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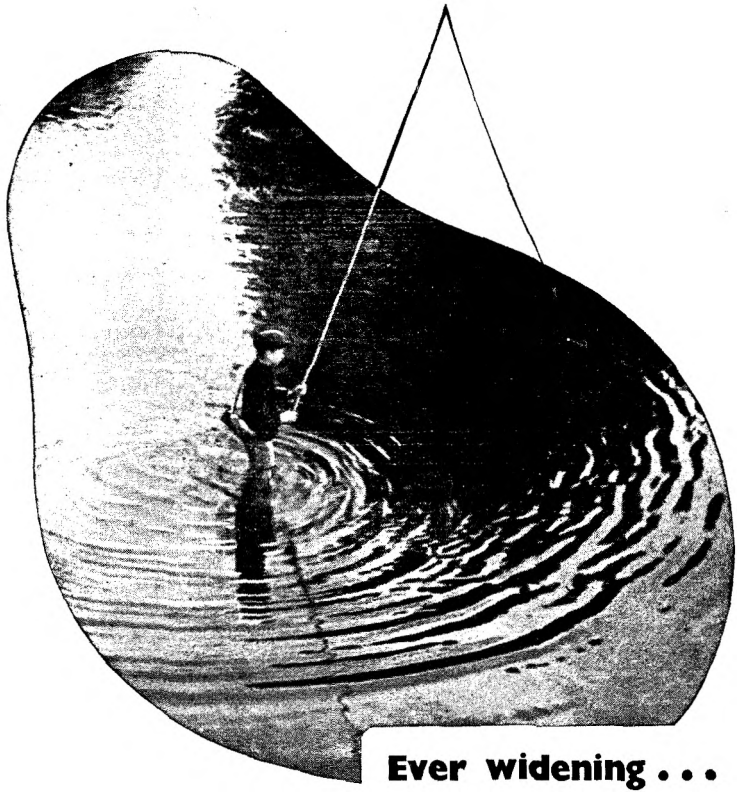

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G. R. BOYES

Chairman, 1953

BRITISH PHARMACEUTICAL CONFERENCE LONDON, 1953

Chairman: G. R. BOYES

CHAIRMAN'S ADDRESS

THE PHARMACIST AND THERAPEUTICS

IN this the Coronation year of Her Majesty Queen Elizabeth II, we are assembled for the 90th meeting of the British Pharmaceutical Conference. This Conference was founded in 1863 through the foresight of men who had the scientific development of pharmacy at heart, and who doubtless realised the contributions which improvements in the practice of pharmacy could make to medicine. Three years previously Oliver Wendell Holmes, who was described by Sir William Osler as the most successful combination of physician and man of letters the world had ever seen, stated in an address to the Massachusetts Medical Society, "I firmly believe that if the whole materia medica as now used could be sunk to the bottom of the sea, it would be all the better for mankind and all the worse for the fishes." Although these words were spoken in America, it is obvious from the records of contemporary British practice that they could have been applied to the materia medica of this country, and that on both sides of the Atlantic Ocean, pharmacy and medicine still retained much mediæval empiricism. A major step towards the advancement of pharmacy was taken shortly before the first full meeting of this Conference, with the publication of the first British Pharmacopœia. This volume replaced the pharmacopœias previously issued separately by the Royal College of Physicians of London, Edinburgh, and Dublin. It was the first attempt to secure, within the British Isles, uniformity of formulæ and methods of preparation for commonly used drugs and galenicals, but its reception by pharmacists was not unattended with criticism. The hope expressed by Henry Deane, President of the Conference for that year, that members of Conference would do all they could to promote and ensure an improvement in every future edition has been abundantly realised. During the whole of its history this Conference has consistently provided opportunities for the exposition and discussion of advances in pharmaceutical practice. The proceedings of the Science Sessions record many original investigations which have led to improvement in methods of preparation, formulation and analytical control of substances used in therapeutics including those that are official in the British Pharmacopœia and the British Pharmaceutical Codex. The eighth British Pharmacopœia (1953) becomes official to-morrow (September 1st) and a new edition of the Codex is expected to be published next year. It is appropriate at this stage in our history that we should review some of the contributions which have been made by pharmacy to the materia medica of to-day.

A memorandum issued in 1952 by the Pharmaceutical Society to the

Minister of Health included the following among the services that should be rendered by the pharmaceutical department of a hospital:—

To obtain and be responsible for the nature and quality of drugs, medicinal preparations, dressings and chemicals such as antiseptics and reagents.

To make preparations to be used in dispensing prescriptions; to prepare other products for medical or surgical use, and to formulate preparations to meet special needs.

To dispense prescriptions. To assist in the development of new methods of treatment.

To assist in efficient prescribing by advising upon the nature and properties of substances used in medicines, and upon the selection of the most suitable substances and forms in which they should be prescribed.

This comprehensive definition of the functions of a pharmacist is applicable in whatever branch of the profession he is engaged. In general terms then, it can be said that the primary function of the pharmacist is to provide the drugs, and preparations of drugs, needed by doctors for the treatment of their patients. These drugs must be in a state of purity and of assured activity, and they must be in a form suitable for the appropriate method of administration, be it by mouth, by parenteral injection, by application to the skin or mucous membrane, or by insertion into one of the body cavities. To the pharmacist, therefore, belongs responsibility for the preparation and quality of drugs, and their formulation into preparations acceptable to both doctor and patient. In order to carry out these functions the pharmacist must obviously have a thorough knowledge of the chemical and physical properties of the substances comprised by the materia medica of to-day, he must be familiar with the analytical methods used in their assay and be able to apply the principles involved in their formulation. To this must be added a basic knowledge of physiology and pharmacology to provide the background for advice upon prescribing.

The Materia Medica

One of the outstanding features of pharmacy and medicine to-day is the wide use that is made of synthetic substances, and of isolated active principles, and the decline in the use of crude extracts, decoctions, infusions and tinctures made from drugs of the plant kingdom.

Although the use of synthetic compounds has increased greatly during the last 10 or 20 years, it had its origin before the beginning of the present century. At the meeting of this Conference held in Liverpool in 1896, the President, William Martindale, described the introduction of synthetic compounds into medicine as a novelty. 20 years later, C. A. Hill in his address as President stated, "Notwithstanding the phenomenal extent to which synthetic drugs have come into use, and despite the increased employment of active principles according as our knowledge of these progresses, the use of the drugs themselves in the form of galenical preparations (whether "standardised" or not) continues to a remarkable and perhaps significant extent. Furthermore, signs are not wanting of a growing recognition of the truth that many a drug and many a food may

contain valuable properties not readily determined by chemical methods. It may be only slowly that the full value of a drug discovered empirically can be stated in scientific terms. Paradoxical as it may seem, the tendency to-day, with advancing scientific knowledge, is to recognise the failure of the active principle to replace the parent drug."

Since these words were spoken, there has been a definite change from nature to the laboratory as the principal source of medicinal substances. This has effected changes in the practice of both therapeutics and pharmacy. The production and supply of simple vegetable drugs and their galenical preparations is a diminishing, though still important, part of pharmaceutical work. The pharmacist in retail practice has less opportunity than had his predecessors of a generation ago to exercise his skill in compounding. He is called upon to an ever increasing extent to supply drugs which, by the very nature of the processes involved in their production, must reach him in a state ready to be administered. The production of insulin, the manufacture of the antibiotics, the sulphonamides, the derivatives of barbituric acid, the sex hormones and the antihistamine drugs, to quote but a few examples, are essentially large scale operations suitable only for specially constructed plant. Compressed tablets, capsules and sterile solutions for injection, which together constitute a large part of dispensed medicines, are prepared more economically on the large manufacturing scale than at the dispensing counter.

Some idea of the growth and magnitude of the industry engaged in the production of synthetic chemicals and isolated principles and of its importance to the life and health of the nation, can be gained from figures published in the *Final Report of the Census of Production for Drugs and Pharmaceutical Preparations for the year* 1948. During the 10 years preceding the year of the Census the number of persons employed in Britain in the industry almost doubled, and its gross output expressed in terms of value, more than trebled. The output of aspirin in all forms rose from about 2 million lb. to 5½ million lb. The production of insulin increased to 6 times the pre-war amount to a total of 4500 million units per annum. In 1948 the production of barbituric acid and its derivatives amounted to 90,000 lb.; the annual production of sulphonamides was approximately 890,000 lb. The production of chloral hydrate was nearly 250,000 lb., and of sex hormones 25,000 oz. Since 1948 there has been a continuous increase of production in volume as well as in value. The production of penicillin, which was only at the rate of 190,000 mega units per week in 1948, had risen to almost 1,300,000 mega units per week by 1952. If we consider that 300,000 units is an average single daily dose, then these figures mean that approximately 625,000 doses were produced each week in 1948, and in 1952 the figure had reached over 4 and a quarter million. In that period too, new drugs such as streptomycin, *p*-amino-salicylic acid and the histamine antagonists have all added to the total production which is now valued at approximately £90 million per annum. Of this amount approximately one-third is exported overseas.

Informative though these figures are, cold statistics can convey but little impression of the contribution made by pharmacy—using the term

in its widest sense—to the national welfare, the advancement of medical science and the saving of human life. That this contribution has been considerable will not be disputed, but it is revealed in its true perspective only when it is examined in the light of those other factors which have influenced medical progress during recent years, and the advances in other branches of scientific endeavour which have been adapted to the requirements of the pharmaceutical laboratory.

On the one hand there has been the awakening of the national conscience to the need for the prevention of disease, and the care of the sick, reflected in the gradual evolution of the Public Health Service, and in particular in the National Health Insurance Act of 1911, and the National Health Service Act of 1946. On the other hand there has been the increasing attention given to research, in clinical medicine and pharmacology, and within the pharmaceutical industry itself. Planned and organised research for the development of new products and the improvement of old ones is now accepted as essential to the progress of pharmacy. Not only do manufacturers examine and attempt to develop discoveries made in their own laboratories, but they must follow very closely the discoveries made by workers in academic centres and always be ready to adapt their methods and their products to the ever advancing flow of knowledge. The result is that the pharmaceutical industry to-day, occupies an unique and essential position in regard to the development of new remedies and the discovery of new therapeutic uses for previously known compounds. As a result many of the substances now official in the British Pharmacopœia, and many more included in the British Pharmaceutical Codex, had their origin in the research laboratories of manufacturing pharmaceutical houses.

This position has been achieved by the application of knowledge gained in the sciences of chemistry, physics, pharmacology and biology to the design of new drugs and the manufacture of pharmaceutical preparations. 40 or 50 years ago almost the only methods employed by pharmacists for making the products needed for dispensing prescriptions were those of maceration or percolation with alcohol and water, decoction, concentration by evaporation and crystallisation. For the most part, reliance was placed on simple methods of volumetric and gravimetric analysis, the determination of melting point, or boiling point, refractive index and specific gravity as guides to purity and activity. Many galenicals were evaluated on their content of total extractive. The adaptation of scientific methods, including fractional precipitation, chromatographic separation, molecular distillation, potentiometry, polarography, spectrophotometry, biological assay and microbiological assay, has provided the tools with which the weapons of modern therapeutics have been fashioned.

Organic Syntheses

The discovery of a new drug with specific therapeutic properties is seldom due to mere chance circumstances, but is often the result of carefully planned and co-ordinated research to which organic chemists, biochemists, pharmacologists, physicians and pharmacists have all

contributed. This research may consist of the routine "screening" of the biological properties of a series of known compounds, or it may be a deliberate attempt to build molecules of predictable pharmacological action. In order to achieve the desired end it is often necessary to examine scores, or probably hundreds, of compounds in order to determine whether the required pharmacological properties are present. Some guidance in the search for new compounds can be obtained from the systematic examination of naturally occurring substances and synthetic compounds of known pharmacological properties and known chemical structure. This will yield information regarding the relationship of chemical structure to pharmacological action. A specific type of biological action is usually associated with a particular basic molecular structure. This activity can be quantitatively modified by alterations of chemical structure which do not involve alterations to the basic configuration. The preparation of a series of homologous compounds or derivatives based on the original primary structure, and a quantitative study of their biological properties must be undertaken in order to find one which possesses a high degree of specific biological activity with only a minimum of unwanted or toxic side effects.

Studies of this type have been used in the development of derivatives of barbituric acid, synthetic œstrogens and other sex hormones, histamine antagonists, sulphonamides, sulphones, antimalarial drugs, neuromuscular blocking agents, ganglionic blocking agents, analgesics and local anæsthetics. It is inevitable that the synthetic pathway to the development of new drugs leads to the production of many compounds which have qualitatively similar pharmacological properties and are used for the same purpose. The doctor is thus able to choose from among a wide range of compounds.

Vegetable Materia Medica

It must not be inferred that vegetable materia medica have been entirely supplanted by the synthetic products of the chemical laboratory. Some examples can be quoted to show that this is far from being the case. There has recently been a revival of interest in the peripheral vasodilator action of preparations of *Veratrum viride* as a means of treating hypertension, and in the dilator effect on the coronary vessels of preparations of *Ammi visnaga* for the relief of angina of effort. These possible clinical applications have led to systematic chemical and pharmacological examination of the constituents of these two drugs, resulting in the case of *Veratrum viride* in the preparation of a stable extract, and, in the case of *Ammi visnaga*, in the isolation of khellin. Liquorice has assumed an importance above that of a demulcent and sweeting agent by the discovery that glycyrrhizinic acid has a pharmacological action on salt and water metabolism closely simulating that of deoxycortone. Many crude vegetable drugs find their way into commerce in large quantities. Senna, aloe, podophyllum, cascara and rhubarb are still extensively prescribed as purgatives. Volatile oils and fixed oils from plant sources are used in many pharmaceutical preparations. We are still dependent upon opium for

supplies of morphine and related alkaloids, on cinchona for supplies of quinine, on ipecacuanha for emetine, on digitalis for cardiac glycosides, on nux vomica for strychnine, on belladonna for atropine and on ergot for ergometrine. Some of these strongholds are already being assailed by the synthetic organic chemist. The synthesis of morphine on a laboratory bench scale has recently been accomplished, and in the case of quinine, atropine and morphine, synthetic alternatives possessing some of the pharmacological properties of the natural alkaloids are available. Studies now in progress on the biogenesis of alkaloids and other plant constituents may be expected to point the way to increasing the yield of these naturally occurring medicinal agents.

The industrial adaptation of the metabolic activities of some micro-organisms has made these members of the plant kingdom important contributors to the materia medica. Moulds and fungi grown under controlled conditions in suitable culture media produce the antibiotics penicillin, streptomycin, aureomycin, terramycin and some others; *Aspergillus niger* grown in a medium containing molasses produces citric acid; cyanocobalamin is produced together with streptomycin as a metabolic product of *Streptomyces griseus*; a microbiological transformation of steroids using *Rhizopus nigricans* has greatly simplified the synthesis of cortisone, and an exo-cellular enzyme produced by the coccus *Leuconostoc mesenteroides* during its growth on a medium containing sucrose is used for the production of the polysaccharide dextran.

Animal Materia Medica

Substances developed in the animal body—hormones, antitoxins, human blood and plasma—are important items of materia medica. The British Pharmacopœia describes methods for the preparation of several derivatives of human blood, and defines tests for their identification and assay. The production of antitoxins contained in the serum of animals, has long been a specialised part of pharmaceutical enterprise. Interest in some of the antitoxins may be renewed, in view of the prevalence of strains of bacteria resistant to the commonly used antibiotics and chemotherapeutic compounds.

Of the hormones produced by endocrine glands, cortisone, and corticotrophin have assumed great importance in therapeutics, although their production in this country is not yet large enough to satisfy all requirements. Cortisone is one of many steroids secreted by the cortex of the adrenal gland. The story of its isolation, chemical characterisation and synthesis, is one of the most fascinating themes of modern organic chemistry. The first synthesis from deoxycholic acid was a long and complicated process involving 32 stages. The total synthesis from *o*-toluidine involved 48 stages. Sterols obtained from vegetable sources—stigmasterol, diosgenin, sarmentogenin and hecogenin—have been investigated as alternative starting materials with a view to shortening the synthesis and increasing the yield of cortisone. Progress along the pathway towards increased production on a commercial scale has been considerably eased by two important recent developments. The first is a method of

biosynthesis in which minced adrenal glands are incubated under ærobic conditions with synthetic deoxycortone, and the second is the microbiological transformation of steroids using *Rhizopus nigricans* to which reference has already been made.

