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The Journal of PHARMACY *and* PHARMACOLOGY

VOLUME VI No. 9

SEPTEMBER, 1954

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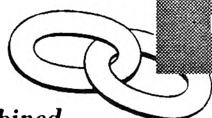

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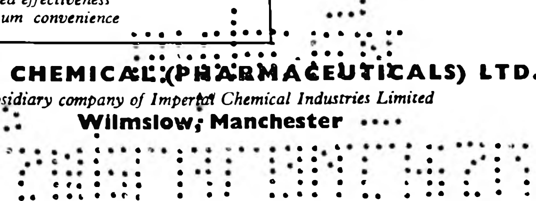

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Annual Subscription 50s. Single Copies 5s.

Vol. VI. No. 9 **September, 1954**

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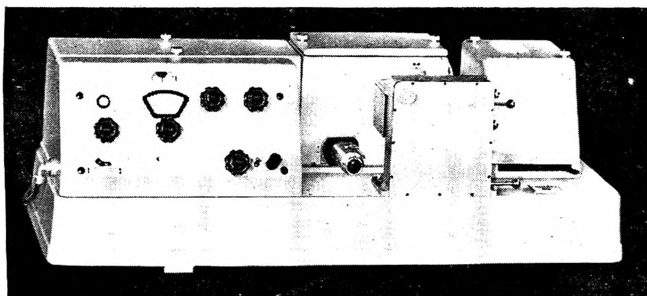

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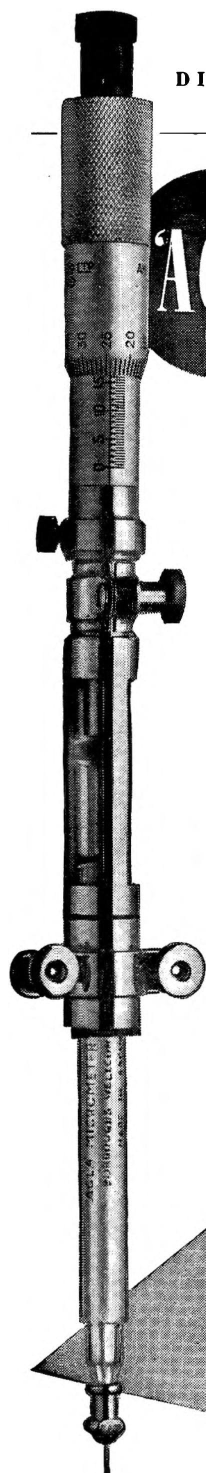

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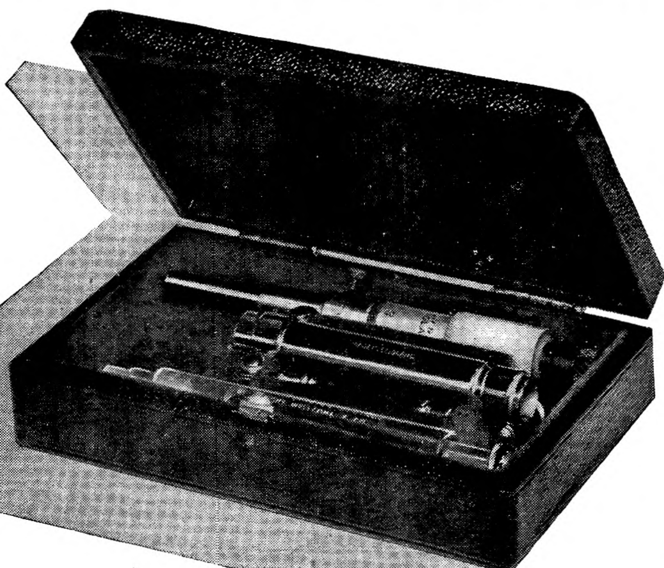

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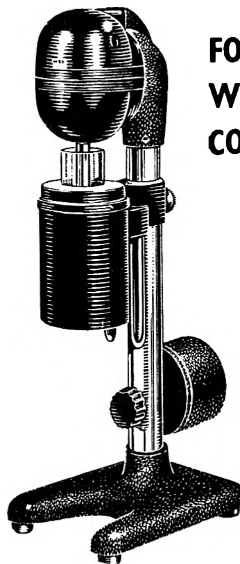

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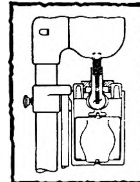
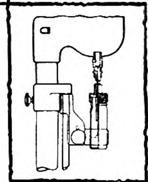


Cross-section showing 10 ml. beaker in position.



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REVIEW ARTICLE

ULTRASONICS IN PHARMACY AND ALLIED SCIENCES

By J. P. Hall, M.Sc., F.P.S.

Lecturer in Pharmacognosy, Bradford Technical College

Introduction

Although nearly forty years have passed since ultrasonic acoustic waves were first used for submarine detection, it is only in recent years that the science of ultrasonics has made its impact on a wide and varied field of research and industrial applications. The literature on the subject, large and scattered as it is, contains references to such diverse uses as ultrasonic soldering and laundering, the modulation of light beams in television systems, the measurement of physical properties of gases and liquids, and echo depth sounding. These and many other interesting applications are outside the scope of the present article which is concerned mainly with the pharmaceutical and allied uses, both actual and potential.

Acoustic vibrations are usually divided into two classes according to frequency. Oscillations with frequencies less than 10 to 20 kc./second are referred to as sonics or audible sound, and those with greater frequencies as ultrasonics or inaudible sound. A frequency of about 10 to 14 kc./second represents the upper limit of audible perception in the adult of the human species, although some animals appear to be receptive to higher frequencies. Whereas audible sound waves of more than a few watts power output cause discomfort and may become unbearable, power outputs of the order of hundreds of watts are readily available from ultrasonic generators designed for high intensity work without apparent harmful effect on the operator. It is important to bear in mind that the laws of sound which are valid for the audible range are also true for ultrasonics, although in the latter case other effects appear which have not been observed in the audible range. While these effects appear to be due mainly to the higher frequencies (or shorter wavelengths), some, particularly the biological and chemical actions, have become evident only because of the relative ease of producing extremely large amplitudes of sound at those frequencies. Although wavelength appears to play some part in biological reactions, a direct correlation has not yet been established and most of the evidence points to sound intensity as one of the most important parameters.

It is convenient at this point briefly to mention one or two fundamentals regarding the propagation and properties of sound vibrations. When a sound wave travels through a given medium the individual particles of that medium execute simple harmonic motion. That is, each particle vibrates backwards and forwards across its normal position of rest. This vibration is said to be longitudinal if it is in the same direction as the propagation of the sound, and transverse if at right angles. Liquids and gases support only longitudinal vibrations whereas solids may support

both. The maximum displacement of each particle from its rest position is called the amplitude, and the total number of excursions per second is known as the frequency. The distance between two particles with the same magnitude and direction of displacement is the wavelength. The velocity of propagation (V), the wavelength (λ), and the frequency (f), are related in the expression $V = f\lambda$. The velocity of propagation is a physical constant of the medium while the wavelength and the frequency are not. Hence if a sound wave travels from one medium at a given velocity to another medium with a different velocity, the wavelength will change but the frequency will remain constant since the latter is determined by the sound source. Table 1 shows the velocity of sound in some commonly used liquids[1].

TABLE I

Liquid	Temperature °C	Density g./ml.	Velocity of sound m./second
Acetone	20	0·790	1190
Benzene	20	0·879	1324
Carbon tetrachloride	25	1·595	926
Chloroform	20	1·488	1002
Ethanol	20	0·789	1168
Transformer oil	25	0·880	1350
Castor oil	25	0·969	1477
Water	25	0·997	1497
Xylol	22	0·877	1352

If the physical properties of the medium are taken into account it is possible to consider the sound wave as an alternating pressure phenomenon. The sound pressure variation with time at any given point in the medium is given by $p = P \sin (2\pi f t)$, where P is the pressure amplitude or maximum pressure obtained at a given point, p is the instantaneous pressure at any time t, and f is the frequency. This pressure variation may be either positive or negative; hence, in a given medium, two points a half wavelength apart may have a pressure difference of twice the maximum pressure or pressure amplitude. These large pressure differences appear to account for many of the biological and chemical actions of ultrasonics. Sound intensity, which is not to be confused with sound pressure, is defined as the energy which passes through unit area in unit time. It is usually measured in watts per square centimetre.

Acceleration and Cavitation

Two prime phenomena, acceleration of particles or molecules, and cavitation, are evident when a liquid is irradiated by high intensity ultrasonics. It is generally accepted that the biological and chemical actions may be explained in terms of these phenomena. The acceleration of particles in the propagating medium is truly enormous. For example, if a sound intensity of about 10 W/sq. cm. is delivered into water at a frequency of about 500 kc./second, a pressure amplitude of $5{\cdot}4 \times 10^6$ dynes/sq. cm., or about 5·4 atmospheres is obtained. Thus the pressure alternates between + 5·4 and – 5·4 atmospheres 500,000 times a second. The amplitude of vibration of the individual water molecules is 1·16 ×

10^{-5} cm., but the acceleration of these particles is $1 \cdot 14 \times 10^8$ cm., or about 1000 km/second^2; this is about 10^5 greater than the acceleration due to gravity. It seems probable that these large accelerations may be largely responsible for the disruption of large molecules referred to later.

If a source of ultrasound is immersed in a trough of cold liquid, say water, and the intensity slowly increased, a point occurs at and beyond which the water becomes violently agitated and appears to boil although still cool. The generally accepted explanation of this phenomenon is that the bubbles appear to be formed by the coalescence of minute dissolved gas nuclei as they move towards the nodes of the wave system. In addition the large negative pressures and hence the large stresses in the liquid cause the latter literally to be torn apart with the formation of hollows or cavities. This is referred to as cavitation. The cavities become filled with dissolved gas or the vapour of the surrounding medium, and on their subsequent collapse produce local pressures of the order of thousands of atmospheres. High local temperatures probably exist as well as tremendous local agitation and it seems possible that electrical potentials are formed in the process.

Two major factors which seem to influence the acoustic intensity required to produce cavitation in any liquid are external pressure and viscosity. In general the higher the viscosity the greater the intensity required. Similarly, if the external pressure upon the liquid is raised so must be the intensity of the applied acoustic beam in order that cavitation may occur. These points are important as it appears that most of the biological and chemical effects are not evident in the absence of cavitation.

The rudimentary principles outlined above are sufficient to indicate that ultrasonics are capable of producing great forces. Small wonder that the subject has been of interest to workers in many fields.

Consideration may now be given to the methods used for producing and applying these forces, bearing in mind that in most of the applications considered in this article the test material is irradiated in solution or suspension in a liquid medium. The propagation of ultrasonic waves in gaseous or solid media is a different problem.

Production of Ultrasonics

Although ultrasonic waves may be produced by, for example, spark discharges, for practical purposes generators of ultrasonics in liquids may be divided into three types, (1) the Pohlmann Whistle, (2) the Magnetostriction Generator, (3) the Piezo-electric Generator.

The Pohlmann Whistle (Fig. 1).—This apparatus[2] and its modifications[3] consist of a nozzle from which a high pressure jet of liquid impinges upon a thin steel blade, which then vibrates at its natural frequency, transmitting the oscillations to the liquid in which it is immersed. A pump is required to drive the jet. For the emulsification of two immiscible liquids the nozzle and blade are immersed in a tank containing one phase, the second phase being forced through the nozzle into the first. The emulsion so formed is re-cycled through the system. (Fig. 2). A frequency of up to about 30 kc./second is obtained with this type of

generator, which therefore operates at the lower end of the ultrasonic spectrum. It has the advantage of comparative cheapness and further research may well produce an apparatus suitable for the large scale production of emulsions, to which end its use is probably limited.

The Magnetostriction Generator. (Fig. 3).—Some ferromagnetic materials such as nickel contract when placed in a magnetic field. A nickel rod, tube or laminate[4] placed inside a solenoid through which flows an alternating current will therefore vibrate in its longitudinal axis. the vibration being strongest when the frequency of this current corresponds to the natural frequency of the material. Ultrasonic waves are transmitted to the liquid in which the rod is immersed, providing, of course,

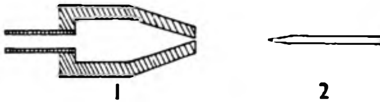

FIG. 1. The Pohlmann whistle.
1. Nozzle. 2. Blade.

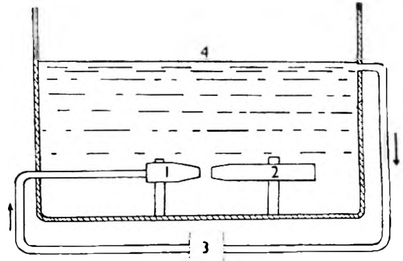

FIG. 2. The Pohlmann whistle as an ultrasonic emulsifier.
1. Nozzle. 2. Blade. 3. Pump. 4. Emulsion.

that the exciting current frequency and the natural frequency of the rod are in the ultrasonic range. The normal frequency range for this type of apparatus is up to about 50 kc./second but laminates have been produced which operate at frequencies of up to 200 kc./second. Eddy current heating produces losses, and another disadvantage is fatigue in the metal which lowers efficiency. This part of the apparatus is, however, readily replaceable. The dimensions of this apparatus are limited by the mechanical properties of the magnetostriction material and as yet it has not been scaled for industrial use. It is nevertheless a useful tool for small scale work and research. Intensities of up to 25 W/sq. cm. cross sectional area of rod have been developed in a commercial model[5]. This type of apparatus forms the basis for the ultrasonic soldering iron.

The Piezo-electric Generator.—In 1880 Pierre and Jacques Curie discovered that certain crystals such as quartz, tourmaline and Rochelle salt exhibited electrical charges upon their surfaces when subjected to mechanical pressure—the so-called "piezo-electric effect." The converse effect, namely that the application of an electrical field of force produces an expansion or contraction of the crystal, was predicted from thermodynamic considerations and later experimentally confirmed by the Curies, and forms the basis upon which the so-called "crystal generators" work. These generators are capable of producing ultrasonic vibrations at much higher frequencies and higher intensities than the types previously mentioned. For this reason many of the investigations into the biological effects

of such vibrations have been carried out with piezo-electric generators[6]. Figure 4 shows the theoretical circuit diagram of a typical apparatus. The crystal, which is supplied with radiofrequency current at high voltage by a valve oscillator (usually a modified Hartley circuit), expands and contracts in sympathy with the applied electric field and transmits vibrations to the surrounding medium in which it is immersed. Maximum intensity is obtained when the frequency of the applied alternating field is the same as the natural or resonant frequency of the crystal, such a condition usually being obtained by tuning the oscillator to the crystal frequency. The upper limit of ultrasonic frequency is determined in particular by the physical properties and type of crystal used. Experiments have been

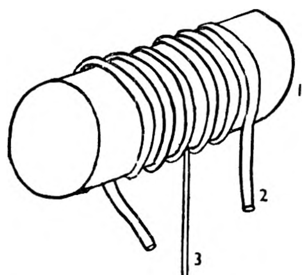

FIG. 3. Magnetostriction device. 1. Nickel rod. 2. Coil through which passes high frequency current. 3. Clamp.

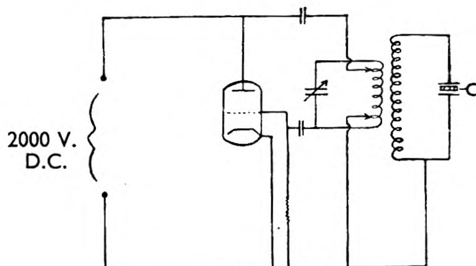

FIG. 4. Theoretical diagram of a typical piezo-electric generator. C. Quartz-crystal.

made with barium titanate[7] and titanate ceramics[8], but quartz is generally used in the form of an X-cut disc with silver electrodes sputtered on to each face. The mechanical strength of piezo-electric crystals has been investigated[9]. If the radiofrequency *current* is too large the crystal may be shattered; power is therefore best applied at high voltage and low current values. However, the higher the frequency the thinner the quartz disc and hence the closer together the electrodes on the crystal surfaces. With the high voltages used (up to 50 to 60 kilovolts in some cases) insulation, arcing and crystal fracture become major problems. Mounting in transformer oil assists in many cases but other ingenious methods of crystal mounting have been devised[10,11,12–21], some[22] for the direct irradiation of aqueous liquids, others for the concentration of the ultrasonic beam by means of spherical mirrors[23–25], or curved quartz crystals[26–28]. Lenses have been used for focussing and "transmission plates" of calculated thickness have been developed[29] for the low loss transmission of ultrasonic energy from one medium to another.

The efficiency of the crystal alone ranges from about 75 to 90 per cent. i.e., about 75 to 90 per cent. of the electrical energy supplied to the crystal is converted into acoustic energy. However the overall efficiency of the whole apparatus including the oscillator is about 20 to 40 per cent., which is about the same order as for the magnetostriction generator. Power levels of up to 500 to 1000 watts have been obtained but at these levels the production of heat in the bath becomes a source of trouble.

The piezo-electric generator, producing as it does high frequency waves of high intensity, has been much used for the irradiation of biological material, for it is at high intensities that many of the now well known effects appear to be exhibited.

Applications

The types of problem within the compass indicated by the title of this article to which ultrasonic acoustic waves have been applied are numerous. They may conveniently be considered under two headings, (1) *Biological* and (2) *Chemical and Physical.*

Biological Applications.—A number of review articles are available on the biological effects of ultrasonic irradiation (e.g., Crawford[5], Dognon and Biancani[30]) and these provide a useful introduction to the subject as a whole, although the latter is now outdated in some respects. In general, the biological material is suspended in an aqueous medium, such as distilled water or normal saline solution, in a tube or ampoule which is placed close to the piezo-electric crystal in a suitable liquid medium such as water or transformer oil. The ultrasonic vibrations propagated by the crystal in this medium are transmitted through the bottom of the tube or ampoule into the suspension. (Fig. 5).

The first observations of the biological action of ultrasonics were made by Langevin in 1917 who noted the sensation of heat produced in the hand when immersed in a liquid irradiated with ultrasonic vibrations. The initial loss of powers of orientation was observed in fish irradiated in a tank of water, further treatment resulting in temporary paralysis which passed off when the beam was switched off. More prolonged exposure resulted in death. Some years later Wood and Loomis[31] reported the destructive action on infusoria and Johnson[32] found that at high intensities a suspension of *Paramœcium* entirely and almost instantaneously disappeared. Harvey and Loomis[33] destroyed certain luminous bacteria, and Williams and Gaines[34] on *E. coli*, Beckwith and Olsen[35] on yeast and Hopwood[36] on vaccinia virus, demonstrated the lethal action of ultrasound on micro-organisms. The effect on yeast[37] appears to be greater with more prolonged exposure but to remain inferior to that exhibited on bacteria. The causative agents of anthrax, dysentery and whooping cough have been destroyed, also *Staphylococcus aureus* and *E. coli*[38,39,40,41]. Grabar and Rouyer[42] at the Pasteur Institute in Paris obtained a 40 per cent. mortality rate on *B. paradysenteriæ* using a low power generator, but a 98 per cent. death rate with one of higher power. Their experiments on a number of bacteria are of interest. Thick suspensions of the micro-organisms in Ringer's solution, normal saline solution or isotonic phosphate solution were placed in a tube of 4 cm. diameter, the bottom being closed by a cellophane membrane. The tube was placed in water over the quartz crystal with the base about 1 cm. from this, and so centred as to obtain maximum agitation within the suspension. An acoustic intensity of 31 W/sq. cm. was produced at the crystal surface and the frequency used was either 320 or 680 kc./second. The suspension was irradiated for definite periods of time, at the completion of which counts were made by plating out suitable

dilutions. Their results are reproduced in Table II. The authors suggest that the mortality rates shown for *B. anthracis* and *B. megatherium* are below the true level because of the liberation of individuals, arising from dis-aggregation of the chains, each giving rise to a colony when plated out. Even so the mortality rate approaches 100 per cent. in three cases. Laporte

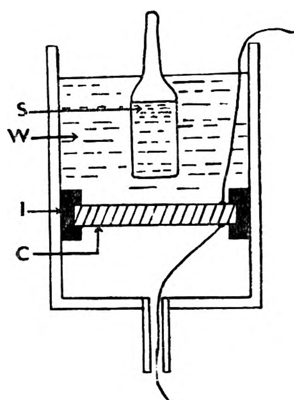

FIG. 5. Quartz crystal and irradiation chamber.

C. Crystal. W. Water
I. Insulation. S. Specimen

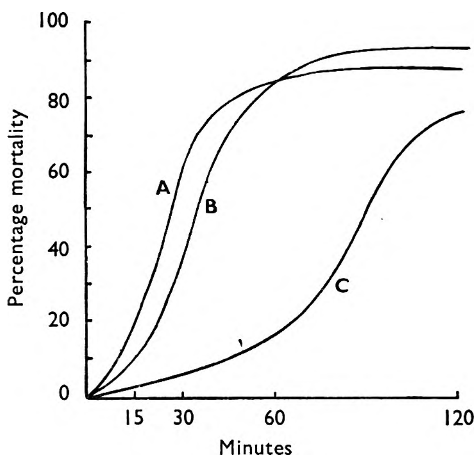

FIG. 6. *Myco. tuberculosis*; percentage mortality of different strains during irradiation with ultrasonics.

A. B.C.G. B. M.6. C. L.A.

and Loiseleur[43] have reported different effects with different strains of *Myco. tuberculosis*, BCG, M6 and LA. Young cultures of age 10 to 15 hours were used, and the results (Fig. 6) show that the rate of disintegration is a function of the time of irradiation. Loiseleur[44] has drawn attention to the similarity of these curves to those obtained by Latarjet[45] by irradiation with ultra-violet light. From results obtained with *E. coli*, Horwood,

TABLE II

Organism	Concentration per ml.	Percentage mortality	Time of exposure minutes	Frequency kilocycles
B. paradysenteriæ	$6 \cdot 2 \times 10^6$	98·0	30	320
B. anthracis	$3 \cdot 5 \times 10^8$	97·5	45	„
B. megatherium, Multilat strain ..	$4 \cdot 4 \times 10^8$	99·7	45	„
Staphylococcus aureus	$40 \cdot 2 \times 10^9$	90·4	45	„
Myco. tuberculosis, BCG strain ..	$3 \cdot 8 \times 10^9$	75·0	75	„
B. dysenteriæ Shigæ	$120 \cdot 8 \times 10^9$	88·0	30	680
Saccharomyces ellipsoideus	$1 \cdot 3 \times 10^9$	85·0	30	„

Norton and Minch[46] have suggested that the bactericidal effect of ultrasonics is a logarithmic function of time of exposure, and that old cultures are more susceptible than young ones.

An important advance has been the extraction of microbial contents[42] and endotoxins[47]. Suspensions of bacteria in distilled water are disintegrated by ultrasonic vibrations. The thick suspensions clear, are centrifuged to throw down the cellular debris and the clear supernatant liquid is filtered through a collodion membrane.

Vaccinia virus[48] and tobacco mosaic virus[49,50] have also been destroyed, and certain bacterial suspensions, after some irradiation, have been shown to remain agglutinable by their anti-sera, but the type of agglutination is modified[51]. Bacteriophage of varying size from 75 mμ to 21 mμ has been destroyed, but the effect was less evident in the size range 8 to 12 mμ[52]. By means of high speed cinematography Harvey and Loomis[53] found that sea urchin's eggs were disintegrated in less than 1/1200th of a second, this being the interval of time between successive frames on the film.

Because of the exploratory nature of this work no cases appear to have been reported in which all the variables of the system have been defined and measured. Hence it is difficult to evaluate much of the research and also to obtain reproducible results. It is even more difficult to make critical comparisons. For example, the measurement of ultrasonic energy actually dissipated within the specimen tube has not proved possible to date, although the use of a transistor to measure small rises in temperature may assist in this problem. The ultrasonic energy is not necessarily distributed evenly throughout the beam. Boyle *et al.*[54] have shown that the greater portion of the energy generated is concentrated in a central diffraction beam arising at a distance from the crystal determined by its radius and frequency and having an angular spread which is also a function of these quantities. Wood and Loomis[6] point out that only when the distance between the vibrating source and the floor of the radiation chamber or tube is an integral number of half wavelengths is the intensity of the radiation a maximum. However, Smith and Stumpf[55] suggest that under these conditions coupling may result which alters the natural frequency of the crystal. Boyle and Rawlinson[56] report that the thickness of this floor is critical in relation to the amount of energy transmitted to the bacterial suspension. Interfering secondary wave trains are also set up from the vibrating walls of the container[57]. The control of heat generated by ultrasonic irradiation is of major importance in experiments with bacteria, for the latter might be destroyed by the heat alone or the action of the waves might be modified. The crystal frequency also varies with temperature. The provision of cooling coils or the addition of ice to the liquid in which the crystal is immersed have been found useful, but a system of thermostatic control seems to be indicated. The type, age and concentration of the bacterial suspension, the medium used and the methods adopted for the determination of the population appear to require definition. Attempts to solve problems of control in quantitative studies of bactericidal effects[53] have not yet succeeded however.

Nevertheless, certain inferences may be drawn from the papers considered above. Most workers e.g.[59,60,61] are of the opinion that the death of biological material is caused by disruption of the cells which electron microscopy shows does occur[62], and protein denaturation probably plays a

part[60]. Oxidation does not appear to be responsible as lethal effects are still observed in the presence of gases other than dissolved air[52,63], although one author[44] suggests that oxygen may play a part. Cavitation does not appear to take place in the absence of dissolved gases and death of the organisms seems not to occur in the absence of this phenomenon. According to Marinesco[64], the disintegration of micro-organisms is caused by the differences in pressure which exist between the nodes and crests of the ultrasonic wave system. These positions are separated by half a wavelength, being closer together when the frequency is greater. This may imply a more effective action at higher frequencies. It is not yet possible to examine this latter hypothesis as present high power generators are limited to an upper frequency limit of about 1 to 3 megacycles/second, imposed by the physical properties and design of the quartz crystal. Examination of many other piezo-electric materials seems to be indicated with a view to raising this limit to much higher frequencies. It appears to be more probable, however, that the large disruptive forces produced by rapidly varying pressures and particle accelerations, previously referred to, play the major part in the lethal activity of ultrasonic waves.

These lethal effects are of interest to pharmacists from the point of view of their possible use as a method for the sterilisation of injections. Most of the bacterial suspensions used so far have consisted of fairly large populations. The results obtained by repetition on small concentrations would probably be interesting, as would the examination of the bacteria not destroyed. The influence of the waves on the medicaments present in the injection would require investigation in each instance in view of the chemical and physical effects reviewed below. Once the variable factors concerned in the application of ultrasonic energy have been defined and controlled, a large scale investigation along these lines appears to be indicated. From the industrial point of view, the major difficulty is one of economics in view of the length of time of irradiation which appears to be necessary and the fact that only one ampoule could be treated at a time with present apparatus. If the time factor could be greatly reduced commercial application may prove to be a worthwhile proposition. Sufficient evidence seems to be presented in the foregoing papers to suggest a fruitful field of research in this direction.

Of interest in the botanical field is the stimulation of plant growth by the treatment of seeds, although a decrease in percentage germination has been recorded by one author[65]. Successful results have been claimed in the treatment of rheumatism and sciatica[66,67]. This may be due to the thermal or vibratory effects or a combination of both. An apparatus for under water massage has been described[68]. The effect of high frequency sound waves on heart muscle and other irritable tissues has been investigated[69]. Ultrasonics have also been applied to problems connected with water purification[70]. In the gastronomic field, the application to the more rapid ripening of cheese and ageing of wines will probably horrify the gourmet.

Chemical and Physical Applications.—The catalogue describing a well-known ultrasonic generator[71] contains, amongst others, a list of the following applications:—induction of molecular rearrangement,

homogenisation of milk, acceleration of chemical reactions, transformation of chemical compounds, flocculation of suspended particles in liquids, emulsification of oil and water[65,72] and mercury and water, increasing the rate of oxidation reactions, dextrinisation of starch, decomposition of gums and gelatin. Oxidation of potassium iodide, hydrogen sulphide and carbon tetrachloride have been demonstrated and the first of these has been used, together with starch, in an attempt to obtain a colorimetric estimation of ultrasonic energy. High polymers have been broken[74] at their co-valent bonds[75]. Other applications are the formation of colloids[76] and their coagulation[77], and the formation of fine grain particles during precipitation. The latter may prove useful in the preparation of photographic emulsions. The clotting time of fibrinogen and the molecular distribution of hæmocyanin have been shown to be considerably modified[78]. Albumin has been split into its component amino-acids and these amino-acids themselves further degraded. Preferential disruption appears to occur in amino-acids containing a cyclic structure (e.g., tryptophane, tyrosine, histidine) and the formation of aspartic acid from histidine is claimed[79]. An interesting and useful feature of these degradations is that the usual methods of acid or alkali hydrolysis are not required and hence the products are obtained in a pure state, no extraneous chemicals having been added.

Critical evaluation of much of this work is difficult for the same reasons which apply to the biological research. The amount of ultrasonic energy required to produce the above effects is not always determined and the operating frequency of the apparatus is sometimes not given. Anomalies occur which require explanation. For example, the emulsification of mercury and water does not require cavitation[72], but these emulsions are not so stable as those of the oil-in-water type which are only produced in the presence of cavitation. Most immiscible liquids are emulsified by ultrasonic irradiation even in the absence of an emulsifying agent. Emulsions may, on the other hand, be cracked by ultrasonics. It seems probable that minute droplets of one phase, formed by the violent disruptive effects of the waves, are thrown into the other phase. If these droplets are sufficiently small an emulsion results. However, the immense forces of acceleration produced in these particles increase their chance of collision and consequent coalescence. This may explain the apparent anomaly.

There seems little doubt that the fracture of macro-molecules is due to these forces of acceleration giving rise to large frictional forces between the macro-molecule and the molecules of the solvent. Presumably, the larger the macro-molecule the greater is its inertia and hence its liability to fracture. Mark[75] has calculated the order of size of these forces when acting on certain bonds and shown them to be greater than the forces of chemical combination. Photochemical reactions have been observed and Frenkel[80] has attempted to explain these by suggesting the formation of electrical charges of opposite sign on opposite walls of the cavitation bubbles. The bubble then acts as a condenser and under certain conditions it is possible that flash over occurs with the production of light,

mainly in the ultra-violet region. Luminescence has been observed to occur in liquids in the dark.

In order to account for oxidation reactions many workers (e.g., Wheyl and Marboe[81]) consider that the aqueous medium is split into oxygen and hydrogen atoms which are electrically charged or chemically unsaturated and hence highly reactive. Peroxides and free hydroxyl groups may also be formed. It is possible to explain the acceleration of so many chemical reactions by postulating the breakdown of the Nernst diffusion layer.

