by the addition of sodium hydroxide (N; 5·5 ml.) and extracted with benzene. The benzene extract after drying with Na_2SO_4 was evaporated under reduced pressure and the residual oily base refluxed with ethyl iodide (2 ml.) for 30 minutes. The residue (0·58 g. 37·3 per cent) remaining after removal of excess reagent under reduced pressure was crystallised from ethanol (charcoal) to yield pale yellow needles of 4-*dioxothiahepta*-2:5-*diene*-1:7-*bis*(*triethylammonium iodide*), m.p. 179° (decomp.). Found: C, 35·8; H, 6·6; N, 4·5; I, 41·8 per cent; $C_{18}H_{36}O_2N_2SI_2$ requires: C, 36·0; H, 6·4; N, 4·7; I, 42·3 per cent.

7-*Ethyl*-7-*thioniatridecylene bis*(*triethylammonium*) *triiodide*. Bis-6-diethylaminohexyl sulphide[15] (1·64 g.) was refluxed with ethyl iodide (3 ml.) for 25 minutes. Removal of excess reagent under reduced pressure and recrystallisation of the product from ethanol gave almost colourless crystals (2·95 g. 76·3 per cent) of 7-*ethyl*-7-*thioniatridecylene bis*(*triethylammonium*) *triiodide*, m.p. 142–143°. Found: N, 3·7; I, 46·6 per cent; $C_{26}H_{59}N_2SI_3$ requires N, 3·5; I, 46·9 per cent.

1:1-*Bisethoxycarbonyl*-4-*diethylaminobutane*. Diethyl malonate (72 g.) was added slowly (30 min.) to a solution of sodium (10·5 g.) in ethanol (320 ml.) at 50°. 3-Chloropropyldiethylamine (67 g.) was added slowly (30 min.) and the mixture then refluxed for a further 2 hours. The bulk of the ethanol was removed by distillation, water (100 ml.) and dilute hydrochloride acid (10 per cent; 200 ml.) added. The solution was saturated with sodium chloride and extracted with ether to remove unchanged diethyl malonate. The solution was made alkaline with sodium hydroxide (20 per cent; 120 ml.) and again extracted with ether. The ether extract was dried with Na_2SO_4, evaporated, and the residual liquid distilled to yield 1:1-bisethoxycarbonyl-4-diethylaminopropane as a colourless oil (55·4 g.; 45 per cent), b.p. 173–176° at 22 mm., $n_D^{23·5}$ 1·4387. Marvel, Zartman and Bluthardt[19] give b.p. 163–170° at 23 mm., n_D^{25} 1·4380.

Ethyl 5-*diethylaminovalerate*. 1:1-Bisethoxycarbonyl-4-diethylamino propane (55·4 g.) was refluxed with concentrated hydrochloric acid (260 ml.) for 2 hours, and then evaporated to dryness under reduced pressure. The yellow crystalline residue was refluxed with ethanol (250 ml.) and sulphuric acid (25 ml.) for 4 hours. The bulk of the ethanol was removed by distillation, the remaining liquid basified with sodium hydroxide solution, and extracted with ether. The ethereal solution was dried with Na_2SO_4, evaporated, and the residue distilled to yield ethyl 5-diethylaminovalerate (20 g.; 49 per cent), b.p. 136–155° at 22 mm., $n_D^{19·5}$ 1·4352. Magidson and Strukov[20] give b.p. 130–131° at 25 mm., n_D^{20} 1·4354. Found: Equiv. titration 202·5. Calc. for $C_{11}H_{23}O_2N$. Equiv. 201.

5-*Hydroxypentyldiethylamine*. Ethyl 5-diethylaminovalerate (12·4 g.) in dry ether (10 ml.) was run slowly into a stirred hot suspension of lithium aluminium hydride (1·5 g.) in dry ether (65 ml.) at a rate sufficient to keep the mixture refluxing (30–40 min.). The reaction mixture was cooled in

ice, and brine added dropwise to decompose the excess lithium aluminium hydride, and then the complex. Sodium hydroxide solution (20 per cent; 50 ml.) was added and the ethereal layer decanted. The residual gel was extracted with ether (2 × 200 ml.), the mixed ethereal solutions dried with Na_2SO_4, the solvent removed, and the residual oil distilled to give 5-hydroxypentyldiethylamine (7·1 g.; 73 per cent), b.p.131–135° at 22 mm., n_D^{23} 1·4512. Synerholm[21] gives b.p. 125–130° at 20 mm., n_D^{20} 1·4544.

Bis-5-diethylaminopentyl sulphide. Excess thionyl chloride (9 ml.) in benzene (20 ml.) was slowly added to a stirred solution of 5-hydroxypentyldiethylamine (14·3 g.) in benzene (100 ml.). The yellow crystalline mass obtained on removal of the solvent and excess reagent was dissolved in water (20 ml.), the solution cooled to 0° and basified with sodium hydroxide solution (30 ml.; 20 per cent). Extraction with ether, drying with Na_2SO_4 and evaporation of the solvent gave crude 5-chloropentyldiethylamine (15·9 g.). The latter in ethanol (8 ml.) was slowly added to a hot solution of anhydrous sodium sulphide (4·4 g.) in water (5 ml.) and ethanol (16 ml.), and the mixture refluxed for 3 hours with continuous stirring. The residual liquid, after removal of the solvent at 100° was poured into brine (50 ml.) and extracted with ether. The ethereal extracts were dried with Na_2SO_4, evaporated, and the residue distilled to yield *bis-5-diethylaminopentyl sulphide*, as a pale yellow oil (3·55 g., 25 per cent), b.p. 200–205° at 1·2 mm., $n_D^{17·5}$ 1·4707. Found: Equiv. titration 157·3. $C_{18}H_{40}N_2S$ requires equivalent 158·3. *Bis-5-diethylaminopentyl sulphide dihydrochloride* from ethanol-ether, m.p. 155–156°. Found: C, 55·8; H, 10·9 per cent; $C_{18}H_{42}N_2SCl_2$ requires C, 55·5; H, 10·9 per cent.

6-*Thiaundecane*-1 : 11-*bis*(*triethylammonium iodide*). Bis-5-diethylaminopentyl sulphide (0·85 g.) was refluxed with excess ethyl iodide for 15–20 minutes. The crystalline deposit gave from ethanol 6-*thiaundecane*-1 : 11-*bis*(*triethylammonium iodide*), m.p. 199–200°. Found: I, 40·8; N, 4·6 per cent; $C_{22}H_{50}N_2SI_2$ requires I, 40·4; N, 4·5 per cent.

6-*Ethyl*-6-*thioniaundecylene bis*(*triethylammonium*) *triiodide*. Bis-5-diethylaminopentyl sulphide (0·85 g.) was refluxed with excess ethyl iodide (4 ml.) for not less than 30 minutes. Removal of excess reagent under reduced pressure, and recrystallisation of the product from ethanol gave 6-*ethyl*-6-*thioniaundecylene bis*(*triethylammonium*) *triiodide*, m.p. 136·5–137·5°. Found: I, 48·3; N, 3·5 per cent; $C_{24}H_{55}N_2SI_3$ requires I, 48·5; N, 3·6 per cent.

6-*Chlorohexyldimethylamine* prepared by the method of Andrews, Bergel and Morrison[12] was obtained as a colourless oil b.p. 69° at 3·5 mm., $n_D^{17·5}$ 1·4467.

Bis-6-dimethylaminohexyl sulphide was prepared from 6-chlorohexyldimethylamine (19·2 g.) by the method described under bis-5-diethylaminopentyl sulphide. *Bis-6-dimethylaminohexyl sulphide* was obtained as a colourless oil (12·8 g. 76 per cent), b.p. 164–165° at 0·75 mm., $n_D^{18·5}$ 1·4742. Found: Equiv. titration 145·4; N, 9·6 per cent; $C_{16}H_{36}N_2S$ requires equiv. 144·3; N, 9·7 per cent.