Corticotrophin (adrenocorticotrophic hormone or A.C.T.H.) is obtained from the anterior lobe of the pituitary gland of pigs, cattle and sheep. Despite the inherent difficulties of the process of extracting the active principle from the glands, considerable progress has been made towards its isolation in pure condition. From pig pituitary glands, a highly active fraction has been obtained by extraction with glacial acetic acid, followed by fractionation with ether and acetone, adsorption on oxycellulose, digestion with pepsin and lyophilisation. This fraction has been termed corticotrophin B. Another product, also of high potency, has been produced from sheep pituitary glands by fractionation of an acid-acetone extract. This fraction has been termed Preparation E. These two preparations differ from one another, and from concentrates of corticotrophin previously described, in their chemical and physical properties. If, as indeed may be the case, either of these substances proves to be a single chemical entity, greater exactitude will be given to the pharmacological and clinical evaluation of the adrenocorticotrophic hormone.

It is just 30 years since the first commercially produced injections of insulin were made available to diabetic patients in Great Britain. In the intervening years much research has been directed to so modify the original soluble insulin as to prolong its action in the body after injection, with the object of controlling the level of blood sugar in patients with diabetes mellitus by a single daily injection. Successive steps towards the achievement of this objective, have been protamine insulin, protamine zinc insulin and globin insulin. During the last few years, workers in several laboratories have re-investigated the effect of zinc ions on the crystallisation of insulin at different *p*H ranges and in the presence of different buffer salts. One outcome of these studies was the crystalline protamine zinc insulin compound at first called N.P.H., to which the approved name isophane insulin has been given. This preparation is used in neutral suspension containing sodium phosphate as the buffering agent.

The most recent development has resulted from the discovery that if an acetate buffer is substituted for the phosphate or citrate buffer previously used for making insulin suspensions, it is possible to precipitate the insulin over a range of *p*H approximating to that of body fluids by the addition of an amount of zinc equivalent to that present in protamine zinc insulin (0·02 mg./10 units). In the absence of citrate or phosphate ions the physical character and the solubility of the zinc insulin compounds depends upon the *p*H of the solution from which it is precipitated. By appropriate modification of the conditions it is possible to precipitate zinc insulin compounds in either amorphous or crystalline forms, containing more zinc in chemical union than is present in soluble crystalline insulin. The two physical forms of this insulin zinc compound differ somewhat in their hypoglycæmic action; that of the crystalline form is much more prolonged

than that of soluble (unmodified) insulin, whereas the amorphous form has a relatively rapid action not unlike that of soluble insulin.

The ideal preparation of insulin for the treatment of diabetes mellitus would be one that is relatively rapid in its effect on absorbed glucose, constant in its action and of sufficiently long duration to enable the blood sugar to be maintained within normal limits by one single daily injection; it should not produce any form of local reaction. The mixture of the amorphous and crystalline zinc compounds suspended in acetate buffer, to which the approved name insulin zinc suspension has been given, may come very close to this conception of the ideal, because it combines rapidity of action with prolongation of effect, and is free from foreign protein. This preparation, which will become available this autumn, will avoid mixing two preparations, one short-acting and the other long-acting.

3 years after the introduction of insulin, therapeutics registered another significant and life-saving advance in the discovery of the effectiveness of raw liver in the treatment of pernicious anæmia. It was not long before extracts containing the active substance or substances were produced, but progress in this field was hampered by lack of knowledge concerning the identity and nature of these active substances and by the fact that there was no laboratory method by which the activity of liver extracts could be assessed—the sole criterion was the clinical response of patients suffering from pernicious anæmia. Attempts to separate clinically active material from inert matter were numerous; for the most part they followed traditional lines of extraction with solvents, enzymatic digestion, precipitation of inert matter with heavy metals or by selective solvents, and charcoal adsorption followed by elution. Gradually it became possible to prepare solutions for injection in which the active material was presented in relatively high concentration, although these still contained considerable proportions of inert matter. The isolation of folic acid from liver and other sources led to the conclusion that this substance was the anti-pernicious anæmia factor, a conclusion subsequently shown on clinical evidence to be erroneous.

It was the application of chromatographic methods of separation and the development of a microbiological method of assay that provided the means for the isolation of the substance now called cyanocobalamin, in a yield of less than 1 g. from 4 tons of liver. The discovery that cyanocobalamin is produced by *Streptomyces griseus* as a metabolite together with streptomycin opened up a new source for commercial production in greater quantity and at less cost.

Analytical Control

Chemical Investigation

For a substance to be suitable for use in therapeutics it is essential that its pharmacological and therapeutic activity must not vary from batch to batch. During the development of a synthetic compound, or the purification of an active principle extracted from its natural source, much information is gained about its physical and chemical characters—solubility, melting point or boiling point, specific rotation, refractive index—and

from data of this type it is possible to draw up specifications of characters and tests by which the substance can be identified, and quantitatively analysed.

The separation of active constituents from their natural sources, and the purification and characterisation of synthetic organic chemicals have been greatly assisted by refinements in methods of chemical and physical analysis. Many of the newer techniques involve the use of costly physical instruments, with the result that the analytical control of the purity of medicinal substances is rapidly becoming the work of the specialist and the capital expenditure needed to instal this modern equipment in a laboratory reaches astronomical amounts. Among the newer techniques which have been employed in the development of new preparations and in the assay of old ones are spectrophotometry, chromatography and polarography.

Absorption spectrophotometry finds wide application for the characterisation of pharmaceutical substances, and for determining their purity and concentration in solution. It is used for the determination of vitamin A, cyanocobalamin, calciferol, some sex hormones, chloramphenicol, many alkaloids and antihistamine drugs.

Absorption in the infra-red region is now assuming importance both for qualitative and quantitative analyses and for providing information about chemical constitution. It was used, for example, in elucidating the chemical structure of cyanocobalamin and of the penicillins.

Two modifications of spectrophotographic methods, namely flame photometry and the "porous-cup" technique, have been applied to the determination of lithium, potassium, iron, silicon, magnesium and other metallic ions in pharmaceutical preparations, and appear to give results of sufficient accuracy to justify their further use.

Chromatography has greatly assisted the isolation and purification of many of the antibiotics. It has provided methods for the separation of noradrenaline from adrenaline; for the fractionation of digitoxin from admixture with other digitalis glycosides and aglycones. Application of this method to the separation of ergot alkaloids, and to the assay of ergometrine and ergometrinine in mixtures, has supplied information about the changes which take place in injection of ergometrine maleate on storage. It has also provided satisfactory methods for the determination of the hyoscine in solanaceous drugs and of strychnine in nux vomica.

The use of a suitable ion exchange resin as the adsorbing column makes it possible to separate the salts of weak organic bases into their component ions. This method has been used for the determination of some alkaloids and local anæsthetics.

Polarography gives satisfactory results in the routine assay of morphine, diamorphine, strychnine and riboflavine contained in injection solutions, tablets, and some galenical preparations. It may also be used for the assay of trace metals in pharmaceutical preparations, and for the determination of iron, arsenic and antimony in their compounds. A polarographic method for the determination of the purity of insulin has been described, and polarographic determination of chloramphenicol in solution buffered at *p*H 4 is said to give results which agree with those obtained by biological assay.

Biological Tests

It is possible to guarantee a constant degree of pharmacological activity from a given weight of most drugs, by rigid control of chemical composition and physical characteristics. There are, however, some drugs, mostly of biological origin, which cannot be reduced to a state of uniform chemical purity, and in which the proportion of active substance to inert matter is liable to vary in different preparations. The biological activity of these substances cannot be precisely predicted from chemical and physical properties and must be controlled by biological assay, in which the response of animals to doses of the substance is compared with the response of a similar group of animals to doses of a standard preparation of the same substance. It is an essential condition of such assays that tests with the sample under examination and with the standard preparation should be carried out at the same time and under identically comparable conditions. Biological assay is required for about 30 substances of the British Pharmacopœia. A laboratory, properly staffed and equipped, where biological assays can be carried out, is therefore an essential auxiliary to manufacturing pharmaceutical plants.

When a new substance is being developed and examined in order to assess its possible use as a therapeutic agent, biological tests are of value, in conjunction with chemical and physical methods as an indication of progressive stages in the isolation and purification of the new substance. Methods of biological assay used in conjunction with chromatography have facilitated the purification of the adrenocotricotrophic hormone, and the isolation and characterisation of the hormones present in adrenal cortex extract. In the case of the former, the method is based on the depletion of ascorbic acid from the adrenal glands of rats after removal of the anterior pituitary gland. A microbioassay, using radioactive isotopes, which measured the ratio of sodium and potassium excreted in the urine of adrenalectomised rats following injection of adrenal cortex extract, combined with paper chromatography, has led to the separation of a new crystalline steroid possessing a high degree of activity on electrolyte metabolism.

Before any new substance can be released for clinical use the pharmacological examination must extend beyond the demonstration of its specific biological activity *in vitro* and *in vivo*. Its rate of absorption, distribution through the body tissues and excretion must be investigated. Information must be obtained concerning its therapeutic index (that is to say the ratio of effective dose to toxic dose), its chronic and acute toxicities, its action on blood pressure, respiration, the central nervous system, the heart, blood, kidney and liver. If the drug is one that is intended to be used for external application its local effects on the skin and mucous membranes. must be studied to ascertain whether it causes irritation and sensitisation. If the substance is shown to have a desirable therapeutic property it must be free from serious side effects before it can be considered acceptable for therapeutic use.

Whether use is made of the whole intact animal, pieces of animal tissue or bacterial cultures as the test object, all methods of biological assay

have one feature in common—that of individual variability. It is therefore necessary to invoke the assistance of statisticians in order to assess the accuracy of the results, and, indeed, to plan the experiments so that reliable information may be obtained with a minimum expenditure of labour and materials. With proper planning of the experiment and appropriate mathematical treatment of the results, biological assay is capable of ensuring uniformity of action between different preparations of the same substance within comparatively small limits of error.

The Formula

A most important part of the pharmacist's work is the formulation of medicinal substances into preparations suitable for administration. It is here that pharmacy is seen both as a science and an art. A science because proper formulation must be based on a knowledge of the physical, chemical and pharmacological properties of the substances to be compounded, and an art because it requires the exercise of skill based on experience. Often this part of the pharmacist's work is taken for granted and it is too little realised that the preparation of a compressed tablet, solution or suspension for injection, an ointment, emulsion or suppository, for example, is possible only after considerable laboratory research. The discussions on the subject of pharmaceutical formulation at recent meetings of the Conference will be still fresh in your memories but it may be appropriate to recapitulate some of the fundamental principles.

The aim of formulation must be to present a drug in a form in which it exhibits its characteristic properties, is safe and convenient to use, and stable for a reasonable period of time under the prevailing climatic conditions.

During the last decade, the practice of pharmacy has been dominated by the need for supplying active preparations of the antibiotics, penicillin, streptomycin, chloramphenicol, and, more recently, aureomycin and terramycin. The dispensing of penicillin solutions and suspensions for parenteral use, and the formulation of creams and ointments for external application, brought into prominence the necessity for observing strict asepsis in manipulation, and revealed numerous incompatibilities which had to be overcome before satisfactory formulation was accomplished. The study and solution of these problems has formed the basis of a number of papers contributed to the Science Sessions of this Conference. The survey of the pharmacy of antibiotics presented at our Symposium Session last year gave an indication of the complexity of these problems, and the manner of their solution.

The problems presented by the formulation of the antibiotics are special examples of problems incidental to the formulation of preparations of any drug for therapeutic use, and their satisfactory solution is reached by the application of the same general principles. In the design of preparations intended for administration by mouth the effect of saliva, the gastric juice and the pancreatic secretion must be taken into consideration, so also must the extent and manner of absorption and excretion of the drug. Palatability and æsthetic appearance are necessary, but are secondary in importance to the stability of the drug in solution or suspension.

The formulation of preparations for parenteral injection presupposes a knowledge of the solubility of the drug in water for injection or other solvent, the *p*H of the solution, the stability of the drug on storage, and on sterilisation, its compatibility with bactericidal compounds which may be used as preservatives, and with sodium chloride or other electrolyte which may be used to render the solution isotonic. Pain on injection, must be avoided, and solutions intended for intravenous injection must not contain pyrogens.

In the preparation of suspensions for intramuscular injection, consideration must be given to those factors which influence the rate of absorption of the drug from the site of injection—particle size, viscosity of the medium, or the presence of an anion radical which will delay the absorption or excretion of the drug.

In the formulation of ointments and creams for external application it is necessary to know whether the drug is intended to pass through the layers of skin, or to remain unabsorbed on the surface.

The formulation of tablets is intimately linked with the rate of disintegration of tablets in the alimentary tract—a matter of great importance if the *pilula perpetua* of Pereira is not to find its counterpart in modern medication.

The Clinical Trial

When a drug has emerged from the scrutiny of pharmacological examination with an indication that it possesses some property that may have practical application in the treatment of disease, and has been formulated into a form suitable for administration, there arises the necessity for testing it on human patients. Ideally every new drug should be submitted to controlled and impartial trial in which its effects on a disease process are carefully observed and the results compared with those of other forms of treatment previously in use for that disease. This method has not always been followed in the past, indeed, it has often not been possible to adopt it. Had it been employed many of the alleged remedies of bygone years would never have seen the light of day. As it is, too many of them have persisted to the present time because of the absence of incontrovertible evidence of their worthlessness. To draw conclusions from insufficient data will lead to erroneous deductions. This error does not belong entirely to a past era. Even to-day claims for therapeutic activity are sometimes based on clinical impressions derived from the observation of one or two patients only!

Ideally the clinical trial should follow the general principle of all biological assays in that it must be carried out on a sufficiently large number of patients and must provide for an adequate series of controls, whenever this can be done without endangering the lives or well-being of patients. If withholding treatment from a patient would mean the difference between survival and death, then clearly no control is possible, other than that supplied by the doctors' clinical impressions, or by the case records of a similar group of patients treated by other methods.

Controlled clinical trials have their greatest usefulness when it is

possible to measure some biochemical or pathological change brought about in response to the administration of the drug, e.g., the blood sugar response to the injection of insulin, the rise in the number of red blood-cells in response to the injection of liver extract or of vitamin B_{12}, the healing of the lesions of pulmonary tuberculosis shown in X-ray photographs during treatment with streptomycin, and the disappearance of parasites from the blood of malaria-infected patients.

By whatever method the clinical evaluation of a drug is attempted, the process requires careful planning, critical examination of the progress of the patients, and systematic recording of the results. It should be conducted by a team of investigators which should include a physician, a biochemist, a pharmacist, and preferably also a statistician. This is essentially the province of the physician, but it is one in which the pharmacist, particularly the hospital pharmacist, can and should collaborate. From it will come the final proof of the successful outcome of the chemical, biological and pharmaceutical research that has preceded it.

Not only do trials on human subjects make it possible to assess the value of a drug in the prevention or treatment of disease, and also to determine the effective dose range, but they reveal side-effects such as nausea, vomiting, headache, giddiness, skin rashes, tinnitus and other symptoms which cannot be detected from animal experiments.

The quantitative evaluation of drugs in man is usually difficult because it is not often possible to obtain continuous and objective records of the drug effect. Furthermore, the number of subjects, the number of observations and the range of doses that can be used are all necessarily limited. Where quantitative methods have been devised they have sometimes revealed that the relative potencies of the members of a group of compounds having qualitatively similar pharmacological actions do not correspond to the deductions made from the results of tests on animals. This is true, for example, of the synthetic œstrogens and of the synthetic muscle relaxants, the relative potencies of which in man differ from their relative potencies in rats. Quantitative methods have been adapted successfully to the study of the antihistamine drugs and the synthetic analgesics of the methadone type.

International Aspects

Many of the substances and methods mentioned in the foregoing brief survey had their origin in countries outside Great Britain. Similarly, many of the discoveries made in our islands have proved of benefit in the relief of human suffering in all parts of the world. Pharmacy like other sciences knows no national boundaries. Its discoveries and methods are applicable the world over. Its difficulties are common to all those who practise it wherever they may be. There is obvious need for the closest collaboration and exchange of information by pharmacists of all nations. Already there is much evidence of the growing recognition of this need. In a world which is rapidly shrinking with the speed of modern travel, we cannot afford to ignore it. The presence in this country during the past summer, and at this Conference, of pharmacists from the

Commonwealth and the Dominions as well as from a number of other countries, will do much to foster this collaboration. The International Pharmaceutical Federation, the Franco-British Pharmaceutical Commission, the International Conference on Military Medicine and Pharmacy and the International Pharmacy Students Federation all provide opportunities for the exchange of views between pharmacists of different nationalities. The invitation given to the International Pharmaceutical Federation to meet in Great Britain during 1955 is a welcome step towards the greater participation of British pharmacists in pharmaceutical affairs beyond the shores of our own islands.