Conclusion

That the science of ultrasonics has come to stay there is no doubt; that it is still in its infancy cannot be denied. Most of the research dealt with in this article can only be considered as exploratory in nature. Considerable pioneer work remains to be done, particularly in the fields of control and measurement of the ultrasonic radiation. It is essential first to establish suitable conditions under which reproducible results are possible. The development of more versatile apparatus capable of producing higher frequencies may produce interesting results, but it is well to bear in mind that solution of problems in this direction may bring new difficulties. For instance, the absorption of ultrasonic energy by a liquid increases as the square of the frequency. Less penetration and greater heat generation will follow.

From a purely pharmaceutical point of view, the following future possibilities suggest themselves:—decreased times for extraction processes, sterilisation of injections and surgical instruments, the breakdown of complex plant constituents without the addition of reagents for the purpose of the elucidation of structure, the investigation of thixotropic substances and gels. The identification of liquids by the measurement of the velocity of ultrasound propagated in them is already possible[72]; the measurement of absorption may also be used, and the technique can be applied to the differentiation of chemical isomers. The velocity of sound in a mixture of liquids depends upon the exact composition of the mixture; here is a pointer to the automatic control of reactions in a continuous system. No doubt many other possible uses will occur to the reader.

My thanks are due to Mr. R. G. Atkinson, F.P.S., for drawing my attention to some of the Russian literature which he kindly undertook to translate. Most of the literature search was conducted whilst the author held an appointment as Assistant Lecturer in Pharmacy at the University of Manchester under Professor H. Brindle, M.Sc., F.R.I.C., F.P.S., to whom thanks are due for his interest and encouragement.

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RESEARCH PAPERS

A SPECIFIC METHOD FOR THE DETERMINATION OF MORPHINE

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Received June 2, 1954

Introduction

Of the many colour reactions which morphine undergoes[1], only a few are applicable to its determination; the most frequently used and one included in the British Pharmacopœia 1953 is the reaction with nitrous acid. Even this reaction is not specific, however, and interference by other phenolic substances and by impurities accompanying morphine has frequently been reported[2]. The disadvantage of the lack of specificity has recently[3] been overcome in a novel manner by the successive reaction of morphine with a series of reagents: the first was iodic acid, which attacked morphine with liberation of iodine; secondly, ammonium carbonate was added which deepened the yellow-brown colour generated in the first stage; the product was then complexed with ferric chloride which produced a reddish violet colour (absorption maximum at 520 mμ) suitable for the absorptiometric and spectrophotometric determination of morphine[4]. This method has found use in toxicological investigations and has been applied to the determination of morphine in opium and opium preparations[5].

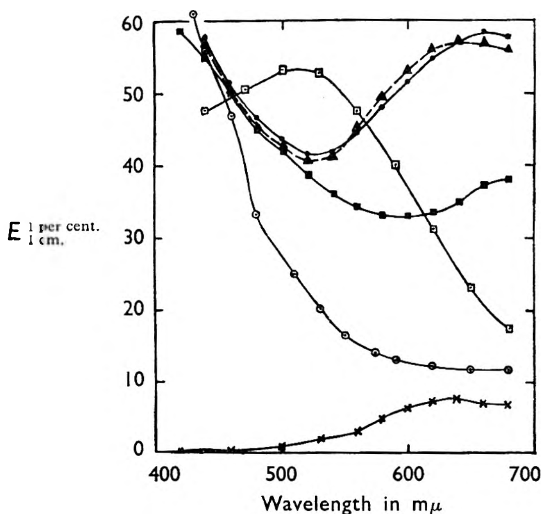


Fig. 1. Absorption curves of the iodic acid—ammonium bicarbonate—metal salt complexes of morphine.
—●—●— Nickel chloride.
—▲—▲— Cupric sulphate.
—▪—▪— Cobaltous chloride.
—⊡—⊡— Ferric chloride.
—⊙—⊙— Solution without metal ions.
—×—×— Morphine-ammonium bicarbonate-cupric sulphate solution (without iodic acid).

This series of reactions was highly specific, and of 2000 substances of pharmaceutical interest examined qualitatively none could be mistaken

for morphine[6], although some, notably apomorphine, physostigmine, and hyoscine gave interfering colours. The complex formation with ferric chloride suffered from some disadvantages, however: thus, the amount of ferric chloride added was critical because an excess gave rise to a gelatinous precipitate that interfered with colorimetry. Moreover, the absorption maximum near 520 mμ was subject to considerable interference by background absorption. These disdavantages were overcome[7] by substitution of nickel sulphate for ferric chloride, when a stable green complex (absorption maximum near 670 mμ) was slowly formed.

It was the purpose of the work reported here to adapt the method leading to the formation of the green complex for routine use, to examine the specificity of the reaction, and to apply it to the determination of morphine in solution, particularly in solutions of natural origin. In the course of this work, coloured complexes were obtained also with copper and cobalt salts (see Fig. 1), but these were less suitable than the nickel complex for routine spectrophotometry.

Development and Application of the Method

1. *Examination of Experimental Conditions*

For the development of the routine method, the experimental variables were investigated in order to find a convenient procedure that would yield the green complex with reproducible and maximum extinction at the absorption maximum. In this work, the effect on the absorption

TABLE I

EFFECT OF VARYING THE CONCENTRATIONS OF IODIC ACID AND OF HYDROCHLORIC ACID ON THE ABSORPTION INTENSITY OF THE GREEN COMPLEX

Reaction time at the first stage was 2 minutes and at second stage 90 minutes. The complexing reagent contained 8·0 per cent. w/v of ammonium bicarbonate, 1·0 N ammonium chloride, and 0·1 per cent. of nickel chloride in 0·20 N aqueous ammonia.

Concentration of Hydrochloric acid in medium — N	Concentration of Iodic acid per cent. w/v	Final pH	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 530 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
0·00	4·0	8·21	39·8	47·8
	6·0	8·14	41·6	53·0
0·02	1·0	—	22·6	29·3
	2·0	—	36·1	44·6
	4·0	8·13	42·8	55·9
	5·0	—	43·3	58·6
	6·0	—	42·8	59·6
0·05	1·0	—	31·8	41·2
	2·0	8·08	40·4	52·8
	4·0	8·04	42·8	58·5
	5·0	—	42·8	58·6
	6·0	8·01	42·1	59·0
0·10	1·0	—	38·8	51·0
	2·0	—	39·2	53·6
	4·0	7·95	38·5	53·2
	5·0	—	37·5	52·8
	6·0	—	36·8	51·9
0·25	1·0	—	27·2	34·4
	2·0	—	30·2	40·3
	5·0	—	22·2	25·8

intensity of varying, in turn, each of the experimental conditions was examined and the final selection of the standard routine procedure (detailed in the experimental section) was made on the basis of the results

TABLE II

EFFECT OF VARYING REACTION TIMES ON THE ABSORPTION INTENSITY OF THE GREEN COMPLEX

At the first stage 4·5 per cent. w/v of iodic acid was added to the morphine solution in 0·05 N hydrochloric acid. The standard complexing reagent was used (see Table I and experimental section).

Reaction time at first (iodic acid) stage minutes	Reaction time at second stage minutes	Final *p*H	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 530 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
	60	—	35·6	48·5
	90	—	36·2	50·0
0·5	120	8·08	36·6	51·3
	190	—	36·8	51·2
	300	8·09	36·4	50·1
	60	—	40·4	56·3
	90	—	42·7	59·1
2·0	120	8·08	43·1	59·4
	190	—	43·1	59·1
	300	8·08	42·0	58·0
	60	—	40·2	54·9
	90	—	41·7	57·6
5·0	120	8·06	42·1	58·5
	190	—	42·0	57·6
	300	8·11	41·5	55·9

TABLE III

EFFECT OF VARYING THE CONCENTRATION OF AMMONIUM BICARBONATE IN THE COMPLEXING REAGENT ON THE ABSORPTION INTENSITY OF THE GREEN COMPLEX

Reaction time at the first stage was 2 minutes and at the second stage 90 minutes. At the first stage 4·5 per cent. w/v of iodic acid was added to the morphine solution in 0·05 N hydrochloric acid. The complexing reagent contained, besides ammonium bicarbonate, 1 N ammonium chloride and 0·1 per cent. of nickel chloride in 0·20 N aqueous ammonia.

Concentration of ammonium bicarbonate per cent. w/v	Final *p*H	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 530 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
4·0	8·16*	39·2	50·9
8·0	8·06	42·4	58·6
12·0	8·04	41·6	57·6

* Final *p*H had to be increased from 7·7, which was well below the optimum, by addition of a few drops of concentrated ammonia solution, before the reaction mixture was adjusted to its final volume.

given in Tables I to VI. In particular, it was found that, in the reaction of iodic acid with morphine, the concentration both of iodic acid and of hydrochloric acid (Table I) and the time of reaction (Table II) were critical. For the subsequent stage, the most consistent results were obtained with a complexing reagent which comprised ammonium bicarbonate and ammonium chloride in aqueous ammonia solution, and in which the nickel salt was incorporated with advantage (*cf.* Tables III, IV and V). The *p*H of the final solution was found to be critical.

It also appeared that the final absorption intensity was higher when chloride (instead of sulphate) ions were present throughout (*cf.* Table VI). Colour development was complete after about 90 minutes (see Table II and Fig. 6).

TABLE IV

EFFECT OF VARIATION IN THE COMPOSITION OF THE COMPLEXING REAGENT ON THE ABSORPTION INTENSITY OF THE FINAL COMPLEX

Time of reaction was 2 minutes at the first and 90 minutes at the second stage. At the first stage, the concentration of iodic acid added was 4·5 per cent. w/v, and the strength of hydrochloric acid 0·05 N. The complexing reagent contained 8·0 per cent. w/v of ammonium bicarbonate and 0·1 per cent. nickel chloride, as well as ammonium chloride and ammonia.

Concentration of ammonia — N	Concentration of ammonium chloride — M	*p*H of reagent	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
0·10	—	8·10	46·9
0·15	—	8·25	47·4
0·20	—	8·35	46·6
0·25	—	8·45	45·7
0·30	—	8·50	44·5
0·20	0·25	8·40	55·0
0·10	1·00	7·90	56·0
0·15	1·00	8·00	58·7
0·20	1·00	8·10	58·7
0·25	1·00	8·15	58·7
0·30	1·00	8·30	57·0
0·30	1·60	8·25	59·8

The complexing reagent and reaction conditions finally selected gave reproducible results of high sensitivity (*cf.* Fig. 5). Moreover, accidental slight variations in the experimental procedure always led to lower results than were obtained by the normal procedure. Fresh solutions of the reagent had to be prepared each day because of its instability. The solid ammonium bicarbonate and the stock solution of ammonia from which the reagent was prepared also tended to be unstable. Another method of preparation of the complexing reagent from more stable materials was therefore examined. The reagent still contained the same concentrations of ammonium and carbonate ions as the original reagent but was prepared from sodium bicarbonate, ammonium chloride and nickel chloride in aqueous sodium hydroxide solution. By the standard reaction procedure, however, it gave a complex of rather lower absorption intensity (see

TABLE V

EFFECT OF VARYING THE CONCENTRATION OF NICKEL CHLORIDE IN THE COMPLEXING REAGENT ON THE ABSORPTION INTENSITY OF THE GREEN COMPLEX

The standard reagents and reaction conditions were used.

Concentration of nickel chloride per cent. w/v	Final *p*H	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 530 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
0·05	8·04	44·4	59·0
0·10	8·00	43·1	59·3
0·20	8·00	42·9	59·1

Table VII). The use of the original reagent for the routine method was therefore continued.

2. *Specificity of the Method*

The specificity of the iodic acid—ammonium carbonate—nickel ion reaction has not hitherto received much attention, although interference by thebaine has been reported[8]; this could not, however, be substantiated in the present investigation, in the course of which two series of substances were submitted to the routine procedure developed. The first series comprised substances such as narceine, narcotine, papaverine, cryptopine,

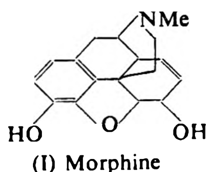
TABLE VI

EFFECT ON ABSORPTION INTENSITY OF SUBSTITUTION OF SULPHATE FOR CHLORIDE IONS IN THE REACTION MEDIUM

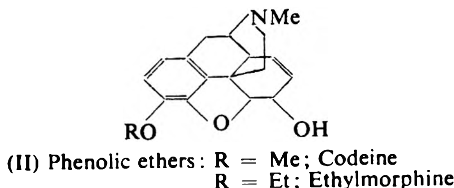
The standard reaction conditions were used.

Acid in initial solution 0·05 N	Ammonium salt 1·0 M	Nickel salt 0·1 per cent. w/v	Final *p*H	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 530 mμ	$E^{1\text{ per cent.}}_{1\text{ cm.}}$ at 670 mμ
sulphuric	chloride	chloride	7·92	40·4	51·4
		sulphate	7·92	40·4	51·4
	sulphate	chloride	8·00	44·5	54·1
		sulphate	8·00	44·5	54·1
hydrochloric	chloride	chloride	8·00	42·4	59·0
		sulphate	8·00	42·4	59·0
	sulphate	chloride	8·00	46·5	59·3
		sulphate	8·00	46·5	59·3

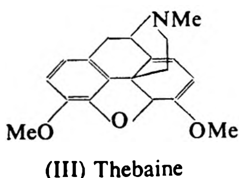
and meconic acid, occurring with morphine in vegetable material; none of these gave a coloured complex. The second series of substances comprised bases closely related to morphine. The natural products codeine, neopine, and thebaine gave no colour when treated by the standard procedure. The other phenolic ethers of morphine examined, namely dihydrocodeine, dihydrocodeinone, ethylmorphine, and morpholinoethylmorphine were also inert, as was diacetylmorphine.



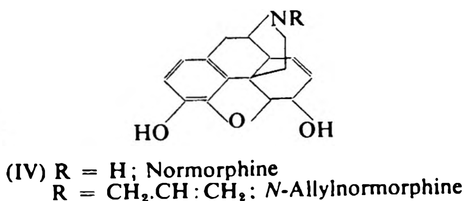
(I) Morphine



(II) Phenolic ethers: R = Me; Codeine
R = Et; Ethylmorphine



(III) Thebaine



(IV) R = H; Normorphine
R = $CH_2.CH:CH_2$: *N*-Allylnormorphine

In contrast, all morphine alkaloids and derived bases with the free phenolic hydroxyl group which were examined reacted with iodic acid and all except pseudomorphine (see below) gave a greenish colour after complex-formation with the ammonium bicarbonate—nickel chloride reagent.

TABLE VII

COMPARISON OF "STANDARD" REAGENT WITH A REAGENT PREPARED FROM MORE STABLE STOCK SOLUTIONS

Reaction conditions were otherwise identical.

Reagent	Composition of reagent			$E_{1\,cm.}^{1\,per\,cent.}$ at 670 mμ		
"Standard"	ammonium bicarbonate 8 per cent. w/v	ammonium chloride 1 M	ammonium hydroxide 0·20 M	58·2	58·4	58·7
"Stable"	sodium bicarbonate 8·4 per cent. w/v	ammonium chloride 2·2 M	sodium hydroxide 0·20 M	57·0	57·1	

The actual colour, i.e., the shape of the absorption curve and the position and intensity of the absorption maximum in the 600 to 700 mμ region, depended on the way in which the morphine molecule had been modified (see Figs. 2 and 3). The substituent at the nitrogen atom seemed to have little effect on complex formation and normorphine and *N*-allylnormorphine gave complexes with maxima at 670 mμ; in contrast, modification of the αβ-unsaturated alcohol system of morphine, by hydrogenation or etherification or both, caused a shift of the maximum to shorter wavelengths or even the complete disappearance of a well-defined maximum. Absence of this system and of the oxygen bridge as in *N*-methylmorphinan and in 3-hydroxy-*N*-methylmorphinan, resulted in inertness in the colour reaction.

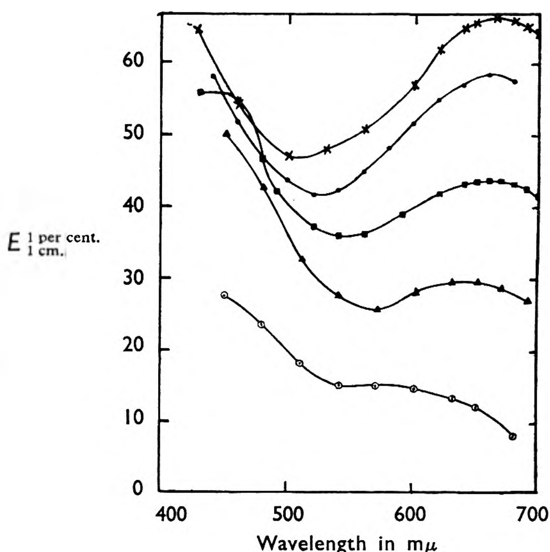


FIG. 2. Absorption curves of the iodic acid—ammonium bicarbonate—nickel chloride complexes of morphine derivatives.

—×—×— *N*-Allylnormorphine.
—●—●— Morphine.
—▪—▪— Normorphine.
—▲—▲— Dihydromorphine.
—⊙—⊙— Dihydromorphinone.

Apomorphine, the heterocyclic system of which differs from that of the morphine alkaloids, gave an orange colour with iodic acid which

on addition of ammonium bicarbonate immediately changed to blue-green: a green precipitate was gradually deposited. This sequence was unaffected by the presence of nickel.

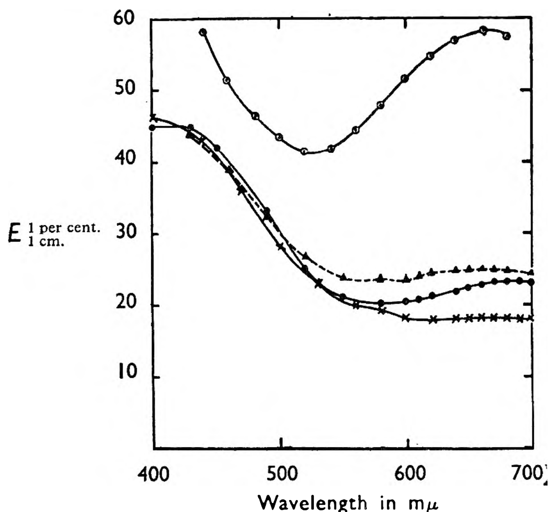


FIG. 3. Absorption curve of the iodic acid—ammonium bicarbonate—nickel chloride complexes of alcoholic ethers of morphine.
—⊙—⊙— Morphine.
—▲—▲— Heterocodeine.
—●—●— Heteroethylmorphine.
—×—×— Dihydroheterocodeine.

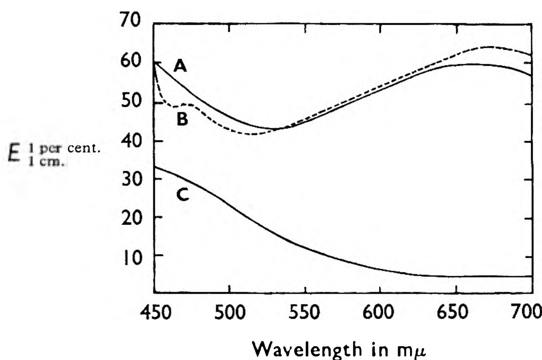


FIG. 4. Absorption curve of the iodic acid—ammonium bicarbonate—nickel chloride reaction product.
A. Morphine complex (on Unicam S.P. 350 spectrophotometer).
B. Morphine complex (on Unicam S.P. 500 spectrophotometer and Hilger Medium Quartz spectrograph).
C. Pseudomorphine product (on Unicam S.P. 350 spectrophotometer).

Pseudomorphine. As discussed above, the naturally occurring contaminants of morphine were inert under the standard conditions for the determination of morphine. Serious interference was encountered only with bases having the full morphine skeleton and a free phenolic hydroxyl group: of these, only the bimolecular pseudomorphine occurs with morphine in natural material. Pseudomorphine, however, does not give a green complex, but the brown colour of the iodic acid reaction product, which is deepended by addition of ammonium bicarbonate and unaffected by nickel (Fig. 4); this colour may be regarded as background absorption. Although the intensity of the absorption in the 500 to 700 mμ region is not high, it was thought of interest to examine the effect of interference by "pseudomorphine-like absorption" on the results of determinations of morphine, and a method of correcting for such background absorption was therefore devised.

It was assumed that the Beer-Lambert laws were obeyed. The wavelengths for extinction measurements were chosen at the maximum and minimum (670 and 530 mμ, respectively) of the absorption curve of the complex, where variation of extinction with wavelength was least. The extinction due to morphine in the mixture was denoted by K and that due to pseudomorphine by k; the observed total extinction at any wavelength, λ,

$$E_2 = \mathrm{K} + \mathrm{k}$$

The ratios of the extinctions at the two wavelengths chosen were denoted by r. Thus the observed ratio $r_o = \frac{E_{670}}{E_{530}}$,

$$\text{for pure morphine } r_m = \frac{K_{670}}{K_{530}}$$

$$\text{for pseudomorphine } r_p = \frac{k_{670}}{k_{530}}$$

$$\text{Then } E_{670} = K_{670} + r_p.k_{530}$$

$$\text{and } E_{530} = \frac{K_{670}}{r_m} + k_{530}$$

$$\text{By substitution } E_{670} = K_{670} + r_p E_{530} - \frac{r_p K_{670}}{r_m}$$

$$\text{Therefore } K_{670} = \frac{r_m\,(E_{670} - r_p E_{530})}{r_m - r_p}$$

$$\text{or} \quad \frac{K_{670}}{E_{670}} = \frac{r_m\,(1 - r_p E_{530}/E_{670})}{r_m - r_p} = \frac{r_m\,(1 - r_p/r_o)}{r_m - r_p}$$

Thus the corrected extinction value for morphine (K_{670}) was determined from the observed extinction (E_{670}) and from the ratio (r_o) of the observed extinction at the maximum and minimum of the absorption curve, the ratios r_m and r_p for pure morphine and pseudomorphine, respectively, having been previously determined from absorption curves.

Values for r_o and for K_{670}/E_{670} calculated are collected in Table VIII, and the effect of applying the correction to three synthetic mixtures of morphine and pseudomorphine is shown in Table IX.

From the results tabulated, it was deduced that interference by pseudomorphine or other substances producing similar background absorption was appreciable only when the amount of impurity exceeded the amount of morphine present. The method of correction adopted then produced results that tended to be somewhat low. The inaccuracy in this method of correction was probably due to the non-applicability of Beer's law to the pseudomorphine absorption and consequent variation in the ratio r_p.

3. *The Determination of Morphine in Opium and Poppy Capsule*

Morphine in opium is generally determined gravimetrically or volumetrically and the extracts obtained therefore have to be submitted

TABLE VIII

CORRECTION FOR BACKGROUND ABSORPTION DUE TO PSEUDOMORPHINE (AND SIMILAR SUBSTANCES)

Ratio r_0 represents the ratio of the extinction at 670 mμ to that at 530 mμ. Ratio K_{670}/E_{670} represents the ratio of the corrected extinction (due to morphine) to the observed extinction at 670 mμ: this is the correction factor.

r_0 (E_{670}/E_{530})	K_{670}/E_{670}	Fraction of total absorption due to morphine per cent.	Approximate content of pseudomorphine per cent.	Approximate error introduced by correction factor per cent.
1·400	1·000	100	Nil	
1·350	0·994			
1·300	0·987			
1·250	0·979			
1·200	0·971			
1·150	0·962			
1·100	0·952			
1·050	0·942	94	50	±0·5
1·000	0·930			
0·950	0·917			
0·900	0·902			
0·850	0·884			
0·800	0·868	87	73	±0·8
0·750	0·847			
0·600	0·765			
0·400	0·565			
0·365	0·501	50	94	±5·5
0·300	0·353			

TABLE IX

DETERMINATION OF MORPHINE IN ADMIXTURE WITH PSEUDOMORPHINE

Composition of mixture		Percentage content of morphine	r_0 (E_{670})/(E_{530})	Morphine found mg./10 ml.	
Morphine mg./10 ml.	Pseudomorphine mg./10 ml.			Without correction	With correction
1·92	Nil	100	1·40	1·92	1·92
1·92	0·96	66·7	1·17	1·93	1·89
1·92	1·92	50	1·00	1·98	1·87
1·92	3·84	33·3	0·83	2·03	1·83
Nil	3·84	0	0·21	—	—

to extensive and time-consuming purification. The specificity of the iodic acid—ammonium bicarbonate—nickel chloride reaction, however, permitted the determination of morphine in the crude extract obtained by slurrying opium with calcium hydroxide and water; adjustment of the *p*H and dilution were the only additional steps before the colour reaction. Some results obtained on 4 random samples of opium are collected in Table X, which also shows the effect of applying the correction for "pseudomorphine-like

TABLE X

DETERMINATION OF MORPHINE IN OPIUM

Sample	Morphine content: Present method, Uncorrected per cent.	Present method, Corrected per cent.	Modified B.P. method* per cent.
1	11·15	10·75	10·79
2	11·3	11·05	10·70
3	10·95	10·70	10·64
4	11·3	11·05	10·87

* Results obtained by Mr. D. R. Wood

colour." The results obtained by the present method, especially when thus corrected, were in satisfactory agreement with the results obtained by a modification of the B.P. method of assay.

Whilst opium contains about 10 per cent. of morphine, the morphine content of poppy capsules is generally between 0·1 per cent. and 0·7 per cent. and the extraction and purification procedures are correspondingly more difficult[9]. Again, a great saving in time and labour was possible by application of the iodic acid-ammonium bicarbonate—nickel chloride reaction, since morphine could be determined in comparatively crude solution. The results obtained were reproducible (see Table XI) and the amount of solid morphine isolated in the laboratory from a bulk sample of poppy capsule agreed satisfactorily with the morphine content indicated by analysis.

TABLE XI

DETERMINATION OF MORPHINE IN POPPY CAPSULE

REPRODUCIBILITY OF RESULTS

Sample	Morphine content per cent.		
	Unicam S.P. 350 Spectrophotometer	E.E.L. Absorptiometer*	
	Operator A	Operator B	Operator C
1	0·24	0·28	0·30
2	0·095	0·095	0·090
3	0·235	0·225	0·235
4	0·23	0·21	0·215
5	0·055	0·055	0·050
6	0·125	0·115	0·120
7	0·10	0·10	0·10

* Analyses by the control department of Messrs. T. & H. Smith, Ltd.

EXPERIMENTAL

Apparatus. Except where otherwise indicated, a Unicam S.P. 350 diffraction grating spectrophotometer was used with 1-cm. absorption cells. The band-width was ca. 30 mμ.

1. *Method*

Reagents. All inorganic reagents were of analytical reagent grade or B.P. quality. The organic bases used complied with B.P. specifications or were purified by the usual methods.

Ammonium bicarbonate (B.P. quality) was stored in stoppered bottles in a desiccator over silica gel.

Morphine was purified by two crystallisations as the hydrogen tartrate and then converted into the hydrochloride.

Boiled-out distilled water was used throughout.

Stock solutions. *A.* 4·5 per cent. w/v iodic acid.
B. 21·4 per cent. w/v ammonium chloride (i.e., 4 M).
C. 1·0 N aqueous ammonia solution.
D. 1·0 per cent. w/v nickel chloride.

Ammonium bicarbonate—nickel chloride reagent. Ammonium bicarbonate (8·0 g.) was shaken with a mixture of stock solutions *B* (25 ml.), *C* (20 ml.), and *D* (10 ml.). On dilution to 100 ml. a clear solution was obtained which was used only on the day of preparation.

Calibration curve. The calibration curve (Fig. 5) was constructed by use of solutions containing known amounts of pure morphine (4·0 to 40·0 mg. of anhydrous base per 100 ml.) in 0·05 N hydrochloric acid. 10-ml. aliquots of solution in a 25-ml. graduated flask were treated with aqueous iodic acid (2 ml. of stock solution *A*) for exactly 2 minutes; then the ammonium bicarbonate—nickel chloride solution (10 ml.) was added and the mixture diluted to 25 ml. Extinction readings were taken at the absorption maximum (670 mμ) after 90 minutes, when colour development was complete (see Fig. 6).

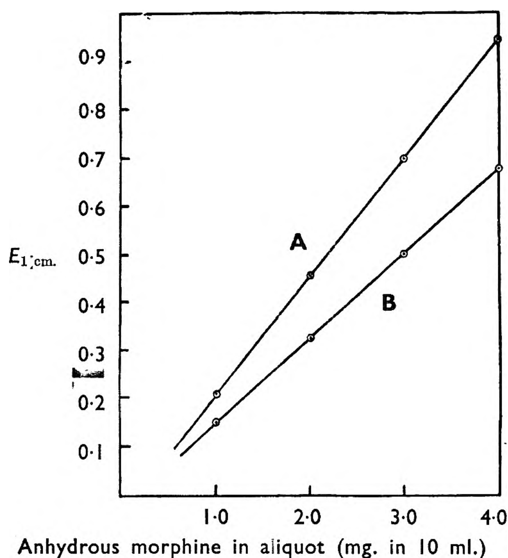


FIG. 5. Calibration curve for morphine (on the Unicam S.P. 350 spectrophotometer).
A. Extinction at 670 mμ (absorption maximum).
B. Extinction at 530 mμ (absorption minimum).

The "blank" solution consisted of a 10-ml. aliquot of the same morphine solution, to which was added, instead of iodic acid, 0·1 N hydrochloric acid (5 ml.) and then the complexing reagent (10 ml.).

To permit application of the correction for "pseudo - morphine - like background colour," similar calibration curves were constructed from extinction readings at the minimum (530 mμ) of the absorption curve, and for pseudomorphine from readings at 530 mμ and at 670 mμ.

For the work here reported the calibration curve for morphine at 670 mμ (Fig. 5) was fitted by the method of least squares to a series of results (collected in Table XII) obtained for 3 different samples of morphine at 5 dilutions. Room temperature during colour development was 20° ± 1·5° C.

Analysis of the results

x = Morphine content (mg. per 10 ml.) of aliquot

y = Extinction ($E_{1\,cm.}$).

Standard error of predicted x, S.E. (x), for y = 0·70 (i.e., in the optimum extinction range) = ± 0·021 (i.e., ± 0·7 per cent.) when x = 3·00. Therefore, 99 per cent. confidence limits for x (13 degrees of freedom, t = 3·01) = 3·00 ± 0·063 (i.e., ± 2·1 per cent.).

The results indicate that Beer's law applies, within the usual limits, over the range of concentrations examined.