7-*Methyl*-7-*thioniatridecylene bis*(*trimethylammonium*) *triiodide*. Bis-6-dimethylaminohexyl sulphide (1·14 g.) was refluxed with methyl iodide

(5 ml.) and ethanol (5 ml.) for 50 minutes. Evaporation under reduced pressure, and crystallisation of the product from ethanol gave yellow crystals (2·2 g. 79 per cent) of *7-methyl-7-thioniatridecylene bis(trimethylammonium) triiodide*, m.p. 170–171°. Found: C, 32·0; H, 6·1; I, 52·6; N, 3·9 per cent. $C_{19}H_{45}N_2SI_3$ requires C, 32·0; H, 6·4; I, 53·3; N, 3·9 per cent.

7-Thiatridecane-1 : 13-bis(trimethylammonium iodide). Bis-6-dimethylaminohexyl sulphide (1·29 g.) was mixed with benzene (6 ml.) and methyl iodide (1 ml.). Separation of a crystalline product commenced within 2–3 minutes, and, after standing overnight, this was separated by filtration and recrystallised from ethanol to yield colourless needles (1·3 g. 51·2 per cent) of *7-thiatridecane-1 : 13-bis(trimethylammonium iodide)*, m.p. 179–180·5°. Found: C, 37·7; H, 7·3; N, 5·0; I, 44·3 per cent; $C_{18}H_{42}N_2SI_2$ requires C, 37·8; H, 7·4; N, 4·9; I, 44·3 per cent.

5-Thianonane-1 : 9-bis(triethylammonium iodide). 5-Thianonane-1 : 9-bis(triethylammonium bromide) (4·3 g.), prepared by the method used by Andrews, Bergel and Morrison[12] for the production of the corresponding methyl analogue, in ethanol (30 ml.) was treated with excess silver carbonate (2·5 g.), and the mixture shaken for 3 hours. After filtration, the filtrate was titrated against a solution of hydriodic acid (11 per cent) until just acid to methyl red. The solution was evaporated to dryness, the colour discharged by addition of minimum amount of sodium thiosulphate solution, and the solution again evaporated. Extraction of the residue with ethanol, and addition of ether gave *5-thianonane-1 : 9-bis-triethylammonium iodide*), m.p. 212–212·5°. Found: I, 41·9; N, 4·7 per cent; $C_{20}H_{46}N_2SI_2$ requires I, 42·3; N, 4·7 per cent.

Attempted preparation of 5-ethyl-5-thionianonylenebis(triethylammonium triiodide. 5-Thianonane-1 : 9-bis(triethylammonium iodide) (0·137 g.) in ethanol (2 ml.) and ethyl iodide (0·5 ml.) was refluxed for 30 minutes. Evaporation under reduced pressure, and crystallisation from ethanol gave unchanged material, m.p. and mixed m.p. 211·5–212°.

Pharmacological

Methods and Materials

Composition of perfusion fluids (g./litre).

Frog Ringer's Solution: NaCl 6·5, $NaHCO_3$ 0·2, KCl 0·138, $CaCl_2$ 0·12, dextrose 1·0. Tyrode's Solution: NaCl 8·0, $NaHCO_3$ 1·0, KCl 0·198, $CaCl_2$ 0·2, $MgCl_2$ 0·1, NaH_2PO_4 0·005, dextrose 1·0. Locke's Solution: NaCl 9·0, $NaHCO_3$ 0·5, KCl 0·42, $CaCl_2$ 0·24, dextrose 1·0.

The following drugs were used: acetylcholine chloride (ACh), (−)-adrenaline hydrochloride (Ad), (−)-noradrenaline hydrochloride (NA), histamine acid phosphate (Hm), tubocurarine chloride (TC), decamethonium iodide (C 10), hexamethonium bromide (C 6), eserine salicylate (eserine), neostigmine methyl sulphate (neostigmine), edrophonium bromide (edrophonium), potassium chloride (KCl), ether, dihexasulphonium triethiodide (DHSE), dipentasulphonium triethiodide (DPSE),

dihexasulphonium trimethiodide (DHSM), 4-dioxothiahepta-2:5-diene-1:7-bis(triethylammonium iodide), 4-dioxothiaheptane-1:7-bis(triethylammonium iodide).

Neuromuscular Blocking Activity

Cat gastrocnemius muscle—sciatic nerve preparation. Cats of either sex weighing between 2·0 to 5·0 kg. were anaesthetised with intraperitoneal pentobarbitone (60 mg./kg.). The gastrocnemius muscle was partially dissected free from the surrounding tissue and the achilles tendon severed at a point near its insertion to the calcaneus. The tendon was attached by means of a strong linen thread to a myograph lever. The sciatic nerve was then partially dissected at a point proximal to the anterior tibial nerve. The nerve was stimulated by means of a Dobbie McInnes square wave generator at a frequency of 4 to 6/minute at 8 to 12 volts, the pulse width being 2 to 3·5 msecs. In any one experiment, frequency, voltage and pulse width were constant except that in some experiments the muscle was also tetanised indirectly with a frequency of 1500/minute and in others the muscle was stimulated directly at 40 volts after it had become unresponsive to indirect stimulation. Drugs were injected into the external jugular vein. In a few experiments contractions of the anterior tibialis muscle or soleus muscle were recorded after sciatic nerve stimulation. The conditions of the experiments were identical with those just described.

Rabbit head drop method. Rabbits of either sex weighing 1·7 to 3·2 kg. were used. Solutions of DPSE, DHSE, and TC, 0·05 mg./ml. in normal saline were administered by infusion into a marginal ear vein at a constant rate of 1·4 ml./minute. Infusion was continued until, following a light tap on the muzzle, the animal was no longer able to raise its head.

Rat diaphragm. The usual method was used[22]. The frequency of indirect stimulation was 6 square pulses per minute at 8 volts and the pulse width was 1 msec. Drugs were left in contact for 3 minutes.

Frog rectus abdominis muscle. Reproducible submaximal contractions of the rectus muscle were induced by ACh (0·1 to 0·2 μg./ml.) or C 10 (2 to 3 μg./ml.) which were left in contact with the tissue for 1·5 minutes. DPSE or DHSE and TC were added 1 minute before the addition of ACh or C 10. In a few experiments rectus muscles from toads were used.

Mouse test. DPSE, DHSE, or TC at different doses were given by intraperitoneal injections to groups of mice weighing 30 to 40 g.

Other Properties

Effects on blood pressure, respiration and nictitating membrane. Cats, chloralosed, 80 to 100 mg./kg., of either sex weighing 1·5 to 3·5 kg. were used. All drugs were administered by injection into the external jugular vein.

Blood pressure was recorded from the common carotid artery, and respiration, by a thread sewn into the skin of the epigastrium, and attached to a recording lever. A solution of 0·2 mg./ml. of DPSE, DHSE or TC was infused into the external jugular vein at a constant rate of 0·8 ml./

minute. Infusion was stopped as soon as respiration failed and artificial respiration begun.

Contractions of the nictitating membrane were elicited by stimulation of the preganglionic fibres of the cervical sympathetic and recorded by conventional methods. Stimulation was by means of square impulses at a frequency of 800 to 1200/minute 8 to 15 volts, pulse width 0·2 to 1·0 msec. In any one experiment frequency, voltage, and pulse width were constant. Two kinds of experiments were made. Intermittent stimulation for 15 to 20 seconds at 3 minute intervals, and continuous tetanisation for an indefinite period. In the former reproducible responses were obtained by stimulating the nerve at 3 minute intervals and drugs were injected 1 minute before stimulation. In the latter, drugs were injected when the recorded response of the nictitating membrane had reached a constant level.

Effects on isolated perfused hearts. Rabbit or kitten hearts were perfused by Langendorff's method[23] with oxygenated Locke's solution at 37°. Outflow was measured by collecting the perfusate into a measuring cylinder at 5 minute intervals. Drugs were administered by injection into the cannula.