The World Health Organisation is to-day taking a greater interest in matters of purely pharmaceutical importance. The necessity for international agreement on standard preparations for use in biological assay has been recognised for many years and has been met by the work of the Permanent Commission on Biological Standardisation set up under the Health Organisation of the League of Nations, and now the World Health Organisation. It is over 50 years since the advantages of using the same name for the same preparation throughout the world were first enunciated at an international conference, but only within the past two or three years has a serious effort to secure such uniformity been made by the establishment of a list of international non-proprietary names for several hundred substances. It is to be hoped that the opposition which has recently arisen in America will not be allowed to vitiate the success of this important contribution to saner pharmacy. In an attempt to extend international agreement on standards for drugs and pharmaceutical preparations, the International Pharmacopœia has been produced by the World Health Organisation Expert Committee on the Unification of Pharmacopœias. The British Pharmaceutical Conference has reason to be proud of the fact that one of its Vice-Chairmen, Dr. C. H. Hampshire, C.M.G., who was Chairman of our meeting in London in 1933, was Chairman of this Expert Committee and directed the labours of those who produced this very material evidence of international pharmaceutical collaboration.

The Pharmacist's Responsibility

In whatever capacity the pharmacist is engaged, be it in the manufacturing laboratory, in the pharmaceutical department of a hospital or in retail business, he or she has a position of great responsibility in regard to the life and health of the community. Under the National Health Service, the pharmacist-contractors of Great Britain dispense more than 200 million prescriptions each year. To this must be added the not inconsiderable number of prescriptions dispensed by hospital pharmacists for both in-patients and out-patients. Medicines are made for the sick, and the pharmacist's ultimate responsibility is to the patient. Every stage in the preparation of a medicine, from the manufacturing laboratory to the dispensing counter, is subject to rigid control—and by control in this context is meant something much more than the analytical control referred to earlier in this Address. It means constant vigilance, checking and cross

checking to avoid errors in manufacturing, compounding and dispensing; errors that might endanger the lives of countless numbers of people if they were perpetrated. To-day, although the emphasis is passing from the bottle of medicine to the compressed tablet, capsule and injection solution, and individual prescriptions have been largely replaced by the standard preparations of the British Pharmaceutical Codex and the National Formulary, or by the finished products of manufacturers, the knowledge of the chemistry and pharmacology of drugs required by the pharmacist, is greater than ever it was at any previous time in our history. As the number of highly potent drugs increases, the pharmacist must be ever more vigilant and his knowledge must keep pace with modern developments.

To equip him to discharge these responsibilities the pharmacist in embryo must undergo a course of training in a recognised teaching institution embracing the basic natural sciences and the specialised subjects of pharmaceutics, pharmaceutical chemistry, pharmacognosy, physiology and pharmacology, and forensic pharmacy. In addition, he must serve a period of articled pupilage to gain an insight into the practice of pharmacy before he is admitted to the register. Thus, the pharmacist acquires a store of knowledge, both academic and practical, which is of the greatest value when it is allied to the knowledge of the physician. Too often in the past has pharmacy been regarded (even by pharmacists themselves) as the mere handmaid of medicine. Rather should it be said that pharmacy is co-partner with medicine, with equal responsibilities, and with equal opportunities to serve the public. Pharmacy and medicine are, in fact, inter-dependent, and together fulfil vitally important functions in the health services of the country.

During the lifetime of this Conference therapeutics has emerged from the darkness of empiricism into the dawning light of an exact science. Pharmacists can be proud of the part which they have played in bringing about that advance, and they can be prouder still of their responsibilities to further it in the future.

SCIENCE PAPERS AND DISCUSSIONS

AN *IN VITRO* EVALUATION OF COMMONLY USED ANTACIDS WITH SPECIAL REFERENCE TO ALUMINIUM HYDROXIDE GEL AND DRIED ALUMINIUM HYDROXIDE GEL

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BECAUSE of the difficulties of comparing antacids by clinical methods the use of *in vitro* methods has been investigated by several workers for assessing the relative efficiencies of antacids.

It has been demonstrated by Rossett and Flexner[1] that the effect of an antacid could be measured by an *in vitro* test and correlated with the effect of the same antacid in the human stomach. They were able to show exact duplication of *p*H changes in man and *in vitro* when a given dose of the antacid was administered. Other workers[2,3] have based their methods of evaluating antacids *in vitro* using modifications of the method of Rossett and Flexner, which measured the *p*H changes which occurred with time when the antacid was added to 100 ml. of hydrochloric acid solution of *p*H 1·4 to which 0·1N hydrochloric acid is added at the rate of 120 ml. per hour. The results obtained show that the method is capable of demonstrating both the extent of the *p*H change and the time during which neutralisation or buffering is effective. Johnson and Duncan[2], Holbert *et al.*[3], Murphy[4], and other workers have expressed the view that the method is of greater value in assessing the efficacy of an antacid than is the acid neutralising test of the B.P.C. and U.S.P. With these workers we agree.

To assess the merits of any method for evaluating materials described as antacids it is necessary to consider the purpose and requirements of antacid therapy.

An ideal antacid should:—(*a*) not be absorbed from the alimentary system, (*b*) be without undesirable laxative or constipating effect, (*c*) be of high neutralising capacity and at the same time rapid in showing initial effect, (*d*) maintain its effect over a prolonged period, (*e*) be non-eructating, (*f*) not cause "acid-rebound."

Acid rebound is caused by the compensatory secretion of parietal hydrochloric acid which occurs when the *p*H of the gastric contents becomes even slightly alkaline[5] and the acid so produced may exceed the original hyperacidity present. Apart from acid-rebound if the gastric contents become alkaline there is a possibility that the mucous membrane of the stomach may be subjected to the strong digestive action of the enzymes, trypsin and erepsin which may enter the stomach by regurgitation from the duodenum[1]. The degree to which gastric acidity should be reduced is an important question on which no general agreement has been reached. It is accepted that except in the presence of pepsin, ulceration of the stomach is not produced by any physiological concentration of acid.

It is known that pepsin is inactive at *p*H values above 3·0. Johnson and Duncan[2] summarised authoritative opinions and concluded that an ideal antacid should buffer at a *p*H within the range of 3·5 to 4 even when the antacid is taken in excess dosage. The *in vitro* method by measuring the *p*H changes which occur under conditions simulating those occuring in the stomach, determines the neutralising or buffering capacity of an antacid in relation to its rate of action and duration of effectiveness and is therefore capable of predicting antacid efficiency within the concept of an ideal antacid. The properties of an antacid in relation to absorption from the alimentary tract, eructation, and effect on the bowel can be determined by theoretical consideration and known effects.

The *in vitro* method we have used in this investigation is essentially a modification of that used by Johnson and Duncan[2] which was described in a paper read before this Conference in 1945. A sample of the antacid in the appropriate dose is added to 150 ml. of artificial gastric juice consisting of hydrochloric acid (0·05N approx.) adjusted to *p*H 1·5 with water and with the addition of 2·0 g. of pepsin per l. This solution corresponds to what would be a hyperchlorhydric level (*p*H 1·0 to 1·5) in the human stomach. The mixture is maintained at 37° C. and continuously stirred. The *p*H is recorded electrometrically, using a glass electrode, after 30 seconds and then at 2, 4, 6, 8 and 10 minutes. 20 ml. of the mixture, representing the physiological loss from the stomach, is then withdrawn and 20 ml. of artificial gastric juice, is added. This procedure is repeated at the end of each 10-minute period. The test is continued until the *p*H readings indicate that the antacid has been neutralised or is no longer effective. The various modifications of this type of test which have been described are, in the main, concerned with the choice of the testing solution and the rate of addition and withdrawal, and there exists a need for a standardised procedure. The addition of pepsin to the artificial gastric juice is important and we have confirmed the observation of Murphy[4] that the antacid effect of the aluminium hydroxide preparations is inhibited to some extent by pepsin.

The method may be used in conjunction with clinical tests to eliminate material and formulations of low antacid activity. It does not give any indication of clinical response to factors other than the antacid effect. For example, it does not indicate protective effect due to coating of the gastric mucosa.

The antacids considered in this investigation have been confined to the non-systemic and non-eructating antacids. The dosage used in each case was the quantity which would maintain a buffering effect for approximately 1 hour under the condition of the test. In the case of the official substances it was found that this dosage was within the limits of the official dosage with the exception of dried aluminium hydroxide gel, the amount of which had to be increased from 0·6 g. to 1·5 g., to obtain the desired effect. The antacids and dosages considered in this investigation are listed in Table I.

Typical results obtained are represented graphically in Figures 1 and 2 and are tabulated in Table II. The curves show maxima and minima for

*p*H with each 10-minute period. For comparison purposes the maxima have been used in compiling the curves shown in Figures 3 to 7.

Examination of the curves in Figures 3 and 4 show that aluminium hydroxide gel B.P.C. (8 ml. dose), dried aluminium hydroxide gel B.P.C. (1·5 g. dose), dihydroxy aluminium aminoacetate (1·5 g. dose) and calcium phosphate B.P. (1·5 g. dose) buffer within the desired clinical range 3·5 to 4 for approximately 60 minutes. Of the other antacids considered aluminium phosphate gel (8 ml. dose), dried aluminium hydroxide gel

TABLE I

THE ANTACIDS AND DOSES CONSIDERED IN THE INVESTIGATIONS

Substance	Dose
Magnesium oxide, light B.P.	0·25 g. and 0·5 g.
Magnesium trisilicate B.P	1·5 g.
Calcium phosphate B.P.	1·5 g.
Aluminium hydroxide gel B.P.C.	120 minims
Dried aluminium hydroxide gel B.P.C.	0·6 g. and 1·5 g.
Dihydroxy aluminium aminoacetate	1·5 g.
Aluminium phosphate gel	120 minims
Bismuth carbonate	1·8 g. and 30 g.
Ion exchange resin A	15 .g
Ion exchange resin B	8 g.
Ion exchange resin C	1 g.

* Ion exchange resin A consists of the mixed potassium and ammonium form of cross-linked polyacrylic (cation) exchange resin.

Ion exchange resin B consists of:—

Alkylene polyamine resin	12 per cent.
Potassium salt of carboxylic resin	29 ,, ,,
Carboxylic resin	59 ,, ,,

Ion exchange resin C consists of:—

Polyamine-methylene resin.

TABLE II

*p*H READINGS WITH LIGHT MAGNESIUM OXIDE AND MAGNESIUM TRISILICATE

Readings obtained by adding 20 ml. of artificial gastric juice and withdrawing 20 ml. of the mixture at 10-minute intervals (*see* Fig. 1)

Period	Magnesium oxide, light B.P. 0·5 g.								
	0–10	10–20	20–30	30–40	40–50	50–60	70–80	80–90	90–100
½ minute	9·16	9·24	9·1	9·06	8·9	8·48	7·7	6·16	2·78
2 minutes	9·61	9·66	9·54	9·54	9·39	9·14	8·88	7·62	2·81
4 minutes	9·66	9·72	9·69	9·69	9·48	9·32	9·07	8·2	2·82
6 minutes	9·7	9·7	9·73	9·73	9·58	9·42	9·18	8·38	2·83
8 minutes	9·7	9·7	9·71	9·71	9·66	9·48	9·24	8·5	2·83
10 minutes	9·7	9·7	9·71	9·71	9·68	9·53	9·3	8·57	2·83
	Magnesium trisilicate, B.P. dose 1·5 g.								
½ minute	1·88	5·37	4·57	3·29	2·81	2·52	2·33		
2 minutes	4·84	6·18	6·0	5·51	3·75	2·70	2·35		
4 minutes	6·38	6·49	6·34	6·15	5·38	2·84	2·38		
6 minutes	6·68	6·64	6·51	6·27	5·95	3·07	2·4		
8 minutes	6·8	6·74	6·62	6·49	6·16	3·26	2·43		
10 minutes	6·88	6·8	6·69	6·58	6·29	3·49	2·46		

B.P.C. (0·6 g. dose), ion exchange resin C (1 g. dose) and bismuth carbonate (1·8 g. and 30 g. dose), fail to raise the *p*H to 3·5. Magnesium trisilicate B.P. (1·5 g. dose), and ion exchange resin A (15 g. dose) and B (8 g. dose) raised the *p*H above 4 but not greater than 7. Light magnesium oxide B.P. (0·25 g. and 0·5 g.) caused an immediate rise to a *p*H approaching 10.

The following observations can be made on these results. Aluminium hydroxide gel B.P.C. (8 ml. dose) is rapid in initial effect and buffers at a *p*H level of 4, maintaining its effect for approximately 60 minutes. Dried aluminium hydroxide gel at the official maximum dose (0·6 g.) is very slow in exerting its effect and the maximum *p*H attained is 2·2 after

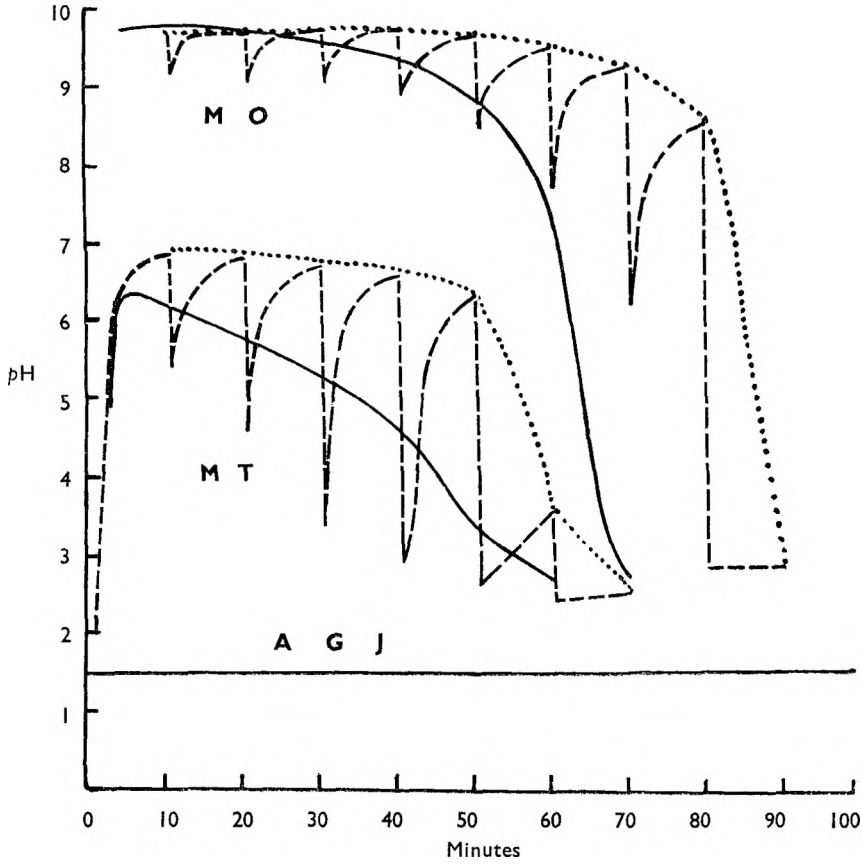

FIG. 1. Curves of light magnesium oxide (MO) B.P. (0·5 g.) and magnesium trisilicate (MT) B.P. (1·5 g.) showing the effect of periodic and continuous addition of artificial gastric juice.

———— Continuous addition (0·5 ml. every 15 seconds).
— — — Periodic addition (20 ml. every 10 minutes).
. . . . . Peak curve.
A G J. *p*H of artificial gastric juice (*p*H 1·5).

40 minutes. Increasing the dosage to 1·5 g. serves to raise the *p*H level to 3·5 and increases the time of effectiveness. With the higher dosage the speed of the initial effect is slightly increased, the maximum *p*H being obtained after 20 minutes, but the dried gel is not comparable with the liquid preparation in this respect. This difference was confirmed with several samples of aluminium hydroxide preparations obtained from various sources. All were found to comply with the B.P.C. requirements.

On examination by the *in vitro* method all gave the typical results shown in Figure 3. The differences between aluminium hydroxide gel and the dried aluminium hydroxide gel is discussed in detail later. Dihydroxy aluminium aminoacetate buffers at the same level as the dried aluminium hydroxide gel at equal dosage (1·5 g.) but is markedly more rapid in

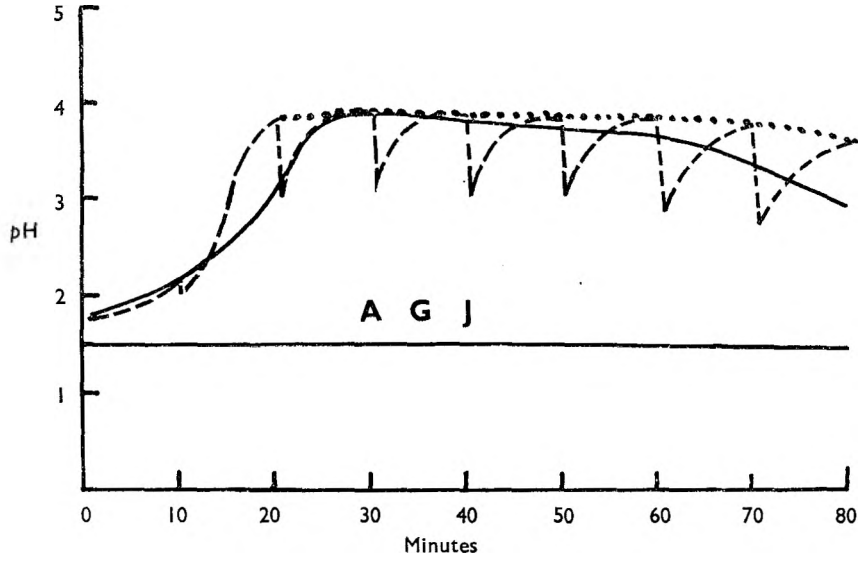

FIG. 2. Curves of aluminium hydroxide gel B.P.C. (1·5 g.) showing the effect of periodic and continuous addition of artificial gastric juice.