Determination of morphine. The solution of unknown morphine content was first adjusted to pH 4 to 5 and then diluted until the morphine concentration was such that extinction readings were within the optimum range on the spectrophotometer (E = 0·3 to 0·7 for the Unicam SP 350); in the course of this dilution the hydrochloric acid concentration was adjusted to 0·05 N. One 10-ml. aliquot was then treated for 2 minutes exactly with iodic acid (2 ml. of stock solution A); the ammonium bicarbonate-nickel chloride reagent (10 ml.) was added, the solution diluted to 25 ml., and the extinction at 530 mμ and 670 mμ determined after 90 minutes. The "blank" was prepared from another 10-ml aliquot. The morphine content was then determined with the aid of the calibration curve at 670 mμ (Fig. 5), and corrected, if so desired, by application of the correction factors (see Table VIII)

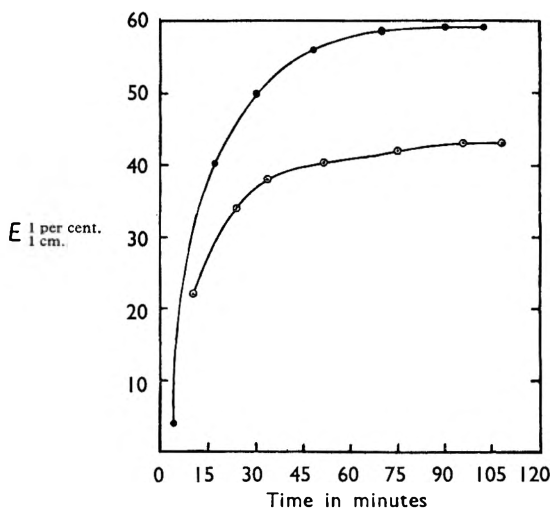


FIG. 6. Variation of extinction of the morphine iodic acid—ammonium bicarbonate—nickel chloride complex with time.
—●—●— Extinction at 670 mμ.
—⊙—⊙— Extinction at 530 mμ.

TABLE XII
DATA FOR CALIBRATION CURVE
VARIATION OF EXTINCTION AT 670mμ WITH MORPHINE CONCENTRATION

Sample	Morphine in aliquot mg./10 ml.	Extinction ($E_{1\text{ cm.}}$)
(i)	0·420	0·07
(ii)	0·425	0·07
(iii)	0·440	0·08
(i)	0·835	0·17
(ii)	0·850	0·17
(iii)	0·880	0·18
(i)	1·670	0·37
(ii)	1·700	0·38
(iii)	1·755	0·40
(i)	2·500	0·58
(ii)	2·550	0·60
(iii)	2·635	0·60
(i)	3·340	0·78
(ii)	3·400	0·80
(iii)	3·510	0·83

Precautions. The following precautions were taken to ensure valid and reproducible results in the routine application of the method:

(i) The pH of the solution (containing the correct amount of hydrochloric acid) was measured before analysis. The pH should be below 1·6. In the presence of buffering impurities, however, the pH may be above 1·6 and the iodic acid reaction will then not be complete within 2 minutes. When necessary, therefore, the morphine was extracted into an organic solvent, such as chloroform-ethanol, re-extracted into acid, and thus freed from buffering impurities.

(ii) The pH of the green solution after analysis was measured. This pH should be in the optimum range of pH 8·00 ± 0·05.

(iii) A standard morphine sample was included in each batch of samples analysed to check that the instrument response and the reagent composition (final pH value) were satisfactory.

2. *Determination of morphine in opium*

Water (25 ml.) was added to opium (5 g.) in a deep evaporating basin. The mixture was warmed and agitated until pasty. Calcium hydroxide (2 g.) was added to the cool paste and the mixture stirred until apparently homogeneous. After suction filtration, the cake was pressed dry, re-slurried with water (25 ml.), and again filtered. Washing by re-slurrying was repeated 4 times more. The combined filtrates were adjusted to pH 4 to 5 with hydrochloric acid and diluted to 500 ml. A 20-ml. aliquot of the resulting solution was treated with 1 N hydrochloric acid (5 ml.) and diluted to 100 ml. This solution was analysed as follows:—

Two 10-ml. aliquots were pipetted into 25-ml. graduated flasks. To one (the "blank") was added 0·1 N hydrochloric acid (5 ml.) and to the other iodic acid (2 ml. of stock solution *A*). After 2 minutes the ammonium bicarbonate–nickel chloride reagent (10 ml.) was added to each solution and the volume adjusted to 25 ml. The absorption intensity was measured after 90 minutes both at the absorption maximum at 670 mμ and at the minimum at 530 mμ, and the morphine content determined by reference to the calibration curves. The correction for "pseudo-morphine-like" background absorption was applied in the usual manner. Results are recorded in Table X.

3. *Determination of morphine in poppy capsule*

Ground poppy capsules (5 g.) were triturated with 10 per cent. w/v aqueous sodium carbonate solution (5 ml.) and kept for 1 hour. The stirred mixture was then treated with 20 per cent. w/v aqueous sodium carbonate solution (5 ml.) and shaken for 1 hour with a solvent mixture (45 ml.) of equal volumes of benzene and *n*-butanol. The resulting mass was suction-filtered and washed with 4 quantities, each of 5 ml. of the solvent mixture. The combined filtrate and washings were extracted with 0·5 N sulphuric acid (20 ml.) and then with water (2 quantities, each of 10 ml.). The aqueous layers were filtered through cotton wool, mixed, adjusted to pH 4 to 5, treated with 0·5 N hydrochloric acid (5 ml.) and diluted to 50 ml. A 10-ml. aliquot of this diluted solution was analysed by the routine procedure (see above). Typical results obtained by this procedure are given in Table XI.

Isolation of morphine from bulk sample of poppy capsule

A sample (500 g.) of ground poppy capsules (containing 0·25 per cent. of morphine by the above method of analysis) was mixed with 10 per cent. w/v aqueous sodium carbonate (500 ml.) and kept for 1 hour with occasional stirring. 20 per cent. w/v aqueous sodium

carbonate solution (500 ml.) was then added, the mass was strongly agitated, and finally Soxhlet-extracted with a mixture of equal volumes of benzene and *n*-butanol for 16 hours. The solvent was removed and the solid further extracted for 8 hours. The organic extracts were separately exhausted with 0·2 N hydrochloric acid and samples of the acidic solutions were analysed: the first extract contained 1·25 g. of morphine by assay (i.e., all the morphine originally present in the capsules), the second extract none. The acidic solution (2300 ml.) containing the morphine was concentrated to small bulk (340 ml.) under water-pump vacuum; the concentrate was made alkaline with concentrated aqueous ammonia solution and extracted with a mixture of chloroform (340 ml.) and ethanol (340 ml.). Eleven further extractions were done with chloroform (340 ml.) and ethanol (160 ml.). The mixed organic extracts were evaporated to dryness under water-pump vacuum, the residue was dissolved in aqueous acid (20 ml.) and the solution adjusted to *p*H 9 when morphine was precipitated. The dry solid (2·535 g.) was analysed both by an extraction method concluding in a volumetric determination and by the iodic acid—ammonium bicarbonate—nickel chloride method: both analyses showed the morphine content of the solid to be 47 per cent. (i.e., the presence of 1·195 g. of morphine; 95·6 per cent. of the morphine content originally found for the capsules). The filtrate was analysed by the latter method which showed a morphine content of 32 mg. (2·6 per cent. of the content determined for the capsules).

4. *Reaction of other substances*: *specificity of the method*

The following substances were submitted to the iodic acid–ammonium bicarbonate–nickel chloride reaction under the standard conditions, and were inert:—narceine, narcotine, papaverine, cryptopine, codeine, neopine, thebaine, dihydrocodeine, dihydrocodeinone, ethylmorphine, morpholinoethylmorphine, diacetylmorphine, *N*-methylmorphinan, 3-hydroxy-*N*-methylmorphinan, and meconic acid. The following substances gave nickel complexes (absorption curves of which are recorded in Figs. 2 and 3) under the standard conditions: normorphine, *N*-allylnormorphine, dihydromorphine, dihydromorphinone, heterocodeine, heteroethylmorphine, and dihydroheterocodeine. Pseudomorphine and apomorphine reacted with iodic acid-ammonium bicarbonate, but the products were unaffected by nickel. Pseudomorphine with iodic acid, gave a brown colour that deepened on addition of ammonium bicarbonate (see Fig. 4 for absorption curve); apomorphine with iodic acid gave an orange colour that changed to blue-green on addition of ammonium bicarbonate: a greenish precipitate was gradually deposited.

5. *Other metal complexes of the morphine-iodic acid reaction product*

In these experiments stock solution *D* (1·0 per cent. w/v nickel chloride) was replaced by (i) 0·5 per cent. w/v cupric sulphate; (ii) 1·0 per cent. w/v cobaltous chloride. The standard procedure was used for the formation of the metal complexes. The iron complex was obtained as

follows: a solution (10 ml.) of morphine in 0·05 N hydrochloric acid was treated first with iodic acid (2 ml. of stock solution *A*) for 2 minutes and then with the ammonium bicarbonate reagent (in absence of stock solution *D*). Then 6 drops of 1·0 per cent. w/v ferric chloride solution were added and the solution was diluted to 25 ml. The "blank" was prepared analogously, the iodic acid solution being replaced by 0·1 N hydrochloric acid (5 ml.). The absorption curves of the complexes are reproduced in Figure 1.

6. *Miscellaneous experiments exploring the reaction mechanism.*

(i) The iodine liberated in the reaction of iodic acid with morphine was rapidly extracted into carbon tetrachloride before addition of the ammonium bicarbonate-nickel chloride reagent. The green complex was formed as usual with the usual absorption intensity.

(ii) Sodium salts were substituted for ammonium salts in all the reagents. No green complex was formed, even when the *p*H of the final solution was adjusted to 8·00.

(iii) Ammonium hydrogen phosphate was substituted for ammonium bicarbonate in the reagent solution and the final *p*H was adjusted to 8·00. A green complex was formed, the absorption intensity of which at the absorption maximum (670 mμ) was much lower than usual ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ was 42·0, as compared with 58·7).

Discussion and Conclusions

The iodic acid-ammonium bicarbonate-nickel chloride method for the determination of morphine has proved satisfactory in routine operation when the critical factors, namely the time of reaction (particularly at the first stage), the hydrochloric acid concentration and *p*H of the solutions analysed, and the composition of the reagent were closely controlled. The great advantages of the method were its specificity and the low absorption intensity of any background colour in the region of maximum absorption of the stable green complex which compensated amply for the additional time required for the colour development of the nickel complex (as compared with that of the iron complex). The sensitivity of the method permitted determination of about 0·75 mg. of morphine in 10 ml. of solution; for about 3 mg. of morphine in 10 ml. of solution the accuracy was within ± 2 per cent.

The method has been applied successfully to solutions containing relatively pure morphine and also to crude extracts of vegetable origin. It can obviously be applied to other problems, such as the limit test for morphine in pharmaceutical materials (e.g., in codeine or morpholinoethylmorphine) or the decomposition of morphine in solution.

The mechanism of the reaction has not yet been elucidated. It has been suggested[3] that an *o*-diphenol may be formed by the action of iodic acid with morphine which forms a complex with ferric chloride under alkaline conditions. For the nickel complex, the *p*H range within which the absorption intensity is at its highest has been found to be very narrow: the intensity falls off rapidly at higher *p*H values, whilst the

complex dissociates completely in acidic solution. The reagent solution must therefore be a good buffer. Moreover, the experimental results obtained in the present work clearly indicate that the presence of ammonium ions is essential and that of carbonate ions is advantageous for complex formation. In this connection it is important that nickel, copper and cobalt which give complexes with absorption maxima in the same spectral region (650 to 670 mμ), also form ammines, and it is probable that the nature of the complexes derived from these metals differs from that of the iron complex for the formation of which ammonium ions are not essential.

The structural features of the morphine molecule affecting complex formation are the phenolic hydroxyl group and the $\alpha\beta$-unsaturated alcohol system (in ring *C*, Formula I). Changes at the nitrogen atom do not affect complex formation greatly. Little evidence on the effect of the oxygen bridge is available; it is of interest, however, that 3-hydroxy-*N*-methylmorphinan (in which this bridge and the unsaturated alcohol system are both absent) does not form the usual complex.

When the free phenolic hydroxyl group is blocked, either by etherification or by esterification, the molecule no longer reacts with iodic acid and no coloured complex is formed in the later stages. Etherification of the alcoholic hydroxyl group does not, of course, inhibit the iodic acid reaction and the fact that modification of the $\alpha\beta$-unsaturated alcohol system of morphine greatly affects the absorption curve of the final complex may probably be best explained in terms of the steric factors involved in complex formation. In morphine (*cis*-fused at C_{13} to C_{14}) the alicyclic ring (ring *C* in Formula I) is at a sharp angle to the rest of the molecule and the alcoholic hydroxyl group is close to the other oxygen atoms in space[10]. In dihydromorphine, where two extra hydrogen atoms are accommodated and the ethylenic linkage is saturated, the alicyclic ring (*C*) is, in consequence, somewhat distorted and complex formation appears to be affected by this change. Interference in complex formation by etherification of the alcoholic hydroxyl group can be explained in terms of the greater volume of the methoxy and ethoxy groups as compared with that of the hydroxy group; the bulkier groups may prevent a sufficiently close approach of the complexing centres to form the normal stable complex. This interpretation is supported by the fact that interference in heteroethylmorphine (ethoxy group) is greater than in heterocodeine (methoxy group) (see also Fig. 3).

Summary

1. The iodic acid-ammonium-carbonate-nickel salt reaction has been adapted for the routine spectrophotometric determination of morphine; the method is sensitive down to about 0·005 per cent. of morphine in solution and 0·03 per cent. of morphine can be determined with an accuracy within $\pm$ 2 per cent.

2. Of the bases occurring with morphine in nature only pseudomorphine interfered weakly and the method has been used for the rapid determination of morphine in opium and poppy capsule.

3. The factors affecting the formation of the green complex are briefly discussed.

The authors wish to thank Mr. E. G. Peppiatt for bringing this reaction to their notice, Mr. D. R. Wood for volumetric analyses and for valuable discussions, Mr. W. E. Simpson for help in the work on poppy capsule, and Mrs. I. P. S. Hardie and Mr. A. B. Veitch for technical assistance. They are greatly indebted to Dr. E. A. Braude for the absorption curve (B) in Figure 4, and to the following who kindly provided valuable samples: Dr. Lyndon F. Small for heterocodeine and heteroethylmorphine, Dr. Marshall Gates for *N*-methylmorphinan, and Messrs. T. & H. Smith Ltd. for *N*-allylnormorphine. Finally they would like to thank Mr. F. J. Bolton for his interest and encouragement and the Directors of J. F. Macfarlan & Co. Ltd., for permission to publish this work.

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THE INHIBITION OF CHOLINE ACETYLASE BY NICOTINE

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Received April 29, 1954

Recent investigations[1] into the action of nicotine on cellular metabolism have shown that the depressant effect of the drug on the respiration of brain-tissue may be attributed to its inhibitory action on the oxidation of pyruvate. A detailed study of the effects of nicotine on isolated steps of the citric acid cycle has revealed that pyruvic dehydrogenase, which catalyses the first step in pyruvate oxidation, is inhibited more strongly than are any of the other enzymes of the pyruvic oxidase system.

It is of interest to compare this action of nicotine with that of cocaine which specifically inhibits the second step of pyruvate oxidation[2].

Evidence has been obtained that some of the pharmacological effects of nicotine are increased by administration of cocaine[3].

Since the formation of active acetate is an essential reaction in the pyruvic dehydrogenase system it becomes of interest to discover whether or not nicotine interferes with other mechanisms in which active acetate is involved. We have therefore investigated the action of nicotine on purified choline acetylase of brain and on the sulphanilamide acetylating system of liver.

Experimental

Effect of nicotine on purified choline acetylase

Choline acetylase was prepared from acetone-dried rabbit brain and purified by dialysis after fractionation with ammonium sulphate according to the method of Nachmansohn, Hestrin and Voripaieff[4]. A boiled extract of washed brewer's yeast was used as the source of coenzyme A.

Mixtures (5·0 ml.), with and without nicotine, containing buffered enzyme solution (1·0 ml. = 250 mg. of dried brain), yeast extract (3·0 ml.) and, in final concentrations, potassium chloride (0·04M), choline (0·02M), acetate (0·02M), cysteine (0·02M), calcium chloride (0·002M), magnesium chloride (0·0002M), adenosine triphosphate (0·003M) and physostigmine (0·001M) were incubated at *p*H 7·0 and 37° C. in nitrogen-filled, stoppered tubes. Samples (2·0 ml.), withdrawn at zero time and after 1 hour, were deproteinised with trichloroacetic acid and, after readjusting the *p*H, were assayed for acetylcholine by the chemical method of Hestrin[5]. Measurements were made at 540 mμ using 1 cm. cuvettes in a Unicam Quartz Spectrophotometer. Nicotine did not interfere with the method.

Results are given in Table I.

TABLE I

EFFECT OF NICOTINE ON CHOLINE ACETYLASE

Experiment	Acetylcholine, μg./g. of dried brain/hour		Inhibition, per cent.
	—	Nicotine (0·015M)	
1	774	430	45
2	645	408	37
3	625	375	40
4	720	382	47

Effect of nicotine on sulphanilamide acetylation

In 3 experiments, nicotine (0·015M) had no effect on the rate of acetylation of sulphanilamide by extract of acetone-dried pigeon-liver as determined by the procedure of Johnson and Quastel[6].

DISCUSSION

The results of the present work show that nicotine, in a concentration which has comparatively little or no effect on most enzyme systems, inhibits appreciably the choline acetylating system of brain-tissue. Since nicotine does not inhibit sulphanilamide acetylation or the synthesis of citrate from acetate by baker's yeast[1], it appears that the drug is not a general inhibitor of all mechanisms in which active acetate is involved and that its action on choline acetylase is a specific one. While the effect on choline acetylase is not in itself sufficiently high to be of marked pharmacological significance, it must be realised that nicotine also inhibits the reaction whereby active acetate is produced from pyruvate and in all probability nerve-tissue depends, primarily if not exclusively, on pyruvate as the acetyl donor for choline acetylation. Since nicotine inhibits the initial step in pyruvate oxidation and, at the same time, stimulates glycolysis[7], the tendency for the local concentration of pyruvate to rise above the normal level would be an additional factor contributing towards the inhibition of acetylcholine synthesis. Nachmansohn and John[8] have shown that pyruvate, in common with other α-ketoacids, is a most powerful inhibitor of choline acetylase.

Evidence has also been obtained that nicotine interferes with coupled phosphorylation[9], thereby adding to the effects which follow from inhibition of the energy-yielding mechanism of carbohydrate oxidation. Under physiological conditions, therefore, the inhibitory action of nicotine on acetylcholine synthesis would be expected to be considerably greater than that due to direct action of the drug on choline acetylase. It has recently been shown, for example, that cocaine, which has no effect on choline acetylase, inhibits markedly the synthesis of acetylcholine in respiring fresh-brain homogenate[10]. In pharmacological opposition to the combined actions of nicotine which suppress acetylcholine synthesis is the ability of the drug to inhibit acetylcholine breakdown. Choline esterase of mammalian brain is inhibited by nicotine[11,12].

SUMMARY

1. Nicotine inhibits the isolated choline acetylase system of brain but has no action on the sulphanilamide acetylating system of liver.

2. Various mechanisms whereby nicotine inhibits acetylcholine synthesis in nerve are discussed.

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THE ROUTINE DETERMINATION OF THE ANTACID EFFICIENCY OF ALUMINIUM HYDROXIDE GELS

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Received April 28, 1954

The test for neutralising capacity specified in the monographs of the British Pharmaceutical Codex 1949 for aluminium hydroxide gel and dried aluminium hydroxide gel does not give a complete picture of the antacid action of the gels for two reasons. Firstly there is a large excess of acid present throughout the test, which is not normally the case *in vivo* and secondly the only determination made is of the total amount of acid neutralised at the end of the test period. This test eliminates the very poor quality dried gels but does not give any information on the rate at which the neutralisation occurs, and this is important in determining the therapeutic value of the gels. Many tests have been devised[1-8] to assess antacid efficiency but nearly all of these have had as their aim the comparison of a wide range of antacid substances. The work reported in this paper was undertaken to devise a routine test for aluminium hydroxide gels only and this limitation of scope has meant that some of the refinements of these general methods could be eliminated, provided that reproducibility could be obtained. The most recent methods published were those described at the British Pharmaceutical Conference 1953 by Armstrong and Martin[6]; Gore, Martin and Taylor[7]; and Brindle[8] and these three will be discussed in more detail as they have put more emphasis on the preparations of aluminium.

The method of Armstrong and Martin demands continuous and close attention for a period as long as 90 minutes, a period which is possibly unreal physiologically and very demanding both of time and labour for routine purposes. In addition, the acid medium contains pepsin, which, being a natural product, cannot be guaranteed to give completely consistent behaviour from one delivery to the next. Finally the adjustment of the initial *p*H of the hydrochloric acid by dilution means that varying strengths of acid will be used and consequently varying quantities will be required to neutralise equivalent amounts of antacid. The use of "room temperature" by Gore, Martin and Taylor is difficult to justify as even in this country it could range from 15° C. to 25° C. and elsewhere the range could be even greater. As will be seen later, close temperature control is essential. The strength of acid used for the initial solution is lower than that used by other investigators and lower than the 0·5N to 0·1N[9,10] usually accepted as the concentration of gastric juice. Brindle pointed out that the artificial gastric juice used by him was variable in its initial *p*H and there is the possibility (which has not been investigated) that there was some effect on the intermediate values found during the test. This

uncertainty cannot be permitted in a test where standards of quality are to be established.

The aim of our investigation was to establish a suitable simple technique for routine use. For this, the following points needed consideration:—(1) The amount and form of the alumina. (2) The quantity and strength of the acid. (3) The use or omission of enzymes and buffering agents. (4) Temperature of test. (5) Rate of stirring. (6) Duration of test. (7) Reproducibility of results. (8) Discrimination.

(1) *Amount and Form of Alumina*

For this work the quantities chosen were the maximum B.P.C. doses, namely 0·6 g. of dried gel and 8 ml. of the liquid, each equivalent to approximately 0·3 g. of Al_2O_3. The form of the alumina should be that in which it is administered, namely liquid—perhaps diluted with water—powder or tablet. For the majority of these experiments dried powder was used, the whole of it having been rubbed through a 100-mesh sieve. The remainder of the experiments were carried out using liquid gel.

(2) *Quantity and Strength of Acid*

The first and perhaps most obvious idea would be to use the quantity present in the stomach but this is actually far from constant. For example Adams, Ensel and Myers[9] found the volume of acid in the fasting stomach could vary from 30 ml. to 300 ml. and Kay[11] has shown that the *average* amount of hydrochloric acid present can range from 70 mg. (as HCl) in normal persons to 265 mg. in duodenal ulcer cases. A better line would be to take a quantity of acid which bears some relation to the amount of alumina used, such as, for example, an equivalent amount, so that the results obtained might also give some indication of neutralising capacity. The dried gel must have a neutralising capacity (B.P.C.) better than 200 ml. of 0·1N acid per g. This is equivalent to a minimum of 240 ml. of 0·05N acid for 0·6 g., so 250 ml. of 0·05N would be a convenient amount to take for the test. The choice of 0·05N for acid strength is probably reasonable although strengths up to 0·1N are mentioned for "appetite juice"[9,10]. The minimum neutralising capacity of the liquid gel is one-tenth that of the dried material so that the most appropriate quantity to take when testing liquid gels would be 6 ml. rather than the maximum dose of 8 ml. However, as the maximum dose was chosen as our guide, the 8 ml. quantity has been retained in these tests, using 250 ml. of 0·05N hydrochloric acid, or its equivalent, in all cases.

(3) *Enzymes and Buffering Agents*

There is some case for the use of enzymes and buffering agents such as pepsin and peptone for the reason that their presence gives a better representation of stomach conditions. There are several arguments against their use, some of which have already been stated but to permit a more definite conclusion to be reached some experiments were made with such additions, and the results are discussed later.

(4) *Temperature of Test*

The natural choice is 37° C., the physiological temperature, and this means that a thermostat bath is required. If, however, the temperature coefficient of the reaction is not considerable then conditions approximating to "room temperature" could be used without precise control. A series of tests was made at different temperatures to establish whether or not close temperature control was necessary.

(5) *Rate of Stirring*

This was an unknown factor in the test and 3 different rates of stirring were tried.

(6) *Duration of Test*

For routine purposes the test should be completed in the minimum of time and in any case it would be meaningless to continue the test for longer than the antacid would be in the stomach. According to Mutch[4], even massive doses of buffering agents do not exert their effect for more than about 1 hour, presumably because of loss to the duodenum. Thus the test should certainly be completed within an hour and, except for experiments on the effects of temperature, all tests were concluded within 60 minutes.

(7) *Reproducibility of Results*

This is an important point and a controlling factor in establishing the exact conditions required. It was confirmed by repeated tests on well-mixed bulk samples.

(8) *Discrimination*

Good discrimination is a valuable characteristic as it enables limits to be set which can easily be maintained. For this reason considerable importance was attached to the need for a test having this property.

Methods of Testing and Apparatus

The simplest form of test would be the addition of alumina gel to a suitable quantity of acid and the measurement of *p*H at intervals. This becomes quite a practical proposition once the characteristic curves of a number of suitable gels have been established, because for further testing, the *p*H at a few chosen time intervals is all that is needed to indicate the quality of a gel. This has been developed as Method I. An alternative procedure which would also give some information on the behaviour of the gel when in considerable excess, would be to simplify the Armstrong and Martin method, by reducing the number of additions of acid and not withdrawing any of the mixture. Thus two additions of 5 ml. of 0·5N acid to 150 ml. of 0·05N acid would give the equivalent of the 250 ml. of 0·05N acid chosen as a suitable amount for the test. The reason for suggesting stronger acid for the additions is that volume and temperature changes would be small and the time taken reduced. This has been developed as Method II.

For all these experiments a 250-ml. squat form Pyrex beaker was used to hold the acid medium and the test was carried out in a thermostat bath. A glass stirrer having two blades, about 3·5 cm. overall diameter, was used to agitate the mix, and the *p*H electrodes, glass and calomel respectively, were arranged so that measurements of *p*H could be made at frequent intervals without interrupting the stirring. The procedure was to measure the acid medium into the beaker, heat to just below the test temperature and put the beaker into the thermostat bath. The stirrer and electrodes were then put into position and the stirrer left running until the correct temperature had been obtained, when the initial *p*H of the solution was measured. The weighed quantity of sample was then added quickly and *p*H readings were taken at measured intervals of time.

EXPERIMENTAL

Method I

The first 2 sets of experiments were planned to test the effects of stirring and temperature and for this series 0·6 g. of dried gel was used in 250 ml. of 0·05N hydrochloric acid.

Rate of Stirring. 3 rates of stirring were tried, 120 r.p.m., which kept the powder in suspension but did not give much turbulence, 240 r.p.m., which seemed to be reasonably vigorous, and 350 r.p.m., which was the fastest speed attainable without splashing. As will be seen from Table I, the variations in rate of stirring have very little effect. The wider difference at 15 minutes is largely due to the velocity of reaction at this stage when only a few seconds are needed for a change of 0·1 *p*H unit.

TABLE I

EFFECT OF STIRRER SPEED ON THE VELOCITY OF REACTION OF ALUMINA GEL WITH ACID

Speed of Stirring r.p.m.	Time in Minutes						
	0	5	10	15	20	25	30
	*p*H readings						
120	1·36	1·60	1·95	2·55	3·55	3·70	3·70
240	1·38	1·61	2·00	2·80	3·65	3·75	3·80
350	1·36	1·61	2·00	2·70	3·65	3·75	3·77

Variations of Temperature. To investigate the need for temperature control, 6 temperatures were chosen, 15°, 20° and 25° C. being used for the "room temperature" set and 32°, 37° and 42° C. for the higher range. The curves given in Figure 1 show quite clearly the considerable effect of temperature differences. The time taken to reach a given *p*H is increased by about 50 per cent. for every 5° C. fall in temperature. Such a high temperature coefficient means that there must not be more than about 0·5° C. variation from the specified temperature, and in view of the time taken to attain full buffering effect at "room temperature" (100 to 180 minutes) there is much advantage in specifying 37° C. One interesting

fact is the rise in the final pH with fall in temperature; at 42° C. the peak pH is 3·7 and at 15° C. it is 4·1.

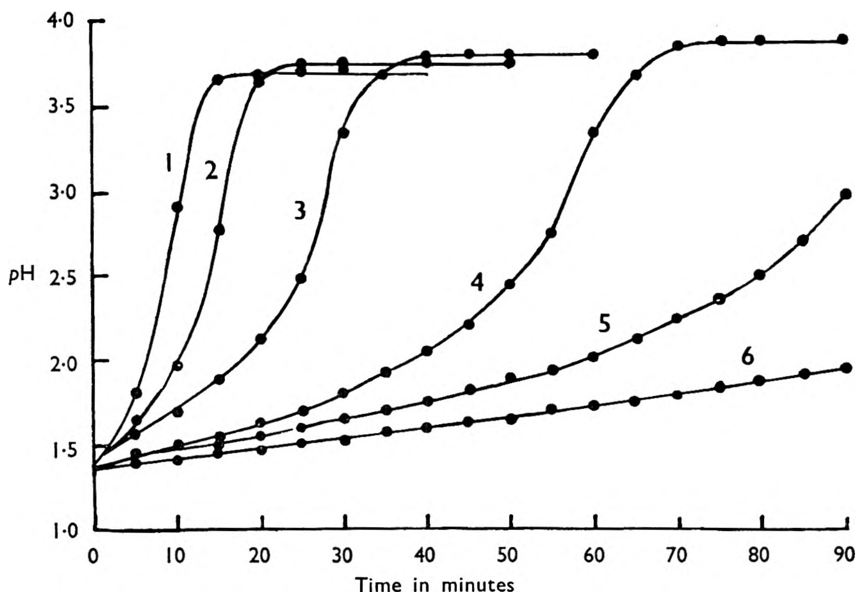

FIG. 1. The effect of temperature on the rate of reaction of dried aluminium hydroxide in 0·05N hydrochloric acid. 1 at 42° C.; 2 at 37° C.; 3 at 32° C.; 4 at 25° C.; 5 at 20° C. and 6 at 15° C.

Discrimination. A number of samples of dried alumina gel from various sources were assayed and their neutralisation capacities determined (see Table II). These were then tested at 37° C. with a stirrer speed of about 240 r.p.m. The curves obtained are given in Figure 2 and show quite clearly that the neutralisation capacity does not give complete information because, for instance, samples B and C, which differ by about 20 per cent. according to the B.P.C. test, have nearly identical reaction rates. Similarly, samples B and E, of similar neutralisation capacity have very different characteristics. The slow action of sample D would render it of doubtful value for the rapid relief of hyperacidity although it conforms to the

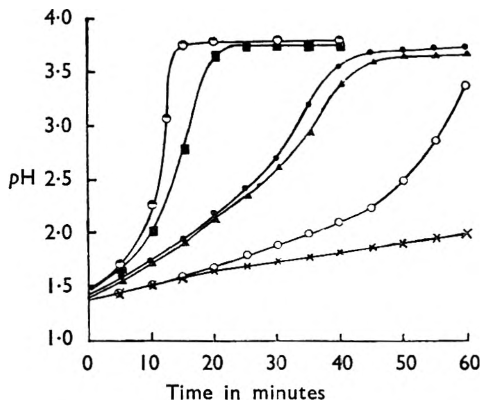

FIG. 2. The reaction rates of various dried aluminium gels, using method I.
■—■ Sample A ×—× Sample D
●—● „ B ○—○ „ E
▲—▲ „ C ◓—◓ „ F

existing requirements of the B.P.C. The spacing of the curves gives a very clear picture of the discriminating power of the test.