Guinea pig ileum. Pieces of the terminal ileum about 4 cm. long were suspended in a 2 ml. bath containing oxygenated Tyrode's solution at 30 ± 0·5°. Reproducible submaximal contractions were obtained to ACh, 0·2 μg./ml., added at 3 minute intervals and left in contact with the tissue for 30 seconds. Drugs were added 1 minute before ACh.

Rabbit duodenum. Pieces of duodenum, about 4 cm. long, were set up in a 40 ml. bath containing oxygenated Locke's solution at 37°. The spontaneous movements of the duodenum were recorded. Drugs were added to the bath at varying intervals of time.

Rat hind quarters. The hind quarters of rats were perfused with oxygenated Locke's solution at room temperature as described by Burn[24]. Outflow was measured by Gaddum's drop recorder and drugs were administered by injection into the cannula.

Results

Neuromuscular Blocking Activity

DPSE and DHSE, 0·1 to 0·4 mg./kg., caused incomplete reversible neuromuscular block. When the dose was increased, 0·5 to 1·0 mg./kg., neuromuscular block was complete but still reversible. Similar results were obtained using TC at similar doses. For all three compounds the duration of block depended upon the dose. After complete block the muscle still responded to direct stimulation (Fig. 1). Potentiation of twitch height was not seen and there were no muscular fasciculations. When muscles partly blocked by DPSE, 0·1 mg./kg., or DHSE, 0·1 mg./kg., were indirectly tetanised, the tension rapidly waned. Similar effects are seen when TC is used but, as is well known, the normal muscle or the muscle partly blocked by C 10 can maintain a tetanus (Fig. 2).

Partial or complete neuromuscular block produced by DPSE or DHSE is rapidly and completely antagonised by intravenous injection of edrophonium, 0·2 to 0·6 mg./kg. Block is also antagonised by neostigmine, 25 to 75 μg./kg., eserine, 30 to 40 μg./kg., and C 10, 0·05 to 0·1 mg./kg.

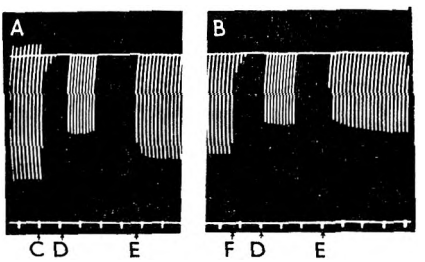

FIG. 1. Direct stimulation of blockaded muscle. Cat, 2·0 kg., female, sciatic nerve-gastrocnemius muscle preparation. Pentobarbitone anaesthesia.

Stimulation of nerve by square impulses, 15 V., frequency 8/min., width 2 msec.

Stimulation of muscle by square impulses, 40 V. frequency, 8/min., width 3·5 msec.

A. DPSE. B. DHSE.

At C, 0·75 mg./kg. of DPSE i.v.

D, Direct stimulation of muscle.

E, 0·5 mg./kg. of edrophonium bromide.

F, 0·75 mg./kg. of DHSE i.v.

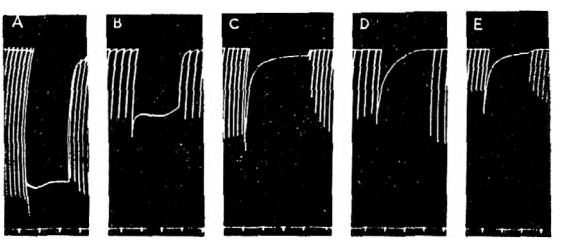

FIG. 2. Comparison of the effects of DHSE, DPSE, TC and C10 on the response of the cat gastrocnemius muscle to indirect tetanisation via the sciatic nerve.

Tetanisation by square impulses, 10 V. frequency 1500/min. during incomplete neuromuscular block, width 3 msec. Time interval 10 sec.

At A, normal response, no drug given.

B, C10 0·02 mg./kg. i.v.

C, DHSE 0·1 ,, ,,

D, TC 0·1 ,, ,,

E, DPSE 0·08 ,, ,,

The edrophonium effect was usually much shorter-lasting than that of neostigmine or eserine (Figs. 3, 4 and 5).

Block produced by DPSE or DHSE, 0·1 to 0·2 mg./kg., is potentiated by ether anaesthesia (Fig. 6). Subsequent doses of the same drug given after complete recovery appeared to produce an increased effect. The first dose of DPSE, 0·4 mg./kg., reduces twitch height by about 50 per cent.

A second and similar dose given after complete recovery of twitch height reduces this by about 80 per cent whilst third and fourth doses reduce it by roughly 95 and 100 per cent. DPSE, DHSE and TC all behave in a similar fashion. DPSE and DHSE do not potentiate one another nor do they potentiate TC. On the other hand the effects of the three compounds are additive. If, for example, DPSE is used to produce neuromuscular

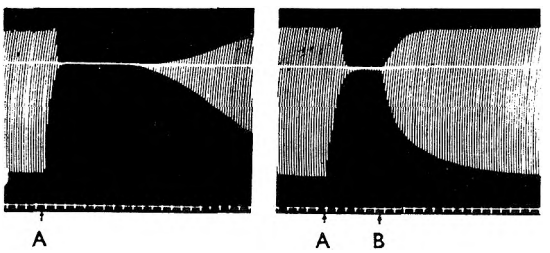

FIG. 3. DHSE-neostigmine antagonism. Cat, 3 kg., female, gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve, 4 square impulses/min. 10 V., width 3 msec. Time interval 1 minute.

At A, 0·5 mg./kg. DHSE i.v.
B, 0·07 ,, neostigmine methyl sulphate i.v.

block, the intensity of effect can be increased by administering DHSE and *vice versa*. In the same way DPSE and DHSE are additive with TC.

Rabbit head drop. In our hands, the average doses to cause head drop in groups of 6 rabbits were, DHSE, 0·36, DPSE, 0·38, and TC, 0·11 mg./

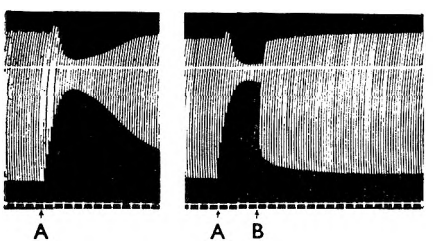

FIG. 4. DPSE-edrophonium antagonism. Cat, 3 kg., female, gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve, 4 square impulses/min., 10 V., width 3 msec. Time interval 1 min.

At A, 0·5 mg./kg. DPSE i.v.
B, 0·35 ,, edrophonium bromide i.v.

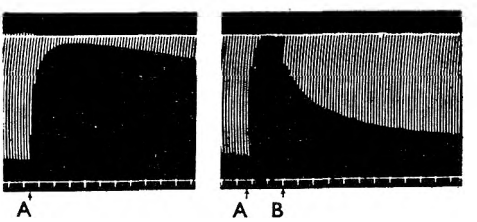

FIG. 5. DPSE-C10 antagonism. Cat, 3·25 kg., female, gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve, 4 square pulses/min. 10 V., width 3 msec. Time interval 1 minute.

At A, DPSE 0·5 mg./kg. i.v.
B, C10 0·15 ,, ,,

kg. When TC was used failure of spontaneous respiration always occurred. With the other compounds it occurred in 2 animals in each group.

Rat diaphragm. DPSE and DHSE, 0·2 mg./ml., reduced the twitch height, but a complete block was not produced, and the effect was always reversible. TC was much more potent on this preparation.

Frog rectus abdominis muscle. Neither DPSE nor DHSE at the doses used had any direct stimulant effect. DPSE and DHSE, 0·5 to 2·5

μg./ml., antagonised contractions induced by 0·1 to 0·2 μg./ml. of ACh or 2 μg./ml. of C 10. TC, 0·1 to 0·5 μg./ml., produced similar effects. Toad muscle was less sensitive to ACh or C 10. On toad muscle preparations DPSE and DHSE, 0·05 to 0·2 mg./ml., antagonised contractions due to ACh, 0·4 μg./ml., or C 10, 3·0 μg./ml.