———— Continuous addition (0·5 ml. every 15 seconds).
— — — Periodic addition (20 ml. every 10 minutes).
. . . . . Peak curve.
A G J. *p*H of artificial gastric juice (*p*H 1·5).

initial effect than the latter, and approaches the speed of aluminium hydroxide gel in this respect. This is due to the availability of the amino grouping for immediate neutralisation, the hydroxy groups reacting slowly as in aluminium hydroxide. Aluminium phosphate gel (8 ml. dose) is rapid in initial effect but fails to raise the *p*H above 2·5. Ion exchange resin C, distributed in the U.S.A. as an antacid, was used at the recommended dosage (1 g.). At this concentration it is low in buffering capacity and has little sustaining power. Bismuth carbonate cannot be regarded as an antacid. The normal dose (1·8 g.) fails to raise the *p*H of the testing solution and high dosage (30 g.) fails to raise the *p*H above 2. Magnesium trisilicate B.P. (1·5 g. dose) is rapid in initial effect and taking the peak curve, buffers at a *p*H level of 6 to 7 for 60 minutes after which time it rapidly loses effect. Although this buffering level is higher than is considered essential, magnesium trisilicate in use would not be likely to cause acid-rebound and may be classed as a good and effective antacid. The ion exchange resins A and B are used therapeutically to correct the electrolyte balance, but they do have an antacid effect and were

accordingly included in this survey. Resin A at the high recommended dosage (15 g.) has a prolonged buffering effect in the region of *p*H 7 and resin B at the recommended dosage of 8 g. buffers at *p*H 5 for approximately 40 minutes. Resin A at smaller dosages buffers at lower *p*H levels and is correspondingly effective for shorter periods. (See Fig. 4.) Magnesium oxide exerts its effect immediately and if in excess raises the *p*H to almost 10. It has little buffering action and achieves its effect

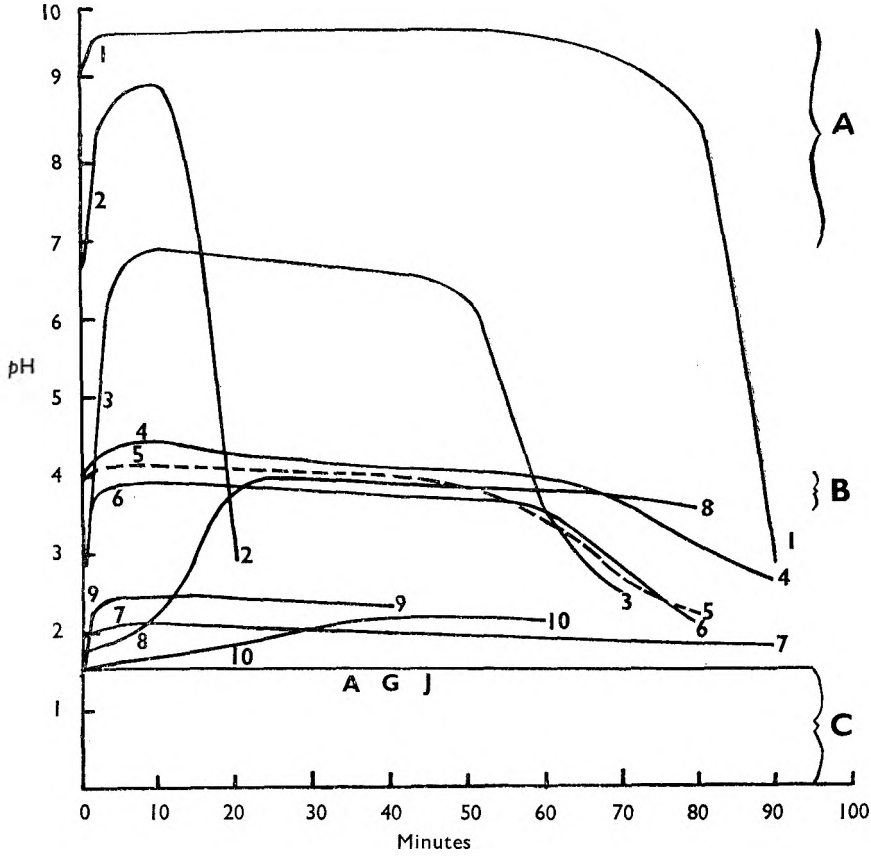

FIG. 3. A comparison of the antacid effect of some commonly used antacids.

1. Light magnesium oxide B.P. (0·5 g.).
2. Light magnesium oxide B.P. (0·25 g.).
3. Magnesium trisilicate B.P. (1·5 g.).
4. Calcium phosphate B.P. (1·5 g.).
5. Aluminium hydroxide gel B.P.C. (8 ml.).
6. Dihydroxy aluminium aminoacetate (1·5 g.).
7. Bismuth carbonate (30 g.).
8. Dried aluminium hydroxide gel (1·5 g.).
9. Aluminium phosphate gel (8 ml.).
10. Aluminium hydroxide dried gel (0·6 g.).

A. Region of acid rebound (*p*H 7 and above).
B. Region of ideal antacid neutralisation (*p*H 3·5 to 4).
C. Hyperchlorhydric region (*p*H below 1·5).
A G J. *p*H of artificial gastric juice (*p*H 1·5).

almost entirely by chemical neutralisation. The duration of effect depends on the amount used. Magnesium oxide when used at a dosage greater than 0·25 g. is an alkalising agent and will cause acid-rebound, and it could be argued that in peptic ulcer therapy even the lower B.P. dose is higher than desirable. It was established that, with the exception

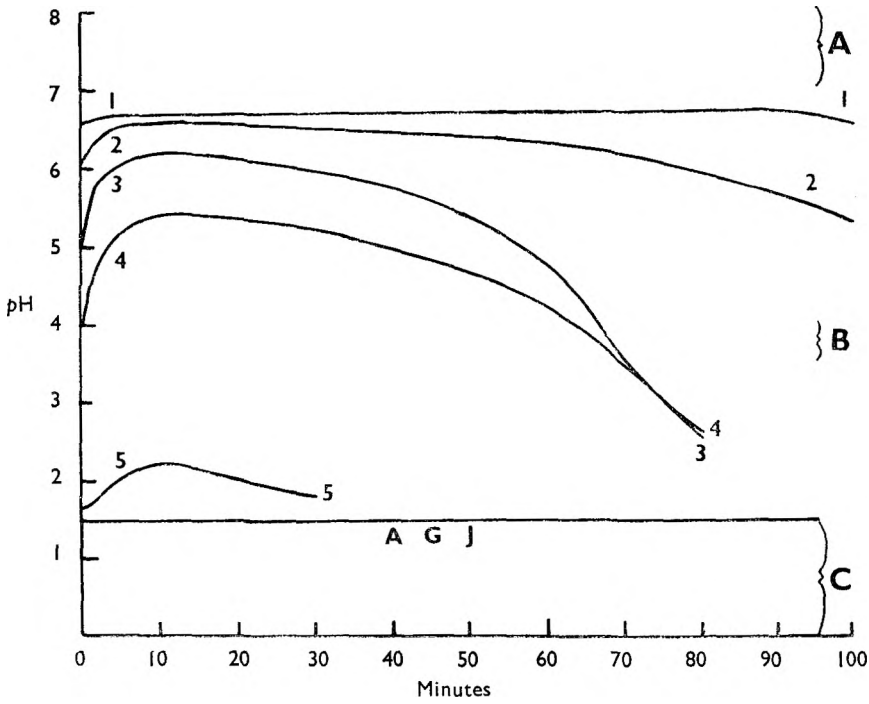

FIG. 4. The antacid effect of some ion exchange resins.

1. Ion exchange resin A (15 g.).
2. Ion exchange resin A (7·5 g.).
3. Ion exchange resin A (3·75 g.).
4. Ion exchange resin B (8 g.).
5. Ion exchange resin C (1 g.).

A. Region of acid rebound (*p*H 7 and above).
B. Region of ideal antacid neutralisation (*p*H 3·5 to 4).
C. Hyperchlorhydric region (*p*H below 1·5).
A G J. *p*H of artificial gastric juice (*p*H 1·5).

of the ion exchange resin and the dried aluminium hydroxide gel (0·6 g. dose), increased dosage prolonged the period of effectiveness without affecting the *p*H level attained.

In conjunction with Figure 3 it is of interest to consider some of the typical curves obtained by plotting the *p*H readings taken at 2-minute intervals. The selected curves are shown in Figures 1 and 2 and contrast the immediately effective or stoichiometric type of antacid as represented by magnesium oxide (Fig. 1) and the buffering type of antacid represented by magnesium trisilicate (Fig. 1) and aluminium hydroxide (Fig. 2). The curve for magnesium oxide shows that further addition of artificial

gastric juice does not appreciably affect the high *p*H level attained indicating that the acid is immediately neutralised. When the excess of magnesium oxide has been used up the curve exhibits a steep decline. The curves for magnesium trisilicate and aluminium hydroxide show that these substances, which act partially by neutralisation and partially by adsorbing hydrogen ions, are more appreciably affected by the further additions of artificial gastric juice but until the antacids are used up they are capable of recovery to the equilibrium *p*H level on each occasion the acid is added. The curves also show that compared with aluminium hydroxide, magnesium trisilicate attains a higher *p*H level, is more

TABLE III

*p*H READINGS WITH ALUMINIUM HYDROXIDE GEL (SEE FIG. 2)

A. Readings obtained by adding 20 ml. of artificial gastric juice and withdrawing 20 ml. of the mixture at 10-minute intervals

Period	Dried aluminium hydroxide gel, B.P.C. 1·5 g.							
	0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80
½ minute	1·75	2·04	3·01	3·05	3·03	3·03	2·78	2·75
2 minutes	1·8	2·2	3·47	3·45	3·45	3·41	3·25	2·85
4 minutes	1·85	2·51	3·76	3·75	3·75	3·72	3·48	3·21
6 minutes	1·92	—	3·83	3·81	3·78	3·76	3·51	3·39
8 minutes	2·0	3·68	3·86	3·82	3·80	3·76	3·73	3·51
10 minutes	2·16	3·82	3·87	3·82	3·82	3·77	3·74	3·59

B. Readings obtained by adding 0·5 ml. of artificial gastric juice and withdrawing 1 ml. of mixture at 15 seconds and 30 seconds respectively

	Dried aluminium hydroxide gel, B.P.C. 1·5 g.							
2 minutes	1·82	2·26	3·46	3·89	3·84	3·74	3·65	3·27
4 minutes	1·89	2·42	3·71	3·88	3·83	3·72	3·60	3·20
6 minutes	1·97	2·61	3·83	3·85	3·82	3·70	3·52	3·18
8 minutes	2·04	2·82	3·89	3·85	3·78	3·69	3·46	3·03
10 minutes	2·14	—	3·89	3·85	3·76	3·67	3·38	3·01

appreciably affected by the additions of the artificial gastric juice and exhibits a steep decline of the *p*H level at the end of the period of effectiveness. This indicates that magnesium trisilicate is more dependent for its antacid effect on its neutralising capacity, due to the magnesium oxide content of the complex, than on its ability to adsorb hydrogen ions.

Figures 1 and 2 also show the effect on these antacids of a more or less continuous addition of artificial gastric juice and continuous withdrawals of the mixture. This more closely simulates the conditions occurring in the stomach. In this modification of the original method used in this investigation, 0·5 ml. of the artificial gastric juice was added every 15 seconds and 1 ml. of the mixture was withdrawn every 30 seconds, the *p*H being recorded at 2-minute intervals. This modified method approximates to the method of Rossett and Flexner[1] which requires a more elaborate technique or apparatus than the original method used in this investigation. The curves obtained from the results of the modified method are superimposed in Figures 1 and 2 on the curves obtained by our original method. As would be expected the curves for *p*H levels lies between the maxima and minima lines of the original curves. The modified curve of magnesium oxide when compared to the "peak curve"

is initially identical but gradually shows an increasingly steeper slope as the reaction proceeds, differing appreciably in *p*H level towards the end of the reaction. The modified curve obtained by the drip method for magnesium trisilicate differs appreciably both in *p*H level and in slope throughout the reaction period, and approximates to what would be the mean curve of the maxima and minima lines obtained by our original method.

TABLE IV

*p*H READINGS WITH VARIOUS ANTACIDS

Period	*Antacid 1	2	3	4	5	6	7	8	9	10
½ minute	9·16	6·8	1·88	4·05	3·9	2·8	1·92	1·75	1·61	1·55
2 minutes	9·61	8·37	4·84	4·25	4·08	3·73	1·98	1·8	2·34	1·56
4 minutes	9·66	8·72	6·38	4·33	4·08	3·86	2·02	1·85	2·42	1·58
6 minutes	9·7	8·86	6·68	4·38	4·08	3·89	2·02	1·92	2·42	1·61
8 minutes	9·7	8·94	6·8	4·4	4·09	3·9	2·05	2·0	2·42	1·64
10 minutes	9·71	9·01	6·88	4·42	4·09	3·9	2·08	2·16	2·42	1·68
20 minutes	9·7	2·84	6·8	4·26	4·08	3·82	2·04	3·82	2·36	1·83
30 minutes	9·71	—	6·69	4·18	4·01	3·80	2·01	3·87	2·3	2·06
40 minutes	9·72	—	6·58	4·08	3·98	3·76	1·96	3·82	—	2·2
50 minutes	9·68	—	6·29	4·03	3·83	3·69	1·91	3·82	—	2·18
60 minutes	9·53	—	3·49	3·93	3·41	3·55	1·88	3·77	—	2·11
70 minutes	9·3	—	2·46	3·69	2·67	2·87	1·82	3·74	—	—
80 minutes	8·57	—	—	3·07	2·25	2·61	1·83	3·59	—	—
90 minutes	2·83	—	—	2·61	—	—	1·8	—	—	—

* See Figure 3 for names and quantities of antacids. Bismuth carbonate 1·8 g. did not raise the *p*H of the testing solution.

TABLE V

*p*H READINGS FOR ION EXCHANGE RESINS

Period	*Ion exchange resin 1	2	3	4	5
½ minute	6·6	6·06	5·08	4·0	1·68
2 minutes	6·68	6·35	5·8	4·82	1·75
4 minutes	6·68	6·46	6·0	4·92	1·96
6 minutes	6·68	6·52	6·08	5·04	2·09
8 minutes	6·69	6·53	6·14	5·04	2·15
10 minutes	6·7	6·57	6·18	5·4	2·2
20 minutes	6·72	6·54	6·1	5·34	2·0
30 minutes	6·68	6·51	5·94	5·18	1·82
40 minutes	6·66	6·45	5·74	4·96	—
50 minutes	6·68	6·37	5·35	4·64	—
60 minutes	6·68	6·28	4·8	4·19	—
70 minutes	6·66	6·09	3·54	3·48	—
80 minutes	6·66	5·9	2·52	2·57	—
90 minutes	6·64	5·64	—	—	—
100 minutes	6·52	5·26	—	—	—

* See Figure 4 for names and quantities of ion exchange resins.

The "drip" curve for dried aluminium hydroxide gel (1·5 g. dose) is almost identical with the "peak curve" differing in a slightly more gradual initial effect and a slightly steeper curve at the end of the period of effectiveness. While no generalisation can be made on these results in relation to the other antacids considered it is felt that the modified method will more closely predict the effect of magnesium trisilicate in actual use and indicates that it would buffer within the region of *p*H 6 to 3·5 for 40 minutes so long as active secretion and withdrawal of gastric juice is taking place.