TABLE II

ASSAYS AND NEUTRALISATION CAPACITIES OF THE DRIED ALUMINA GELS

	Sample					
	A	B	C	D	E	F
Assay—Al_2O_3, per cent. ..	50·5	50·3	50·5	50·4	50·0	50·8
Neutralisation Capacity (ml. of 0·1N acid/g.) ..	258	270	219	210	270	280

Enzymes and Buffers. Another series using 0·05N acid containing 0·15 per cent. each of pepsin, peptone and sodium chloride as used by Brindle shows very much less difference between the various samples and much lower *p*H values even after 60 minutes. Similarly, when 3 of these samples were tested in acid containing 0·15 per cent. of pepsin alone, which is similar to the Armstrong and Martin acid medium, the slowing down of the reaction gave a less distinct classification. These results are compared in Table III. Thus from the point of view of routine testing the omission of pepsin and similar materials is an advantage as differences can be more readily detected and, as Rossett and Flexner[1] showed, comparable results are obtained from *in vivo* tests and *in vitro* tests using hydrochloric acid without the addition of physiological substances.

TABLE III

RATE OF REACTION WHEN ENZYMES AND SIMILAR SUBSTANCES ARE ADDED TO THE ACID MEDIUM. METHOD I

(a) 250 ml. of 0·05N hydrochloric acid with 0·15 per cent. each of pepsin, peptone and sodium chloride.

	Time in Minutes					
	0	10	20	30	40	60
Sample	*p*H readings					
A	1·44	1·64	1·80	1·94	2·06	2·31
B	1·40	1·57	1·63	1·72	1·80	1·94
D	1·40	1·56	1·62	1·72	1·82	2·00
E	1·46	1·56	1·60	1·62	1·67	1·76

(b) 250 ml. of 0·05N hydrochloric acid with 0·15 per cent. of pepsin.

A	1·42	1·66	1·92	2·20	2·48	3·15
D	1·36	1·52	1·59	1·67	1·73	1·90
E	1·38	1·50	1·54	1·58	1·62	1·72

Reproducibility. 4 additional tests were made on sample A and also on samples B and E. The results are given in Table IV. The tests on stirring rate were made on sample A and Table I could accordingly be considered as a further set of figures. It will be seen that close agreement can be obtained. The poorest results were obtained with sample E, a relatively inactive gel, at the point of most rapid change of *p*H.

Method II

For the second type of test, in which there was an initial excess of antacid, 150 ml. of 0·05N acid was used at the start and after 20 minutes 5 ml. of 0·5N acid was added, followed 10 minutes later by a further 5 ml., giving a total quantity of acid equivalent to 250 ml. of 0·05N as before. The same samples of dried gel were tested as above and the curves obtained are given in Figure 3. Although the shape of the curves is quite different, the relative positions of the lines is unchanged except that sample E now

approaches closely to the behaviour of B and C in the final 15 minutes of the test. Considering the test as a whole, however, the gels would be given the same relative placings.

TABLE IV

REPRODUCIBILITY OF METHOD I

Sample	Time in Minutes								
	0	5	10	15	20	30	40	50	60
	*p*H readings								
A	1·38	1·66	2·00	2·75	3·66	3·75			
A	1·38	1·64	2·00	2·85	3·67	3·80			
A	1·36	1·63	2·00	2·73	3·70	3·78			
A	1·40	1·64	1·96	2·49	3·56	3·71			
B	1·40	1·59	1·76	1·94	2·18	2·99	3·65	3·72	3·74
B	1·39	1·58	1·74	1·94	2·17	3·04	3·66	3·70	3·72
B	1·35	1·56	1·76	1·96	2·17	2·88	3·64	3·70	3·73
B	1·40	1·61	1·77	1·96	2·19	2·89	3·63	3·72	3·75
E	1·36	1·44	1·50	1·60	1·70	1·90	2·16	2·59	3·42
E	1·35	1·41	1·49	1·61	1·71	1·88	2·13	2·38	3·10
E	1·36	1·44	1·52	1·61	1·72	1·94	2·20	2·58	3·43
E	1·38	1·43	1·51	1·60	1·70	1·88	2·12	2·41	2·86

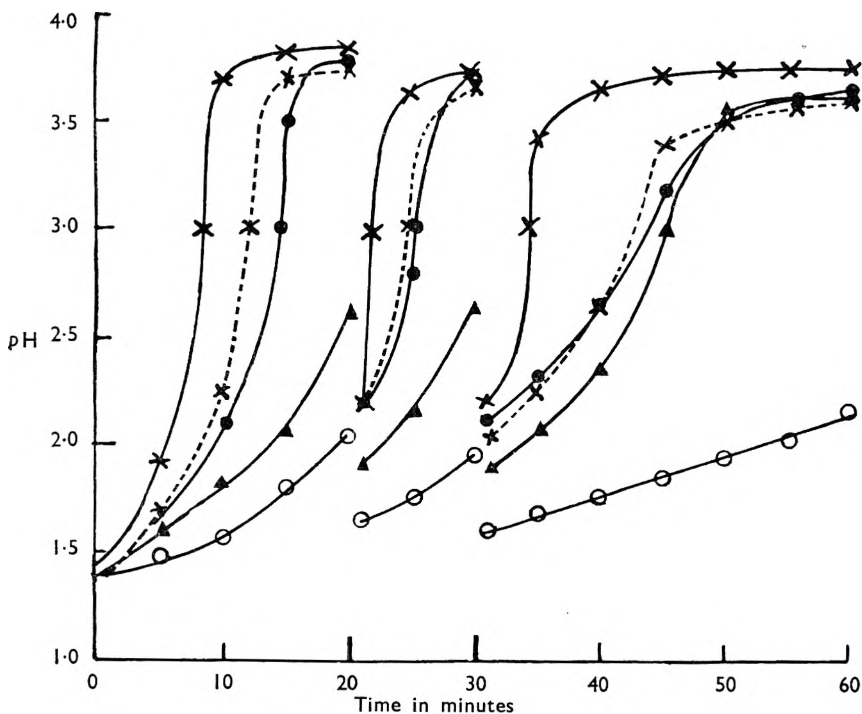

FIG. 3. The reaction rates of various dried alumina gels using method II. To avoid confusion the lines joining the 20 and 21 minute and the 30 and 31 minute points, have been omitted.

× —— × Sample A
× - - - × ,, B
● —— ● ,, C
○ —— ○ Sample D
▲ —— ▲ ,, E

Enzymes and Buffers

Only 3 samples were tested by Method II with added pepsin and with added pepsin, peptone and sodium chloride (in the 0·05N acid only). The effect on the reaction rate was closely similar to that found in Method I as will be seen in Table V.

TABLE V

RATE OF REACTION WHEN ENZYMES AND SIMILAR SUBSTANCES ARE ADDED TO THE ACID MEDIUM. METHOD II

(a) 150 ml. of 0·05N hydrochloric acid with 0·15 per cent. each of pepsin, peptone and sodium chloride.

Sample	Time in Minutes							
	0	10	20	21	30	31	40	60
	*p*H readings							
A	1·38	1·76	2·07	1·71	1·95	1·65	1·85	2·30
D	1·36	1·57	1·71	1·52	1·59	1·42	1·52	1·71
E	1·38	1·55	1·61	1·42	1·49	1·35	1·45	1·61

(b) 150 ml. of 0·05N hydrochloric acid with 0·15 per cent. pepsin.

Sample	0	10	20	21	30	31	40	60
A	1·36	1·90	3·30	1·97	2·81	1·90	2·30	3·45
D	1·39	1·62	1·77	1·55	1·72	1·52	1·59	1·90
E	1·36	1·56	1·72	1·50	1·66	1·48	1·59	1·86

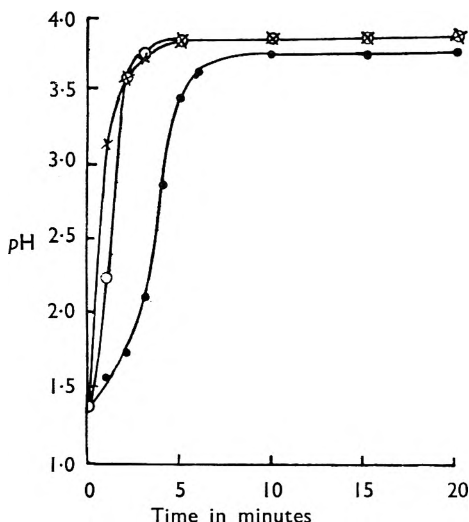

FIG. 4. The reaction rates of 3 samples of liquid alumina gels using method I.
●—● Sample 1.
×—× ,, 2.
○—○ ,, 3.

Liquid Gels

3 liquid gels were tested by Method I, without the addition of pepsin etc. The weight per ml. of liquid gel is about 1·015 g. to 1·020 g. so that a negligible error is introduced by using 8 g. instead of 8 ml. The gel was diluted with 8 ml. of water before addition so that more complete transference could be achieved. This is quite permissible as the gel is usually diluted before administration. Figure 4 gives the results and shows that the reaction is so rapid that little distinction is possible between different samples.

DISCUSSION

For the purposes of a routine test the use of 0·05N hydrochloric acid rather than artificial gastric juice is quite permissible because the aim of the test is to establish the relation between different samples of closely similar

material. The use of additives aimed at producing artificial gastric juice have been shown to give no advantage in the performance of such a test and are probably a disadvantage as they reduce the overall range of the readings and hence the discrimination. If they had a significant effect on the relative placings of the samples these disadvantages would be offset but no such effect was found.

Taking the maximum B.P.C. dose of gel as a starting point, a convenient form of test is the first one described. The alternative form of test, where a considerable excess of antacid is present during the first 20 minutes, has attractions at first sight as it seems to be closer to the conditions existing *in vivo*. For routine purposes it seems to offer no advantage as it would need an elaborate set of limits. The *p*H would have to be specified at several time intervals if the test is to have real value.

Thus the most suitable method of testing is as follows: Take 8 g. of liquid gel or 0·6 g. of dried gel (or equivalent amounts if in another form) and add quickly to 250 ml. of 0·05N hydrochloric acid at 37° ± 0·5° C. in a 250 ml. beaker provided with a stirrer and electrodes for *p*H measurement. Maintain brisk agitation and determine the *p*H at intervals. The *p*H of the acid, which should have a factor between 0·98 and 1·02 should be between 1·35 and 1·40 at 37° C. Suitable limits for dried gel could be, for example, that a *p*H not less than 3·0 should be attained in 20 minutes and a *p*H not less than 3·5 in 30 minutes. At no time should the *p*H exceed 4·0. For the liquid gel the corresponding values could be a *p*H not less than 3·5 in 10 minutes and not more than 4·0 at any time.

SUMMARY

(1) *In vitro* methods for assessing the therapeutic value of antacids have been discussed in relation to the routine evaluation of aluminium hydroxide gels.

(2) Experiments based on these tests and the conclusions drawn from the discussion have been carried out with a view to establishing a suitable routine test.

(3) A form of test has been given in detail and limits have been suggested.

We thank the Directors of John Wyeth and Brother Ltd. for permission to publish this work and express our indebtedness to the Wyeth chemists in the United States whose work formed the basis for the proposed routine method.

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ON THE METABOLISM OF SOME AROMATIC NITRO-COMPOUNDS BY DIFFERENT SPECIES OF ANIMAL

PART II. THE ELIMINATION OF VARIOUS NITRO-COMPOUNDS FROM THE BLOOD OF DIFFERENT SPECIES OF ANIMAL

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Received April 15, 1954

INTRODUCTION

KING and Harvey[1] have shown that the rat can eliminate dinitro-*o*-cresol from the blood at different rates and that these depend on several normal and artificial variables. The present communication records the elimination rates of some aromatic nitro-compounds from several species of animal.

EXPERIMENTAL

Four substances were chosen for experiment, partly because of their chemical relationship and partly because they present some occupational risks. The first, *p*-nitrophenol, is a moiety of the *p*-nitrophenyl diethyl thiophosphate molecule (parathion, E605). This is widely used as a pesticide. The second substance, 2: 4-dinitrophenol, was once used as an explosive in the First World War. It was also used therapeutically as a metabolic stimulant, and is very similar to the third substance 4 : 6-dinitro-*o*-cresol. The latter has been studied in the rat, rabbit and man, and considerable information exists on its elimination rates by the 3 species (King and Harvey[2,3]). The fourth substance, 2: 4-dinitro-α-naphthol was once used as its sodium salt as a colouring pigment, more commonly known as Martius Yellow, but the discovery of its toxic properties prevented its further employment in this respect. All 4 compounds possess the *p*-nitrophenolic group, but more important, 3 of them possess the 2: 4-dinitrophenolic group which appears to be essential for a specific form of biological activity. The animals selected for this study were the mouse, the rat, the guinea-pig and the rabbit. All of these animals are employed in laboratories where toxicological assays are performed. Also a few small West African monkeys were used, but the observations made on these were not sufficient for detailed statistical evaluation. *p*-Nitrophenol was estimated by the method of Lawford and Harvey[4], dinitrophenol and dinitro-α-naphthol were estimated essentially by the method of Parker[5] modified by Harvey[6] for dinitro-*o*-cresol in whole blood. The wavelengths at which solutions of the sodium salts of these compounds in methyl ethyl ketone were measured were 4200 Å for dinitrophenol, 4300 Å for dinitro-*o*-cresol, 4500 Å for dinitro-α-naphthol. Solutions for administration by oral feeding or by intraperitoneal injection were made by dissolving the substances in 0·5 per cent. sodium chloride solution and 0·5 per cent. sodium bicarbonate solution to give final concentrations of the substance of 0·5 or 1·0 per cent. In the case of dinitro-α-naphthol the administration was carried out at about 37° C.

because of the relative insolubility of its sodium salt and its tendency to separate out in solutions stronger than 0·5 per cent. LD50 values were determined essentially by the method employed by Harvey[7,8], in studies on dinitro-*o*-cresol and associated compounds. As far as possible similar strains of animal were used throughout. This rule was not easy to observe in the case of rabbits. These consisted very largely of "scrub" groups, usually lop-eared or half lop-eared types obtained from various sources. Statistical analyses for variance, and the method employed for the determination of regression lines—elimination rates—(*b*) were essentially as described by Emmens[9].

RESULTS

These are given in Tables I, II and III, and in Figure 1. Table I gives the absolute slope values (*b*) of the nitro-compounds for the mouse, rabbit, guinea-pig and rat following administration of the substances by stomach tube (oral) and by intraperitoneal injection. Table II summarises the results in the form of ratios of *b*, with rat = 1 or dinitro-*o*-cresol = 1. Table III compares LD50 values with absolute slope values and their ratios. Figure I summarises the experiments carried out on the monkeys and compares them with those of the rat.

TABLE I

ABSOLUTE RATES OF ELIMINATION (*b*) OF THE FOUR NITRO-COMPOUNDS

Animal	Method of dosage	Substance mol. wt. solubility in water g./100 ml.	*p*-nitrophenol 139 1·6 (25° C.)	2:4-dinitrophenol 184 0·56 (18° C.)	4:6-dinitro-*o*-cresol 198 0·024 (19° C.) (a)	2:4-dinitro-α-naphthol 234 0·004 (18° C.)
Mouse	Oral	S (b)	30	36	44	
		T	30	36	44	(c)
		b	− 0·90 ± 0·06	− 0·098 ± 0·033	− 0·036 ± 0·004	
	Intra-peritoneal	S	24	24	28	20
		T	24	24	28	20
		b	− 1·24 ± 0·12	− 0·21 ± 0·014	− 0·04 ± 0·002	− 0·012 ± 0·006
Rabbit	Oral	S	4	6	6	4
		T	64	24	30	16
		b	− 0·43 ± 0·036	− 0·010 ± 0·02	− 0·045 ± 0·001	− 0·061 ± 0·02
	Intra-peritoneal	S	5	6	3	4
		T	45	24	15	20
		b	− 0·78 ± 0·006	− 0·22 ± 0·0009	− 0·077 ± 0·0109	− 0·087 ± 0·02
Guinea-pig	Oral	S		16	16	20
		T	(c)	16	16	20
		b		− 0·12 ± 0·017	− 0·032 ± 0·001	− 0·051 ± 0·004
	Intra-peritoneal	S		16	20	16
		T	(c)	16	20	16
		b		− 0·135 ± 0·017	− 0·021 ± 0·003	− 0·04 ± 0·004
Rat	Oral	S	4	6		4
		T	32	24	−0·01	24
		b	− 0·190 ± 0·012	− 0·062 ± 0·009		− 0·015 ± 0·0006
	Intra-peritoneal	S	5	6	(d)	4
		T	25	24	−0·02	20
		b	− 0·80 ± 0·06	− 0·122 ± 0·008		− 0·021 ± 0·001

NOTES.—(a) See Harvey[7]. (b) S = number of animals; T = number of blood samples. These were always arranged in equal groups. Thus when S = 4 and T = 64, there were 16 equal sample groups. All mice and guinea-pigs gave one sample each so S = T. Groups were spaced fairly evenly over time necessary for total elimination. This was determined approximately by preliminary experiments involving a small number of animals. (c) Values too scattered to give a satisfactory regression line. (d) See King and Harvey[1].

TABLE II

COMPARISON OF RATES OF ELIMINATION (*b*)

(A) By Animal Species : Rat = 1

Substance		Mouse		Rabbbit		Guinea-pig		Rat
p-nitrophenol	Oral	4·9	>	2·3	>	(N.A.)	>	1·0
	Intra-peritoneal	1·5	>	1·0	≃	(.NA.)	≃	1·0
2:4-dinitro-phenol	Oral	1·5	<	1·6	<	2·0	>	1·0
	Intra-peritoneal	1·7	<	1·8	>	1·1	>	1·0
4:6-dinitro-*o*-cresol	Oral	3·6	<	4·5	>	3·2	>	1·0
	Intra-peritoneal	2·0	<	3·8	>	1·0	=	1·0
2:4-dinitro-α-naphthol	Oral	(N.A.)		4·0	>	3·4	>	1·0
	Intra-peritoneal	6·0	>	4·1	>	1·9	>	1·0

(B) By Compounds : dinitro-*o*-cresol = 1

Animal		*o*-nitrophenol		2:4-dinitrophenol		2:4-dinitro α-naphthol		4:6-dinitro-*o*-cresol
Mouse	Oral	25·0	>	2·7	>	(N.A.)	>	1·0
	Intra-peritoneal	31·0	>	5·2	>	3·0	>	1·0
Rabbit	Oral	9·5	>	·22	>	1·3	>	1·0
	Intra-peritoneal	10·0	>	3·0	>	1·3	>	1·0
Guinea-pig	Oral	(N.A.)		3·9	>	1·6	>	1·0
	Intra-peritoneal	(N.A.)		6·5	>	2·0	>	1·0
Rat	Oral	19	>	6·2	>	1·5	>	1·0
	Intra-	40	>	6·1	>	1·1	>	1·0

TABLE III

COMPARISON OF LD50 VALUES AND ELIMINATION SLOPE RATIOS

(A) For dinitro-*o*-cresol for the four species

	Mouse		Rabbit		Guinea-pig		Rat
Absolute LD50 values (mg./kg.)	24·0 24·2 (b) 26·0		23·5		22·5		28·5 (b)
Ratio (Rat = 1)	0·8	<	0·7	=	0·7	<	1·0
Elimination slope ratio	2·0	<	3·8	>	1·0	≃	1·0

(B) For all 4 nitro-compounds for mice and rats (all in mg./kg.)

	p-nitro-phenol		2:4-dinitro-phenol		4:6-dinitro-o-cresol		2:4-dinitro-α-naphthol
MICE:							
Absolute LD50 values (mg./kg.)	107·6		26·0		24·0 24·2 26·0 (b)		55
Ratio (dinitro-*o*-cresol = 1)	4·3	>	1·0	=	1·0	<	2·2
Elimination slope ratio (dinitro-*o*-cresol = 1)	31·0	>	5·2	>	1·0	<	3·0
RATS:							
Absolute values	97·0		32·7		28·5		47·5
Ratio (dinitro-*o*-cresol = 1)	3·4	>	1·3	>	1·0	<	1·7
Elimination slope ratio (dinitro-o-cresol = 1)	40·0	>	6·1	>	1·0	<	1·1

NOTES.—(a) All substances administered by intraperitoneal injection for slope ratios and for determination of LD50 values.

(b) See Harvey.[7]

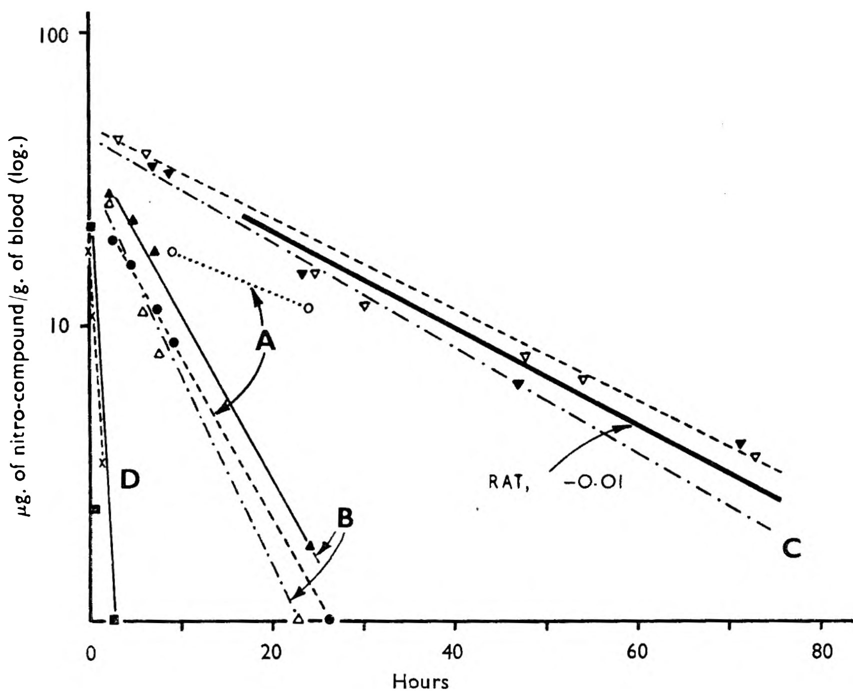

FIG. 1. Elimination of nitro compounds by the monkey.

A. Dinitro-α-naphthol
B. Dinitrophenol
C. Dinitro-*o*-cresol
D. *p*-Nitrophenol

Symbol	Dose and route	
× - - - ×	20 mg./kg. *p*-nitrophenol oral	one monkey
■——■	20 mg./kg. *p*-nitrophenol intraperitoneal	
△-·-·-△	15 mg./kg. 2:4-dinitrophenol oral	one monkey
▲——▲	15 mg./kg. 2:4-dinitrophenol intraperitoneal	
○ · · · · ○	15 mg./kg. dinitro-α-naphthol oral	one monkey
●- - - -●	15 mg./kg. dinitro-α-naphthol intraperitoneal	
▽ - - - ▽	20 mg./kg. dinitro-*o*-cresol oral	one monkey
▼-·-·-▼	20 mg./kg. dinitro-*o*-cresol intraperitoneal	one monkey

DISCUSSION

Three main conclusions can be made from the results. First, that the 4 substances are eliminated in the following order of descending speed: *p*-nitrophenol, dinitrophenol, dinitro-α-naphthol, and dinitro-*o*-cresol. Secondly, that the animals eliminate these compounds from the blood in the following descending order of efficiency: mouse, rabbit, guinea-pig, rat and monkey. Thirdly, that the ratios of the LD50 values are smaller and more uniform in rats and in mice for all 4 compounds than are the corresponding ratios of their elimination rates. An attempt was made to correlate eliminating power with species and with the substances studied. thus at first it was thought that there might be a correlation between the animals' basal metabolism and their ability to detoxicate the substances administered. Brodie, Proctor and Ashworth[10], in an extensive study of

the basal metabolic rates of mature animals of different species, have shown that the basal metabolism tends to vary with the 0·73 power of the body weight, also that there is a linear relation between the logarithm of the body weight and the logarithm of the basal metabolism of the animal. The value increases with increasing size. Thus, the order of the animals studied would be mouse, rat, guinea-pig and rabbit. Nevertheless, the present study indicates that no such relationship exists. Quite clearly, an explanation of the quantitative differences observed will have to be investigated in a further study of the specific excretion and detoxication mechanisms of the 4 species. Although the elimination rates appear to increase with diminishing molecular weight and with increasing solubility, there is a reversal in the case of dinitro-*o*-cresol and dinitro-α-naphthol. On the whole the former compound is more slowly excreted than the latter.

Of the four substances studied dinitro-*o*-cresol is undoubtedly the most important from the occupational-hazard aspect. *p*-Nitrophenol can be placed next to dinitro-*o*-cresol in importance because of its derivation from parathion and because of the wide use of this pesticide. The ready release of this last substance by the hydrolysis of parathion *in vivo* suggested that its estimation in the blood of workers at risk to parathion or its analogues might be a useful monitoring device.

However, the results of the work reported in the present communication indicate that the removal of *p*-nitrophenol from the blood of animals is very rapid. In fact most doses are eliminated completely from the blood within 2 hours of administration. No information is available on the clearance of *p*-nitrophenol from the blood of man, but it seems probable that unless estimations are made immediately after exposure the results will not be very valuable. A more practical approach appears to be in the estimation of urinary *p*-nitrophenol. This has been applied by Lieben, Waldman and Krause[11] in environmental studies on tobacco pickers. The results of these workers, although interesting, do not contain sufficient information from which to derive excretion rates. Clearly, further information on this aspect is highly desirable.

The evidence presented in this and the previous communication (King and Harvey[1]) emphasises that it is essential to include elimination experiments of the type described in any survey of a toxic substance. This is particularly important if one of the ultimate objects of the assay is to determine whether the substances will accumulate in man as the result of small but repeated exposures (Harvey[12]).

Summary

1. Four aromatic nitro-compounds have been studied, and it has been found that they are eliminated from 4 species of laboratory animals in the following descending order of speed: *p*-nitrophenol $>$ 2:4:dinitrophenol $>$ 2:4-dinitro-α-naphthol $>$ 4:6-dinitro-*o*-cresol.

2. In the 4 species of animals employed the eliminating efficiency in descending order of speed is mouse $>$ rabbit $>$ guinea-pig $>$ rat.

3. A few experiments on West African monkeys reveals that the elimination rates of the 4 substances are of the same order as for the rat.

4. A comparison of the ratios of the LD50 values and of the elimination rates indicates that the LD50 values are much closer numerically than are the elimination rates. The latter exhibit some divergence.

Our thanks are due to Miss Jean Peal for her constant and reliable assistance and to Miss Audrey Mackrill and Miss June Welsher for help with the animals, also to Mr. H. Garling of the Human Nutrition Research Unit, Medical Research Council, for assistance in the preparation of the two diagrams in this and the previous paper.

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AN INVESTIGATION INTO THE EFFECTS OF 2:2-BIS-(*p*-CHLOROPHENYL)-1:1-DICHLOROETHANE (D.D.D.) ON THE MOUSE ADRENAL CORTEX

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Received April 29, 1954

2:2-Bis-(*p*-chlorophenyl) -1 : 1-dichloroethane (D.D.D.), an agricultural insecticide and an analogue of D.D.T., was reported by various workers[1-7] to produce severe cytotoxic atrophy of the zona fasciculata region of the adrenal cortex of the dog when administered over prolonged periods. Verne and Wegmann[8] observed histological damage in the rat adrenal cortex after the administration of the compound during a period of 4 to 6 weeks. Brown[9], working on adult rats maintained on a diet containing 0·1 per cent., reported that the animals rapidly developed signs of some adrenal dysfunction, a decreased response to cold stress being one of the symptoms.

On the other hand several workers were unable to demonstrate adrenocortical atrophy in similar experiments on rats and rabbits[2,3,10,11]. Recently Stoner[12] reported failure to produce degeneration of the zona fasiculata after the injection of the drug in oily solution to rats and mice and, in further experiments based on the study of regeneration of the rat adrenal cortex after medullectomy, he showed that the drug did not exert any striking cytotoxic effect. Stoner suggested that the success of experiments in the dog might be due to the greater susceptibility of the adrenal cortex to toxic agents in a species in which spontaneous adreno-cortical atrophy can occur[13]. Sheehan, Summers and Nichols[14] reported on its use in Cushings's syndrome and concluded that the drug did not produce any significant clinical effect on the condition nor did it produce any permanent adrenal atrophy.

In view of the confusion and contradiction of reports in the literature it was decided to investigate further the effects of the drug on the adrenal cortex of the mouse to determine whether cortical atrophy could be produced in that species. In a previous publication it has been demonstrated that adrenalectomised mice are hypersensitive to cold stress[15]. It was therefore decided to determine the effect of prolonged administration, both orally and parenterally, on the survival time of mice exposed to cold stress, using the survival time of the treated animals as a relative index of adrenocortical atrophy.

Methods

Groups of white male weanling mice were maintained on a diet of crushed M.R.C. cubes (diet 41) into which had been incorporated 0·1 per cent. of the recrystallised commercial drug (Rhothane); further groups

fed on the cube diet alone were injected subcutaneously thrice weekly with 20 mg./100 g. of body weight as a 2 per cent. solution in arachis oil. The volume of the injected solution was adjusted weekly to allow for the increase in body weight of the treated groups. Control groups of normal untreated animals and groups of untreated and recently adrenalectomised animals were included in the experiment.

The doses were based on studies performed by Haag, Finnegan, Larson, Dreyfuss, Main and Riese[11], who reported that the symptoms of toxicity were a diminished rate of growth and general emaciation occurring with doses considerably less than the lethal dose. In order to ensure that the dose administered in the current experiment did not produce a general toxic effect, groups of mice were maintained on a diet containing 0·1 per cent for the duration of the experiment and their daily body weight and general condition was compared with untreated control animals. Treatment was continued for 6 weeks, the groups of treated, control and adrenalectomised animals being subjected to cold stress (2·5° C. ± 1·5° C.) in a refrigerator at 2 weeks, 4 weeks and 6 weeks from the commencement of the treatment. The procedure for adrenalectomy and subjection of mice to cold stress has been given elsewhere[15].

Results

The mean survival times of groups of 9 to 12 treated, control and adrenalectomised animals stressed at 2, 4 and 6 weeks from commencement of treatment are given in Table I. The standard error of the mean is given for each value.