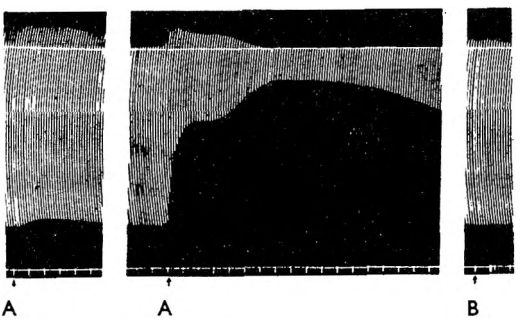

FIG. 6. Ether potentiation of DHSE. Cat, 3·25 kg., male, gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Indirect stimulation via sciatic nerve, 4 square impulses/min., 10 V., width 3 msec. Time interval 1 min.

At A, 0·1 mg./kg. DHSE i.v.
B, 0·1 ,, ,, ,, with ether anaesthesia.

Mouse test. Intraperitoneal injection of DPSE and DHSE, 1·0 to 1·35 mg./kg., caused flaccid paralysis. This was followed by failure of respiration which was the apparent cause of death because the heart continued to beat for some time after the respiration had ceased. At low doses, 1·0 mg./kg., there was partial muscular paralysis and some embarrassment of the respiration. DHSE appeared to be slightly more potent in causing respiratory paralysis in mice. The LD50 of DHSE was 1·2 $\pm$ 0·1 mg./kg. and that of DPSE was 1·3 $\pm$ 0·1 mg./kg.

Other Properties

Neither DPSE or DHSE, 0·5 mg. to 1·0 mg./kg., caused a fall or rise in the blood pressure of the anaesthetised cat. In contrast, TC, 0·5 mg./kg., caused the blood pressure to fall. Neither antagonism nor potentiation was shown by DPSE or DHSE to the characteristic effects of ACh, 0·4 to 1·0 μg./kg., Ad, 0·4 to 2·0 μg./kg., NA, 0·4 to 2·0 μg./kg., or Hm, 0·4 to 1·0 μg./kg.

DPSE, 0·1 mg./kg., caused a slight relaxation of sustained contractions of the nictitating membrane induced by electrical stimulation of preganglionic fibres of the cervical sympathetic. After this compound, 0·5 to 0·8 mg./kg., there was also a slight reduction of the height of contraction of the nictitating membrane following indirect intermittent stimulation. DHSE on the other hand caused neither relaxation of the tetanised membrane, 0·1 mg./kg., nor a reduction in the height of contraction after intermittent stimulation, 0·25 to 0·5 mg./kg. TC was much more potent on both of these preparations.

Continuous infusion of both DPSE and DHSE caused respiratory paralysis. If administration of the drug was stopped when respiration had ceased, then in some animals spontaneous respiration began again, but as a rule artificial respiration had to be given. This always produced a complete recovery. The approximate dose of DPSE to paralyse respiration was 0·76 mg./kg.; that of DHSE was 0·92 mg./kg. The dose of TC to cause paralysis of respiration was 0·48 mg./kg.

Neither DPSE or DHSE, 0·2 to 2·0 mg., had any effect on the rate, amplitude or outflow of the isolated perfused heart of the rabbit or kitten. No effect was observed on the peristaltic movements of the isolated rabbit duodenum at doses of 20 μg./ml. Very large doses, 0·25 mg./ml., antagonised ACh induced contractions of the guinea pig ileum.

Both these compounds, 0·2 to 1·0 mg., caused vasoconstriction in the isolated perfused rat hind quarters but they were much less potent vasoconstrictors on this preparation than TC (50 to 250 μg.).

Discussion

Both DPSE and DHSE are neuromuscular blocking agents which appear to act in a similar manner to TC. We were unable to show evidence of a depolarising action such as is shown by C 10. Both compounds are about equipotent with TC on the gastrocnemius muscle of the cat, but are less potent on the rat diaphragm and frog rectus muscle and have a higher head-drop dose. They appear to lack ganglion blocking activity and not to release histamine. They are less potent than TC in paralysing respiration in the cat. Tris-quaternary compounds have been shown to possess curare-like properties[11,17]. The tris-nitrogen compound gallamine has about one fifth of the potency of TC; it lacks ganglion-blocking activity[25] and does not cause significant histamine release[26], and is not potentiated by ether anaesthesia[27]. It has an atropine-like vagolytic action[10,11,27]. The compounds we have studied appear to have a great deal in common with gallamine. They act by a similar mechanism and do not cause ganglion block or histamine release unless given in massive doses. In addition they have the advantage that they do not appear to alter the heart rate. Like TC, their action is potentiated by ether anaesthesia.

We have also studied the ganglion-blocking and neuromuscular blocking properties of three other compounds (IV, $n = 3$), V, VI ($n = 6$, R = Me). None possess neuromuscular-blocking activity. DHSM was occasionally observed to have slight ganglion-blocking activity but the other compounds were almost inert.

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DISCUSSION

The paper was presented by MR. J. J. LEWIS.

The CHAIRMAN. Had the authors any theory for the failure to quaternise in the case of the butyl sulphide, were the sulphonium salts very soluble in water and was there any information regarding stability?

PROFESSOR K. BULLOCK (Manchester). Had the authors tried the *in vitro* anticholeresterase activity of the compounds.

DR. G. E. FOSTER (Dartford). Since sulphonal and trional were sulphones, had the compounds under discussion any soporific value.

In reply MR. LEWIS said that the *in vitro* anticholeresterase activity of the compounds had not been tried and no soporific properties had been observed.

DR. J. B. STENLAKE had not been able to obtain a satisfactory explanation why the third quaternary atom could not be introduced in the butyl derivatives. The compounds were all very soluble and he had experienced no difficulty in preparing any solution required for pharmacological tests. As regards stability, compounds with *NNN* quaternary groups were stable, but he was uncertain about the *NSN* compounds.

British Pharmaceutical Conference

NINETY-FOURTH ANNUAL MEETING, BRISTOL, 1957

REPORT OF PROCEEDINGS

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H. HUMPHREYS JONES, F.P.S., F.R.I.C., Liverpool.
T. E. WALLIS, D.Sc., F.P.S., F.R.I.C., F.L.S., London.
H. BRINDLE, M.Sc., F.P.S., F.R.I.C., Manchester.
B. A. BULL, F.P.S., A.R.I.C., Nottingham.
NORMAN EVERS, Ph.D., F.R.I.C., Ware.
A. D. POWELL, M.P.S., F.R.I.C., Nottingham.
H. BERRY, B.Sc., Dip.Bact. (London), F.P.S., F.R.I.C., Eastbourne.
H. B. MACKIE, B.Pharm., F.P.S., Brighton.
G. R. BOYES, L.M.S.S.A., B.Sc., F.P.S., F.R.I.C., London.
H. DAVIS, C.B.E., B.Sc., Ph.D., F.P.S., F.R.I.C., London.
J. P. TODD, Ph.D., F.P.S., F.R.I.C., Glasgow.
K. BULLOCK, M.Sc., Ph.D., F.P.S., F.R.I.C., Manchester.

Honorary Treasurer:

H. TREVES BROWN, B.Sc., F.P.S., London.

Honorary General Secretaries:

H. G. ROLFE, B.Sc., F.P.S., F.R.I.C., London.
E. F. HERSANT, B.Pharm., Ph.D., F.P.S., F.R.I.C., London.

Other Members of the Executive Committee:

The CHAIRMAN of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain (*ex officio*).
The PRESIDENT of the Pharmaceutical Society of Ireland (*ex officio*).
The PRESIDENT of the Pharmaceutical Society of Northern Ireland (*ex officio*).
The EDITOR of the *Journal of Pharmacy and Pharmacology* (*ex officio*).
The CHAIRMAN and Honorary Secretary of the Local Committee (*ex officio*).