It is apparent from the results obtained that based on the equivalent aluminium oxide content, dried aluminium hydroxide gel B.P.C. is less

effective than the aluminium hydroxide gel B.P.C. This feature has been previously observed by Adams *et al.*[6] and Johnson and Duncan[2]. Figure 5 shows in detail that at the maximum recommended dose of 0·6 g. dried aluminium hydroxide gel (Al_2O_3 content 0·33 g.) is almost devoid of antacid property contrasting markedly with the efficient antacid properties of the aluminium hydroxide gel B.P.C. at the B.P.C. equivalent dosage of 8 ml. (Al_2O_3 content 0·37 g.). To obtain a similar antacid effect to that produced by 8 ml. of the liquid gel 1·5 g. of the dried gel was required.

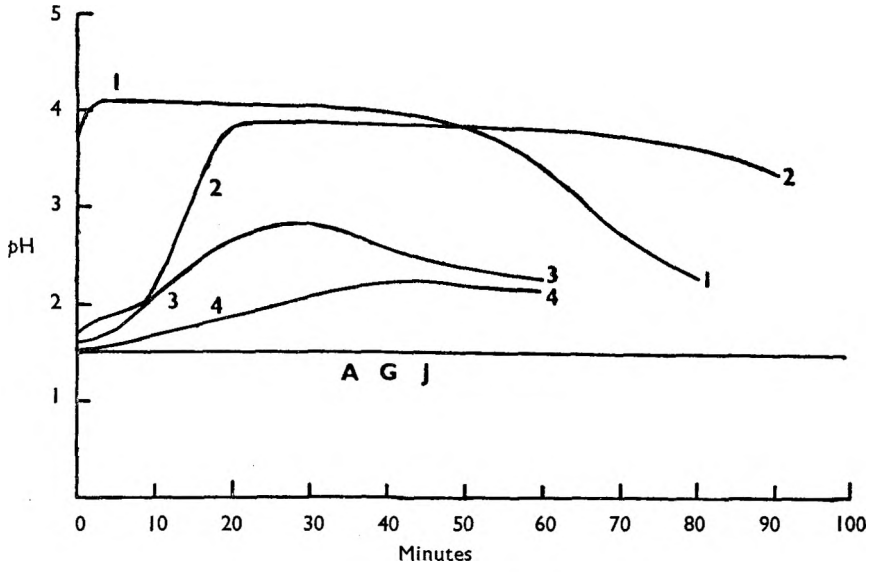

FIG. 5. The difference in antacid effect between aluminium hydroxide gel B.P.C. and dried aluminium hydroxide gel B.P.C.

1. Aluminium hydroxide gel B.P.C. 8 ml. ≡ 0·37 g. Al_2O_3.
2. Dried aluminium hydroxide gel B.P.C. 1·5 g. ≡ 0·825 g. Al_2O_3.
3. Dried aluminium hydroxide gel B.P.C. 0·6 g. + 7·75 ml. of water and heated at 70° C. for 5 minutes.
4. Dried aluminium hydroxide gel B.P.C. 0·6 g. ≡ 0·33 g. Al_2O_3.

A G J. *p*H of artificial gastric juice (*p*H 1·5).

At this increased dosage the dried gel requires 20 minutes to obtain approximately the same *p*H level, as that which is produced almost at once by the liquid gel.

These two samples were subjected to the B.P.C. neutralising capacity test at equivalent amounts, based on the aluminium oxide content. 5·65 g. (required 5·647 g.) of aluminium hydroxide gel B.P.C. and 0·5 g. of dried aluminium hydroxide gel B.P.C. respectively, were added to 150 ml. of 0·1N hydrochloric acid. The mixture was stirred continuously and maintained at 37° C. for 1 hour. After cooling the amount of acid neutralised was determined by titration with 0·1N sodium hydroxide using bromophenol blue as the indicator. In addition to the B.P.C. test the *p*H of the reaction mixture was recorded at 2-minute intervals during the period of the test. The *p*H readings obtained are shown graphically

in Figure 6. The aluminium hydroxide gel (5·65 g.) neutralised 136 ml. of 0·1N hydrochloric acid and the dried aluminium hydroxide gel B.P.C. (0·5 g.) neutralised 127 ml. of 0·1N of hydrochloric acid under the conditions of the B.P.C. test. In both cases the B.P.C. requires the neutralisation of not less than 100 ml. of hydrochloric acid. (The B.P.C. directs that 5 g. of the liquid gel be taken for the acid neutralisation test.)

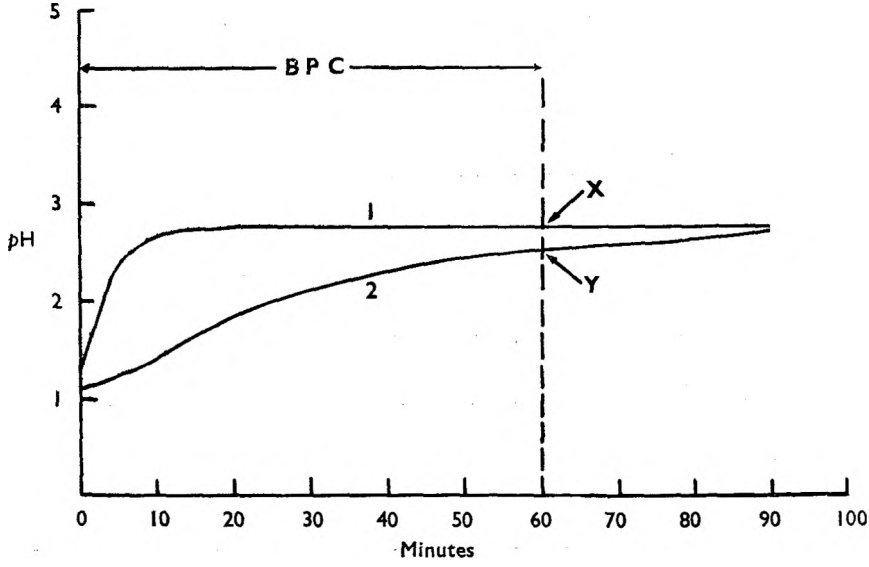

FIG. 6. *p*H changes occuring during the period of the acid neutralising test of the B.P.C. in the case of aluminium hydroxide gel B.P.C. and the dried gel.

1. Aluminium hydroxide gel B.P.C. 5·65 g. ≡ 0·261 g. Al_2O_3.
2. Dried aluminium hydroxide gel B.P.C. 0·5 g. ≡ 0·26 g. Al_2O_3.

X. 136 ml. of 0·1N hydrochloric acid neutralized.
Y. 127 ml. of 0·1N hydrochloric acid neutralized.
B.P.C. Period of B.P.C. test.

The acid neutralising capacities of the two preparations at equivalent amounts should be identical but as indicated by the B.P.C. test there is a difference in acid neutralising capacity. Examination of the *p*H curves in Figure 6 show that this is due to dried gel requiring more than 1 hour for neutralisation, whereas the liquid gel requires 20 minutes for complete neutralisation. A repeat experiment showed that the dried gel required 90 minutes for complete neutralisation. This curve is represented in Figure 6. The marked difference in the low *p*H level attained by the dried gel, as shown by the *in vitro* test, is due to the progressive loss of unreacted dried aluminium hydroxide gel in the repeated withdrawals of the reaction mixture. These withdrawals, however, represent physiological loss from the stomach.

It was thought that a difference in effectiveness, in relation to the speed of action, may be due to a difference in particle size associated with the degree of hydration and surface properties. Particle size determination by a microscopic technique was found to be unsatisfactory, the particle

size being beyond the size of resolution. Electron micrographs revealed that in the dried gel the particles were aggregated and that the individual particle size would be of the order of 0·1 μ. The use of agents such as sodium tartrate, wetting agents, etc., did not cause any appreciable deflocculation. It is not of course possible to examine the liquid gel by electron microscope without altering its character.

Consideration was given to the affect of wetting and hydration of the dried gel but treatment of 0·6 g. of the dried gel by stirring with water for 1 hour in the cold did not appreciably affect the curve for antacid effect.

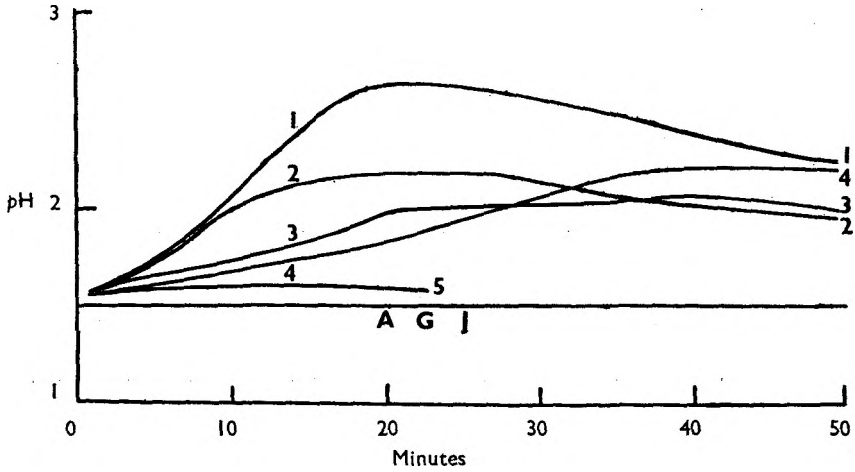

FIG. 7. A comparison of the antacid efficiency of dried aluminium hydroxide gel when hydrated at various temperatures. In each case 0·6 g. of the dried gel was heated with 7·75 ml. of water for the period indicated prior to testing.

1. 70° C. for 5 minutes.
2. 70° C. for 1 hour (*a*) and 90° C. for 5 minutes (*b*).
3. 50° C. for 5 minutes.
4. Material at room temperature.
5. 90° C. for 30 minutes (*a*) and 1 hour (*b*).

A G J. *p*H of artificial gastric juice (*p*H 1·5).

By heating dried aluminium hydroxide gel with water partial hydration can be effected with increased antacid efficiency. The amount of heat applied, governed by volume, time and temperature, is critical, as overheating may cause the deterioration or even complete loss of antacid efficiency. This effect is shown graphically in Figure 7, and the curve showing optimum efficiency achieved by hydration is shown for comparative purpose in Figure 5.

SUMMARY

Conclusions

1. An *in vitro* method has been used to compare the relative efficiencies of antacids in respect of the *p*H changes, rate and time of effectiveness when tested under conditions simulating those occurring in the stomach. Further work is indicated to establish a standardised procedure.

2. Of the antacids considered aluminium hydroxide gel B.P.C., dried

aluminium hydroxide gel B.P.C., dihydroxy aluminium aminoacetate, and calcium phosphate B.P. at appropriate dosage buffer at the desired clinical range of *p*H 3·5 to 4.

TABLE VI

*p*H READINGS FOR ALUMINIUM HYDROXIDE PREPARATIONS

Period	*Preparation 1	2	3	4
½ minute	3·9	1·75	1·55	1·55
2 minutes	4·08	1·8	1·6	1·56
4 minutes	4·08	1·85	1·68	1·55
6 minutes	4·08	1·92	1·79	1·61
8 minutes	4·09	2·0	1·92	1·64
10 minutes	4·09	2·16	2·06	1·68
20 minutes	4·08	3·82	2·64	1·83
30 minutes	4·01	3·87	2·84	2·06
40 minutes	3·98	3·82	2·57	2·22
50 minutes	3·83	3·82	2·36	2·18
60 minutes	3·41	3·77	2·25	2·11
70 minutes	2·67	3·74	—	—
80 minutes	2·25	3·59	—	—
90 minutes	—	3·36	—	—

*See Figure 5 for the quantities of aluminium hydroxide preparations used.

TABLE VII

*p*H READINGS OBTAINED DURING THE B.P.C. ACID NEUTRALISING TEST

(*see* Fig. 6)

Period	Dried aluminium hydroxide gel, B.P.C. 0·5 g. 0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90
2 minutes	1·13	1·5	1·88	2·1	—	2·45	—	—	—
4 minutes	1·18	1·57	1·96	2·2	2·34	2·46	—	—	—
6 minutes	1·24	1·68	2·0	2·24	2·36	2·46	—	—	—
8 minutes	1·31	1·74	2·04	2·28	2·4	2·49	—	—	—
10 minutes	1·4	1·84	2·08	2·29	2·42	2·50	2·58	2·64	2·72

Period	Aluminium hydroxide gel, B.P.C. 5·65 g. 0–10	10–20	20–30	30–40	40–50	50–60	60–70	70–80	80–90
2 minutes	1·71	2·71	2·78	—	—	—	—	—	—
4 minutes	2·2	2·74	2·78	—	—	—	—	—	—
6 minutes	2·43	2·76	2·78	—	—	—	—	—	—
8 minutes	2·58	2·76	2·78	—	—	—	—	—	—
10 minutes	2·66	2·78	2·78	2·78	2·78	2·78	2·78	2·78	2·78

TABLE VIII

*p*H READINGS FOR DRIED ALUMINIUM HYDROXIDE GEL

Period	*Preparation 1	2 (*a*)	2 (*b*)	3	5 (*a*)	5 (*b*)
½ minute	1·55	1·61	1·58	1·59	1·58	1·56
2 minutes	1·6	1·66	1·75	1·6	1·6	1·58
4 minutes	1·68	1·74	1·83	1·63	1·6	1·57
6 minutes	1·79	1·82	1·90	1·68	1·6	1·57
8 minutes	1·92	1·91	1·98	1·72	1·6	—
10 minutes	2·06	2·0	2·03	1·78	1·6	—
20 minutes	2·64	2·17	2·18	2·0	1·57	—
30 minutes	2·84	2·12	2·15	2·07	—	—
40 minutes	2·57	2·02	2·08	2·06	—	—
50 minutes	2·36	1·93	2·0	2·0	—	—
60 minutes	2·25	1·84	—	1·98	—	—

*See Figure 7 for quantities and treatment of aluminium hydroxide.

3. Magnesium trisilicate B.P. is an effective antacid buffering at a slightly higher *p*H level of 6 to 3·5.

4. Bismuth carbonate cannot be considered an antacid.

5. Magnesium oxide B.P.C. at the lower B.P. dosage (0·5 g.) is an alkalising agent and in use is likely to cause acid re-bound.

6. A difference in antacid effectiveness between aluminium hydroxide gel B.P.C. and the dried gel at equivalent dosage has been demonstrated.

7. The B.P.C. acid neutralising test does not indicate the difference in antacid effectiveness of the aluminium hydroxide preparations.

In conclusion we wish to thank Mr. A. W. Bull and Mr. C. A. Hill for helpful suggestions and advice, and the Directors of Boots Pure Drug Co. Ltd., for permission to publish this paper.

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THE EVALUATION OF BUFFER ANTACIDS, WITH PARTICULAR REFERENCE TO PREPARATIONS OF ALUMINIUM

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The treatment of gastric hyperacidity by the administration of substances with antacid properties has engaged the attention of numerous workers in the clinical and related fields. The trend of medical opinion has been to move away from the idea of complete neutralisation of the gastric fluid with the straightforward antacids, and to turn to the use of buffer substances with the object of controlling the *p*H at a more physiologically desirable level. The endeavour has been to remove hyperacidity whilst avoiding the stimulation of acid rebound, or the risks of alkalosis, respectively associated with temporary or prolonged over-neutralisation.

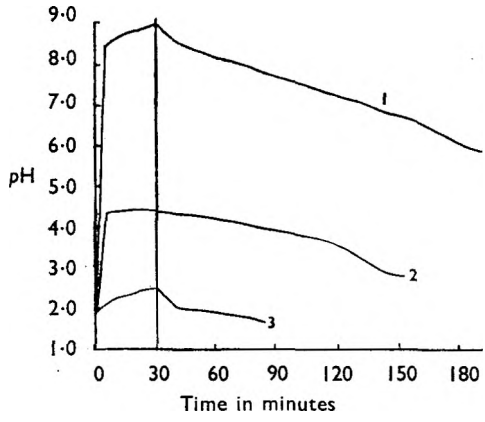

Fig. 1. Type of response when the described method is applied to:—

1. Light magnesium carbonate.
2. Aluminium glycinate.
3. Magnesium trisilicate.

From the pharmaceutical view-point it is a simple matter to ensure that the clinician is provided with sodium bicarbonate of a standard antacid quality, but to ensure a similar service in the case of buffer substances presents something of a problem. Of these buffer types of antacid it is probable that the aluminium preparations are among the most widely used and there can be little doubt that a determination of acid-neutralising capacity as laid down in the B.P.C. 1949 and U.S.P. XIV, for aluminium hydroxide gel, is an inadequate measure of the therapeutic value of the antacids in question, at least so far as the dried forms are concerned.

This type of test was criticised by Holbert, Noble and Grote[1,2] when assessing aluminium preparations and they suggested an alternative procedure based on a modification of a method designed by Johnson and Duncan[3] to simulate conditions in the stomach. These methods were dependent on measuring the rate of change of *p*H of an acid solution in the presence of the antacid in question, and thus took account of the time factor and the reactivity of the antacid under standard empirical conditions. Various procedures based on this principle have been employed by other workers (Mutch[4,5]; Hammarlund and Rising[6,7] and Murphey[8]) in comparing the status of antacids, mainly of the insoluble type.