TABLE I

THE EFFECT ON THE SURVIVAL TIME OF MICE EXPOSED TO COLD STRESS (2·5° C. ± 1·5° C)

Treatment	Mean survival time of groups of 9 to 12 mice expressed in hours ± standard error of the mean		
	Duration of treatment		
	2 weeks	4 weeks	6 weeks
Adrenalectomised control group.	3·05 ± 0·31	3·11 ± 0·37	3·18 ± 0·23
Normal control group.	5·40 ± 0·38	5·45 ± 1·1	5·08 ± 0·75
Oral 0·1 per cent. of drug in diet.	5·50 ± 0·84	5·25 ± 0·81	5·60 ± 0·92
Parenteral 20 mg./100 g. of body weight thrice weekly subcutaneously.	6·35 ± 0·54	6·60 ± 1·2	6·04 ± 0·89

Statistical treatment of the results given in Table I shows that although after 2, 4 and 6 weeks' treatment the mean survival times of the untreated adrenalectomised groups were significantly different from their respective normal control groups, the treated groups at no time differed significantly from the normal controls. It was therefore considered that at no stage of the experiment did the treatment simulate the condition of adrenalectomy; i.e., it appeared that no significant degree of adrenocortical atrophy had taken place.

General toxic symptoms due to the oral administration of the drug were not evident in the treated groups as indicated by the general condition and the increase in body weight of the animals over the period of the investigation, as compared with that of the untreated controls.

To provide additional information concerning the effect of the drug on the adrenal glands themselves as distinct from the effects of the drug on a function of the glands (the response of the animal to cold stress) the adrenals were removed from each animal, in the above experiment, as soon as possible after death. The adrenals after removal were preserved in formol-saline for a suitable period and when fully hardened they were carefully dissected free from fat and other extraneous tissue and finally weighed. The combined weight of the right and left adrenal in each animal was expressed in terms of mg. of adrenal gland per 100 g. body weight of the animal.

Table II shows the results of this investigation; the mean adrenal weight in mg. per 100 g. of body weight for a group of 9 to 12 animals is given together with the standard error of the mean.

TABLE II

THE EFFECT ON THE ADRENAL WEIGHTS OF MICE

Treatment	Mean adrenal weight of groups of 9 to 12 mice expressed as mg. of adrenal per 100 g. of body weight ± standard error of the mean		
	Duration of treatment		
	2 weeks	4 weeks	6 weeks
Normal control group.	21·55 ± 1·21	22·33 ± 1·26	18·29 ± 1·36
Oral 0·1 per cent. of drug in diet.	21·90 ± 0·86	19·76 ± 0·94	16·85 ± 0·76
Parenteral 20 mg./100 g. of body weight thrice weekly subcutaneously.	22·18 ± 0·82	21·12 ± 1·51	17·32 ± 1·63

Statistical treatment of the results given in Table II show that treatment for either 2, 4 or 6 weeks failed to produce a significant decrease in the mean adrenal weight per 100 g. of body weight of the treated groups as compared with that of the normal controls. It was therefore assumed that the drug had failed to produce any significant adrenal atrophy.

DISCUSSION

The results of this investigation indicate that, under the experimental conditions observed, the drug administered in the diet in a concentration of 0·1 per cent. or injected subcutaneously thrice weekly in a dosage of 20 mg./100 g. of body weight, for periods of up to 6 weeks, failed to produce an effective atrophy of the mouse adrenal cortex as indicated by the two criteria, the survival time of the treated animal exposed to cold stress (2·5° C. ± 1·5° C.) and the adrenal weights of the treated groups.

The conclusions are in agreement with previous published data based on experiments with rabbits, rats and mice[2,3,10,11,12]. They are, however,

contrary to the results of experiments on dogs [1-7] and also to the decreased response to cold stress in treated rats observed by Brown[9] using similar experimental procedure to that detailed above.

SUMMARY

1. White male mice maintained on a diet containing 0·1 per cent. of 2:2-bis-(*p*-chlorophenyl)-1:1-dichloroethane and mice injected subcutaneously thrice weekly with an oily solution at a dosage of 20 mg./100 g. of body weight, for periods up to 6 weeks failed to exhibit a significantly decreased response to cold stress as compared with the untreated controls.

2. The drug did not significantly decrease the adrenal weights of the treated groups.

I should like to thank Professor G. A. H. Buttle and Dr. J. R. Hodges for their constant interest in this investigation and Miss E. M. Howard for her generous and invaluable assistance.

The drug (Rhothane) used for this experiment was kindly supplied by Charles Lennig and Company (Great Britain) Ltd., and Dr. H. B. Stoner of the Serum Research Institute.

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COMPARATIVE STUDIES OF METHODS OF EVALUATING ANTIBACTERIAL SUBSTANCES

PART I. EVALUATION OF BACTERIOSTATIC ACTION

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Received May 28, 1954

METHODS of evaluating antibacterial substances have been reviewed by Reddish[1,2] and Berry,[3] and the established methods justly criticised. Until more is known of the actual chemical and/or physical nature of the bacteria-bactericide reactions the evaluation of antibacterial substances is likely to remain empirical, but so diverse are the natures of different antibacterial substances that their modes of action must be different. It would seem then, that the study of the dynamics of a particular disinfecting system by two or more of the available methods would indicate whether the different methods are in fact measuring the same phenomenon. This series of papers presents the results of attempts to do this.

INTRODUCTION

The distinction between bacteriostatic and bactericidal action is confused. Marshall and Krenoff[4] defined bacteriostasis as "A concept of those conditions in which living bacteria, under the influence of a definite chemical agent, are induced to multiply at any rate less than normal." Parkinson[5] postulated two types of bacteriostasis, a true bacteriostasis in which there is an absolute arrest of growth and the bacteria die of senescence, and a dynamic bacteriostasis in which the organisms are killed at a rate equal to or greater than that at which they reproduce. Price[6] maintained that it was futile and unrealistic to try to differentiate between bacteriostatic and bactericidal agents, the two apparent effects being in fact due to the differences in resistance of individual bacteria, and instanced the use of antagonists to bactericidal agents to prove this point.

Since this work is an initial attempt at the comparison of techniques of measuring bacteriostatic action, the bacteriostatic strength of a substance will be taken as that strength which prevents further growth of the organism, and the investigation of antagonists will be left to a future study.

The traditional methods of ascertaining bacteriostatic strength are of three types, a liquid culture method and two plate methods. In the first the bacteriostatic agent is diluted with a nutrient broth and the liquid inoculated with a test organism and the culture examined for growth turbidity after incubation. In the second method an agar plate is seeded with a test organism and the size of a zone of inhibition of growth, caused by diffusion of the bacteriostatic from a cup or cylinder placed on the

plate, is measured. In the third, the bacteriostatic agent can be incorporated in the solid medium and growth or no growth of a culture streaked on the surface is recorded.

The second method has been used in the assay of antibiotics and the factors affecting the results have been examined and discussed by Cooper and Woodman.[7]

The third method has been used mainly in a qualitative way to ascertain the bacteriostatic "spectra" of various agents. The first method is the method of choice for ascertaining the strengths of bacteriostatics to be incorporated in injections, in multidose containers, and other pharmaceutical preparations to prevent the growth of micro-organisms in the preparations. This method cannot be easily used with bacteriostatic agents which cause a precipitate or cloudiness when diluted with the nutrient medium as is the case with some quaternary ammonium compounds and formulated disinfectants of the lysol and black and white fluid types. Nor has the existence of any correlation between the results of the first and third methods been examined.

EXPERIMENTAL

Organisms

1. *Pseudomonas æroginosa* (Syn. *Pseudomonas pyocyanea*) N.C.T.C. 8203.
2. *Echerichia coli* I (Syn. *Bacterium coli*) N.C.T.C. 8196.
3. *Salmonella typhi* N.C.T.C. 160.
4. *Staphylococcus aureus* N.C.T.C. 4163.
5. *Shigella dysenteriæ* type 1. N.C.T.C. 8217.
6. *Bacillus anthracis* N.C.T.C. 8234.
7. *Streptococcus fæcalis* N.C.T.C. 370.
8. *Mycobacterium smegmatis* N.C.T.C. 8159.
9. *Neisseria catarrhalis* N.C.T.C. 5483.
10. *Bordetella bronchiseptica* (Syn. *Hæmophilus bronchisepticus*) N.C.T.C. 452.
11. *Corynebacterium diphtheriæ, mitis.* N.C.T.C. 3989.

Bacteriostatic Substances

(*a*) Solids

Phenol, A.R. quality.
6-Chloro-3-hydroxytoluene (chlorocresol B.P.).
Phenylmercuric acetate B.P.C.
Cetyltrimethylammonium bromide (Cetrimide B.P.C.).

(*b*) Formulated preparations.

Solution of cresol with soap. B.P. (Lysol).
Solution of chloroxylenol. B.P. (Roxenol).
A commercial "black fluid."
A. 10. 39.* (A formulated quaternary ammonium compound.)

* Supplied by Messrs. Airkem Ltd.

Medium

The medium was peptone 1 per cent., Lab. Lemco 1 per cent., sodium chloride 0·5 per cent. in distilled water. For the solid medium this was gelled with 2 per cent. of Davis agar. In the majority of cases the medium was prepared double strength and diluted with an equal quantity of the bacteriostatic diluted with sterile water.

Apparatus

In all experiments the inoculum consisted of 1 drop from a No. 22 gauge needle on the special pipette designed by Cook and Yousef.[8] In order to check on the variability of the volume of 1 drop the following experiment was performed.

Experiment. Using the same needle the weight of one drop of distilled water was estimated by weighing ten lots of 10 drops, 10 lots of 5 drops, 10 lots of 2 drops and 10 lots of 1 drop. A series of 10 lots of 1 drop of distilled water from each of 6 different needles was then weighed.

Results. The results are summarised in Tables I and II.

TABLE I

VARIABILITY OF WEIGHT OF 1 DROP OF DISTILLED WATER FROM 1 PIPETTE

	Number of drops weighed			
	1	2	5	10
Mean weight of 1 drop in mg.	16·70	16·74	16·80	16·79
Standard deviation	0·19	0·21	0·05	0·05
95 per cent. fiducial limits	±0·14	±0·15	±0·04	±0·03

TABLE II

ANALYSIS OF VARIANCE OF WEIGHT OF 1 DROP OF DISTILLED WATER ESTIMATED USING 6 DIFFERENT PIPETTES

Source of variance	d.f.	Sum of squares	Mean square
Between pipettes	5	2·9135	0·5827
Between weighings	9	0·5308	0·0590
Residual	45	4·1282	0·0917
Total	59	7·5725	

Table II shows that the greatest source of variance is the between-needle variance but the total variance is small and the 95 per cent. fiducial limits using all the 60 readings are 16·83 to 17·02 mg. and so the size of 1 drop from any one pipette can be taken as 1/59 ml.

METHODS

Liquid Dilution Method. 10 ml. of medium was made by aseptically diluting 5 ml. of double strength culture medium with 5 ml. of the bacteriostatic solution in sterile distilled water. One drop of a 24-hour culture of the test organism was added as inoculum. The tube was then incubated for 48 hours at 37° C. and examined for growth. Controls of uninoculated tubes were also set up.

TABLE III

Bacteriostatic		Phenol					Chlorocresol					Phenylmercuric acetate					Cetrimide				
Percentage strength		2	1	10^{-1}	10^{-2}	10^{-3}	0·2	10^{-1}	10^{-2}	10^{-3}	10^{-4}	0·05	10^{-2}	10^{-3}	10^{-4}	10^{-5}	0·5	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Organism	Liquid or solid method																				
Ps. pyocyanea	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	+	+	+	P	P	P	+	–
	Solid	–	–	+	+	+	–	–	+	+	+		–	+	+	+	+	+	+	+	+
Bact. coli	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	+	+	P	P	P	+	+
	Solid	–	–	+	+	–	–	–	+	+	+		–	–	+	+	–	–	+	+	+
Salm. typhi	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	+	+	P	P	P	+	+
	Solid	–	–	+	+	–	–	–	+	+	+		–	–	+	+	–	–	+	+	+
Staph. aureus	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	+	+	+	–	–	+	+	+		–	–	–	–	–	–	–	+	+
Shig. dysenteriæ	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	±	+	P	P	P	+	+
	Solid	–	–	+	+	+	–	–	+	+	+		–	–	+	+	–	–	–	+	+
B. anthracis	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	+	+	+	–	–	+	+	+		–	–	±	–	–	–	–	+	+
Strept. fæcalis	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	+	+	+	–	–	+	+	+		–	–	±	–	–	–	–	+	+
N. catarrhalis	Liquid	–	–	–	+	+	–	–	–	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	–	+	+	–	–	–	+	+		–	–	±	–	–	–	–	+	+
H. bronchisepticus	Liquid	–	–	–	+	+	–	–	+	+	+	P	–	–	+	+	P	P	P	–	+
	Solid	–	–	±	±	+	–	–	–	+	+		–	–	±	±	–	–	–	+	+
Corynebact. diphtheriæ	Liquid	–	–	–	±	+	–	–	+	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	±	±	+	–	–	+	+	+		–	–	–	–	–	–	–	–	+
Myco. smegmatis	Liquid	–	–	+	+	+	–	–	+	+	+	P	–	–	–	–	P	P	P	–	+
	Solid	–	–	+	+	+	–	–	+	+	+		–	–	–	–	–	–	–	–	+

Bacteriostatic		A.10.39					Roxenol					Black fluid					Lysol				
Dilution		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Organism	Liquid or solid method																				
Ps. pyocyanea	Liquid	P	+	+	+	+	P	P	+	–	+	P	P	P	+	+	P	P	–	+	+
	Solid	–	+	+	+	+	–	+	+	+	+	–	+	+	+	+	–	–	–	+	+
Bact. coli	Liquid	P	–	–	+	+	P	P	–	+	+	P	P	P	+	+	P	P	+	+	+
	Solid	–	–	–	+	+	–	–	–	+	+	–	–	–	+	+	–	–	+	+	+
Salm. typhi	Liquid	P	–	+	+	+	P	P	+	+	+	P	P	P	+	+	P	P	+	+	+
	Solid	–	–	+	+	+	–	–	+	+	+	–	–	+	+	+	–	–	+	+	+
Staph. aureus	Liquid	P	–	–	+	+	P	P	–	+	+	P	P	P	–	+	P	P	+	+	+
	Solid	–	–	–	+	+	–	–	+	+	+	–	–	–	–	+	–	–	+	+	+
Shig. dysenteriæ	Liquid	P	–	–	+	+	P	P	+	+	+	P	P	P	+	+	P	P	–	+	+
	Solid	–	–	+	+	+	–	–	+	+	+	–	–	+	+	+	–	–	+	+	+
B. anthracis	Liquid	P	–	–	+	+	P	P	–	+	+	P	P	P	–	+	P	P	–	+	+
	Solid	–	–	–	+	+	–	–	–	+	+	–	–	–	–	+	–	–	–	+	+
Strept. fæcalis	Liquid	P	–	–	+	+	P	P	–	+	+	P	P	P	–	+	P	P	–	+	+
	Solid	–	–	–	–	–	–	–	–	+	+	–	–	–	–	+	–	–	–	+	+
N. catarrhalis	Liquid																				
	Solid																				
H. bronchisepticus	Liquid	P	–	+	+	+	P	P	+	–	–	P	P	P	+	+	P	P	–	+	+
	Solid	–	+	+	–	–	–	–	+	–	+		–	+	–	+	–	–	–	+	+
Corynebact. diphtheriæ	Liquid	P	–	–	–	+	P	P		+	+	P	P	P	–	+	P	P	–	–	+
	Solid	–	–	–	+	+	–	–	–	–	+	–	–	–	–	+	–	–	–	+	+
Myco. smegmatis	Liquid	P	–	–	+	+	P	P	–	–	–	P	P	P	–	+	P	P	–	+	+
	Solid	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	+	+

Showing results of bacteriostatic screening tests using liquid dilution and solid dilution methods. – = no growth, + = growth, P = precipitate prevented reading after 48 hour incubation.

Solid Dilution Method. 20 ml. of medium was made by aseptically adding 10 ml. of bacteriostatic solution in distilled water to 10 ml. of double strength agar medium melted and cooled to 65° C. This medium was poured into a sterile petri dish and when solidified was dried for 1 hour in an incubator with the lid raised. One drop of a 24-hour culture of the test organism was dropped onto the dried plate as in a Miles and Misra count. When the drop had been absorbed into the medium the plate was incubated at 37° C. for 48 hours and then examined for colony development.

TABLE IV

		Phenol				A.10.39					Roxenol		
	Percentage strength	0·20	0·18	0·16	0·14	0·10	0·08	0·06	0·04	0·02	0·10	0·08	0·06
Bact. coli ..	Liquid	− − − − −	− − − − −	+ + + + +	+ + + + +	+ − − − −	+ − − − −	+ + + − −	+ + + + +	+ + + + +	− − − − −	+ − − − −	+ + + + +
	Solid	− − − − −	− − − − −	+ + + + +	+ + + + +	+ ± ± ± −	+ + + + +	+ + + + +	+ + + + +	+ + + + +	− − − − −	+ + + + +	+ + + + +
	Percentage strength	0·16	0·14	0·12	0·10	0·14	0·12	0·10	0·08	0·06	0·16	0·14	0·12
Shig. dysenteriæ	Liquid	− − − − −	− − − − −	+ + + + +	+ + + + +	− − − − −	+ + + − −	+ + + − −	+ + + − −	+ + + + +	− − − − −	− − − − −	+ + + − −
	Solid	− − − − −	− − − − −	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +	− − − − −	− − − − −	+ + + + +
	Percentage strength	0·16	0·14	0·12	0·10		15·0	10·0	4·0	2·0	9·0	8·0	7·0
Ps. pyocyanea ..	Liquid	− − − − −	− − − − −	+ + + − −	+ + + + −								
	Solid	− − − − −	− − − − −	+ + − − −	+ + + + +		− − − − −	+ + + − −	+ + + + +	+ + + + +	− − − − −	+ + + + +	+ + + + +

Showing results of bacteriostatic tests using liquid dilution and solid dilution methods, − = no growth, + = growth, after 48 hours incubation.

The above two methods were carried out simultaneously. The initial ranging experiments were carried out using a tenfold dilution of the bacteriostatic agent with all the test organisms. The experiment was then repeated using a closer range of dilutions of some of the bacteriostatic solutions and each test carried out in triplicate. An even closer range of dilutions was used against *Bact. coli*, *Shig. dysenteriæ* and *Ps. pyocyanea* and carried out in quintuplicate against phenol, A. 10.39 and roxenol B.P. With the latter two bacteriostatic agents against *Ps. pyocyanea* it was only

possible to perform the test by the solid method since liquid dilutions were turbid.

Counting Method. This method was used with *Bact. coli*, *Shig. dysenteriæ and Ps. pyocyanea* against one strength of phenol, one strength of Roxenol and two strengths of A.10.39.

The method used was to set up 5 tubes for each test organism. The first tube contained 10 ml. of sterile water, the second 10 ml. of quarter strength Ringer's solution, the third tube 10 ml. of broth with added bacteriostatic, the fourth tube sterile water plus bacteriostatic and the fifth tube quarter strength Ringer's solution plus bacteriostatic. Viable

TABLE V

Tube	Inoculum	Counts after incubation for			
		0 hours	5 hours	10 hours	50 hours
Control (water)	*Bact. coli*	$8 \cdot 3 \times 10^5$	$1 \cdot 0 \times 10^6$	$1 \cdot 8 \times 10^6$	$2 \cdot 8 \times 10^6$
" (Ringer)		$7 \cdot 9 \times 10^5$	$2 \cdot 6 \times 10^6$	$3 \cdot 8 \times 10^6$	$3 \cdot 0 \times 10^6$
Phenol 0·18 per cent in broth ..		$8 \cdot 3 \times 10^5$	$6 \cdot 2 \times 10^5$	$5 \cdot 0 \times 10^5$	0
" " " water ..		$8 \cdot 0 \times 10^5$	$2 \cdot 1 \times 10^6$	$3 \cdot 5 \times 10^6$	59
" " " Ringer		$7 \cdot 1 \times 10^5$	$3 \cdot 5 \times 10^5$	$2 \cdot 4 \times 10^5$	637
Control (water)	*Shig. dysenteriæ*	$2 \cdot 2 \times 10^5$	$1 \cdot 9 \times 10^5$	$2 \cdot 2 \times 10^4$	$7 \cdot 3 \times 10^3$
" (Ringer)		$3 \cdot 2 \times 10^5$	$7 \cdot 0 \times 10^5$	$4 \cdot 3 \times 10^6$	$3 \cdot 2 \times 10^5$
Phenol 0·12 per cent. in broth ..		$2 \cdot 8 \times 10^6$	$1 \cdot 7 \times 10^6$	$4 \cdot 2 \times 10^4$	0
" " " water ..		$2 \cdot 1 \times 10^5$	$1 \cdot 1 \times 10^5$	$7 \cdot 0 \times 10^3$	0
" " " Ringer		$3 \cdot 1 \times 10^5$	$2 \cdot 1 \times 10^6$	$2 \cdot 6 \times 10^6$	$3 \cdot 9 \times 10^4$
Control (water)	*Ps. pyocyanea*	$1 \cdot 0 \times 10^6$	$3 \cdot 1 \times 10^6$	$5 \cdot 8 \times 10^6$	$7 \cdot 2 \times 10^6$
" (Ringer)		$1 \cdot 6 \times 10^6$	$1 \cdot 4 \times 10^7$	$1 \cdot 5 \times 10^7$	$1 \cdot 2 \times 10^7$
Phenol 0·14 per cent. in broth ..		$3 \cdot 1 \times 10^6$	$6 \cdot 5 \times 10^6$	$1 \cdot 1 \times 10^7$	$3 \cdot 0 \times 10^7$
" " " water ..		$2 \cdot 2 \times 10^6$	$3 \cdot 4 \times 10^5$	$8 \cdot 9 \times 10^6$	$5 \cdot 5 \times 10^4$
" " " Ringer		$8 \cdot 9 \times 10^5$	$3 \cdot 6 \times 10^6$	$2 \cdot 9 \times 10^6$	$6 \cdot 4 \times 10^5$
Control (water)	*Bact. coli*	$1 \cdot 3 \times 10^6$	$5 \cdot 0 \times 10^5$	$1 \cdot 6 \times 10^6$	$3 \cdot 4 \times 10^6$
" (Ringer)		$1 \cdot 3 \times 10^6$	$5 \cdot 7 \times 10^6$	$1 \cdot 0 \times 10^7$	$6 \cdot 5 \times 10^6$
Roxenol 0·08 per cent. in broth		$1 \cdot 7 \times 10^5$	$2 \cdot 5 \times 10^5$	$5 \cdot 5 \times 10^6$	$1 \cdot 3 \times 10^7$
" " " water ..		$1 \cdot 1 \times 10^6$	$1 \cdot 2 \times 10^7$	$1 \cdot 3 \times 10^6$	$6 \cdot 7 \times 10^4$
" " " Ringer		$1 \cdot 3 \times 10^6$	$1 \cdot 6 \times 10^6$	$9 \cdot 9 \times 10^5$	$5 \cdot 0 \times 10^3$
Control (water)	*Shig. dysenteriæ*	$2 \cdot 1 \times 10^5$	$3 \cdot 0 \times 10^5$	$2 \cdot 6 \times 10^6$	$7 \cdot 8 \times 10^3$
" (Ringer)		$3 \cdot 1 \times 10^5$	$9 \cdot 3 \times 10^5$	$1 \cdot 2 \times 10^6$	$1 \cdot 1 \times 10^6$
Roxenol 0·12 per cent. in broth		$3 \cdot 7 \times 10^6$	$3 \cdot 9 \times 10^6$		$1 \cdot 1 \times 10^7$
" " " water		$3 \cdot 0 \times 10^6$	$2 \cdot 3 \times 10^5$	$2 \cdot 2 \times 10^6$	$2 \cdot 0 \times 10^6$
" " " Ringer		$3 \cdot 2 \times 10^5$	$3 \cdot 3 \times 10^5$	$1 \cdot 5 \times 10^6$	$5 \cdot 9 \times 10^5$
Control (water)	*Ps. pyocyanea*	$4 \cdot 7 \times 10^5$	$8 \cdot 3 \times 10^5$	$1 \cdot 1 \times 10^6$	$1 \cdot 6 \times 10^7$
" (Ringer)		$1 \cdot 2 \times 10^6$	$9 \cdot 7 \times 10^6$	$1 \cdot 7 \times 10^7$	$2 \cdot 2 \times 10^7$
Roxenol 8·0 per cent. in broth		$1 \cdot 0 \times 10^5$	$1 \cdot 0 \times 10^3$	$3 \cdot 4 \times 10^2$	0
" " " water		0	0	0	0
" " " Ringer		0	0	0	0
Control (water)	*Bact. coli*	$1 \cdot 5 \times 10^6$	$1 \cdot 6 \times 10^6$	$6 \cdot 8 \times 10^6$	
" (Ringer)		$1 \cdot 4 \times 10^6$	$2 \cdot 9 \times 10^6$	$1 \cdot 4 \times 10^7$	
A.10.39 0·08 per cent in broth ..		$1 \cdot 4 \times 10^6$	0	0	
" " " water ..		$3 \cdot 9 \times 10^4$	0	0	
" " " Ringer		$9 \cdot 4 \times 10^5$	0	0	
Control (water)	*Shig. dysenteriæ*	Estimate	$3 \cdot 4 \times 10^6$	$3 \cdot 2 \times 10^5$	
" (Ringer)		$3 \cdot 0 \times 10^5$	$1 \cdot 6 \times 10^6$	$1 \cdot 8 \times 10^6$	
A.10.39 0·16 per cent in broth			0	0	
" " " water ..			0	0	
" " " Ringer			0	0	
Control (water)	*Ps. pyocyanea*	$1 \cdot 1 \times 10^6$	$2 \cdot 2 \times 10^6$	$4 \cdot 2 \times 10^5$	
" (Ringer)		$3 \cdot 5 \times 10^4$		$1 \cdot 6 \times 10^7$	
A.10.39 10 per cent. in broth ..		0	0	0	
" " " water ..		0	0	0	
" " " Ringer ..		0	0	0	

Showing results of counts performed on bacteriostatic test solutions after various times of incubation at 37° C.

counts were performed on each tube after 0, 5, 10 and 50 hours incubation at 37° C. The counts were performed in quintuplicate on tenfold dilutions by the Miles and Misra overdried plate method.

Results

Table III shows the results of the initial ranging tests. Table IV shows the results using closer ranges on 3 organisms with 3 bacteriostatic agents.

A viable count was performed in quintuplicate on 24-hour cultures of each of the 3 principal organisms using the Miles and Misra technique and gave the following results:—

Bact. coli	$9{\cdot}8 \times 10^8$ organisms per ml.	(p for $\chi^2 = 0{\cdot}7$).
Shig. dysenteriæ	$2{\cdot}2 \times 10^8$,, ,,	(,, ,, $= 0{\cdot}7$).
Ps. pyocyanea	$2{\cdot}4 \times 10^{10}$,, ,,	(,, ,, $= 0{\cdot}3$).

Using an inoculum of 1 drop per 10 ml. of test solution this was equivalent to inoculum levels of approximately

Bact. coli	$1{\cdot}5 \times 10^6$ organisms per ml.
Shig. dysenteriæ	$4{\cdot}0 \times 10^5$,, ,,
Ps. pyocyanea	$4{\cdot}0 \times 10^7$,, ,,

Table V summarises the results of the counting technique.

The results using A.10.39 were obtained using strengths which had shown no growth in the dilution methods and these results showed that for the counting technique these strengths were more than bacteriostatic and so the experiment was repeated using weaker solutions and also single drops from the reaction tube were plated out at intermediate times. The results are summarised in Table VI.

TABLE VI

		Count after incubating for							
	Inoculum	0	½ hour	1 hour	1½ hours	2 hours	2½ hours	5 hours	10 hours
Control	*Bact. coli*	$1{\cdot}5 \times 10^6$	—	—	—	—	—	$8{\cdot}5 \times 10^5$	$5{\cdot}3 \times 10^5$
0·06 per cent. A.10.39									
in broth		$1{\cdot}5 \times 10^6$	—	—	$< 10^4$	—	$< 10^4$	$6{\cdot}5 \times 10^3$	$2{\cdot}0 \times 10^2$
in water		$1{\cdot}1 \times 10^6$	—	—	$1{\cdot}3 \times 10^3$	—	0	0	0
in Ringer		$1{\cdot}4 \times 10^6$	—	—	$1{\cdot}8 \times 10^2$	—	0	0	0
Control	*Shig. dysenteriæ*	$3{\cdot}9 \times 10^5$	—	—	—	—	—	$2{\cdot}6 \times 10^5$	$1{\cdot}0 \times 10^5$
0·08 per cent. A.10.39									
in broth		$3{\cdot}7 \times 10^5$	—	$< 10^4$	—	$< 10^4$	—	$8{\cdot}1 \times 10^3$	$3{\cdot}0 \times 10^3$
in water		$3{\cdot}5 \times 10^5$	—	600	—	0	—	0	0
in Ringer		$3{\cdot}3 \times 10^5$	—	0	—	0	—	0	0
Control	*Ps. pyocyanea*	$3{\cdot}8 \times 10^6$	—	—		—	—	$1{\cdot}6 \times 10^6$	$4{\cdot}5 \times 10^5$
8·0 per cent. A.10.39									
in broth		$2{\cdot}1 \times 10^5$	1770	—	0		0	0	0
in water		0	0	—	0	—	0	0	0
in Ringer		0	0	—	0	—	0	0	0

Discussion

If the definition of bacteriostasis outlined in the introduction is adopted then results of preliminary tests using tenfold dilutions show that there is a definite correlation between the solid dilution and liquid dilution methods of evaluating the bacteriostatic values for the given agents

under the stated conditions. It is suggested that for preliminary screening of an antibacterial agent the solid dilution techniques can be recommended as being much more economical of medium and apparatus, since up to 12 different organisms can be tested on one 4-inch petri dish. The technique would need slight modification for use with organisms that tend to spread over the surface of the plate as for example *Proteus vulgaris*.

The extension of the methods for use with narrower ranges of bacteriostatic agent dilutions also shows the same correlation between the 2 methods in cases of 2 of the agents used, phenol and roxenol, where a range as close as 1 part in 5000 was used. The results with A.10.39, the formulated quaternary ammonium compound, however, were not so precise, a range of 1 part in 1000 being necessary to decide between growth and no growth, and in this case the solid dilution method shows growth in more concentrated mixtures than with the liquid dilution method. Two possible reasons can be suggested to account for this difference. If the quaternary compound exerts its bacteriostatic effect by a mechanism differing from that of the phenolic compounds, then it would be expected that the distribution of the resistances of the bacteria to these two mechanisms would be different and account for the scatter of the results with A.10.39. A second possible explanation is that the media used contain an antagonist to the quaternary ammonium compound and this antagonist is not uniformly distributed throughout the media and the solid medium contains more than the liquid medium.

Ps. pyocyanea has shown itself much more resistant to the bacteriostatics tested than all the other organisms, with the exception in the case of phenol. This resistance makes the evaluation of the activity against *Ps. pyocyanea* by the fluid method impossible with many of the formulated antibacterial substances and therefore the solid dilution method is selected as the technique for use.