*E. A. BROCKLEHURST, M.P.S., J.P., Hull.
K. R. CAPPER, B.Pharm., Ph.D., F.P.S., D.I.C., London.
J. W. FAIRBAIRN, B.Sc., Ph.D., F.P.S., A.R.I.C., F.L.S., London.
G. E. FOSTER, B.Sc., Ph.D., F.R.I.C., Dartford.
D. C. GARRATT, B.Sc., Ph.D., F.R.I.C., Nottingham.
*G. H. HUGHES, M.P.S., Colwyn Bay.
J. B. LLOYD, M.P.S., Manchester.
W. MITCHELL, B.Sc., Ph.D., F.R.I.C., London.
*GRANVILLE SHAW, F.P.S., Long Eaton.

* Members nominated by the Council of the Pharmaceutical Society of Great Britain.

PROCEEDINGS OF CONFERENCE

BRISTOL, 1957

THE OPENING SESSION

The opening session of the Conference was held in the Conference Hall of the Council House, Bristol, on Monday, September 2, with Mr. D. W. Hudson, President of the Conference (President of the Pharmaceutical Society) in the Chair. On the platform were the Chairman of the Conference (Dr. F. Hartley), the Lord Mayor of Bristol (Alderman P. W. Raymond), the Chairman and Secretary of the Local Committee (Mr. D. F. Baldwin and Mr. E. George), the Honorary General Secretaries the Honorary Treasurer, together with members of the Conference Executive Committee.

The President introduced the Lord Mayor, who welcomed the Conference to Bristol. The President thanked the Lord Mayor on behalf of the Conference for his welcome.

The President then handed over the further conduct of the Conference to the Chairman (Dr. F. Hartley), who delivered his address entitled "Steroids in Pharmacy and Medicine," which is printed in full in the *Journal of Pharmacy and Pharmacology*, 1957, **9**, pages 705–729.

On the proposition of Mr. T. C. Denston, seconded by Mr. H. Steinman, the Conference accorded a hearty vote of thanks to the Chairman for his address.

CIVIC AND ACADEMIC RECEPTION

On the evening of Monday, September 2, a Joint Civic and Academic Reception was given at the City Museum and Art Gallery. The guests were received by the Lord Mayor of Bristol and the Lady Mayoress, the Vice-Chancellor of the University and Lady Morris. A dance was held at the Berkeley after the reception.

THE SCIENCE SESSIONS

Meetings were held on Monday, Tuesday, Wednesday and Friday, September 2, 3, 4 and 6, at The Royal Fort or the Engineering Laboratories, the Chairman presiding. During the sessions the following 22 papers were communicated:—

1. Losses of Bacteriostats from Injections in Rubber-closed Containers. By A. Royce, F.P.S. and G. Sykes, M.Sc., F.R.I.C.
2. The Purity of Chloroform B.P. By A. C. Caws, B.Sc., A.R.I.C. and G. E. Foster, B.Sc., Ph.D., F.R.I.C.
3. Some Properties of Mixed Sols of Lecithin and Lysolecithin. By L. Saunders, B.Sc., Ph.D., F.R.I.C.
4. The Application of Infra-red Spectrophotometry to the Examination of Essential Oils. Part I. Cineole in Lavender Oil. By A. H. J. Cross, B.Sc., A. H. Gunn and S. G. E. Stevens, B.Sc., F.R.I.C.
5. The Vitamin B_{12} Potency of Pharmaceutical (Including Dietetic) Products Estimated by the Ochromonas Method. By F. Wokes, B.Sc., Ph.D., F.P.S., F.R.I.C. and M. H. Woollam.
6. The Preparation and Antifungal Activity of Some Salicylic Acid Derivatives. By L. V. Coates, D. J. Drain, B.A., F.R.I.C., K. A. Kerridge, B.Pharm., Ph.D., F.P.S., F. June Macrae, B.Sc. and K. Tattersall, B.Sc.
7. The Resistances of Vegetative Bacteria to Moist Heat. By B. A. Wills, B.Pharm., Ph.D., M.P.S., A.R.I.C.
8. Pharmacological Properties of Hydrallazine, Dihydrallazine and some Related Compounds. By S. M. Kirpekar, M.Sc.Tech. (Bombay) and J. J. Lewis, M.Sc., F.P.S.
9. The Rheology of Oil-in-Water Emulsions. II. The Microscopical Appearance of Emulsions in Laminar Flow. By Arnold Axon, M.S., B.Pharm., F.P.S.

10. The Spectrophotometric Determination of $\alpha\beta$-Unsaturated Aldehydes and Ketones with Girard-T Reagent. Part I. Essential Oils. Part II. Keto-steroids. By J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C. and W. D. Williams, B.Pharm., F.P.S.

11. The Determination of Morphine in Opium and some of its Galenical Preparations. By D. C. Garratt, D.Sc., Ph.D., F.R.I.C., C. A. Johnson, B.Pharm., B.Sc., F.P.S., A.R.I.C. and Cecilia J. Lloyd, B.Sc.

12. The Preparation and Progestational Activity of some Alkylated Ethisterones. By A. David, M.R.C.S., L.R.C.P., D.I.H., F. Hartley, B.Sc., Ph.D., F.P.S., F.R.I.C., D. R. Millson, B.Sc. and V. Petrow, D.Sc., Ph.D., F.R.I.C.

13. Some Effects of Increasing Stillhead Surface Area on Liquid Entrainment During Distillation. By D. Train, M.C., B.Pharm., B.Sc.(Eng.), Ph.D., A.C.G.I., D.I.C., F.P.S., F.R.I.C., A.M.I.Chem.E. and Bayardo Velasquez-Guerrero, M.Pharm.

14. Alpha- and Beta-Prodine Type Compounds; Configurational Studies. By A. H. Beckett, B.Sc., Ph.D., F.P.S., F.R.I.C., A. F. Casy, B.Sc., Ph.D., F.P.S., A.R.I.C., G. Kirk, B.Sc., M.P.S. and J. Walker, B.Pharm., F.P.S., A.R.I.C.

15. The Inactivation of Pyrogens by Gamma Radiation. By T. D. Whittet, B.Sc., F.P.S., F.R.I.C., D.B.A. and W. Hutchinson, M.P.S.

16. The Pharmacology of some Hydroxybenzyl*iso*quinoline Derivatives. By J. J. Lewis, M.Sc., F.P.S. and M. S. Zoha, M.B., B.S.

17. The Oxidation of Solubilised and Emulsified Oils. I. Oxidation of Benzaldehyde in Potassium Laurate and Cetomacrogol Dispersions. By J. E. Carless, B.Pharm., M.Sc., Ph.D., F.P.S. and J. R. Nixon, B.Pharm.

18. The Use of Oxidised Cellulose for the Determination of Strychnine in Pharmaceutical Preparations. By D. A. Elvidge, B.Sc., A.R.I.C. and K. A. Proctor, M.Sc., F.R.I.C.

19. Observations on the Intravenous Toxicity and Chelating Activity of Sodium Stibogluconate. By J. Green, B.Sc., Ph.D., A.R.I.C., A. C. T. Hickman, B.Sc., A.R.I.C., Helen M. Sharpe, B.Sc. and E. G. Tomich, B.Sc., F.R.I.C., M.I.Biol.

20. The Non-Aqueous Titration of Phenolic Compounds. By J. Allen, A.R.I.C. and E. T. Geddes.

21. A Comparative Examination of some Samples of Digitoxin. By E. H. B. Sellwood, B.Pharm., F.P.S., A.R.I.C.

22. Some *NSN*-Tris-Quaternary Neuromuscular Blocking Agents. By D. Edwards, B.Sc., F.P.S., A.R.I.C., J. J. Lewis, M.Sc., F.P.S., J. B. Stenlake, B.Sc., Ph.D., F.P.S., A.R.I.C. and M. S. Zoha, M.B., B.S.