The purpose of the present paper is to focus further attention upon this problem and to illustrate the use of a somewhat simplified procedure which experience has shown to be of considerable value in assessing antacid properties. Particular attention is directed to the desirability of reconsidering the standards for dried aluminium hydroxide gel.

Suggested Test Conditions

1·0 g. of sample, passing a 100 mesh B.S.S. screen, is added to 200 ml. of water and 3 ml. of N hydrochloric acid. The mixture is agitated continuously by mechanical stirrer to maintain a uniform suspension of the insoluble material. The *p*H of the mixture is determined at intervals of 5 minutes over a period of 30 minutes, the measurement being made electrometrically *via* electrodes remaining *in situ* in the liquid. Thereafter, 1 ml. of N hydrochloric acid is added at intervals of 10 minutes and the *p*H determined immediately prior to each successive addition. This procedure is continued for a period depending upon the rate at which the *p*H falls and the minimum target set for the particular antacid material under test. The whole operation is carried out at room temperature.

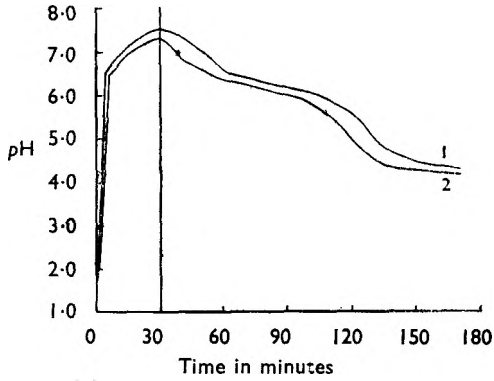

Fig. 2. Depressant tendency upon antacid activity of 1 per cent. of Karaya gum in a tablet containing magnesium carbonate, calcium carbonate and dried aluminium hydroxide gel.
1. Antacid mixture without gum.
2. Antacid mixture with gum.

During the first few minutes of the test the wettability of the powder is a potential factor. This, however, is not normally significant and is reduced to a minimum by rapidly levigating with a little of the test solution and rinsing the suspension into the vessel. It has been found unnecessary and indeed undesirable to use wetting agents.

Comments on the Suggested Conditions

Amongst other properties, the efficacy of an antacid depends upon the rate at which it can exert its effect on the gastric juice, the *p*H to which it will raise the juice when the antacid is present in excess, and its capacity to maintain the *p*H in the face of continued secretion of fresh juice. An attempt is made to assess the first two of these factors in the initial 30-minute period of the described test, whilst the subsequent part of the test takes into account the capacity factor.

The strength of the initial acid solution is such that the *p*H is approximately 1·8. This is chosen as a reasonable approximation of the order of values found in the untreated hyperacidic stomach as indicated by control measurements in fractional test meals[9]. The choice of volume of this

solution, and the strength and rate of addition of acid in the second part of the test, must obviously be on a much more speculative basis, but it is suggested that the chosen conditions have a practical bearing upon their *in vivo* counterparts. The interval between *p*H readings will obviously influence the sensitivity of the test in so far as the recorded points will generally not be equilibrium values. It is clear that the selectivity of the test will diminish as the interval between additions of acid is increased. The 10-minute interval was found to be about the minimum time consistent with expedience in adding acid, measuring the *p*H, and allowing reasonable accuracy in timing, upon which the reproducibility of the results very largely depends. The weight of sample taken should bear some relationship to customary dosage and the 1 g. quantity advocated is considered to be generally consistent with the preparations under discussion.

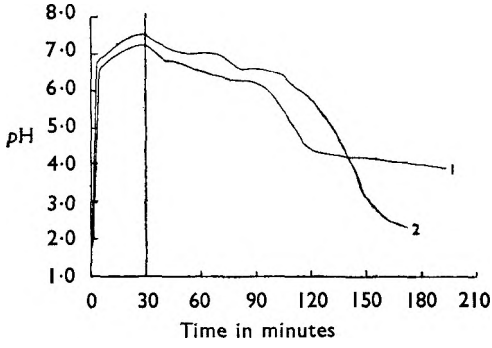

FIG. 3. Loss of buffer activity in the region of *p*H 4, in one of 2 antacid mixtures, due to an adverse condition in manufacture. The aluminium contents of the mixtures were of a similar order.

1. Mixture of magnesium carbonate, calcium carbonate and aluminium hydroxide.
2. Similar mixture to 1 in which the aluminium hydroxide is inactivated.

The suggested conditions do not demand any special elaboration in the laboratory, in that there is no elevated temperature control, and there are no intermittent withdrawals of the test liquid. These two conditions have been taken into account in methods previously described by other workers, with the object of closely simulating conditions in the stomach, but in our opinion the practical significance of these two factors is doubtful.

General Value of the Test

A graphical representation of the results of a test affords a very useful pictorial characterisation of an antacid, or mixture of antacids, in relation to probable clinical behaviour. For example, Figure 1 shows the curves obtained with light magnesium carbonate, dihydroxy aluminium aminoacetate, N.N.R. ("Aluminium Glycinate") and magnesium trisilicate.

The curves in Figure 1 for magnesium carbonate and aluminium glycinate illustrate the types of response when the described method is applied to a strong neutralising agent and a highly active buffer substance, respectively. From current concepts of antacid therapy it would be expected that any substance which gave a curve of the first type would tend to stimulate acid rebound and to have a comparatively brief antacid effect, whilst a substance giving the second type of curve should avoid these disadvantages. The third curve typifies the response obtained when

a slow acting weak buffer substance is submitted to the prescribed conditions. In the case of a mixture of the various classes of antacid, a composite curve is obtained which shows at a glance whether one or other type of action is likely to predominate.

A study of such curves has been found to be of great value in considering the formulation of antacid products, not only in respect of the selection of active components and assessing the promptness, type, and duration of action, but in providing a sensitive measure of the influence upon antacid activity of various factors during manufacture and storage. Figure 2, for example, shows the depressant tendency upon antacid activity of 1 per cent. of Karaya gum in a tablet containing magnesium carbonate, calcium carbonate and dried aluminium hydroxide gel. The curves reproduced in Figure 3, obtained from two antacid mixtures similar to that in Figure 2, show the loss of buffer activity in the region of *p*H 4 due to adverse conditions in manufacture. The aluminium contents of these two mixtures were of a similar order.

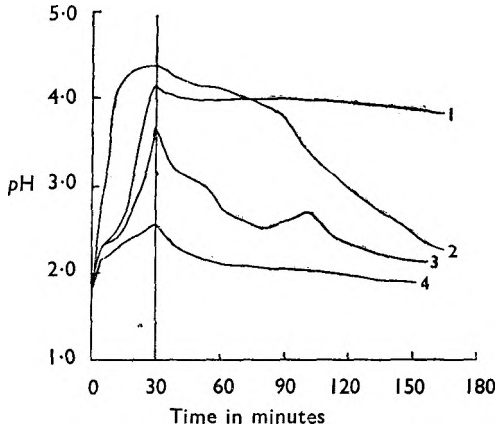

FIG. 4. Difference in reactivity and buffer capacity of 4 samples of B.P.C. dried aluminium hydroxide gel.

ALUMINIUM PREPARATIONS

It is proposed to confine attention to dried aluminium hydroxide gels and aluminium glycinate. The former are the subject of monographs in the B.P.C. 1949 and U.S.P. XIV, and a monograph is devoted to the latter antacid in New and Nonofficial Remedies 1952 (N.N.R.). In each case an evaluation of antacid activity is based on a measurement of the amount of acid absorbed under standard conditions by allowing the sample to stand in contact with an excess of acid for 1 hour at 37°C in the B.P.C. and U.S.P. tests, and for 10 minutes at room temperature in the N.N.R. test. In all cases the residual acid is measured by titration with alkali using bromophenol blue as indicator.

From the purely analytical aspect the end-point is ill-defined because of the strong buffer action of the aluminium salt present. It is suggested that if this type of test should be retained it would be preferable to specify an electrometric procedure.

In addition to the acid absorption test for aluminium glycinate the N.N.R. monograph describes a test designed to measure rate of reaction. This test specifies that the *p*H must exceed 3 when about 0·2 g. has been in contact with 25 ml. of 0·1N hydrochloric acid for 10 minutes, the suspension being shaken during the first 5 minutes. Disregarding for the moment any criticism of this test, the imposition of this additional standard implies

that the acid absorption type of test is not wholly adequate in assessing antacid value, and it would seem desirable that some form of test which specifically takes the rate of reaction into account should likewise be extended to the dried aluminium hydroxide gels.

It is instructive to consider the curves reproduced in Figure 4, which were obtained by the method described by us, when applied to 4 samples of dried aluminium hydroxide gel, all of which passed the B.P.C. neutralising capacity test. The actual acid absorptions of samples (1) to (4) determined by the B.P.C. procedure and expressed in terms of ml. of acid consumed per g. were 282, 266, 231 and 200, respectively. From an inspection of the curves in their entirety, it is evident that the antacid activities of these samples are very widely divergent. Sample (1) is clearly superior as an antacid buffer. Sample (4) is very poor both in regard to promptness of action, as indicated in the first part of the test, and capacity to sustain the *p*H at an effective level, as shown by the second stage of the test. Sample (3) approaches sample (1) in its promptness of action, but it is evidently much less effective in its buffering capacity. The curve for sample (2) indicates a high rate of reaction but a comparatively weak buffer capacity, and its general shape suggests the presence of residual sodium carbonate, for which the B.P.C. monograph does not impose any limit. The U.S.P. pays some attention to the presence of strong alkali by prescribing a limit for *p*H of the filtrate from an aqueous suspension of the sample.

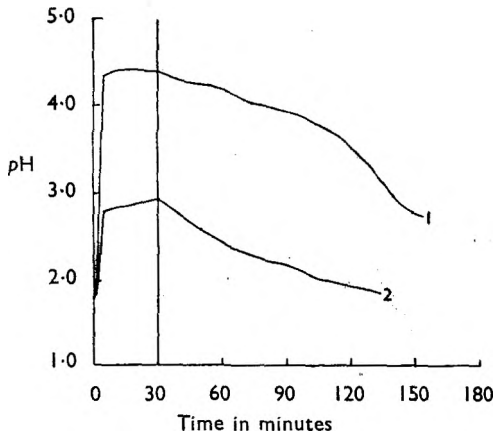

FIG. 5. Responses to the described test from samples of:—
1. Aluminium glycinate.
2. Dried aluminium hydroxide gel and glycine mixture of equivalent aluminium and nitrogen content to 1.

Figure 5 shows the responses to the described test from samples of aluminium glycinate and a mixture of dried aluminium hydroxide gel and glycine such that the aluminium and nitrogen contents were the same in both samples. These two curves show that the aluminium glycinate is a greatly superior antacid to the physical mixture of the parent components. These two samples were also subjected to the acid absorption tests as prescribed in the B.P.C. and N.N.R. monographs and to the N.N.R. reaction rate test. The results are shown in Table I.

The acid absorption figures in Table I illustrate the influence of conditions upon the degree of discrimination exhibited by this type of test. It is plain that the conditions prescribed in the N.N.R. monograph are much more stringent than those of the B.P.C. It is also evident that the reaction rate test is a useful criterion of activity.

Discussion

From data presented in this and other communications, it would seem desirable to consider whether the B.P.C. standard for antacid activity of dried aluminium hydroxide gel is adequate. It is tentatively suggested that if the B.P.C. test is to be retained in its present form, it might be well to consider raising the lower limit for acid absorption from 200 to 250 ml. of 0·1N hydrochloric acid per g. It would also seem desirable to adopt a test for reactivity on the lines of those used for aluminium glycinate or aluminium phosphate gel U.S.P.

Measurements of the properties mentioned above are integrated in the test at present described. So far as dried aluminium hydroxide gel is concerned it is suggested that reasonable standards would be *p*H of 3·5 to 4·5 after the initial 30 minutes and a total acid consumption of not less than 10 ml. of N hydrochloric acid before the *p*H falls below 3·5, corresponding to 100 minutes under the conditions of the test. In selecting the various critical conditions for this type of test, it is obvious that the possible variations are almost infinite. The choice of the actual conditions described was dictated by an attempt to compromise in order of importance between therapeutic significance and expedience in the laboratory.

TABLE I

SHOWING THE RESPONSES TO B.P.C. AND N.N.R. TESTS OF (1) A SAMPLE OF ALUMINIUM GLYCINATE AND (2) A MIXTURE OF GLYCINE AND DRIED ALUMINIUM HYDROXIDE GEL WITH THE SAME ALUMINIUM AND NITROGEN CONTENTS AS SAMPLE (1).

Sample	Acid absorption test ml. of 0·01N HCl/g.		*p*H obtained in N.N.R. reaction rate test
	B.P.C.	N.N.R.	
1	183	152	3·9
2	159	42	1·6

In considering any test for antacid activity such as the measurement of neutralising capacity as prescribed in the B.P.C., or the type at present described, it is important not to overlook the fact that such measurements are concerned solely with the ability of a substance to neutralise hydrochloric acid. No account is taken of the multiplicity of factors likely to arise in the stomach such as the influence of pepsin. It is merely submitted that the suggested procedure, in measuring both promptness and duration of buffer action, is more discriminating and informative than that used in the B.P.C. monograph in estimating the clinical potentialities of dried aluminium hydroxide gel.

The authors record their thanks to Miss A. Braddick for performing much of the practical work associated with this investigation, and to the Directors of Beecham Research Laboratories Limited for permission to publish.

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THE CHEMICAL EVALUATION OF ANTACIDS

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To assess the value of an antacid for medicinal purposes the obvious method is to study the effect on the gastric secretion in the stomach of the human subject. This subject may be a normal person or one with hyperacidity or, if emphasis is placed on clinical use, one suffering from an ulcerous condition. It is very difficult however to secure reliable information by this method. There are very wide variations in the constitution of the gastric juice of the same individual even within a short space of time and according to Mutch[1] critical assessment of the relative healing or prophylactic virtues of antacids is impossible.

It may well be that the results of well designed chemical tests will supply more reliable information than *in vivo* experiments which might at the first sight appear to be more desirable. Such chemical tests, however, should approach as nearly as possible the normal conditions which might exist in the stomach of a patient who is taking an antacid to control hyperacidity. The acid absorption test for magnesium trisilicate of the British Pharmacopœia 1953 consists in adding to the antacid a considerable excess of 0·05N hydrochloric acid and stirring continuously at 20° C. for 4 hours and determining the amount of acid neutralised by titrating the excess with 0·05N alkali. The neutralising capacity test for aluminium hydroxide gel of the British Pharmaceutical Codex 1949, is similar but the reaction is carried out at 37° C. for one hour. These tests are of value for the purpose for which they were designed, i.e., to ensure that the medicinal substance has a certain minimum capacity to neutralise acid. No attempt is made to approach the conditions under which the antacid will be expected to act medicinally except that the British Pharmaceutical Codex directs that the test be carried out at about body temperature, 37° C. If the results of such tests are used as criteria for evaluating antacids it is possible that very erroneous conclusions may be reached. The conditions of these tests differ from those occurring in actual use in that there is (*a*) a considerable excess of acid at all times during the test and this may not be the case in the stomach, (*b*) the effect of buffers etc. present in the stomach is not allowed for, (*c*) in the stomach there is a continuous and variable secretion of acid and formation of other substances, and (*d*) there is continuous removal of the stomach contents to the duodenum.

These factors must have a considerable effect upon the *in vivo* action of antacids and must be considered when attempts are made to correlate the results of chemical tests with the probable action in the stomach. Hammarlund and Rising[2] added hydrochloric acid at intervals of 30 minutes to the antacid and recorded the *p*H every 5 minutes for 3 hours. Alstead[3] used human gastric juice for his tests and Mutch[1] also added hydrochloric

acid in small portions at intervals to an excess of antacid and determined the *p*H at frequent intervals. Mutch[4] measured the resulting *p*H when varying amounts of acid and antacid were left in contact for different periods.

The present writer is not here concerned with the clinical value of antacids in ulcerous conditions where such considerations as their influence on peptic digestion, laxative effect and protective action on the gastric mucosa would have to be taken into account; neither is he concerned with the properties of mixtures of antacids, nor in examining a large variety of antacids. The object of the work here described was to devise a comparatively simple chemical test which would enable some assessment to be made of the neutralising power of an antacid which would take into account, (*a*) the presence of buffers similar to those in gastric juice, (*b*) body temperature, (*c*) rate of neutralisation, (*d*) ultimate *p*H when antacid is in excess. These are important factors in the clinical use of antacids which do not enter into the official tests.