In comparing the counting method with the dilution methods only 0·14 per cent. phenol against *Ps. pyocyanea*, 0·08 per cent. roxenol against *Bact. coli*, and 0·12 per cent. roxenol against *Shig. dysentariæ* can be said to be bacteriostatic. In all other cases the dilutions which were bacteriostatic by the dilution methods are bactericidal when tested by the counting method.

The suspensions of the 3 organisms in distilled water and quarter strength Ringer solution were relatively stable over the 50-hours period, in fact the latter permitted a small amount of growth.

Table VI shows that with 0·06 per cent. of A.10.39 against *Bact. coli* and 0·08 per cent. against *Shig. dysenteriæ* the rate of kill is much quicker in water and Ringer's solution than in broth, this lends weight to the assumption that the broth contains some substances which is antagonistic to the antibacterial action of A.10.39. This fact together with the bactericidal effects shown by the other substances supports Price's[6] hypothesis that there is no true bacteriostasis and Parkinson's definition of dynamic bacteriostasis. The differences in rate of kill shown by the counting methods results supports the theory that the different antibacterial substances act in different ways even against different organisms and that

the various methods used for evaluating are in all probability measuring different reactions. To clarify this situation some method should be evolved, for the counting technique, of ensuring that the effect of the bacteriostatic agent is completely suppressed in the dilutions taken from the reaction mixture before the actual count is made. This has not been done in the above work but is under consideration.

Summary

1. Three methods of measuring bacteriostasis have been examined; dilution of the bacteriostatic agent with a liquid culture medium, incorporation of the agent in a solid medium, and a counting method.

2. A fair degree of correlation has been shown to exist between the first two methods.

3. The counting method has given support to Price's[6] hypothesis that there is no real difference between bacteriostatic and bactericidal actions.

The author wishes to express his thanks to Mr. A. Edwards for his technical assistance in carrying out this work.

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COMPARATIVE STUDIES OF METHODS OF EVALUATING ANTIBACTERIAL SUBSTANCES

PART II. EVALUATION OF BACTERICIDAL ACTION. A COMPARISON OF AN EXTINCTION METHOD WITH A COUNTING METHOD

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Received May 28, 1954

INTRODUCTION

IN this paper the bactericidal action of phenol on *Bacterium coli* is examined by the method of Berry and Bean[1] and the results compared with those obtained by a counting technique.

THE LOGLOG ANALYSIS OF EXTINCTION DATA

Using the extinction data provided from this laboratory, it has been shown by Mather[2] that, using the loglog transformation of the proportion of negative samples at each contact time, the time when there is on the average one surviving organism per unit volume can be calculated. The method of analysis permits estimation of the sampling variances of the mean weighted loglog and the slope of the regression of loglog proportion of negative samples upon contact time. The standard error of an estimate of the killing time may be computed. The calculation of the regression line relating loglog to time follows the same course as probit analysis, but with the use of different weighting coefficients. The most informative observations are those where the proportion, p, lies between 0·2 and 0·3. The single mean survivor time is that at which $p = 0{\cdot}3679$, corresponding to loglog $= 0$.

Estimation of the mean single survivor time necessitates some modifications in the design of an experiment from that originally described. The reliability of an estimate will be improved by performance of as many replicate determinations as possible within an experiment and by reducing the intervals between contact times as far as possible subject to an adequate range being provided around the anticipated killing time. With aqueous solutions of phenols an adequate number of proportions of negative samples will not usually be found if the contact time intervals exceed 1/7 of the killing time. The most convenient structure of an experiment was usually found to consist of between 15 and 20 replicates at 6 contact times.

During the period of 5 consecutive days in which the percentages of survivors at various contact times were determined, as described in the following section, the single mean survivor time with 1·10 per cent. phenol was determined. The results of the experiment are shown in Table II and the values of the loglogs (y) are shown plotted against contact times in Figure 1, in which A represents the line best fitted by

inspection. By reading the value on the abscissa corresponding to $y = 0$ an estimate of 45·4 minutes was obtained for the mean single survivor time. A first approximation to the loglog relationships was made, following exactly the method described by Mather, from which an analysis of variance of the new relationship yielded the information expressed in Table I.

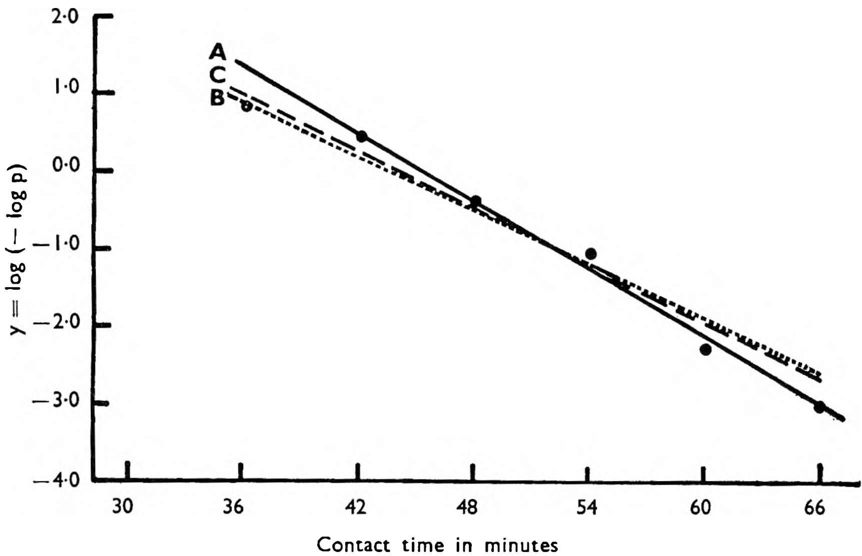

FIG. 1. The relationship between y (log[−log p]) and contact times and the calculation of the regression of y upon x: line best fitted by inspection, A; first approximation, B; and second approximation, C. Data for the exposure of *Bact. coli* to 1·10 per cent. phenol.

This variance ratio with the appropriate degrees of freedom corresponds to a probability level between 0·01 and 0·001, so that the regression is highly significant. The sampling variances of mean working loglog and slope were calculated as $\bar{y}_w = -0{\cdot}18189 \pm 0{\cdot}02657$ and $b = -0{\cdot}11442$

TABLE I

Source of variance	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Regression ..	27·62456	1	27·62456	$n_1 = 1$ 40·1547 $n_2 = 4$
Residual	2·75182	4	0·68795	
Total	30·37638	5		

± 0·00047. The revised approximation to the loglog relationship was $Y = -0{\cdot}18189 - 0{\cdot}11442\ (X - 45{\cdot}6870)$, this giving the line B in

Figure 1. The standard error of an estimate was calculated from the expression:

$$S_{x1} = \left(\frac{1}{b^2}\left\{\frac{1}{\Sigma(nw)} + \frac{(x_1 - \bar{x})^2}{\Sigma[nw(x-\bar{x})^2]}\right\}\right)^{\frac{1}{2}},$$

and the new value of the mean single survivor time found to be 44·090 ± 1·457 minutes.

The cycle of computation was repeated in order to derive a second approximation to the relationship. The residual variance was thereby further reduced to 0·321, and the estimates of mean working loglog and slope recalculated as

$$\bar{y}_w = -0{\cdot}1092 \pm 0{\cdot}0232 \text{ and } b = -0{\cdot}1214 \pm 0{\cdot}00030$$

The second approximation to the relationship was

$$Y = -0{\cdot}1092 \pm 0{\cdot}12114\,(X - 45{\cdot}3434),$$

from which the line C in Figure 1 was obtained. The final estimate of the mean single survivor time was 44·444 ± 1·264 minutes, and this was used for the correlation with contact times required to give other percentages of survivors, which is described below. The second approximation has brought about small reductions in the residual variance and in the standard error of an estimate; however, as Mather found, the estimate based on the use of a line fitted by inspection yields a result lying within the standard errors of the estimates based on the corrected regressions.

Correlation between Extinction Data and Percentages of Survivors at Various Contact Times with Aqueous Solutions of Phenol

The use of the loglog analysis of extinction data and the development by Finney[3] of a loglog analysis of the data of dilution series suggested that a relation might exist between the mean single survivor time and the contact times required for varying percentages of survivors determined by dilution series. The establishment of such a correlation would signify that coefficients of disinfectant action based on end-point methods were of comparable reliability with those based on counts of bacterial survivors. Moreover, a comparison of these two methods was desirable, rather than a comparison of extinction results with direct colony counts, because any tendency of the organisms to clump in the presence of the solution of bactericide would be expected to lead to a similar discrepancy in both methods, thus making possible a more direct comparison. This consideration, coupled with the fact that the same form of analysis would be used for data of both methods, was thought to more than outweigh the loss of accuracy inherent in counts by dilution series as against direct colony counts.

Experimental

1. *Scheme.* The percentages of survivors were determined by adding 10 drops of a standardised suspension of a 24-hour growth of *Bact. coli*

to 5 ml. of a 1·10 per cent. phenol solution contained in a 60 ml. glass-stoppered bottle, which had previously been immersed in a water bath maintained at a temperature of 20° ± 0·05° C. The contents of the bottle were mixed by rotation and the bottle replaced in the water bath. After a fixed time exposure a sample of 10 drops of the reaction mixture was withdrawn and diluted by successive factors of 4, over a range exceeding that dilution at which one survivor could exist. 6 consecutive dilutions considered to lie around the end-point were selected and 5 drops of each inoculated into each of 10 aluminium-capped test-tubes containing 5 ml. of sterilised broth of the same composition and same batch preparation as that used in estimation of the single survivor time. The inoculated tubes were placed at once in a water bath maintained at 37° C. and afterwards incubated at the same temperature for 48 hours before reading the results. Further periods of incubation of up to 2 months resulted in no further decrease in the proportion of negative samples.

Each day experiments were carried out using exposures of varying times—3 or 4 different times each day. In order to express the results as percentages of survivors, an initial count was made by adding 10 drops of bacterial suspension to 5 ml. of water, diluting 10 drops of this with a single large volume, and diluting a sample of this by successive factors of 4. A correction, however, has to be applied for the larger volume of a drop of the reaction mixture as compared with the drop volume of the phenolic reaction mixture.

2. *Details*

(i) *The Dilutions.* In the beginning of the work these were made with sterilised distilled water. 25 drops of a previous dilution was added to 75 drops of water in a sterilised test-tube, the mixture shaken, and 25 drops of this dilution removed to the next tube. It was found that such dilutions took a considerable time to perform, and that although the percentages of survivors appeared to decrease logarithmically with time after exposures of up to 15 minutes, the decrease was very much more steep with longer exposures. It was considered that damaged organisms might not survive in water over the period between preparation of all dilutions and their inoculation into the broth. Dilutions were afterwards prepared using sterilised broth. The numbers of survivors at all time intervals were increased and a strong correlation found to exist between the logarithms of the percentages of survivors and contact times over a range of exposures from 5 to 25 minutes.

The first dilution was always obtained by addition of 10 drops of reaction mixture to 5 ml. of broth before commencing the dilutions by factors of 4. This was chosen in order that the phenol might be well diluted immediately on expiry of the period of exposure. In the case of the "blank" exposures which used water in place of the phenol solution, the first dilution was made by the addition of 10 drops to 350 ml. water, giving a 1 : 2000 dilution.

Owing to the variations in the drop volumes of solutions of phenol

diluted with broth, these dilutions further diluted with broth, and of water diluted with broth, the drop volumes of water, 1·10 per cent. phenol solution and the broth were determined by the method described by Withell[4], and the drop volumes of mixtures of these were determined. This enabled graphs to be plotted recording (*a*) decrease in drop volume as water was continuously diluted with broth; (*b*) decrease in drop volume as 1·10 per cent. solution of phenol was diluted with broth. In both cases only two corrections need be applied, for after 2 dilutions the discrepancies are smaller than the errors due to variation in drop volumes delivered by different dropping pipettes.

(ii) *The Dropping Pipettes.* Prepared from 5 mm. diameter glass tubing, one end being drawn out and ground to fit a "Record" hypodermic needle as described by Cook and Yousef[5]. The needles were ground and the tips polished to give a square and completely smooth end. The drop weights of water delivered from all the needles prepared for use were determined, taking 20 weighings from each needle. The results, when subjected to an analysis of variance, showed a significantly greater variation in the performance of different needles than in the performance of one needle. However, this difference is of little practical significance, since the difference between the highest and lowest needle means scarcely exceeded 2 per cent. of the weight delivered—comparing favourably with the performance of different delivery pipettes.

Results and Treatment

The treatment of the results of the dilution series closely followed that described by Finney. The proportions of media showing no growth at each dilution were observed and the corresponding value of the loglog found. A rough estimate of the bacterial density may be obtained by plotting the loglog against the natural logarithms of the dilution factors. The value of the dilution factor corresponding to a loglog of 0 is the dilution required to yield one survivor, and its value is thus the estimated number of organisms contained in a five drop sample of the reaction mixture.

Such estimations were found to be inaccurate owing to the fact that seldom did an experiment show more than 3 dilutions, sometimes only 2, with a proportion of negative samples. The remaining dilutions gave all positive or all negative samples. The method of calculation described by Finney was therefore used in all cases to obtain the final result. This consisted in estimating a series of working loglogs, Y, corresponding to the proportions of negative samples in each of 5 dilution series. The values of weighting coefficients, w, and working deviates, η, were found by reference to the tables published by Finney, and from these a correction factor, $\bar{\eta} = \frac{\Sigma nw\eta}{\Sigma nw}$, computed and subtracted from the values of Y to give a new series of corrected working loglogs. The process was repeated until the correction became sufficiently small to be ignored. In this case no further calculation was employed where the correction was

reduced to below 0·005, this usually requiring 2 or 3 cycles of computation. An example of the calculation is given below:—

EXPERIMENT PS/46

EXPOSURE TO 1·10 PER CENT. PHENOL FOR 15 MINUTES

Dilution Factor	p	Empirical Loglog	Y	nw	η	Y	nw	η
1/4	0	—	1·0	5·2204	−0·3679	1·1	4·7080	−0·3329
1/16	0·5	−0·366	−0·4	4·7057	−0·0386	−0·3	4·9999	0·0659
1/64	0·9	−2·250	−1·8	1·5201	0·3735	−1·7	1·6650	0·4401
1/256	1·0	—	−3·2	0·3994	1·0207	−3·1	0·4404	1·0229
1/1024	1·0	—	−4·6	0·1000	1·0050	−4·5	0·1105	1·0056
				11·9456			11·9238	

$$\Sigma nw = -1{\cdot}0263 \qquad \Sigma nw\eta = 0{\cdot}0566$$

$$\bar{\eta}\text{ (1st cycle)} = -\frac{1{\cdot}0263}{11{\cdot}9456} = -\,0{\cdot}0859;\ \text{value of Y at 1/16 dilution} = -\,0{\cdot}3141$$

$$\bar{\eta}\text{ (2nd cycle)} = +\frac{0{\cdot}0566}{11{\cdot}9238} = +\,0{\cdot}0047;\ \text{value of Y at 1/16 dilution} = -\,0{\cdot}3188$$

$$\text{Log}_e - 0{\cdot}3188 = 0{\cdot}7270$$

Hence density per sample volume = 11·632.

Initially 10 drops of reaction mixture were diluted with 5 ml. broth, corresponding to a further dilution factor of 37·23. The density per 10 drops of reaction mixture is therefore $11{\cdot}632 \times 37{\cdot}23 \times 2 = 8{\cdot}66 \times 10^2$. The density per 10 drops at zero time determined on the same day was found to be $12{\cdot}64 \times 10^6$, giving a percentage of survivors after 15 minutes exposure of 0·0063 per cent.

TABLE II

DEATH OF *Bact. coli* ON EXPOSURE TO 1·10 PER CENT. PHENOL

Series/Time (minutes)	36	42	48	54	60	66
1	+	+	+	−	−	−
2	+	+	+	+	−	−
3	+	−	−	−	−	−
4	+	+	+	+	+	+
5	+	−	−	−	−	−
6	−	+	+	−	−	−
7	+	+	+	+	−	−
8	+	+	+	−	−	−
9	+	+	+	+	−	−
10	+	+	−	−	+	−
11	−	−	+	−	−	−
12	+	+	−	+	−	−
13	+	+	+	−	−	−
14	+	+	−	−	−	−
15	+	+	−	−	−	−
16	+	−	−	−	−	−
17	+	+	+	+	−	−
18	+	+	−	−	−	−
19	+	+	−	−	−	−
20	+	+	−	−	−	−
Total of negative samples	2	4	10	14	18	19
p	0·1	0·2	0·5	0·7	0·9	0·95
$y = \log(-\log p)$	0·834	0·476	−0·366	−1·031	−2·250	−2·970

In all, 18 bacterial densities were estimated on 4 consecutive days: 4 of these consisted of the estimates of densities at zero time and the remainder were estimates of the survivors after varying periods of exposure. The results of these experiments are recorded in Table III.

In all, 10 estimations of the density of the original bacterial suspension had been made, these having been carried out on different days on freshly prepared and standardised suspensions. The results were a follows, expressed as densities per ml.:—

2.366×10^9, $2{\cdot}322 \times 10^9$, $1{\cdot}680 \times 10^9$, $1{\cdot}923 \times 10^9$, $2{\cdot}095 \times 10^9$, $2{\cdot}353 \times 10^9$, $2{\cdot}508 \times 10^9$, $2{\cdot}549 \times 10^9$, $2{\cdot}503 \times 10^9$, $1{\cdot}819 \times 10^9$.

Mean density $= 2{\cdot}211 \times 10^9 \pm 0{\cdot}3146 \times 10^9$.

This shows a reasonable degree of reproducibility both of standardisation of the suspension and of estimation of the densities.

Analysis of the Results

Figure 2 shows the logarithms of percentages of survivors plotted against the logarithms of the corresponding contact times with 1·10 per cent. phenol solution. On inspection, it was considered possible that there might be sufficient variability between the results obtained on different days that four significantly different relationships could be established. This variation was thought to be explained largely by variations in the densities of the original suspensions. Since only one single mean survivor time had been determined and since no initial count had been made on the suspension used for estimation of the single survivor time during the week occupied by these experiments, it was decided to express it as 4 percentages corresponding with the densities estimated on each of the other 4 days. These are shown as the 4 almost coincident points at the bottom of the graph. The problem then was to treat the results as 4 separate relationships, to ascertain whether the 4 regression lines differ significantly in slope, and if no such difference was indicated, to determine whether the 4 regression lines could be regarded as adequately represented by one coincident line (Tippett[6]).

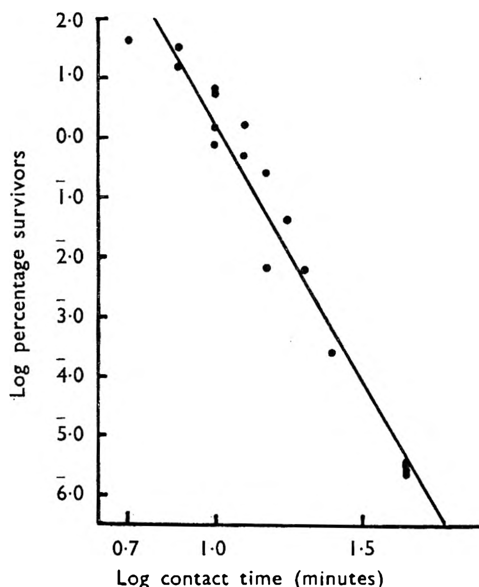

Fig. 2. The relationship between percentages of survivors and contact times after exposure to 1·10 per cent. phenol solution.

TABLE III

EXPOSURE OF *Bact. coli* TO 1·10 PER CENT. PHENOL SOLUTION OVER VARYING TIME INTERVALS

Experiment	Contact time (minutes)	Density per 10 drops of reaction mixture	Percentage survivors
PS/38	0	$13·98 \times 10^6$	—
PS/39	10	$2·036 \times 10^5$	1·455
PS/40	12·5	$6·984 \times 10^4$	0·499
PS/41	20	$8·70 \times 10^2$	0·00622
PS/42	25	33·27	0·000237
PS/43	0	$13·742 \times 10^6$	—
PS/44	7·5	$2·060 \times 10^6$	14·99
PS/45	10	$9·754 \times 10^4$	0·710
PS/46	15	$8·660 \times 10^2$	0·00630
PS/47	0	$9·937 \times 10^6$	—
PS/48	7·5	$3·176 \times 10^6$	31·96
PS/49	10	$6·070 \times 10^5$	6·109
PS/50	12·5	$1·602 \times 10^5$	1·613
PS/51	17·5	$4·170 \times 10^3$	0·0419
PS/52	0	$10·378 \times 10^6$	—
PS/53	5	$4·641 \times 10^6$	44·72
PS/54	10	$5·626 \times 10^5$	5·421
PS/55	15	$2·712 \times 10^4$	0·261

First, the correlation coefficients and residual sums of squares were calculated for each relationship, coding the values of the ordinates by addition of 7 to the index of the logarithm.

(i)

$\Sigma^2 x = 41·520625$ $\quad \Sigma^2 y = 547·861433$

$\Sigma x^2 = 8·565273$ $\quad \Sigma y^2 = 132·436315$

$\Sigma x \Sigma y = 150·822907$ $\quad \Sigma xy = 27·733522$

$\Sigma(x - \bar{x})^2 = 0·261148$

$\Sigma(y - \bar{y})^2 = 22·864028$

$\Sigma(x - \bar{x})(y - \bar{y}) = -2·431059$

$r = -0·9949$

Residual S. Sq. $= 0·232999$

(ii)

$\Sigma^2 x = 22·079849$ $\quad \Sigma^2 y = 449·870463$

$\Sigma x^2 = 5·864064$ $\quad \Sigma y^2 = 138·716550$

$\Sigma x \Sigma y = 99·664798$ $\quad \Sigma xy = 21·931006$

$\Sigma(x - \bar{x})^2 = 0·344102$ $\quad \Sigma(y - \bar{y})^2 = 26·273934$

$\Sigma(x - \bar{x})(y - \bar{y}) = -2·985193$

$r = -0·9928$

Residual S. Sq. $= 0·376447$

(iii)

$\Sigma^2 x = 34·372189$ $\quad \Sigma^2 y = 939·184058$

$\Sigma x^2 = 7·229236$ $\quad \Sigma y^2 = 218·836626$

$\Sigma x \Sigma y = 179·671401$ $\quad \Sigma xy = 32·636754$

$\Sigma(x - \bar{x})^2 = 0·354797$

$\Sigma(y - \bar{y})^2 = 30·999854$

$\Sigma(x - \bar{x})(y - \bar{y}) = -3·297526$

$r = 0·9943$

Residual S. Sq. $= 0·352211$

(iv) $\Sigma^2 x = 20{\cdot}455991$ $\Sigma^2 y = 590{\cdot}879350$
$\Sigma x^2 = 5{\cdot}586893$ $\Sigma y^2 = 178{\cdot}090972$
$\Sigma x \Sigma y = 109{\cdot}940997$ $\Sigma xy = 23{\cdot}809900$
$\Sigma(x - \bar{x})^2 = 0{\cdot}472895$ $\Sigma(y - \bar{y})^2 = 30{\cdot}371134$
$\Sigma(x - \bar{x})(y - \bar{y}) = -3{\cdot}675349$
$r = -0{\cdot}9698$
Residual S. Sq. $= 1{\cdot}806251$

$$\text{Total residual variance} = \frac{0{\cdot}232999 + 0{\cdot}376447 + 0{\cdot}352211 + 1{\cdot}806251}{18 - 8}$$
$$= 0{\cdot}276791$$

Total residual S. Sq., assuming one regression coefficient, but different means of y for a given value of x

$$= \frac{\Sigma^2(x - \bar{x}_1)(y - \bar{y}_1)}{\Sigma(x - \bar{x}_1)^2} + \frac{\Sigma^2(x - \bar{x}_2)(y - \bar{y}_2)}{\Sigma(x - \bar{x}_2)^2} + \ldots$$
$$\frac{[\Sigma(x - \bar{x}_1)(y - \bar{y}_1) + \Sigma(x - \bar{x}_2)(y - \bar{y}_2) + \ldots]^2}{\Sigma(x - \bar{x}_1)^2 + \Sigma(x - \bar{x}_2)^2 \ldots}$$
$$= 107{\cdot}741002 - 107{\cdot}115618$$
$$= 0{\cdot}625384$$

There are $4 - 1 = 3$ degrees of freedom, so the estimate of this residual variance is 0·208461. This is smaller than the residual variance for the separate regression coefficients, so that the samples could be considered as being derived from a population having a regression of common slope. Next, the total of 18 results were pooled and the residual sum of squares calculated.

$\Sigma x^2 = 27{\cdot}245466$ $\Sigma^2 x = 463{\cdot}462534$ $\Sigma(x - \bar{x})^2 = 1{\cdot}497547$
$\Sigma y^2 = 668{\cdot}080463$ $\Sigma^2 y = 9914{\cdot}326390$ $\Sigma(y - \bar{y})^2 = 117{\cdot}284558$
$\Sigma xy = 106{\cdot}111182$ $\Sigma x \Sigma y = 2143{\cdot}57617$
$\Sigma(x - \bar{x})(y - \bar{y}) = -12{\cdot}976383$
$r = -0{\cdot}979136$ $b = -8{\cdot}665092$

Regression Equation: $Y = 5{\cdot}5317 - 8{\cdot}6651\,(X - 1{\cdot}196)$
Residual S.Sq. from common regression $= 4{\cdot}843001$ for $18 - 2$
$= 16$ degrees of freedom
Residual S.Sq. from separate regressions $= 2{\cdot}76791$ with 10 degrees of freedom

The difference between the two residual sums of squares is 2·07509, with $16 - 10 = 6$ degrees of freedom, giving a variance estimate of 0·3458. This may be tested against the residual variance for the separate regressions, the variance ratio being 1·249. This ratio, with the appropriate number of degrees of freedom, corresponds to a probability level of above 0·2. Hence the residual variance from the common regression is not significantly greater than that from the separate regressions, and the

4 separate relationships can be confidently regarded as being represented by one coincident regression line.

Discussion

A correlation between the percentages of survivors after varying periods of contact with an aqueous solution of phenol and the percentage of survivors corresponding with the mean single survivor time, as estimated from extinction data, has been demonstrated. This leads to the conclusion that the evaluation of bactericidal activity by an extinction method, provided the results are subjected to the loglog analysis, will yield as much information as can be derived from the use of counting methods in the region of virtual sterilisation. It is realised that estimations of bacterial densities by dilution series as employed in this investigation are not of comparable accuracy with direct colony counts; and it has been pointed out that such estimates were used in view of the equal tendencies to clumping of organisms in the two methods used.

Inspection of the log. per cent. survivors–log. contact time regression (Fig. 2) might lead to the conclusion that the deviations are due to the relationship being in fact sigmoid. If this were the case it might be expected that a more satifactory correlation would be evident on plotting the values of the percentages of survivors on a probit scale. Accordingly, the percentages of survivors were converted into probits.

In order to compare the significance of the two regressions, the data for each relationship were pooled, but the values of the percentages of survivors corresponding to the mean single survivor time were not included. In the case of the logarithmic survivor-time curve, the slope was calculated as − 7.7908 and the correlation coefficient found to be − 0·9287, with 12 degrees of freedom. The value of the contact time corresponding to the single survivor percentage was found to be 54·95 minutes, whereas the observed value was 44·44 minutes. With the probit per cent. survivors—log. contact time regression, the slope was calculated as − 6·6319, the correlation coefficient as − 0·9548, with 12 degrees of freedom, and the single survivor contact time as 30·69 minutes, as compared with the observed 44·44 minutes.

These results indicate that the probit relationship provides the more highly significant regression over the largest part of the curve, and the existence of the probit relationship is evidence of a log-normally distributed resistance to the bactericide amongst the test organisms, which has been demonstrated by many workers who have employed colony counting methods. However, the fact that the experimentally observed value of the single survivor time is smaller than the value calculated on the basis of the logarithmic regression appears to be reasonable on the grounds that sampling variations in obtaining a viable organism at the extreme of the curve would lead to an apparently shorter contact time than that expected; on the other hand, the probit regression permits calculation of a single survivor time smaller than that observed. In neither case can the discrepancy be regarded as serious since the limits of error of an estimate of a contact time at this extreme will be wide.

Evaluation of bactericidal activity by the extinction method described leads to a general economy in time and apparatus over counting methods. Moreover, the treatment of results is relatively simple and the results are rapidly read, so that it is conceivable that this method might well be applied to the routine testing of bactericidal activity, giving estimates of equal reproducibility to those of any other methods now in common use.

Summary

A correlation between the mean single survivor time and percentages of survivors at a series of shorter contact times as estimated by serial dilution counts has been established.

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THE ESTIMATION OF BACTERICIDAL ACTIVITY FROM EXTINCTION TIME DATA

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Received May 5, 1954

The most frequently used methods for the evaluation of bactericidal activity involve either the measurement of extinction times or the enumeration of surviving organisms after varying periods of exposure to the bactericide. In general, counting techniques are preferred, for they can provide information on the velocity of the reaction over a great range of the time-survivor curve, whereas extinction methods yield information at but one point of the reaction and concerning only the overall velocity of the complete bactericidal action. Furthermore, the accuracy and degree of reproducibility of estimates of bactericidal activity based on survivor counts are usually acknowledged to be superior to those based on extinction data, a belief which might be attributed to the paucity of replication, the use of inadequately small sampling intervals and the failure to take account of sampling variations in the usually employed experimental designs for extinction time determinations.

Counting methods possess some obvious disadvantages. They are tedious in performance, a consideration of importance in the routine testing of bactericides, and are not of universal application. Such methods could not be used, for example, where the test organisms themselves tend to aggregate into clumps or chains or where there is a tendency for the bactericide to agglutinate the organisms. Colony counts obtained from samples of agglutinated organisms would be meaningless. Hence, when there is a tendency for agglutination to occur during the bactericidal reaction, recourse must be made to methods other than counting.

The authors were presented with this problem when they attempted to evaluate the bactericidal activities of solutions of soaps. They found that solutions of potassium laurate agglutinated test organisms with which they were inoculated, the phenomenon being more marked with some organisms than with others. Microscopical examination revealed that a strain of *Bacterium coli* (N.C.T.C. No. 5933) was not seriously agglutinated by the soap, but *Pseudomonas pyocyanea* (N.C.T.C. No. 1999) was agglutinated to an extent which was considered to completely invalidate the use of counting methods.

In the face of this difficulty, a new method for the estimation of extinction times was adopted. It is the purpose of this communication to describe the method and to point out the advantages which it possesses over other methods for the determination of extinction data. The principal feature of the method is that of sampling the reaction mixture *immediately after mixing the organisms with the bactericide.* The samples are transferred to sterilised tubes, maintained at a controlled temperature, where the reaction is allowed to proceed until quenched by the

addition of a sufficient volume of sterilised broth to render the bactericide inactive. Other features of the method are its performance in replicate, the mean of several extinction times so obtained being utilised: the use of a dropping pipette for sampling the reaction mixture in place of the more usual platinum loop; and the use of relatively narrow limits—between 1/5 and 1/10 of the anticipated death times—for the exposure time intervals.