The papers are printed in full with reports of discussions in the *Journal of Pharmacy and Pharmacology*, 1957, **9**, pages 814 to 1016.

THE SYMPOSIUM SESSION

A symposium on "Surgical Dressings" was held on Thursday, September 5. The CHAIRMAN presided. Two introductory papers were presented by Dr. J. Scales and Mr. D. E. Seymour. The meeting is reported in the *Journal of Pharmacy and Pharmacology*, 1957, **9**, pages 785 to 813.

PROFESSIONAL SESSIONS

With the President of the Conference, Mr. D. W. Hudson, in the chair, professional sessions were held on the mornings of Tuesday, September 3, when Mr. A. Aldington read a paper on "Efficiency in Pharmaceutical Practice," and Friday, September 6, when Drs. K. R. Capper and J. G. Dare read a paper on "Dispensing Tolerances in Liquid Medicines." Full reports of the papers and discussions were published in *The Pharmaceutical Journal*, 1957, **179**, 175–180, 204–206, 231–239, 262–264.

THE CLOSING SESSION

The closing session of the Conference was held on Friday, September 6, in the Great Hall of the University, Bristol, the Chairman presiding.

Vote of Thanks to the Local Committee

The Chairman called on Miss E. Button to propose a vote of thanks to the Local Committee. This was seconded by Mr. G. T. M. David. The Chairman then presented to the Bristol Branch an embossed minute book provided from the Bell and Hills Fund. Mr. D. F. Baldwin (Chairman of the Local Committee) replied to the vote of thanks and acknowledged the gift.

Annual Report

Mr. H. G. Rolfe presented the following Annual Report of the Executive Committee.

Your Executive have pleasure in presenting the ninety-fourth Annual Report.

Membership.—In addition to Members, Honorary Members and Students of the Pharmaceutical Society, together with the Members of the Pharmaceutical Society of Northern Ireland, the Conference includes 70 members elected by the Executive who are not members of these Societies.

Obituary.—The Executive report with deep regret the death, since the last meeting, of Mr. P. F. McGrath, President of the Pharmaceutical Society of Ireland, a member of the Executive, and Chairman of the Local Committee for the Dublin Conference, 1956.

Conference Research Papers.—Twenty-eight papers were submitted, two were withdrawn and twenty-two have been accepted for presentation to the Conference. The Executive thank the authors for their contributions.

Dublin Meeting, 1956.—The report of the meeting of the Conference at Dublin was published in the 8th Volume of the *Journal* of *Pharmacy and Pharmacology*. The papers and discussions at the professional sessions were published in the *Pharmaceutical Journal*, Series IV, Volume 123.

Journal of Pharmacy and Pharmacology.—The Executive has been represented on the Editorial Board by the Chairman (Dr. F. Hartley), Professor K. Bullock and the Senior Honorary General Secretary.

Future Meetings.—An invitation will be presented at this meeting for the Conference to meet in Llandudno during the week commencing September 15, 1958.

Several branches of the Society have made preliminary enquiries regarding the possibility of entertaining the Conference in future years and the Executive are grateful for all these offers of hospitality.

Officers and Executive of the Conference.—Your Executive has nominated the following Officers for 1957–1958:—

Chairman: G. E. Foster, *Vice-Chairmen:* R. R. Bennett, H. Deane, H. Humphreys Jones, T. E. Wallis, H. Brindle, B. A. Bull, Norman Evers, A. D. Powell, H. Berry, H. B. Mackie, G. R. Boyes, H. Davis, J. P. Todd, K. Bullock and F. Hartley. *Honorary Treasurer:* H. Treves Brown. *Honorary General Secretaries:* H. G. Rolfe and E. F. Hersant.

Other members of the Executive:—

As more than six persons were nominated for membership of the Executive, an election was held during this meeting of the Conference. The result is that the following will be the elected members of the Executive for 1957–1958—D. C. Adamson, A. W. Bull, K. R. Capper, J. W. Fairbairn, W. H. Linnell and J. B. Lloyd.

The above persons, together with the President of the Conference (the President of the Pharmaceutical Society of Great Britain *ex officio*), the three persons nominated by the Council of the Pharmaceutical Society of Great Britain, namely, the persons for the time being holding the office of Vice-President, immediate past President and Chairman of the Organisation Committee, together with the following *ex officio:* The Chairman of the Executive of the Scottish Department, the President of the Pharmaceutical Society of Ireland, the President of the Pharmaceutical Society of Northern Ireland, the Editor of the *Journal* of *Pharmacy and Pharmacology*, the Chairman of the Local Committee and the Honorary Local Secretary, will form the Executive for 1957–1958.

Dr. R. E. STUCKEY proposed the acceptance of the report and the election of officers of the Conference for the ensuing year. Dr. N. J. Harper seconded.

Dr. G. E. FOSTER thanked the Conference on behalf of the newly-elected officers.

TREASURER'S REPORT

Mr. H. TREVES BROWN presented and proposed the adoption of the following Report and Statement of Accounts for the year 1957:—

During the year ended 31st December, 1956, the income from investments, together with the donations of £25 from the Pharmaceutical Society of Northern Ireland and from the Pharmaceutical Society of Ireland, amounted to £94 8*s.* 6*d.* The total expenditure for the year amounted to £38 16*s.* 0*d.*, leaving a surplus of £55 12*s.* 6*d.* to carry forward.

With the addition of this surplus the Accumulated Fund on 31st December, 1956, amounted to £1,926 15*s.* 10*d.* In addition, the Balance Sheet contains the Local Committee Fund, consisting of a donation of £250 from the London Committee, 1953, held by the Executive to assist a Branch if necessary in the initial stages of preparation for a Conference. These two funds, shown in the Balance Sheet as amounting together to £2,176 15*s.* 10*d.*, include the original donation from Alderman Clayton of Consols, to the nominal value of £1,610, and 3 per cent. Savings Bonds, 1960–1970, to the nominal value of £200. The market values of these investments on 31st December, 1956, were £845 and £157 respectively. The first reference to the Clayton donation in a treasurer's report as published in the Yearbook of Pharmacy was in the year 1926 and it is noteworthy that the market value at that time was stated to be £880, so that the decline in value since the investment has been held by the Conference is relatively small. The investments are of course held primarily for interest and the market value is not of great importance.

The subscriptions from elected members, including a composition fee from the Pharmaceutical Society of Northern Ireland, amounted to £127, and were credited to the account of the *Journal of Pharmacy and Pharmacology*.

The Executive much appreciates the assistance given to the Conference by the Pharmaceutical Society of Great Britain in meeting the cost of clerical assistance kindly placed at its disposal and sundry other expenses arising out of the Conference.

At the Conference last year attention was drawn to the fact that the accounts then presented had not been audited. This was in accordance with the practice for a number of years but obviously it is undesirable that the omission should continue. Accordingly, at the request of the Executive, Mr. J. C. Hanbury and Mr. T. Heseltine agreed to audit the accounts for the year 1956 and the Executive thank them for their services.