Experimental

After a number of trials of different concentrations of hydrochloric acid and different buffers it was finally decided that an artificial gastric juice which would be suitable for experimental use would be 0·05N hydrochloric acid containing 0·15 per cent. each of pepsin, peptone and sodium chloride. It was advisable to preserve it with about 0·5 per cent. v/v of chloroform by shaking with a slight excess of chloroform. This artificial gastric secretion had an average *p*H at 38° C. of 1·5. The *p*H of different batches varied between 1·45 and 1·55 probably due to some variation in the pepsin and peptone. Because of this variation in the initial *p*H of the fluid it was better to take account of the change of *p*H on addition of the antacid in preference to the actual *p*H readings. The general plan of the experimental work as finally decided after many trials of different conditions was to add a 20 per cent. excess of the antacid to 100 ml. of the artificial secretion at 38° C. and record the change of *p*H at frequent intervals keeping the liquid briskly stirred. It will be shown that it was possible to obtain a fairly accurate figure for the amount of acid neutralised in any given time.

Relationship of p*H change to amount of acid neutralised.* The correspondence between the recorded *p*H change and the amount of acid neutralised was determined by heating 100 ml. of the artificial juice to 38° C. and titrating with 0·5N sodium hydroxide added in small portions with constant stirring and recording the change in the *p*H of the fluid. It was realised that these were not conditions which would be directly comparable with those occuring during the actual test of the antacid. Sodium chloride was formed as the result of the neutralisation of the acid and the volume was increased eventually by 10 per cent. Neither of these circumstances might occur during the actual testing of an antacid but it was considered that the differences would be negligible for all practical purposes. In any case although a knowledge of the proportion of acid neutralised by the antacid might be useful information, the change of

*p*H produced is probably of greater significance. The result is shown graphically in Figure 1.

A number of titrations were carried out and satisfactorily concordant results were obtained. The degree of accuracy varied somewhat in different parts of the titration but the variation never exceeded ±5 per cent., the figures being much more consistent as neutrality was approached.

Comparison of artificial with human gastric secretion. Samples of human gastric secretion showed considerable variation of *p*H with alkali treatment even when there was not much difference in their "hydrochloric acid" acidity. In order to determine if the artificial secretion would behave roughly similarly to the natural, a number of samples of human gastric juice of about 0·05N acidity were selected and titrated with 0·5N sodium hydroxide under the same conditions as those just described for the artificial secretion. There was a considerable variation between individual samples but the average gave a result very similar to those for the artificial juice (see Fig. 1). It is evident from these curves that in testing an antacid it should be possible to determine with considerable accuracy the percentage of hydrochloric acid neutralised in any given time when the antacid is added to a definite amount, in these experiments 100 ml., of the artificial gastric secretion. Although no allowance has been made for the "emptying" of the stomach the information so gained should be very valuable when a comparison is made of the probable relative efficiencies of antacids.

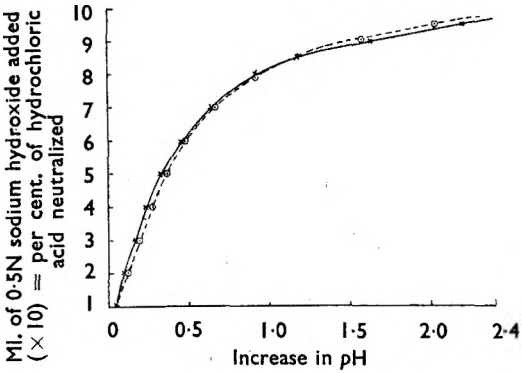

FIG. 1. Titration curves of sodium hydroxide against human and artificial gastric secretion.

——×——×— Artificial secretion.
– – ○ – – ○ – Human secretion.

As explained above, the object of the work here described was to devise a test for antacids for which the conditions could be standardised, which would supply information regarding the probable speed of action and ultimate *p*H in the stomach. For some clinical purposes rapid neutralisation of the stomach acid may be required, for others a steady, slow neutralisation may be desirable. Again in some conditions it may be that a final *p*H of about 2 to 2·5 or alternatively 5 to 6 is required and this *p*H attained either rapidly or slowly.

It is suggested that the test described below would supply most of the information required and should be of value in comparing the antacids now in use, and also for the evaluation of new antacids or mixtures.

Method of Testing

After a number of trials the following conditions and apparatus appeared to offer the most advantages and fewest disadvantages.

100 ml. of the artificial gastric secretion described above is placed in a 150-ml. beaker and immersed in a water bath at 38° C. to a depth equal to the level of the liquid inside the beaker. The liquid is kept constantly stirred and the electrodes of a *p*H meter immersed in it. The *p*H meter may be of the automatic recording type but this is not essential. When the *p*H and the temperature are constant, the latter at 38° C., the antacid is added and the *p*H read at frequent intervals. The amount of antacid to be added is arbitrary but for each experiment described below a 20 per cent. excess over the "neutralisation value" of the antacid was used. This neutralisation value was determined by adding a weighed amount of the antacid to 100 ml. of 0·05N hydrochloric acid at 38° C. taking care that there will be finally an excess of about 50 ml. of the acid. The temperature was kept at 38° C. by immersing the beaker in a water bath and the mixture was thoroughly stirred continuously. After four hours or when the reaction was complete the mixture was filtered, if necessary, and after washing the filter paper and residue, if any, the excess of acid titrated with standard sodium hydroxide using bromophenol blue as indicator. The term "neutralisation value" for the antacid as used in this paper was the weight of the antacid required to neutralise 100 ml. of 0·05N hydrochloric acid under these conditions. At the elevated temperature in the presence of a considerable excess of acid and absence of buffers it may be assumed that the figure obtained for the amount of acid neutralised is a maximum one. This often differed markedly from the result which would be obtained by the electrometric titration in the presence of buffers when a large excess of acid is not used, these being the conditions of the test.

Details and results of tests. A selection was made of a number of antacids which were obtained in the ordinary way either from the makers or through wholesale suppliers. The samples therefore are representative of those which would normally be used by patients. In each case the "neutralisation value" was determined by the method given above and 20 per cent. above this quantity added to 100 ml. of the artificial gastric secretion. The *p*H was read at frequent intervals, usually 1-minute intervals at the beginning of the experiment, but at wider intervals later when a small *p*H change does not represent as much acid neutralised.

The Tables give the increase of *p*H at intervals of 10 minutes for 30 minutes and then after the first hour. Since the initial *p*H of the liquid was about 1·5 the approximate actual *p*H can be calculated by adding 1·5 to the figure for the increase in *p*H.

Magnesium trisilicate. 3 samples of magnesium trisilicate were first tested and the results up to 1 hour are given in Table I and are expressed graphically in Figure 2. The experiments were usually carried out over a 3-hour period but the significant time was the first 60 or 90 minutes. Table I gives the results up to 60 minutes and the Figure 2 up to 90 minutes. The approximate actual *p*H at any stage can be calculated by adding 1·5 to the figure given for the increase in *p*H. The approximate percentage

of hydrochloric acid neutralised is obtained from the graph given in Figure 1.

It is interesting to note the variation between the 3 samples. Sample C was a specimen which had been stored for about 12 months but samples A and B were of recent manufacture. It is not suggested that there had

TABLE I

INCREASE IN *p*H VALUE AND PERCENTAGE OF HYDROCHLORIC ACID NEUTRALISED ON ADDING 20 PER CENT. EXCESS OF MAGNESIUM TRISILICATE TO ARTIFICIAL GASTRIC SECRETION AT 38° C.

Antacid	Neutralisation value	After 10 minutes		After 20 minutes		After 30 minutes		After 60 minutes		Final *p*H with 100 per cent. excess of antacid
		*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	
Magnesium trisilicate:										
A	0·4393 g.	0·30	47·5	0·58	67·5	0·86	78	2·00	95	7·5
B	0·4358 g.	0·50	62·5	1·02	82·5	1·52	90	3·36	100	
C	0·4586 g.	0·32	50·0	0·53	65·0	0·64	70	0·82	77	

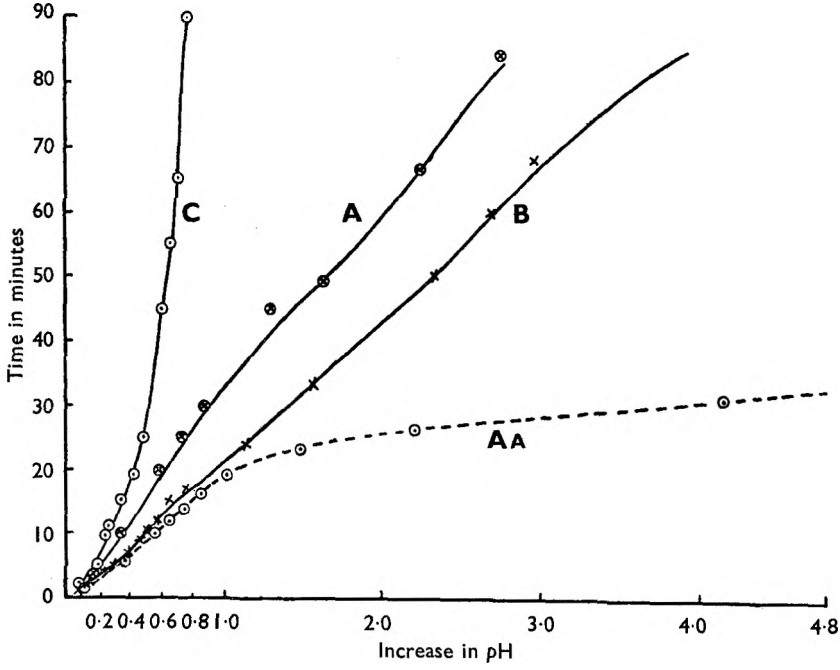

FIG. 2. Increase in *p*H on addition of magnesium trisilicate.

A. Sample A.
AA. Sample A without buffer.
B. Sample B.
C. Sample C.

been any deterioration in the case of C since no figures were available for its original behaviour. For all practical purposes samples A and B had neutralised all the acid in 1 hour but C had neutralised only 77 per cent. and the reaction was proceeding very slowly. The final *p*H in all the cases was about 7. Near the conclusion, sufficient magnesium trisilicate was added to give an excess of 100 per cent., but this had little effect upon the final *p*H value which was still 7·0 to 7·5. It will be noticed that when the increase in *p*H is plotted against the time the graph is practically a straight line until there is almost full neutralisation of the acid.

TABLE II

INCREASE IN *p*H VALUE AND APPROXIMATE PERCENTAGE OF HYDROCHLORIC ACID NEUTRALISED ON ADDING 20 PER CENT. EXCESS OF VARIOUS ANTACIDS TO ARTIFICIAL GASTRIC SECRETION AT 38° C.

Antacid	Neutralisation value	After 10 minutes		After 20 minutes		After 30 minutes		After 60 minutes		Final *p*H with 100 per cent. excess of antacid
		*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	
Aluminium phosphate suspension	18·85 g.	1·02	82	1·04	83	1·07	84	1·12	85	3·24
Dried aluminium hydroxide gel (A) ..	0·1829 g.	0·17	30	0·24	40	0·32	49	0·45	60	3·88
do. supplied as B.P.C. (B) ..	1·144 g.	0·98	81	1·76	91	1·93	93	1·98	94	3·82
Aluminium hydroxide gel	8·707 g.	1·40	88	2·14	95	2·32	96	2·40	98	4·76
Magnesium phosphate	0·511 g.	2·36	96	2·88	98	3·13	100	3·28	100	5·45
Bismuth carbonate ..	1·159 g. to 1·179 g.	0·0	0	0·0	0	0·0	0	0·0	0	1·53

Other antacids. Tests with the artificial gastric secretion were carried out on a few other slow-acting antacids. Sodium bicarbonate, calcium carbonate, magnesium hydroxide and others which act quickly were not tested at this stage because the results could be forecasted with considerable accuracy. It is intended to apply the test to all possible antacids including mixtures in the near future. The results of the antacids tested are given in Table II and graphically in Figures 3 and 4.

Many more readings were taken than are shown in Table II and in Figures 3 and 4, and tests were repeated to ensure that the results were reproducible.

Aluminium phosphate. A proprietary suspension stated to contain 7·5 per cent. of aluminium phosphate was used. It will be noted that the initial reaction was very rapid, the equivalent of about 78 per cent. of the

hydrochloric acid being neutralised in 3 minutes and 81 per cent. in 7 minutes. Thereafter, however, there was little further action. With about 100 per cent. excess of the suspension the final *p*H was 3·24.

Dried aluminium hydroxide gel. The neutralising capacity of sample B was much below the minimum standard of the British Pharmaceutical Codex. A sample of the minimum B.P.C. standard would have a "neutralising value" of 0·25 g. whereas of sample B 1·144 g. was required. It was therefore less than $\frac{1}{4}$ of the minimum "official" strength. As judged by the neutralisation curve it would appear to be efficient but a

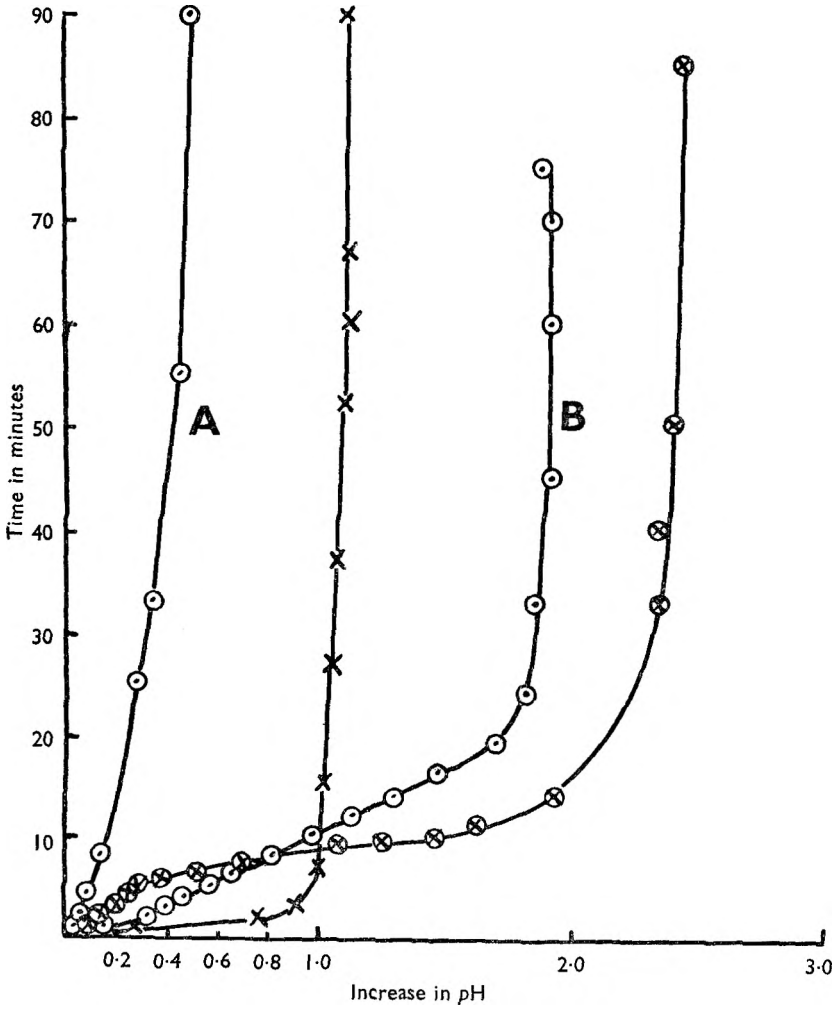

FIG. 3. Increase in *p*H on addition of antacids.

—×—× Aluminium phosphate.
—⊕—⊕ Aluminium hydroxide gel.
—⊙—⊙ Dried aluminium hydroxide gel, samples A. and B.

large dose would be required. The neutralisation "curves" for both samples show the usual straight line until practically all the acid had been neutralised.

Aluminium hydroxide gel; *supplied as B.P.C.* The neutralisation was more rapid than with the dried gel and a higher final *p*H was reached.