During the development of the method several different concentrations of phenol were used as the bactericidal solution. Phenol was selected because its behaviour as a bactericide has been extensively studied and it was known to yield reproducible results when subjected to other methods of evaluation. If the assessment of activity of phenol by the proposed technique accorded with that revealed by numerous established and accepted techniques, then the method could be assumed to be satisfactory.

Method

(i) *Cultivation of Test Organisms.* The strain of *Bacterium coli* (N.C.T.C. No. 5933) was maintained by freeze-drying. At monthly intervals a freeze-dried culture was opened and transferred to a slope of peptone agar. After 24 hours' incubation, four "sub-master" slopes were prepared from this "master" slope, and each day for 14 days slopes were inoculated from a "sub-master" slope. The slopes so prepared were used in the experiments from the fourth to the fourteenth day, when a fresh "sub-master" slope was introduced.

The peptone agar was prepared by gelling a peptone broth, described by Needham[1], with 2 per cent. of bacteriological agar. The broth contained 1 per cent. of "Oxoid" peptone and 0·5 per cent. of sodium chloride, the solution being adjusted to *p*H 7·3 by the addition of sodium hydroxide.

(ii) *The Bacterial Suspension.* The 24-hour growth of the organisms was washed from the surface of 3 agar slopes with a few ml. of sterilised quarter strength Ringer's solution, as recommended by Wilson[2] and used by Berry and Michaels[3]. The suspension thus obtained was centrifuged at 2000 r.p.m. for about 1 minute in order to precipitate small fragments of agar removed from the slopes and then was lightly shaken with sterilised glass beads to ensure complete dispersion of the organisms. The volume of the suspension was adjusted to contain about 2000×10^6 *Bact. coli* per ml., a density corresponding to that found in a 24-hour culture of Needham's broth. This adjustment was made with the aid of a photoelectric absorptiometer.

(iii) *Preparation and Inoculation of the Bactericide.* The solutions of phenol used in the experiments were prepared in water distilled from a heavily tinned still fitted with an all-glass condensing system. Water from the same still was also used in the preparation of the nutrient media and the Ringer's solution. 5 ml. quantities of the phenol solutions were introduced into 60 ml. glass-stoppered "Pyrex" bottles, which were placed for at least 20 minutes in a water bath maintained at $20° \pm 0·1°$ C.

The inoculation of the phenol solutions with the suspension of *Bact. coli* was at first performed using a 1 ml. graduated pipette. The pipette was

rinsed by drawing the suspension into it and rejecting several times. 0·2 ml. was then added to the bactericide solution, which was agitated by gentle rotation for 10 seconds in order to effect uniform distribution of the organisms throughout the solution. It was afterwards considered preferable to make the addition of the suspension by means of a dropping pipette, adding 10 drops, which was equivalent to approximately 0·18 ml.

(iv) *Sampling the Reaction Mixture.* As soon as possible after mixing the organisms with the bactericide, a portion of the reaction mixture was withdrawn into a dropping pipette, which was clamped vertically and fitted with a rubber teat. The standard dropping pipettes were prepared by the method described by Withell[4]. Six drops of the reaction mixture, delivered from the pipette at one second intervals, were transferred to each of a series of sterilised aluminium-capped test-tubes, which had been immersed in a water bath maintained at $20° \pm 0·1°$ C. for at least 20 minutes. Care was taken that the drops fell upon the bottom of the tube. The tubes were replaced in the water bath immediately after inoculation.

The reaction between the bactericide and the test organisms was allowed to proceed in the tubes at the controlled temperature. After predetermined time intervals, tubes were removed from the water bath and the reaction was quenched by the addition of 5 ml. of sterilised broth. Immediately after quenching, each tube was placed in a water bath maintained at 37° C., and at the end of the experiment the tubes were transferred to an incubator and examined for evidence of growth after 3 days. The importance of incubating the tubes immediately after the addition of broth was proved when preliminary tests, with as many as 20 replicate experiments performed on one day, showed quite clearly that tubes left in the laboratory for an hour or so before being transferred to the incubator gave an extinction time estimate considerably shorter than did experiments in which the tubes were immediately incubated. It appears that the bactericide damages a proportion of the organisms without killing them and that rapid transferrence to a favourable environment at optimal growth temperature allows the organisms to make good their recovery before death supervenes. A large volume of literature has appeared on the errors inherent in methods of evaluating bactericides, but little importance has been attached to the above factor.

Illustration of the Method Using Solutions of Phenol

The experiments were performed with 8 different concentrations of phenol, one experiment with the greatest possible replication being performed on one day with one concentration of phenol. It might be objected that it would be fundamentally more sound to perform a number of experiments with several different phenol concentrations but with very limited replication during one day. However, such a procedure would tend to obscure between-replicates variability, for the variations observed by performing many experiments on different days would probably have been attributed to day-to-day variations in the susceptibility of the test organisms. Unpublished results since obtained from these laboratories indicate that, on the evidence of determinations of mean single survivor

times with the same concentration of phenol performed over many days, day-to-day ("subculture") variations are small relative to the standard error of an estimate of the extinction time. Variations between "master" cultures were found to be significantly greater than those within "master" cultures.

Typical results are set out in Tables I and II, which show the extinction times of *Bact. coli* in 1·0 per cent. and 1·15 per cent. w/v phenol respectively. The lowest extinction time observed in 1·0 per cent. phenol was 60 minutes

TABLE I

DEATH TIME OF *Bact. coli* IN 1·0 PER CENT. W/V PHENOL SOLUTION

Experiment	Time in minutes								Estimated death time Minutes
	40	50	60	70	80	90	100	110	
1	+	+	+	+	+	−	−	−	90
2	+	+	+	−	−	−	−	−	70
3	+	−	+	−	−	−	−	−	70
4	+	+	+	−	−	−	−	−	70
5	+	+	+	−	−	−	−	−	70
6	+	+	−	+	−	−	−	−	80
7	+	+	+	−	+	−	−	−	90
8	+	+	+	−	−	−	−	−	70
9	+	+	−	−	−	−	−	−	60
10	+	+	−	−	−	−	−	−	60
11	+	+	+	−	−	−	−	−	70
12	+	+	−	+	−	−	−	−	80
								TOTAL	880
								MEAN DEATH TIME	73·3 minutes

TABLE II

DEATH TIME OF *Bact. coli* IN 1·15 PER CENT. W/V PHENOL SOLUTION

Experiment	Time in minutes								Estimated death times minutes
	12	14	16	18	20	22	24	26	
1	+	+	+	+	+	−	−	−	22
2	+	+	+	−	−	−	−	−	18
3	+	+	+	−	+	−	−	−	22
4	+	+	+	+	−	+	−	−	24
5	+	+	+	+	−	−	−	−	20
6	+	+	+	+	−	−	−	−	20
7	+	+	−	+	−	+	+	−	26
8	+	+	+	+	−	−	−	−	20
9	+	+	+	+	+	−	−	−	22
10	+	+	+	−	+	−	−	−	22
11	+	+	+	+	−	−	−	−	20
12	+	+	+	+	−	−	−	−	20
13	+	+	+	−	+	+	−	−	24
14	+	+	+	+	+	+	−	−	24
15	+	+	+	−	−	−	−	−	18
16	+	+	+	−	−	−	−	−	18
17	+	+	−	−	−	−	−	−	16
18	+	+	−	+	−	−	−	−	20
								TOTAL	376
								MEAN DEATH TIME	20·9 minutes

and the highest 90 minutes, whilst in 1·15 per cent. phenol the times were 16 and 26 minutes respectively. Thus with both of these solutions the maximal extinction time for an individual replicate estimation was approximately 150 per cent. of the minimal estimated time. Similar ratios

of maximal to minimal times were observed with the other concentrations of phenol examined. For each phenol concentration the mean extinction time was calculated by dividing the sum of the individual extinction times by the number of replicate determinations performed. In deciding the individual extinction times a convention was adopted that if no growth was obtained after a given period of exposure in all replicates, then any growth obtained thereafter was neglected.

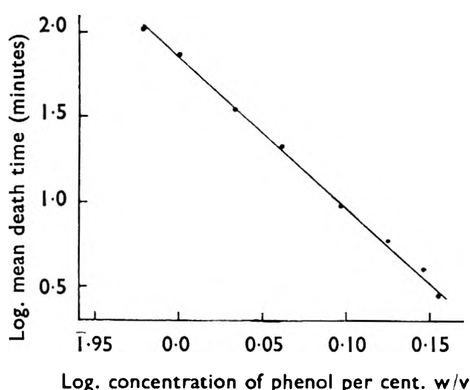

FIG. 1. The relationship between mean death times of *Bact. coli* and concentrations of phenol.

Table III records a summary of the experiments performed with the eight concentrations of phenol, and Figure 1 shows the relation between concentrations and extinction times plotted on a logarithmic scale. The obvious close approximation to a linear relationship over the range of concentration used is in good agreement with the results described by Phelps[5] and by many other workers in more recent years.

TABLE III

THE DEATH TIMES OF *Bact. coli* IN AQUEOUS SOLUTIONS OF PHENOL

Phenol concentration per cent.	Log phenol concentration	Mean death time ($\bar{x}$)	Log. mean death time	Number of replicate determinations	Standard deviation	Coefficient of variation
		Minutes				
0·952	$\bar{1}$·9780	104·5	2·0155	11	6·578	6·32
1·000	0·0000	73·3	1·8653	12	9·427	12·85
1·080	0·0333	34·6	1·5391	15	5·306	15·30
1·150	0·0607	20·9	1·3201	18	2·512	12·03
1·250	0·0969	9·6	0·9823	20	1·624	16·91
1·333	0·1248	5·9	0·7723	12	0·576	9·73
1·400	0·1461	4·0	0·6020	20	0·623	15·81
1·428	0·1547	2·8	0·4471	15	0·160	5·71

DISCUSSION

After the performance of only a few experiments it became obvious that the results of any one determination were by no means perfectly reproducible. In a number of replicate determinations there was always a scatter of the end-points. This is a factor which has not been stressed sufficiently in reports of extinction data, and may well be due to the fact that many workers have sampled their reaction mixtures at intervals of similar duration to those specified in the Rideal–Walker or Food and Drugs Administration methods. Such sampling time intervals as 2·5, 5, 7·5 and 10 minutes or 5, 10 and 15 minutes constitute a large proportion of the anticipated death time and will tend to obscure any between

replicates variations. In the present series of experiments the time intervals between samples was reduced to between 10 and 20 per cent. of the expected extinction time. It is the use of these narrow sample intervals which serves to account for the sampling variations at the extreme end of the time-survivor curve and which gives the method here described a greater degree of accuracy than all previous commonly used extinction methods.

Withell[6] has shown that simultaneous death does not occur when bacteria are introduced into a disinfectant solution. The logarithms of the resistance of the individual organisms to the bactericide are distributed normally: towards the end of the disinfection process only the most highly resistant organisms remain alive. These organisms of high resistance represent only a very small proportion of the initial inoculum and the number remaining viable decreases only slowly with time, especially in the more dilute disinfectants. Thus a sample removed towards the end of the bactericidal reaction may or may not contain a survivor, although there may be many survivors in the bulk of the solution. The between-replicates variation, estimates of which have been expressed as coefficients of variation for each phenol concentration in Table III, can therefore be explained in terms of variation in resistance among the last survivors and variations in sampling. Provided this is realised, and the structure of the experiment be so designed that the variation may be estimated, there appears to be no *prima facie* reason as to why the extinction method should not provide valid and reproducible data.

Previous methods of determination of extinction times have usually involved sampling the reaction mixture by means of a platinum loop. It is well known that, even in the hands of experienced operators, the volume of reaction mixtures sampled by means of a loop may vary widely from a mere film across the loop to a large pendant drop. For a given solution, the sample volume withdrawn with a loop depends upon the angle at which the loop is withdrawn from the liquid, upon effects of heating when the loop is repeatedly sterilised, and upon the skill of the operator. Estimates of the variations between samples delivered by means of dropping pipettes have been given by Withell[4] and, in the case of pipettes fitted with needles, by Cook and Youssef[7]; these reveal that the variations in drop volumes from different pipettes, where samples of 10 or 20 drops are delivered, is unlikely to exceed 2 per cent.

The method of sampling employed possesses another advantage. Owing to the small number of survivors remaining towards the end of the disinfection process, the possibility of obtaining a representative sample of the reaction mixture at this point will be increased by taking as large a sample as possible, the only limitation to the sample size being the convenience of the volume of broth required to render the bactericide inactive by dilution. In practice, the 6-drop samples withdrawn from the reaction mixture correspond to a volume exceeding 0·1 ml., and hence are greater than those withdrawn by means of a loop. The sampling error, of course, would be minimised by taking several simultaneous samples. This was not possible in the experimental procedure adopted,

but it was possible to take similar samples from a number of experiments performed under identical conditions.

The relatively simple experiment which has been outlined will yield a mean extinction time estimate representative of a high percentage mortality of the inoculum. A more accurate statistical treatment of the authors' results has been described by Mather[8], who has calculated the mean single survivor time with respect to a sample volume of the reaction mixture, together with estimates of sampling variation and standard error of an estimate. However, for most practical purposes the simple calculation of the mean extinction time may be considered adequate.

Summary

1. A new method for the determination of extinction time data is described.

2. The method has been employed for the disinfection of *Bact. coli* by aqueous solutions of phenol over a range of concentrations and death times ranging from 2 to over 100 minutes.

3. The use of extensive replication, short sampling intervals, and constant sample volumes withdrawn immediately after inoculation of the bactericide are among the chief features of the technique.

4. Estimates of the mean extinction time obtained from the data are of reproducibility within limits sufficiently close to invalidate other extinction time methods, and are of comparable value with those obtained by the use of any other techniques.

5. The method has been satisfactorily applied to systems containing water-insoluble phenols solubilised in solutions of soaps (Berry and Bean[9]) where clumping of the test organisms might completely invalidate other methods of estimation of bactericidal activity.

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MELTING POINTS WITH DECOMPOSITION AND THE HEAT STABILITY OF ATROPINE SULPHATE

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Received May 3, 1954

WHEN an organic substance commences to decompose before it melts, the melting point is lowered by the products of decomposition, and depends on the time the sample has been subjected to the decomposition temperature. When the m.pt. forms part of an official specification, the results of different analysts may be widely divergent, and may lead to disputes regarding quality. Some alkaloid salts, and particularly sulphates, are sensitive to small variations in m.pt. procedure, and a study has been made of these factors as they affect atropine sulphate. The heat stability, or time taken for the substance to melt at a constant temperature well below its normal m.pt., is a useful indication of purity.

INTRODUCTION

A decomposition m.pt. will be higher the faster the substance is taken through the critical range of temperature. Thus, Kempf[1] observed that tyrosine melted at 280° C. when heated slowly, and at 314 to 318° C. when heating was rapid. Decomposition varies in nature and degree, until it reaches the point of explosion, as noted by Willgerodt[2] for iodoxybenzene. The apparatus of Dennis and Shelton[3] overcomes, to some extent, the difficulties inherent in the capillary tube method. The sample is laid in a thin trail along a copper bar heated at one end to establish a temperature gradient, and the temperature at the point at which the substance melts is measured by a thermocouple formed by touching the bar with a constantan wire. This apparatus was improved by Kofler[4], who called it a "heat bank," and among the m.pts. given by Kofler and Sitte[5] are acetylsalicylic acid, 143° C. (135° to 138° C.), arginine, 260° C. (238° C.) and morphine, 260° C. (254° C.), the figures in brackets being measured by the conventional method in capillary tubes. Since the latter is official in the various Pharmacopœias, it has been used exclusively in this investigation.

ALKALOIDAL SALTS AND DERIVATIVES

All types of decomposition occur in the alkaloid field, from the darkening of aurichlorides and the frothing of amino-oxides to the explosion of certain picrates. Acetylatropine methylnitrate, immersed at 140° C. and heated at 3° C. per minute, melted sharply at 148° C. Acetylatropine methylbromide, with the same rate of heating, was strikingly anomalous. Immersed at 215° to 220° C. it melted at 223° C., lost acetic acid, resolidified, and melted again as apoatropine methylbromide at 265° C.

When immersed at 210° C., loss of acetic acid occurred before melting and the sample-remained solid to 265° C.

At elevated temperatures, atropine sulphate loses water, forming apoatropine sulphate. It is stable indefinitely at 50° to 60° C., but the B.P. test shows apoatropine after some weeks at 100° to 110° C., and after 2 hours at 150° C., and it is probable that a more profound decomposition also takes place. Homatropine sulphate is even more heat sensitive than the atropine salt, although it contains mandelic acid in place of tropic acid, and a similar dehydration cannot occur, whilst atropine hydrochloride, with the sulphate radical absent, is considerably more stable.

Official Standards and Methods

The methods and definitions of the B.P. 1953 regarding m.pts., differ from those of the U.S.P. XIV, and so do the standards for atropine sulphate. The sulphuric acid baths are similar, and the capillary tubes of the same dimensions, but the former specifies immersion 10° C. below the expected m.pt., and a heating rate of 3° C. per minute, whilst the latter requires immersion 30° C. below, and a heating rate of 3° C. per minute reduced to 1° C. per minute over the final 3 degrees. Apart from the manipulative difficulty of effecting this change of rate with precision, the U.S.P. sample is heated for a total of 12 minutes, against $3\frac{1}{3}$ minutes in the B.P. test. This is reflected in the official m.pt. standards, the lower limits being 188° C. and 191° C. respectively.

Variability of Results

The difficulty in obtaining agreement on the m.pt. of atropine sulphate is illustrated by a series of tests made on one sample in 3 different laboratories, the means of several determinations in each case being 188° C., 190° C. and 191·5° C., the B.P. method being adopted for all tests. This led to a statistical investigation, in which 6 separate measurements were made by each of 8 experienced analysts on a B.P. sample of zero rotation. Using the B.P. method, the mean of the 48 tests was 191·4° C., with a range of 2·5° C. and a standard deviation 0·5° C. A similar series under U.S.P. conditions gave a mean of 188·9° C., range 4·2° C. and standard deviation 0·75° C. Simplication of the U.S.P. method by continuing the 3° C. per minute rate up to the time of melting raised the m.pt. 1° C., and reduced the range. A check series of 48 determinations on hyoscine hydrobromide, which does not decompose, was made by the same team, giving a range of only 1° C., and standard deviation 0·3° C. Analysis of the results for atropine sulphate shows that, if the chance of a sample being wrongly approved is not to exceed 0·001, the least acceptable mean of two measurements must exceed the B.P. lower limit by 1·4° C., and the U.S.P. by 2·2° C.

Effect of Variable Factors

Preliminary series, each of 10 tests, showed that the m.pt. was not appreciably affected by the use of soda-glass or Pyrex capillaries, by variations in their diameter from 0·5 to 1·5 mm., by method of packing,

or by the rate of stirring the bath. The two factors of importance were the method of drying, and, to a predominant degree, the rate (and consequently, the time) of heating.

(*a*) *Method of Drying.* Atropine sulphate crystallises with one molecule of water and in the B.P. melting point test the sample is dried for 15 minutes at 135° C. The U.S.P. requires 4 hours at 105° C. Comparative test series gave the same mean m.pt. after 15 minutes at 135° C. and 2 hours at 105° C., but a fall of 0·8° C. when the time at 105° C. was extended to 4 hours.

(*b*) *Rate of Heating.*

TABLE I

M.PT. OF ATROPINE SULPHATE IN B.P. APPARATUS WITH INCREASING RATE OF HEATING

Heating rate ° C./minute	Immersed at ° C.	Time heated Minutes	M.pt. ° C.
1	180	8	188
2	180	5	190
3	180	3·8	191·4
4	180	3	192
5	180	2·7	193·4
10	175	2·0	195·3
20	170	1·35	197

When the time of heating falls below 1·5 minutes melting is delayed, since heat transfer through the capillary takes an appreciable time.

ATROPINE SULPHATE: STABILITY TESTS

When the bath is held at a constant temperature somewhat below the normal m.pt. the sample melts after a definite time which depends upon its purity. Each of the times in Table II is the mean of 6 measurements, which did not vary more than 7 per cent. on either side of the mean. The atropine sulphate was the B.P. sample of m.pt. 191·4° C. The figures for this sample in Table II may be compared with the results for one which failed to comply with the B.P. standard, and melted at 190° C. This melted in 17 minutes at 175° C., 6·3 minutes at 182° C., and 3·9 minutes at 184° C.

TABLE II

TIME TAKEN FROM IMMERSION TO MELTING AT VARIOUS TEMPERATURES

Temperature ° C.	Mean time to melting Minutes
175	34·5
180	16·0
182	10·0
184	8·3
186	7·3
188	5·4
190	4·2
192	2·4
193	2·1
194	1·8
195	1·4
196 and over	1·0

From this, and from Table I, the true m.pt of this sample, if decomposition products do not interfere, is apparently close to 195° C.

OTHER ALKALOIDAL SALTS

The hydrobromides and hydrochlorides of hyoscine, hyoscyamine and homatropine have m.pts. which are not greatly affected by small variations

in time and rate of heating. In Table III the stability of atropine sulphate is shown to be much less than that of the hydrochloride. Homatropine sulphate, in which dehydration of the mandelic acid cannot occur, is even less stable than atropine sulphate. It is concluded that the sulphate radical has a profound destructive effect on the ester-alkaloids, and that the instability of atropine sulphate is not explained simply by the formation of apoatropine.

TABLE III

HEAT STABILITY OF ALKALOIDAL SALTS COMPARED. TEMPERATURE OF BATH CONSTANT

Number of ° C. between immersion temperature and m.pt.	Time in minutes to melting for		
	Atropine sulphate	Atropine hydrochloride	Homatropine sulphate
9	10·0	—	12·1
7	8·3	27·0	7·0
5	7·3	21·0	4·7
3	3·2	14·0	1·5
Actual m.pt.	191·4°	171·5°	220·0°

SUMMARY

1. Decomposition melting points are affected mainly by rate and time of heating, and this has been illustrated for atropine sulphate.

2. In two series of tests on atropine sulphate, m.pts. following the B.P. technique were more concordant than those carried out according to the U.S.P.

3. The results were less divergent with experienced analysts, but, even with the B.P. technique, considerable replication is desirable to ensure a reliable figure.

4. The sulphates of these ester-alkaloids appear to be more heat-sensitive than the hydrobromides and hydrochlorides.

5. The "heat stability" of decomposable substances may give a useful indication of purity.

The authors are grateful to the Directors of Drug Houses of Australia Ltd. for permission to publish these results.

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A PRELIMINARY NOTE ON THE ALKALOIDS OF *ASPIDOSPERMA EXCELSUM* BTH.

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Received June 17, 1953

THE results of work on the alkaloids of the barks of *Aspidosperma spp.* have recently been reported[1,2,3,4]. Some preliminary observations have now been made on the actions of the alkaloids of the bark of *Aspidosperma excelsum* Bth.

The total alkaloids were extracted from the dried powdered bark by classical methods and purified. These appeared to contain indolic bases. A 1 per cent. solution of the alkaloidal hydrochlorides in the appropriate saline was used.

The pharmacological properties of the total alkaloids of *Aspidosperma excelsum* bark were investigated by the methods outlined in previous papers[1,2,3,4]. These were qualitatively almost identical with those of the total alkaloids of *Aspidosperma oblongum* ADC[1,2] which were also found to contain indolic bases[5]. Thus the alkaloids of *Aspidosperma excelsum* antagonised the spasmogenic actions of acetylcholine, histamine and barium on smooth muscle preparations of the guinea-pig and rabbit small intestine, and antagonised the spasmogenic actions of acetylcholine on the frog rectus abdominis muscle. On the frog and rabbit isolated perfused hearts, we observed depression of tonus, amplitude and frequency, and a well marked auricular-ventricular block. On the perfused blood vessels of the rabbit's ear and rat's hind quarters, there was reversal of the constrictor action of 0·1 μg. to 1·0 μg. of adrenaline hydrochloride. Antagonism to the vasodilator action of acetylcholine was shown on the perfused blood vessels of the rabbit's ear. 0·5 to 1·0 mg. of the alkaloids had a dilator effect on the blood vessels of the rabbit's ear, but constricted those of the rat's hind quarters. In the chloralosed cat the alkaloids caused a prolonged lowering of the blood pressure (Fig. 1) and there was reversal of the pressor reponse to adrenaline (Fig. 2). Antagonism was shown to the pressor response to adrenaline hydrochloride on the spinal cat, but no adrenaline-reversal was seen. In both chloralosed and spinal cats antagonism to adrenaline could be overcome by adminstration of larger doses of adrenaline. The alkaloids (2 mg. by intraperitoneal injection) were found to lower the body temperature of normal mice, weighing 24 to 25 g.) by 3° C., and at a dose level of 3 mg. to protect mice against the lethal effects of a potentially toxic dose of adrenaline hydrochloride. A local anæsthetic action was shown using the frog plexus anæsthesia method[6,7]. No antimalarial action could be shown against *Plasmodium*

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berghei in mice, by 5 mg. of the total alkaloids given by intraperitoneal injection.

When investigating the actions of the alkaloids upon the electrocardiogram of rats and mice anæsthetised with pentobarbitone, a reversible bundle branch block was observed after intraperitoneal injection of the drug. This effect was not observed with the *Aspidosperma oblongum* alkaloids.

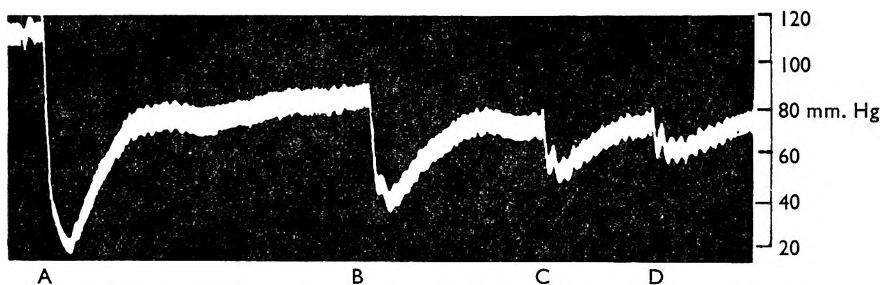

FIG. 1. Effects of the total alkaloids of *Aspidosperma excelsum* on the arterial blood pressure of a chloralosed cat weighing 2·8 kg. At "A" 5 mg., at "B" 2·5mg., at "C" 1·0 mg., and at "D" 0·5 mg. of the total alkaloids was injected into the jugular vein.

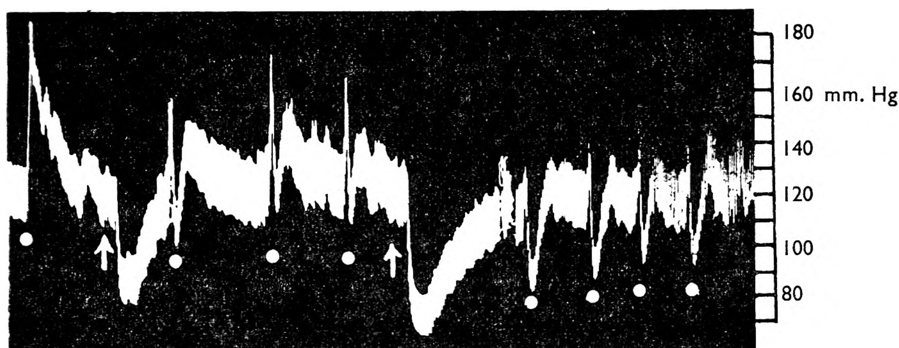

FIG. 2. Effects of the total alkaloids of *Aspidosperma excelsum* on the pressor response to injected adrenaline hydrochloride of a chloralosed cat weighing 3·0 kg. At white dots 4 μg. of adrenaline hydrochloride was injected into the jugular vein. At the arrows 5 μg. of alkaloids was injected.

In frogs 1 mg. of the total alkaloids when injected into the dorsal lymph sac caused miosis, depression of respiration and muscular weakness. Recovery ensued. In mice subcutaneous injection of 100 mg. per kg. caused general depression of movement and activity; the eyes were closed and the pupils slit-like. Recovery ensued. Chemical and pharmacological studies on these alkaloids are being continued.

We thank Mr. D. B. Fanshawe, Conservator of Forests, British Guiana, for the bark; Major J. Buchanan, of Messrs A. C. Cossor, Ltd., for technical advice with the Cossor 1314 E.C.G.; Dr. L. G. Goodwin for supplying us with a strain of *Plasmodium berghei* and for advice. We are especially indebted to Mr. G. E. Trease, Director of Pharmaceutical Studies, University of Nottingham, who aroused our interest in the work.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Adrenocortical Steroids, Determination of. E. Heftmann and D. F. Johnson. (*Analyt. Chem.*, 1954, **26**, 519.) A method for the separation of 6 active adrenocortical hormones by partition chromatography on silicic acid columns is presented. The method of preparation of the column is given, partition taking place between the stationary water phase and a mobile petroleum ether-dichloromethane phase which contained increasing proportions of dichloromethane, the adrenocortical steroids being eluted in the order of increasing polarity. Details are given for the separation of the steroids deoxycorticosterone, dihydrocorticosterone, cortisone, hydrocortisone and corticosterones B and S; identification in the eluates was by the sulphuric acid test and by ultra-violet absorption assay of the fractions. The separation of steroids was automatic, using a fraction collector. Small amounts of adrenocortical steroids could be determined in the presence of a large excess of other adrenal steroids. R. E. S.

Amino-acids, Determination of. J. F. Roland and A. M. Gross. (*Analyt. Chem.*, 1954, **26**, 502.) A simple method, employing monodimensional paper chromatography, is given for the resolution of 16 amino-acids with two solvent systems. 2-Butanol-3 per cent. ammonia (3 to 1) provided good resolution of the amino-acids lysine, arginine, alanine, proline, tyrosine, valine, methionine, *iso*leucine, leucine, and phenylalanine as individual entities in 48 hours; aspartic-glutamic-cystine, serine-glycine, and histidine-threonine were resolved according to the method of Block (*Analyt. Chem.*, 1950, **22**, 1327) using 72 per cent. phenol. Additional systems are described specially for the resolution of histidine and tryptophan. Measurement of the maximum colour density of the individual spots on the chromatograms was found to be satisfactory for most amino-acids, although high results were usually obtained for lysine, arginine, aspartic and glutamic acids; more accurate results were obtained by the area density procedure. Determination by the method, of the amino-acid composition of β-lactoglobulin were found to agree with reported values. The method was also used for the identification of μg. quantities of peptides separated by paper chromatography.
R. E. S.