H. TREVES BROWN,
Honorary Treasurer.

BRITISH PHARMACEUTICAL CONFERENCE ACCOUNT

INCOME AND EXPENDITURE ACCOUNT, 1956

Expenditure	£	*s.*	*d.*	*Income*	£	*s.*	*d.*
Gavel—memento to Pharmaceutical Society of Ireland	5	17	6	Interest on 2½% Consols net	23	3	0
Replica of Chairman's Badge and engraving	7	7	6	Interest on 3% Savings Bonds	6	0	0
Expenses of Speakers	25	7	0	Interest on P.O. Savings Bank Account	15	5	6
Cheque stamps		4	0	Donation from Pharmaceutical Society of Northern Ireland	25	0	0
Surplus carried to Balance Sheet	55	12	6	Donation from Pharmaceutical Society of Ireland	25	0	0
	£94	8	6		£94	8	6

BALANCE SHEET AT 31ST DECEMBER, 1956

Liabilities	£	*s.*	*d.*	*Assets*	£	*s.*	*d.*
Accumulated Fund, as at 31.12.55	1,871	3	4	Investments at cost (*a*) £1,610 2½% Consols (Donation by the late Alderman Clayton of Birmingham) (Market value at 31st December, 1956: £845)	1,250	0	0
Add: Surplus 1956	55	12	6	(*b*) £200 3% Savings Bonds 1960–70 (Market value at 31st December, 1956: £157)	200	0	0
	1,926	15	10	Stock of Replicas (4) of Chairman's Badge	27	10	0
Local Committee Fund:— Donation from London Committee, 1953	250	0	0	Post Office Savings Bank Account	651	13	6
				Cash at Westminster Bank	47	12	4
	£2,176	15	10		£2,176	15	10

Audited and found correct
J. C. HANBURY
T. HESELTINE
23rd July, 1957

The President seconded, and the Report was adopted.

British Pharmaceutical Conference

INAUGURAL MEETING HELD AT NEWCASTLE-ON-TYNE IN 1863

Years	*Places of Meeting*	*Presidents*	*Local Secretaries*
1864	BATH ..	HENRY DEANE, F.L.S.	J. C. POOLEY.
1865	BIRMINGHAM ..	HENRY DEANE, F.L.S.	W. SOUTHALL, JUN.
1866	NOTTINGHAM ..	PROF. BENTLEY, F.L.S.	J. H. ATHERTON, F.C.S.
1867	DUNDEE ..	PROF. BENTLEY, F.L.S.	J. HODGE
1868	NORWICH ..	DANIEL HANBURY, F.R.S.	F. SUTTON, F.C.S.
1869	EXETER ..	DANIEL HANBURY, F.R.S.	M. HUSBAND.
1870	LIVERPOOL ..	W. W. STODDART, F.C.S.	E. DAVIES, F.C.S. J. DUTTON (Birkenhead).
1871	EDINBURGH ..	W. W. STODDART, F.C.S.	J. MACKAY, F.C.S.
1872	BRIGHTON ..	H. B. BRADY, F.R.S.	T. GLAISYER.
1873	BRADFORD ..	H. B. BRADY, F.R.S.	R. PARKINSON, PH.D.
1874	LONDON ..	THOS. B. GROVES, F.C.S.	M. CARTEIGHE, F.C.S.
1875	BRISTOL ..	THOS. B. GROVES, F.C.S.	J. PITMAN.
1876	GLASGOW ..	PROF. REDWOOD, F.C.S.	A. KINNINMONT
1877	PLYMOUTH ..	PROF. REDWOOD, F.C.S.	R. J. CLARK.
1878	DUBLIN ..	G. F. SCHACHT, F.C.S.	W. HAYES.
1879	SHEFFIELD ..	G. F. SCHACHT, F.C.S.	H. MALEHAM.
1880	SWANSEA ..	W. SOUTHALL, F.L.S.	J. HUGHES.
1881	YORK	R. REYNOLDS, F.C.S.	J. OWRAY.
1882	SOUTHAMPTON	PROF. ATTFIELD, F.R.S.	O. R. DAWSON.
1883	SOUTHPORT ..	PROF. ATTFIELD, F.R.S.	WM. ASHTON.
1884	HASTINGS ..	J. WILLIAMS, F.C.S.	F. ROSSITER.
1885	ABERDEEN ..	J. B. STEPHENSON.	A. STRACHAN.
1886	BIRMINGHAM ..	T. GREENISH, F.C.S.	CHAS. THOMPSON.
1887	MANCHESTER ..	S. R. ATKINS, J.P.	F. B. BENGER, F.C.S.
1888	BATH	F. B. BENGER, F.C.S.	H. HUTTON.
1889	NEWCASTLE-ON-TYNE	C. UMNEY, F.I.C., F.C.S.	T. M. CLAGUE.
1890	LEEDS ..	C. UMNEY, F.I.C., F.C.S.	F. W. BRANSON, F.C.S.
1891	CARDIFF ..	W. MARTINDALE, F.C.S.	ALFRED COLEMAN.
1892	EDINBURGH ..	E. C. C. STANFORD, F.C.S.	PETER BOA.
1893	NOTTINGHAM ..	OCTAVIUS CORDER.	C. A. BOLTON.
1894	OXFORD ..	N. H. MARTIN, F.L.S., F.R.M.S.	H. MATTHEWS.
1895	BOURNEMOUTH	N. H. MARTIN, F.L.S., F.R.M.S.	STEWART HARDWICK.
1896	LIVERPOOL ..	W. MARTINDALE, F.C.S.	T. H. WARDLEWORTH. H. O. DUTTON (Birkenhead).
1897	GLASGOW ..	DR. C. SYMES.	J. A. RUSSELL.
1898	BELFAST ..	DR. C. SYMES.	R. W. MCKNIGHT. W. J. RANKIN.
1899	PLYMOUTH ..	J. C. C. PAYNE, J.P.	J. DAVY TURNEY.
1900	LONDON ..	E. M. HOLMES, F.L.S.	W. WARREN. HERBERT CRACKNELL.
1901	DUBLIN ..	G. C. DRUCE, M.A., F.L.S.	J. I. BERNARD.
1902	DUNDEE ..	G. C. DRUCE, M.A., F.L.S.	W. CUMMINGS.
1903	BRISTOL ..	T. H. W. IDRIS, M.P., F.C.S.	H. E. BOORNE.
1904	SHEFFIELD ..	T. H. W. IDRIS, M.P., F.C.S.	H. ANTCLIFFE.
1905	BRIGHTON ..	W. A. H. NAYLOR, F.I.C., F.C.S.	W. W. SAVAGE. C. G. YATES.
1906	BIRMINGHAM ..	W. A. H. NAYLOR, F.I.C., F.C.S.	C. THOMPSON.
1907	MANCHESTER ..	THOS. TYRER, F.I.C., F.C.S.	W. KIRKBY.
1908	ABERDEEN ..	ROBT. WRIGHT, F.C.S.	W. F. HAY.
1909	NEWCASTLE-ON-TYNE	J. G. TOCHER, B.Sc., F.R.I.C.	T. M. CLAGUE. H. W. NOBLE.
1910	CAMBRIDGE ..	FRANCIS RANSOM, F.C.S.	A. A. DECK. T. J. MALLETT.
1911	PORTSMOUTH ..	W. F. WELLS.	T. O. BARLOW. T. POSTLETHWAIT.
1912	EDINBURGH ..	SIR EDWARD EVANS, J.P.	THOS. STEPHENSON.
1913	LONDON ..	JOHN C. UMNEY, F.C.S.	W. J. UGLOW WOOLCOCK.
1914	CHESTER ..	E. H. FARR, F.C.S.	R. CECIL OWEN, B.Sc.
1915	LONDON ..	E. SAVILLE PECK, M.A.	———
1916	LONDON ..	DAVID HOOPER, LL.D., F.R.I.C.	———
1917	LONDON ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	———
1918	LONDON ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	———
1919	LONDON ..	W. KIRBY, M.Sc., F.C.S.	———
1920	LIVERPOOL ..	CHAS. ALEX. HILL, B.Sc., F.R.I.C.	H. HUMPHREYS JONES, F.R.I.C.
1921	SCARBOROUGH	E. SAVILLE PECK, M.A.	E. R. CROSS.
1922	NOTTINGHAM ..	PROF. H. G. GREENISH, Dès. Sc., F.I.C.	E. C. CARR.