Cadmium and Magnesium, Determination of, with Disodium Ethylenediamine-tetra-acetate. E. G. Brown and T. J. Hayes. (*Analyst*, 1954, **79**, 220.) The simultaneous determination of cadmium and magnesium by titration with a solution of disodium dihydrogen ethylenediaminetetra-acetate containing zinc sulphate is described. Initial attempts at the titration of a cadmium sulphate solution at *p*H 6·8 with disodium ethylenediaminetetra-acetate with Solochrome Black W.D.F.A. as indicator were unsuccessful since the addition of disodium ethylenediaminetetra-acetate solution did not give a definite end-point change from purple to pure blue; titrations in both maleic acid, sodium maleate buffer, ammonium acetate buffer solutions and at various *p*H values between 7·5 and 8·0 were also unsatisfactory. It was found that a mixture of zinc and cadmium sulphates could be quantitatively titrated to a sharp Solochrome Black end-point at *p*H 6·8, the final titration representing the sum of the

two metals; the result was confirmed for several molecular ratios of zinc and cadmium, including one with a very small proportion of zinc to cadmium. It was also found possible to determine cadmium and magnesium on the same solution by titration first at *p*H 6·8 (cadmium) and then at *p*H 10 (magnesium) by the use of this disodium zinc ethylenediaminetetra-acetate reagent, provided that the molecular ratio of magnesium to cadmium was not greater than unity. A mechanism for the reaction is advanced based on the assumption that the cadmium chelate is more stable than the zinc chelate, free cadmium ions displacing zinc ions from the zinc chelate. R. E. S.

Glycerol, Colorimetric Determination of. H. D. Reese and M. B. Williams. (*Analyt. Chem.*, 1954, **26**, 568.) Glycerol solutions (1 to 13,000 μg. per ml.) are mixed with a quantity of standard potassium dichromate solution and sulphuric acid is added. After heating for 5 minutes in a boiling water bath the reaction mixture is diluted to a known volume, an aliquot is added to a sulphuric acid/*s*-diphenylcarbazide mixture and the resulting product again diluted to volume. The light absorption, determined at 540 mμ, is then measured within 10 minutes and the amount of glycerol obtained from calibration curves previously obtained. The wide range of glycerol concentrations which can be analysed by this colorimetric method necessitates several dichromate reagents, the concentrations of which are given. Blank determinations are variable and should be repeated from time to time. A table is given showing the results of 100 analyses by the method. R. E. S.

Quaternary Ammonium Compounds and Certain Tertiary Amines, Volumetric Determination of. E. D. Carkhuff and W. F. Boyd. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 240.) Small quantities of quaternary ammonium salts and tertiary amines may be estimated by titration with sodium lauryl sulphate or dioctyl sodium sulphosuccinate solution in the presence of sulphuric acid and chloroform. The method is rapid and may be applied to samples containing about 0·025 per cent. of cetylpyridinium chloride, β-diethyl 1-*cyclo*hexyl*cyclo*hexanecarboxylate hydrochloride (dicyclomine hydrochloride), etc. A solution of 1·2 g. of sodium lauryl sulphate in 1000 ml. of water is standardised against the compound undergoing analysis, for example, as follows. Mix 20 ml. of dicyclomine hydrochloride solution with 10 ml. of water, 5 ml. of dilute sulphuric acid, 20 ml. of chloroform and 1 ml. of methyl yellow indicator solution, and shake vigorously. Titrate with sodium lauryl sulphate solution shaking after each addition until the chloroform layer develops the first orange tint. The sample for assay, equivalent to 10 to 25 mg. of dicyclomine hydrochloride is then similarly titrated. Aqueous solutions containing 10 mg. in 50 ml., or chloroform solutions containing 10 mg. in not more than 30 ml. may be assayed, but accuracy is difficult to maintain at greater dilutions. G. B.

Yohimbine, in the Presence of Other Alkaloids, Determination of. C. Stainier and C. Lupière. (*J. Pharm. Belg.*, 1954, **36**, 3.) The following method depends upon the hydrolysis of yohimbine and precipitation of the resulting yohimbic acid with reineckate. Tablets containing phenobarbitone, papaverine hydrochloride, ergotoxine ethanesulphonate, atropine sulphate and yohimbine hydrochloride were shaken with water and chloroform to remove phenobarbitone. The aqueous liquid was extracted with chloroform in the presence of sodium carbonate, and the chloroform solutions, containing yohimbine,

evaporated. The residue was dissolved in ethanol and heated with 0·5 N ethanolic potassium hydroxide under a reflux condenser for 30 minutes, evaporated *in vacuo*, and the residue dissolved in water, extracted with chloroform to remove other alkaloids, neutralised and treated with sulphuric acid and ammonium reineckate. After allowing to stand overnight, the precipitate was filtered off, dissolved in acetone, and the optical density determined at 525 mμ. From this figure the quantity of yohimbine in the tablets was calculated.

G. B.

ESSENTIAL OILS

Essential Oils, Auto-oxidation of. L-E. Fryklöf. (*Farm Revy.*, 1954, **53**, 317, 361.) For the determination of peroxides in essential oils, 1·00 g. of the oil, in a stoppered flask, is dissolved in 20 ml. of citric acid reagent (10 g. of citric acid dissolved in 60 ml. of tertiary butanol and diluted with 35 ml. of carbon tetrachloride). Air is removed by passing carbon dioxide, and then 1 ml. of saturated sodium iodide solution is added. After standing for 20 minutes in the dark, 50 ml. of water is added and the iodine is titrated with 0·01 N. thiosulphate. Results are calculated to a peroxide value, representing the number of ml. of thiosulphate solution equivalent to 1 g. of the sample. Comparative tests under conditions of accelerated oxidation showed the following results for a number of oils.

Oil	Time, in hours, to attain peroxide value of 5
Turpentine ..	10
Lemon	50
Fennel	220
Lavender ..	330
Peppermint ..	840
Bergamot ..	1000
Anise	1450
Rosemary ..	1500
Clove	3500
Cinnamon ..	6750

With the exception of the first two of these, oxidation makes little change in the physical constants of the oil, although changes in the odour can be observed. Under favourable conditions of storage oxidation was slight, but it was much greater with stock bottles from which material was removed at intervals. Turpentine may be to a considrable extent protected from oxidation by the addition of nordihydroguaiaretic acid, propyl gallate or butyl hydroxyanisate, and the effect of these substances is increased by the addition of 0·01 per cent. of citric acid or ethylenediaminetetra-acetic acid.

G. M.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Arsenic in Biological Materials, Determination of. R. J. Evans and S. L. Bandemer. (*Analyt. Chem.*, 1954, **26**, 595.) The material under examination is mixed with saturated magnesium nitrate solution and ashed overnight a

600° C. in a muffle furnace. The ash is dissolved in dilute hydrochloric acid and the arsenic distilled as arsine, which is collected in an iodine solution. The arsenic content of the solution is finally determined by developing the blue molybdenum compound of arsenic and reading the colour at 840 mμ. Good recoveries of arsenic added to egg homogenate and either fresh or dried liver tissue were obtained, and the values were reproducible; the method was also applied to eggs, chicken liver, breast muscle, leg muscle, skin and dried pig tissues. Complete recovery of arsenic on distillation was not obtained, but recovery was constant at 87 to 90 per cent., so that standard curves were prepared by distilling known quantities of arsenic. R. E. S.

Fluorine in Biological Material, Estimation of. P. Venkateswarlu and D. Narayana Rao. (*Analyt. Chem.*, 1954, **26**, 766.) It was found that reliable results on biological materials were obtained if a preliminary sulphuric acid distillation was performed; this was necessary owing to the presence of iron. Silica in plant materials also interfered with fluorine recovery; the interference could be reduced by preliminary distillation of the unashed sample, or by fusion of the lime-ashed sample with sodium hydroxide before distillation of the fluorine from perchloric acid. The use of magnesium oxide as an adsorbent for the fluoride ions (*Analyt Chem.*, 1950, **22**, 441) considerably aided fluoride analysis. R. E. S.

Glycogen in Tissues, Determination of. A. Kemp and A. J. M. Kits van Heijningen. (*Biochem. J.*, 1954, **56**, 646.) A micromethod for the determination of glycogen in tissues is described. The tissue is extracted with a solution of trichloroacetic acid at 100° C. and the glycogen in the extracts is determined, without previous hydrolysis, by the colorimetric method described by Mendel *et al.* (*Biochem. J.*, 1954, **56**, 639). Pure glycogen dissolved readily in the deproteinising solution, but only part of the glycogen could be extracted from the tissues with a cold solution of trichloroacetic acid; all of the glycogen could however be brought into a solution by grinding the tissues with trichloroacetic acid solution and then heating the suspension for 15 minutes at 100° C. Glucose present in the tissues, although usually small, is extracted and determined with the glycogen by this method and procedures are described for the determination of both glycogen and glucose in muscle and liver. Only glucose 1-phosphate of the glycogen metabolites containing a hexose molecule gives the colour reaction. R. E. S.

CHEMOTHERAPY

Germine, Synthetic Hypotensive Esters from. F. L. Weisenborn, J. W. Bolger, D. B. Rosen, L. T. Mann, Jr., L. Johnson and H. L. Holmes (*J. Amer. chem. Soc.*, 1954, **76**, 1792.) A number of synthetic esters of high hypotensive activity were prepared by selective and stepwise esterification of germine. The methods for the preparation of mono-, di-, tri and tetraesters are described. Evidence is presented indicating that direct acylation of germine introduces the acid radicals on the same hydroxyl groups found esterified in the natural di- and tri-esters. All the synthetic tetraesters were essentially inactive, while the triesters of germine were the most active. The results indicated that, four- or five-membered α-branches acid radicals are necessary for appreciable hypotensive activity with the restriction that the over-all dimension of the germine ester molecule lies close to an optimum value. None of the synthetic esters showed a significantly more favourable emetic ratio than the natural ones. A. H. B.

PHARMACOGNOSY

***Claviceps purpurea*, Culture of.** A. G. Paul, W. J. Kelleher and A. E. Schwarting. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 205.) A medium containing 2 per cent. of mannitol and 1 per cent. of casein hydrolysate together with mineral material was inoculated from slant cultures of the organism and incubated at 21 to 22° C. on a reciprocal shaker. Sub-cultures were incubated for 72 hours on the shaker, after which solutions of indole, indole and DL-serine, L-tryptophane and α-amino-*N*-methyltryptophane were added. These cultures were incubated for 24 hours. The mycelia were separated centrifugally, washed and incubated on a reciprocal shaker in a solution of the test compound in saline solution. The cultures were freeze-dried and extracted by the U.S. National Formulary method for the determination of total alkaloids of ergot, the extracts being concentrated and analysed chromatographically. Mycelium was separated from the replacement cultures and the filtrate submitted to chromatography on paper, using a mixture of butanol, glacial acetic acid and water (4:1:5). The chromatograms were sprayed with a 2 per cent. solution of *p*-dimethylaminobenzaldehyde in hydrochloric acid which gave a pink colour with indole, and blue with tryptophane and amino-*N*-methyltryptophane. Quantitative determinations of these substances were made spectrophotometrically. The various compounds used, representing several structural moieties of lysergic acid, had no effect on the production of ergot alkaloids in the organism. In the absence of organic material, indole in a solution containing 50 μg./ml. was completely utilised in 24 hours and 25 μg./ml. of tryptophane appeared in the solution. Added L-tryptophane and amino-*N*-methyltryptophane were partially utilised. When the organism was grown on a tryptophane-free medium, it could be shown that growth was significantly inhibited by the addition of tryptophane. G. B.

***Digitalis ferruginea*, A Preliminary Phytochemical Investigation of.** R. M. Appel and O. Gisvold. (*J. Amer. pharm. Ass., Sci. Ed.*, 1954, **43**, 215.) An aqueous extract of the fresh leaves was treated with methyl *iso*butyl ketone. From the aqueous mother liquor, saponin, chiefly tigonin, was extracted in a quantity representing 0·42 per cent. of the weight of the leaves. The methyl *iso*butyl ketone extract was separated into ether-soluble and ether-insoluble fractions. The ether-insoluble material yielded a crystalline glycoside, apparently α-acetyldigoxin. From the ether-soluble fraction, an amorphous glycoside was obtained which appeared to be digitoxin. Attempts to separate a mixture of digoxin, acetyldigoxin and the crystalline and amorphous glycosides obtained from *Digitalis ferruginea*, by the use of paper chromatography with a mixture of methyl *iso*butyl ketone, methanol and water (10:1·8:5) or a constant-boiling mixture of methyl *iso*butyl ketone and water as solvents were not successful. The amorphous glucosides gave a positive reaction in the periodate-benzidine test. G. B.

Rutin, Presence of, in *Rheum* Species. L. Hörhammer and K. Müller. (*Arch. Pharm. Berl.*, 1954, **287**, 126.) In a systematic paper chromatographic examination of the Polygonacæa, the leaves and flowers of all species of *Rheum* examined contained a flavone with an R_F value of 0·35. This was identified as

rutin. The species in question, with yields of rutin obtained from them, are given below

Species	Date of collection	Yield of rutin per cent.
R. emodi	July	0·32
R. officinale	July	1·30
R. palmatum ..	July	3·10
R. pruinosum ht. ..	July	0·61
R. rhaponticum ..	July	0·70
R. ribes	July	—
R. undulatum ..	June	—
R. wittrockii	July	—

G. M.

PHARMACOLOGY AND THERAPEUTICS

Anticholinergic Agents, Comparative Effects of, on Human Gastric Secretion. J. A. McGowan, Jr. and M. Stanley. (*J. Lab. clin. Med.*, 1954, **43**, 359.) Atropine and 10 quaternary ammonium compounds were compared for their anticholinergic activity in diminishing gastric acid secretion in man. The quaternary compounds tested were methanthelinium, propantheline, oxyphenonium bromide, prantal, Squibb compounds 2963 (dimethylethyl 3-*n*-propyl benzilate ammonium bromide) 3199 (*N*-diethylaminoethyl-*N*-methylbenzilamide methobromide) 2998 (2-hydroxyethyl methylpiperidinium bromide, 1 benzoyl*cyclo*propane carboxylate) 3505 (di*iso*propyl [2-hydroxyethyl] methylammonium bromide, 1-benzoyl*cyclo*propanecarboxylate) 2806 (2-ethyl [1-benzoyl*cyclo*propanecarboxylate] methyldiethyl ammonium bromide) and Roche compound Ro2-3773 (1-methyl-3-benzyloyl-oxyquinuclidinium bromide). The subjects were 134 hospitalized patients, 25 of whom were controls, 43 per cent. of the test group and 48 per cent. of the controls having peptic ulcers, active or healed. The drugs were studied by the double meal technique where a standard meal was followed after 15 minutes by intubation and complete aspiration of gastric contents. The drug was then given orally or parenterally and the meal procedure repeated one hour later. The drugs were also compared by their depression of basal acid secretion in patients with gastric hypersecretion, but the control responses here were more variable than with the double meal method. Achlorhydria was seen only infrequently after large oral doses of the more potent agents, but smaller parenteral doses of most of the drugs achieved this ideal more often. As might be expected, achlorhydria was seen more often in studies on basal secretion. Despite claims to the contrary no drug appeared to have a clear superiority over the others in relation to side effects (xerostomia mydriasis, blurring of vision, and bladder and intestinal dysfunction). The approximate single oral doses which will reduce the basal acid secretion in patients with hypersecretion, or food-stimulated secretion in normals by 20 per cent or more:—Atropine, 1·3 mg.; oxyphenonium, 10 to 25 mg.; Ro2–3773, 10 to 35 mg.; propantheline, 30 to 60 mg.; Squibb 2963, 75 to 125 mg.; methanthelinium and Squibb 3199, 100 to 150 mg.; prantal, Squibb 3505, 2998 and 2806; 400 to 700 mg. G. P.

Cyanocobalamin; Nasal Instillation and Inhalation in Pernicious Anæmia. R. W. Monto and J. W. Rebuck. (*Arch. intern. Med.*, 1954, **93**, 219.) This is a report on the treatment of 12 patients with pernicious anæmia in relapse by both inhalation and nasal instillation of cyanocobalamin. Cyanocobalamin in isotonic sodium chloride solution without a preservative was employed. The maximum hæmopoietic effect was elicited with a solution containing 100 μg. per ml.; 1000μg. in 1/10 ml. by volume of lactose powder was used for administration as a dust. Nasal instillation was performed in 0·5 ml. volume divided between the two nostrils and given in the usual manner of nasal liquid medicaments. In each of the 12 patients a satisfactory hæmatological and clinical response was obtained. Maximum reticulocyte increase occurred between the 8th and 10th days of treatment and varied between 18 and 44 per cent. according to the severity of the anæmia. The bone marrow picture reverted from a megaloblastic arrest to normal erythropoiesis, and the peripheral blood findings reflected this improvement. A single inhalation of 100 μg. of cyanocobalamin in 1 ml. of isotonic sodium chloride solution produced a reticulocyte elevation to 18 per cent. in a patient whose initial erythrocyte count was 1,020,000, and a similar installation effected a reticulocytosis of 37 per cent. in another patient whose original red cell count was 2,880,000. In addition, the condition of 20 patients with pernicious anæmia in remission has been maintained by this therapy for periods varying up to 18 months. S. L. W.

Folic Acid, Danger of Polypharmaceutical Preparations Containing. C. P. Lowther. (*Brit. med. J.*, 1954, **1**, 564.) A woman aged 46 complained of stiffness of her knees and after various treatments had been tried it was found that the hæmoglobin level was 48 per cent. The administration of iron was recommended and a preparation containing iron and folic acid with vitamin B and liver extract in capsule form was prescribed. Considerable improvement in the patient's condition occurred but, 3 months later, on examination after 10 days in hospital following a fall she was found to have a spastic paraplegia and eventually subacute combined degeneration of the cord was diagnosed. Anahæmin, 4 ml. on alternate days, and cytamen, 100 μg. daily, brought about rapid hæmatological and neurological improvement although the patient had still a very slight ataxia when last seen. The inclusion of folic acid in polypharmaceutical preparations is condemned on four grounds. It temporarily improves the blood picture and subacute combined degeneration of the cord is likely to be misdiagnosed since it rarely occurs without anæmia. The relief of the general symptoms of pernicious anæmia reassures both patient and doctor and masks the deterioration of the nervous system. Folic acid deficiency occurs infrequently and is not needed in multivitamin or iron preparations. It is still uncertain whether folic acid actually precipitates cord degeneration. H. T. B.

Gastric Secretory Function, Tubeless Method. H. M. Pollard, A. Carballo and R. J. Bolt. (*J. Lab. clin. Med.*, 1954, **43**, 340.) In a series of studies on the determination of gastric secretory function in man, the authors have re-examined the method introduced by Segal *et al.* (*Proc. Soc. exp. Biol., N.Y.*, 1950, **74**, 218) which depends upon the release of quinine in the stomach from the quininium salt of a cation-exchange resin, diagnex; the quinine is absorbed from the small intestine, approximately one-third being excreted in the urine. Determination of this urinary quinine was then made using the ether-sulphuric acid method of Kelsey and Geiling. For the test the patients were fasted overnight and the fasting urine collected. A further urine sample was collected one hour

after giving 250 mg. caffeine sodium benzoate in water. 2 g. of the quininium resin compound was then given in water and urine collected after one and two hours. These two samples were analysed and the second-hour sample results compared with the conventional gastric analysis involving intubation and histamine stimulation of secretion. The test was very reliable for the detection of achlorhydria, but was not quantitative enough for routine gastric acid determinations. In the 76 patients who underwent the tests, there was some degree of correlation between disease state and urinary quininium, duodenal ulceration being associated with hypersecretion of acid and gastric ulceration, with normal or low secretion. Gastric carcinoma was attended by low normal or anacidity and pernicious anæmia by persistent achlorhydria. The test is much simpler than uropepsin determination and is particularly useful where psychogenic or organic illness precludes the use of gastric intubation. Also, in patients with partial gastrectomies and gastro-enterostomies the intubation methods is unreliable whereas there is no loss of accuracy of the quinine method. As a diagnostic aid the usefulness of the test is limited except where a negative result is sought. The analysis of the second-hour sample would seem unnecessary except in such cases and would further simplify the test. G. P.

Lead Poisoning, Monocalcium Disodium Ethylenediaminetetra-acetate in. H. L. Hardy, H. B. Elkins, B. P. W. Ruotolo, J. Quinby and W. H. Baker (*J. Amer. med. Ass.*, 1954, **154**, 1171.) Monocalcium disodium ethylenediamine tetra-acetate (calcium versenate), a chelating agent forming unionised complexes with heavy metals, was tried in 3 patients with chronic lead intoxication. The compound was administered daily for 5 to 7 days by intravenous injection during 2 hours in doses of 3 to 4 g. dissolved in 450 to 600 ml. of 5 per cent. aqueous dextrose solution. The urinary excretion of lead and of coproporphyrin was determined before and during treatment. In each patient the urinary excretion of lead was about 30 times greater during treatment, and there was a decrease in coproporphyrin excretion. When treatment was stopped, lead excretion returned to pre-treatment values; coproporphyrin excretion continued to decrease in 2 of the patients but increased in the third until after a second course of calcium versenate. Before treatment all the patients were anæmic, with stippled cells, increased resistance of the red cells to hypotonic sodium chloride and increased mechanical fragility. The treatment resulted in a rise in the hæmoglobin level and disappearance of abnormalities in the cells. No untoward effects were observed. H. T. B.

***iso*Nicotinyl Hydrazides, Toxicity in Pulmonary Tuberculosis.** E. O. Coates, G. L. Brickman and G. M. Meade. (*Arch. intern. Med.*, 1954, **93**, 541.) Results obtained in a series of 77 patients confirm previous reports that iproniazid (*N*-*iso*nicotinoyl-*N′*-*iso*propylhydrazine) is considerably more toxic than isoniazid and suggest, in addition, that side-effects are more frequent and more severe when it is given in combination with streptomycin or *p*-aminosalicylic acid. Termination or interruption of therapy, or reduction in dosage because of toxicity, was necessary for 45 per cent. of those receiving iproniazid, and only 9 per cent. of those given isoniazid. A standard dose of 4 mg./kg. was employed for both isoniazid and iproniazid. Therapy was continued for a minimum of 6 months in 41 patients, for from 2 to 6 months in 22 patients, and for less than 2 months (discontinued because of toxicity) in 14 patients. Toxic reactions occurred in two main categories, those related to the autonomic nervous system and those apparently related to the central nervous system and the

peripheral nerves. In the first category were constipation, postural hypotension, dryness of the mouth, urinary difficulties, sweating, impotence, bradycardia, and increasing dyspnœa. In the second category were mental changes, muscular irritability, paræsthesias. Purpura, accompanied by hæmoptysis, occurred in one patient taking isoniazid and two taking iproniazid. A case of toxic encephalitis and one sudden death (ventricular fibrillation) may have been related to therapy with iproniazid. "Withdrawal" symptoms (nightmares, muscular twitchings, and difficulty with micturition) were observed in 64 per cent. of 14 patients in whom iproniazid therapy was abruptly terminated; these were not relieved by substitution of isoniazid . The authors conclude that in spite of some apparent superiority over isoniazid in its benefits upon the symptoms of pulmonary tuberculosis, iproniazid appears too toxic for general use.

S. L. W.

Noradrenaline, Excretion of, in Urine in Hypertension. U. S. von Euler, S. Hellner and A. Purkhold. (*Scand. J. clin. Lab. Invest.*, 1954, **6**, 54.) Estimates of noradrenaline in the urine of 500 cases of hypertension of unknown origin were made. In 60 per cent. of these the noradrenaline content was within normal limits, in 20 per cent. it was not significantly increased above the normal and in the remaining 20 per cent. it was significantly increased. This increased noradrenaline excretion in some cases of essential hypertension may be important in the pathogenesis of the disease. The increase may come from the adrenergic nerves, in particular the vasomotor fibres. M. M.

Plasma Substitutes. W. d'A. Maycock. (*Brit. med. Bull.*, 1953, **10**, 29.) Dextran possesses most of the desirable properties of a plasma substitute. After infusion, molecules small enough to enter the glomerular filtrate are excreted in a few hours; the remainder leave the blood at a uniform decremental rate of about one-third per day and pass into the tissues. It thus disappears from the body almost completely in a short time and no histological changes attributable to it have been observed. Dextran is metabolised but the metabolic process is not known. Dextran solutions have two disadvantages. They are likely to cause rouleaux formation, thus interfering with compatibility tests, and certain individuals are sensitive to dextran and may exhibit severe reactions. Solutions of animal gelatin have been investigated experimentally and clinically. The native gelatin molecules are degraded by autoclaving, the molecular size varying with the time of heating. The solution originally tested (mean mol. wt. about 33,000) gelled at room temperature; the next preparation (mean mol. wt. about 19,000) gelled at 12° to 15° C. Both these forms are rapidly excreted by the kidneys. More recently a polymerised form (oxypolygelatin), with a gelling point of 10° to 13° C., and a fluid gelatin, with a gelling point of 0° to 4° C. have been under trial. All these gelatins are non-antigenic and are free from harmful effects—apart from causing rouleaux formation—but their clinical value in the treatment of oligæmia has not yet been established. The place of polyvidone among the plasma substitutes has still to be determined. It has been shown that it is not metabolised to any significant degree, that molecules with a mol. wt. of less than 25,000 are excreted very rapidly, and that those with mol. wt. between 25,000 and 40,000 are all excreted within a few days. Molecules with weights greater than 110,000 are probably retained in the tissues for a long time, possibly for years. Histological evidence of storage of polyvidone and of certain regressive changes in human tissues many months after administration have been reported. These observations suggest that polyvidone solutions should not contain molecules of greater mol. wt.

than 60,000 to 70,000, the majority having a mol. wt. of 25,000 to 40,000. The retention of such a solution in the blood stream would be short-lived; it is estimated that in the normovalæmic patient the plasma volume expansion would be only 50 to 60 per cent. of the infused volume after 6 hours and only about 30 to 35 per cent. after 12 hours. Since all plasma substitutes fail in one way or another to meet all the requirements and until it is known what importance should be attached to their lack of many of the properties of plasma, they should be used cautiously, though they have an important part to play where transfusion services are lacking, in national emergencies, and during the temporary absence of blood or plasma. S. L. W.

Primidone, Clinical Evaluation of. D. Sciarra, S. Carter, C. I. Vicale and H. H. Merritt. (*J. Amer. med. Ass.*, 1954, **154**, 827.) Primidone (5-phenyl-5-ethylhexahydropyrimidine-4-6-dione), an anticonvulsant drug closely related to phenobarbitone, was administered to 121 patients with one or more types of convulsive seizures. The ages of the patients ranged from 5 to 71 years, the majority being between 20 and 40. All seizure types were represented, but grand mal predominated. In every case primidone was added to the medicaments that the patients were already receiving. The drug was administered in tablets of 0·25 g., the usual starting dose being 250 mg. daily. This was increased by one tablet daily at approximately weekly intervals to the point of therapeutic or toxic effect. The therapeutic effects could be evaluated in 72 of the 121 patients; this group was followed for periods ranging from 1 to 18 months. The period of observation was 1 to 11 months for 28 patients and 12 to 18 months for 44 patients. In the remaining 49 patients it was not possible to evaluate the anticonvulsant effect accurately, owing to toxic reactions or because follow-up data were inadequate. The attacks were entirely controlled in 7 patients (10 per cent.), reduced in frequency in 31 (43 per cent.), and unchanged in 34 (47 per cent.). The greatest benefit occurred when the seizures were of the grand mal, psychomotor, minor or focal types; no improvement was noted in any of the patients with petit mal. Side effects occurred in 65 of the 79 patients, but none was serious. Drowsiness and ataxia were the two most frequent symptoms and made it necessary to discontinue the administration of primidone in 25 patients. In a third of the patients side-effects developed with a daily dose of 0·75 g. or less, and two-thirds of the patients manifested side-effects with a daily dose of 1·25 g. or less. In the remainder the toxic manifestations appeared when the daily dose exceeded 1·25 g. S. L. W.

Vitamin K as Antagonist to Anticoagulants. T. Hilden and O. Munck. (*Scand. J. clin. Lab. Invest.*, 1953, **5**, 361). The purpose of this study was to examine the effect of synthetic vitamin K, menadione, on dicoumarol, ethyl biscoumacetate and phenylindanedione under uniform conditions. The examinations were made on three groups of 8 patients undergoing treatment with one or other of these anticoagulants. Before the vitamin K experiments were made it was ensured that the prothrombin concentrations were at fairly constant concentrations. The menadione was given in a dose of 100 mg. by mouth, in one series of experiments, and in the same dose by intramuscular injection in a second series. Administration of the maintenance dose of the anticoagulant was continued while vitamin K was administered. Menadione was shown to exert an equally potent effect, whether given by mouth or intramuscularly, on all the anticoagulants employed, though the effect varies considerably from one person to another. Vitamin K_1, administered by mouth in a similar dosage, was also found to exert a uniform effect on the three anticoagulants, though its effect was more pronounced than that of menadione. S. L. W.

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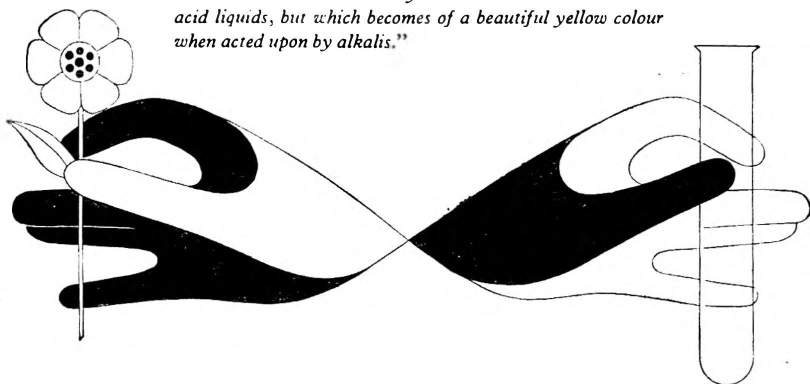

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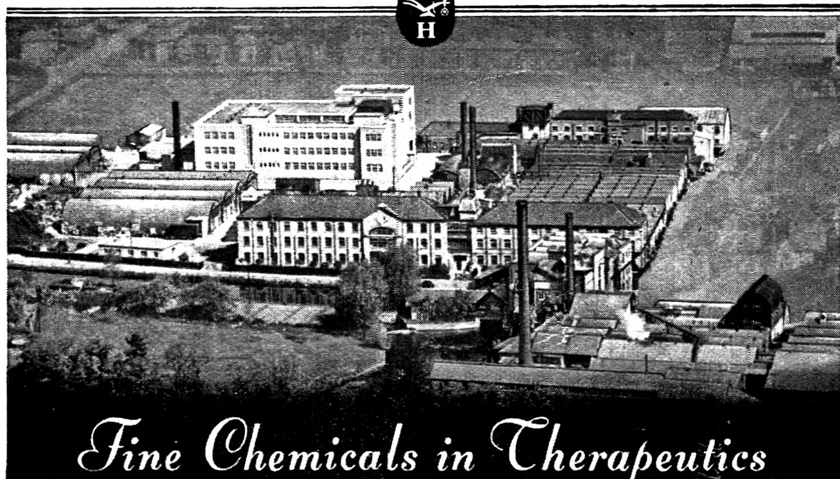

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