Years	*Places of Meeting*	*Chairmen*	*Local Secretaries*
1923	LONDON ..	F. W. GAMBLE.	W. J. U. WOOLCOCK, C.B.E.
1924	BATH	EDMUND WHITE, B.Sc., F.I.C.	P. J. THOMPSON W. H. HALLETT.
1925	GLASGOW ..	EDMUND WHITE, B.Sc., F.I.C.	P. M. DUFF.
1926	LEICESTER ..	D. LLOYD HOWARD, J.P.	J. BARKER.
1927	BRIGHTON ..	D. LLOYD HOWARD, J.P.	F. W. BURGESS.
1928	CHELTENHAM ..	R. R. BENNETT, B.Sc., F.R.I.C.	P. JAMES.
1929	DUBLIN ..	R. R. BENNETT, B.Sc., F.R.I.C.	V. E. HANNA.

Years	Places of Meeting	Chairmen	Local Secretaries
1930	CARDIFF ..	J. T. HUMPHREY.	J. MURRAY.
1931	MANCHESTER ..	J. H. FRANKLIN.	R. G. EDWARDS.
1932	ABERDEEN ..	H. SKINNER.	H. M. DUGAN.
1933	LONDON ..	C. H. HAMPSHIRE, M.B., B.S., B.Sc., F.R.I.C.	H. N. LINSTEAD.
1934	LEEDS ..	C. H. HAMPSHIRE, M.B., B.S., B.Sc., F.R.I.C.	G. C. CRUMMACK. J. F. SIMON.
1935	BELFAST ..	F. W. CROSSLEY-HOLLAND, L.M.S.S.A.	D. L. KIRKPATRICK.
1936	BOURNEMOUTH	HAROLD DEANE, B.Sc., F.R.I.C.	V. J. SCAMPTON.
1937	LIVERPOOL ..	T. EDWARD LESCHER, O.B.E.	W. E. HUMPHREYS.
1938	EDINBURGH ..	J. RUTHERFORD HILL, O.B.E.	C. G. DRUMMOND.
1939	BIRMINGHAM ..	J. RUTHERFORD HILL, O.B.E.	D. J. RUSHTON.
1940	LONDON ..	H. HUMPHREYS JONES, F.R.I.C.	———
1941	LONDON ..	A. R. MELHUISH.	———
1942	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1943	LONDON ..	T. E. WALLIS, D.Sc., F.R.I.C., F.L.S.	———
1944	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1945	LONDON ..	H. BRINDLE, B.Sc., F.R.I.C.	———
1946	LONDON ..	B. A. BULL, A.R.I.C.	———
1947	TORQUAY ..	B. A. BULL, A.R.I.C.	T. D. EVANS.
1948	BRIGHTON ..	NORMAN EVERS, PH.D., F.R.I.C.	A. WILSON.
1949	BLACKPOOL ..	NORMAN EVERS, PH.D., F.R.I.C.	P. VARLEY. T. A. DURKIN.
1950	GLASGOW ..	A. D. POWELL, F.R.I.C.	A. OFFICER.
1951	HARROGATE ..	H. BERRY, B.Sc., Dip. Bac. (London), F.R.I.C.	R. W. JACKSON.
1952	NOTTINGHAM ..	H. B. MACKIE, B.Pharm.	W. E. NEWBOLD. Miss G. M. WATSON.
1953	LONDON ..	G. R. BOYES, L.M.S.S.A., B.Sc., F.R.I.C.	J. M. ROWSON.
1954	OXFORD ..	H. DAVIS, C.B.E., B.Sc., PH.D., F.R.I.C.	T. R. HARDY.
1955	ABERDEEN ..	J. P. TODD, PH.D., F.R.I.C.	G. L. DICKIE.
1956	DUBLIN ..	K. BULLOCK, M.Sc., PH.D., F.R.I.C.	D. J. KENNELLY.
1957	BRISTOL ..	F. HARTLEY, B.Sc., PH.D., F.R.I.C.	E. GEORGE.

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1940 to 1947, T. MARNS.
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1947 to H. G. ROLFE, B.Sc., F.R.I.C.
1953 to E. F. HERSANT, B.Pharm., PH.D., F.R.I.C.

Closing Session (continued).

PLACE OF MEETING FOR 1958

Mr. H. NEVILLE on behalf of the Anglesey, North Caernarvonshire and Colwyn Bay Branch, extended an invitation to hold the Conference in Llandudno in 1958.

Mr. K. HOLLAND proposed that the invitation be accepted, and Dr. D. TRAIN seconded. The vote was put to the meeting and unanimously carried.

VOTE OF THANKS TO CHAIRMAN

Mr. H. G. ROLFE proposed a vote of thanks to the Chairman.

Mr. R. M. MCCRONE seconded. The vote was put to the meeting by the President and carried with acclamation.

Dr. HARTLEY briefly responded.

British Pharmaceutical Conference

CONSTITUTION AND RULES

1. The British Pharmaceutical Conference shall meet annually for the discussion of subjects relative to the science of pharmacy, for the promotion of friendly reunion amongst pharmacists and those interested in pharmacy, and generally for the furtherance of the objects sought to be obtained under the Royal Charter of Incorporation granted to the Pharmaceutical Society of Great Britain and the several Pharmacy Acts.

2. The Conference shall consist of:—

(*a*) members, honorary members and student-associates of the Pharmaceutical Society of Great Britain;

(*b*) members of the other Pharmaceutical Societies within the British Commonwealth of Nations which desire to be associated with the work of the Conference and have made an agreement with the British Pharmaceutical Conference whereby an annual subscription shall be paid by their Society in lieu of individual subscriptions from members; and

(*c*) persons at home and abroad interested in the advancement of Pharmacy who, not being qualified for membership of the Pharmaceutical Society of Great Britain or one of the other Societies associated with the work of the Conference, have been nominated in writing by a member of the Conference, and elected by the Executive.

3. The Officers of the Conference, who shall collectively constitute the Executive Committee, shall be:—A Chairman, Vice-Chairmen, one Honorary Treasurer, two Honorary General Secretaries, together with three members of the Council of the Pharmaceutical Society of Great Britain, and six other members of the Conference. Of the six other members nominated annually by the outgoing Executive the two members who have had the longest period of continuous service shall be ineligible for re-nomination for one year. The President of the Pharmaceutical Society of Great Britain shall be *ex officio* a member of the Executive Committee and President of the Conference. The Chairman of the Executive of the Scottish Department of the Pharmaceutical Society of Great Britain, the President of the Pharmaceutical Society of Ireland, the President of the Pharmaceutical Society of Northern Ireland, the President of any other Pharmaceutical Society the members of which are members of the Conference, the Editor of the *Journal of Pharmacy and Pharmacology*, the Chairman of the Local Committee, and the Honorary Local Secretary shall be *ex officio* members of the Executive Committee.

4. The Officers of the Conference shall be selected at each annual meeting by those present. Nominations for membership of the Executive Committee may be made at the annual meeting by the outgoing Executive and shall include nominations which have been received by the Honorary Secretaries in writing at least twenty-eight days before the annual meeting.

5. The Chairman of the Conference shall give the inaugural address, preside over the meetings for the reading of scientific papers and take the chair at the meetings of the Executive Committee.

6. Members, honorary members and student-associates of the Pharmaceutical Society of Great Britain shall not be required to pay a separate subscription to the British Pharmaceutical Conference; they shall be entitled, on application, to receive the issues of the *Journal of Pharmacy and Pharmacology*, as published, on preferential terms.

Members of any other Pharmaceutical Society, who are members of the Conference by virtue of the arrangement between their Society and the British Pharmaceutical Conference, shall not be required to pay a separate subscription to the British Pharmaceutical Conference: they shall be entitled, on application, to receive the *Journal of Pharmacy and Pharmacology*, as published, on preferential terms.

Other members elected by the Executive shall pay a subscription of 35*s*. annually, which shall entitle them, on application, to receive the *Journal of Pharmacy and Pharmacology*, as published. Subscription shall become due on January 1, and membership shall cease if subscriptions are not paid by June 1.

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