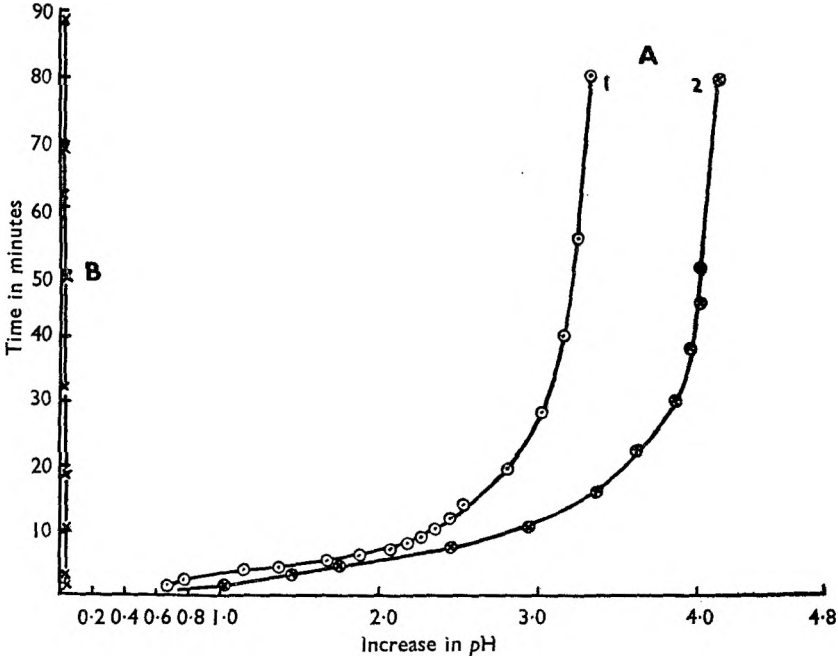

FIG. 4

A. Magnesium phosphate.
   1. Artificial secretion.
   2. Human gastric secretion.
B. Bismuth carbonate.

The minimum B.P.C. standard is equivalent to a neutralisation value of 2·5 g. This sample was well below the B.P.C. standard. Judging by these results it would appear that there are samples of aluminium hydroxide gel and dried aluminium hydroxide gel on the market which are not of a satisfactory standard.

Magnesium phosphate. This is the most rapid of the "slow acting" antacids which were tried. Neutralisation of the hydrochloric acid present was practically complete and a *p*H of 3·5 was reached in 5 minutes. For comparison human gastric secretion equivalent to 100 ml. of 0·05N hydrochloric acid was used and the result is given in Table III and graphically in Figure 4. This sample of human secretion was more acid than the artificial, 70·3 ml. being the equivalent of 100 ml. of 0·05N. It was to be expected therefore that the neutralisation of the more concentrated acid would be more rapid. It would appear possible from the graph that the human secretion in this case was not so well buffered as the artificial. Even with this sample of human secretion which was somewhat

unusual the general plan of the reaction was very similar to that occurring with the artificial secretion.

Bismuth carbonate. Several samples of the light and medium varieties were tried. Certain samples gave a very slight initial *p*H increase representing about 5 per cent. acid neutralisation but in most cases there was no appreciable effect upon the *p*H value. 5 g. of a sample which gave a slight initial rise in *p*H was boiled with successive quantities of 100 ml. of distilled water. The first 100 ml. of the washings had *p*H 8·65 and required 0·53 ml. of 0·1N hydrochloric acid to neutralise it to methyl orange. There was a gradual decrease in the volume of acid required for each washing portion, the second requiring 0·4 ml., the third 0·3 and the fourth 0·2 ml. It would appear probable, therefore, that the slight initial reaction of some samples was due to the presence of soluble alkali as an impurity in the bismuth carbonate. It was decided, therefore, that

TABLE III

NEUTRALISATION OF HYDROCHLORIC ACID IN ARTIFICIAL GASTRIC SECRETION AND HUMAN GASTRIC SECRETION

	After 2 minutes		After 3 minutes		After 5 minutes		After 10 minutes	
	*p*H increase	Hydrochloric acid *neutralised* per cent.	*p*H increase	Hydrochloric acid *neutralised* per cent.	*p*H increase	Hydrochloric acid *neutralised* per cent.	*p*H increase	Hydrochloric acid *neutralised* per cent.
Artificial gastric secretion ..	0·90	78	1·20	85	1·8	92	2·35	96
Human gastric secretion ..	1·4	88	1·60	90	2·1	95	2·95	100

under the conditions of this test pure bismuth carbonate had no neutralising action upon the artificial gastric secretion. 100 per cent. excess of bismuth carbonate had no effect upon the original *p*H after more than one hour's contact.

The result with bismuth carbonate is interesting as throwing into sharp relief the fact that the results of an "acid absorption" test such as that of the British Pharmacopœia for magnesium trisilicate or a "neutralising capacity" test as that of the British Pharmaceutical Codex for aluminium hydroxide gel may lead to very erroneous conclusions if considered as criteria for evaluating antacids. 1·159 g. to 1·179 g. of bismuth carbonate was capable of neutralising 100 ml. of 0·05N hydrochloric acid when tested by a method similar to these but double that amount (100 per cent. excess) had no appreciable neutralising effect after 2 hours when added to 100 ml. of artificial stomach secretion containing hydrochloric acid of 0·05N concentration at 38° C. thus approaching nearer to actual conditions in the stomach. This is not to say that bismuth carbonate is valueless in cases of gastric or duodenal ulcer but its efficiency as an antacid is obviously open to very grave doubt.

Comparison of the effect of magnesium trisilicate on unbuffered 0·05N hydrochloric acid and the artificial stomach secretion. A test was carried out under the same conditions as previously but using 100 ml. of

unbuffered 0·05N hydrochloric acid and the figures obtained for the *p*H change and percentage of hydrochloric acid neutralised compared with those given by 100 ml. of the artificial stomach secretion (buffered 0·05N hydrochloric acid) using the same weight of the same sample of magnesium trisilicate. The results are given in Table IV and Figure 2.

It will be noticed that neutralisation was much more rapid in the case of the unbuffered hydrochloric acid. This result emphasises the desirability of using buffered hydrochloric acid when evaluating antacids.

TABLE IV

COMPARISON OF THE ACTION OF MAGNESIUM TRISILICATE ON UNBUFFERED 0·05N HYDROCHLORIC ACID AND ON ARTIFICIAL STOMACH SECRETION (BUFFERED 0·05N HCL)

Antacid	Neutralisation value	After 10 minutes		After 20 minutes		After 30 minutes		After 60 minutes		Final *p*H with 100 per cent. excess of magnesium trisilicate
		*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	*p*H increase	Hydrochloric acid neutralised, approximately per cent.	
Magnesium trisilicate buffered 0·05N HCl	0·4393 g.	0·30	47·5	0·58	67·5	0·86	78	2·00	95	7·05
Magnesium trisilicate unbuffered 0·05N HCl	0·4393 g.	0·56	67	1·10	84	3·8	100			7·50

DISCUSSION

From the results obtained when various antacids are added to the artificial gastric secretion it is considered that the proposed fluid is a suitable medium for testing and comparing antacids. The actual conditions in the stomach are simulated as nearly as possible. No allowance is made for continued secretion of gastric fluid and the removal of the stomach contents. These vary enormously in the living body and could scarcely be reproduced in a test in which the conditions should be capable of standardisation. If it is desired an allowance could be made for these changes since the test gives information regarding the rate at which the acid is neutralised and the state of the liquid at any time is ascertainable. Pepsin and peptone may not be ideal buffers to use but they are probably the nearest readily available substances which can be regarded as imitating the buffers etc. in normal gastric secretion. Standard pepsin and peptone are unobtainable but variations within limits are not important if increase in *p*H on addition of the antacid is considered and not the actual *p*H.

The results with bismuth carbonate and the comparison of the artificial medium with unbuffered 0·05N hydrochloric acid indicate that some standard method of testing antacids is desirable.

In clinical practice there are considerable differences in the conditions which it is desired to bring about in the alimentary tract of patients. Sometimes, the object is to remove only the excess of acid as in simple hyperacidity. Here an antacid which reacts slowly and leaves a final

*p*H of perhaps 2 to 2·5 is required. Peptic action could still occur at this *p*H. Alternatively it may be desirable to prevent peptic action but not to alkalise the stomach contents, in which case a final *p*H of about 4 to 5 would be required and a slow-acting antacid suitable. In the case of actual pain a quick acting antacid to give rapid relief combined with a slow one to maintain the condition would probably be used. In general, modern practice is against the use of soluble alkalis as tending to produce the so-called acid rebound. It is suggested that valuable information is made available by a test such as the one given above. Further in the case of old and new antacids some information can be deduced as regards efficient dosage. Although the amount and rate of secretion of hydrochloric acid varies very much, an average figure may be taken to be the equivalent of about 3 ml. of 0·05N acid per minute during waking hours. Therefore, twice the "acid equivalent" mentioned above should be taken every hour if full neutralisation is required. If the doses are more widely spaced some idea of the conditions likely to occur can be obtained from a consideration of the dose used and the ascertained rate of neutralisation when 20 per cent. excess over the "acid equivalent" is used in the above described test.

Summary

1. It is suggested that a standard test using buffered hydrochloric acid should be used for the evaluation of antacids.

2. The solution suggested for the test is an artificial gastric secretion consisting of 0·05N hydrochloric acid buffered with 0·15 per cent. each of pepsin, peptone and sodium chloride.

3. An "acid neutralisation value" of the antacid is obtained by subjecting it to the action of an excess of 0·05N hydrochloric acid at 38° for 4 hours. The acid neutralisation value is the weight of antacid which will neutralise 100 ml. of 0·05N hydrochloric acid.

4. An excess of 20 per cent. of the antacid is added to 100 ml. of the artificial stomach secretion at 38° C. and the change of *p*H noted at frequent intervals. Results are obtained for the rate of neutralisation of the acid.

5. The importance of using buffered hydrochloric acid is stressed and comparisons are made between the results with buffered and unbuffered acid and also with human gastric secretion.

6. A number of antacids are tested and results are given for the rate of neutralisation of the acid and for the final *p*H obtained.

I wish to thank Dr. R. W. Fairbrother, D.Sc., M.D., F.R.C.P., Director of the Department of Clinical Pathology and Mr. H. Varley, M.Sc., A.R.I.C., Chief Biochemist in the same Department, for procuring for me a number of samples of human gastric secretion.

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DISCUSSION

The three papers on antacids were discussed together.

The first paper was presented by MR. J. ARMSTRONG, the second by MR. D. N. GORE, and the third by PROFESSOR H. BRINDLE.

MR. N. L. ALLPORT (London) was surprised by the general agreement that bismuth carbonate had little antacid value. Having examined a number of aluminium hydroxide gel preparations, he had often been worried because frequently a certain amount of sodium carbonate was left in them; there did not seem to be any test in the B.P.C. for its presence. It would be interesting to know whether by application of the tests suggested by the authors, sodium carbonate could be detected in washed aluminium hydroxide gel.

DR. W. MITCHELL (London) said that making allowances for slight differences in the techniques of the authors, it was clear that various specimens of magnesium trisilicate behaved differently. Had the authors any information concerning the magnesium trisilicate used? At least two types of material were available, light and relatively dense, and although the B.P. suggested that magnesium trisilicate could be made by reacting magnesium sulphate with sodium silicate, it could also be made by reacting magnesia with silica gel. The latter was the more attractive method because it obviated the necessity of washing out soluble by-products. Both products complied with the B.P. tests including total acid absorption, but how they behaved *in vivo* was another matter.

MR. A. W. BULL (Nottingham) asked whether the low level of the curve for magnesium trisilicate in Figure 1 of Mr. Gore's paper was due to the fact that the experiment was carried out at room temperature whereas in the other two papers the temperature was 37° C. Further clarification of the influence of conditions of manufacture of aluminium hydroxide on its antacid properties was needed. Dried aluminium hydroxide gel was slower in attaining a satisfactory *p*H level than "liquid" gel but it was excellent for maintaining an effective *p*H level. It would be interesting to know whether superimposition of the curves for individual constituents of a compound antacid mixture, for example, of the curve for a quick acting antacid such as magnesia on the curve for aluminium hydroxide gel, would make it possible to predict the behaviour of the mixture. Theoretically one would expect the mixture to show quick attainment of a satisfactory *p*H level and prolonged activity.

DR. G. E. FOSTER (Dartford) drew attention to the wide range of the amounts of different antacids quoted in the National Formulary as being required to deal with the daily output of hydrochloric acid by the stomach. The figure for bismuth carbonate of 136 g., indicating its poor antacid value, appeared to be substantiated by the papers under discussion. It was somewhat surprising, if bismuth carbonate possessed such poor qualities, that it should have attained the reputation it had enjoyed for many years in the treatment of gastric complaints. Did the *in vitro* test bear any relationship to its clinical efficiency? In the course of his work he had made aluminium hydroxide gels for the purpose of absorbing

enzymes and other biological products, and he recalled some papers by Willstätter and co-workers published in 1923 in which conditions for the preparation of different types of aluminium hydroxide gel were described. He had found that the gels produced had remarkably different absorptive properties, and it would be interesting for their antacid properties to be tested in order to ascertain which method of preparation gave the most efficient product.

MR. J. H. OAKLEY (London) said that the B.P.C. gave no guidance as to how aluminium hydroxide gel should be made. Many different starting materials were allowed, and there was no indication of the concentrations of the solutions to be employed, the temperature at which these solutions should be mixed, nor of the order of mixing. Further, the process used in washing the gel could modify the physical texture of the product. It would be interesting to learn whether the authors had noticed any appreciable differences in the behaviour of different batches of the liquid gel such as those which had apparently been noticed in the case of the dried gel. Referring to mixture of magnesium hydroxide, he said he would expect, from the information contained in the papers, that its neutralising effect would be very small and transient, and he asked whether the authors could give any information on the type of curve one might expect for the product. It was a common practice to mix oils, such as liquid paraffin, with antacids, and he desired to know whether that would delay the onset of and prolong the antacid action.

MR. R. L. STEPHENS (Brighton) said that possibly the action of bismuth had been explained by Mr. Armstrong when he pointed out that the purpose of an antacid was to prevent the action of pepsin. If the *p*H were brought to the level at which pepsin was no longer active, no damage would occur to the stomach wall. He had recently found that Mist. Bismuth. Co. cum Pepsin., even when the *p*H was reduced to 2 or lower, would not digest white of egg. The pepsin appeared to be completely inactivated. That was a possible clue to the value which was attached to bismuth for many years. He suggested that sufferers from indigestion might find greater relief from something which would inactivate the pepsin and leave the hydrochloric acid relatively alone. A common example of a mixture of antacids was Mist. Mag. Trisil. Co. in which sodium bicarbonate and magnesium trisilicate appeared together. He had found that with some grades of magnesium trisilicate the mixture had a *p*H substantially that of sodium bicarbonate, namely 8·4, and others appeared to react, some excess of carbon dioxide was removed, and a solution of sodium carbonate resulted with a *p*H of about 10. It would be of interest to know whether the authors had encountered samples of magnesium trisilicate which gave a *p*H of 10 when mixed with sodium bicarbonate.

MR. C. J. EASTLAND (London) confirmed that the antacid power of dried aluminium hydroxide gel decreased with the prolonged application of heat more or less in proportion to the temperature to which it was exposed. One practical issue was the effect of storage at high temperatures, such as obtained in the tropics, on liquid preparations of aluminium

hydroxide gel. Even at 37° C. something like 25 per cent. of the potency of the preparation was lost after three months. The effect of heat might account for the great variation in the acid neutralising powers of different samples of dried gel. With regard to the question of acid rebound, a paper was recently published in the *Lancet* describing the introduction of sodium bicarbonate and magnesium trisilicate as test antacids into the fasting stomachs of patients. It was stated that no evidence was found of rebound secretion of acid within the period of the test which was admittedly short, namely, 2½ to 3 hours.

MR. G. R. WILKINSON (London) said that the percentage of alumina in the preparations under test, particularly the dried gel, varied. He had found that if it were dried to, say, 52 per cent. of Al_2O_3, the activity might fall to 10 per cent. of that of the same gel if it were dried to 48 per cent. of Al_2O_3. The powders which were used were very slow in their reaction rate. Using a similar test, he was obtaining a *p*H of 3 to 3·2 within 5 minutes of addition, rising to between 3·4 and 3·5 within 10 minutes, and it would be useful to have the authors' comments on that point. The sources from which the alumina was derived had a marked influence on its reaction rate, because in his view the gel, when dried, was not aluminium hydroxide. Other ions had an important part to play in its activity, and also later in its buffering action. Referring to testing for sodium in aluminium hydroxide gel, since saccharin and sodium benzoate were permitted as flavouring and preservative, there might be something of the order of 0·3 per cent. of sodium ions present which was considerably greater than the amount of sodium which was left behind after washing. It would be interesting to know why *p*H 1·5 was used as a basis for the test set out in Mr. Armstrong's paper. In his opinion *p*H 1 was more convenient. On the question of storage in the tropics, he had found that if the storage conditions and containers were satisfactory, the material would retain its activity over a period of years. With the liquid preparation, if the temperature exceeded 40° C. gelling took place, but there did not appear to be any serious diminution in its acid neutralising capacity.

MR. C. E. WATERHOUSE (Southport) stated that of the two grades of magnesium trisilicate on the market, one had a bulk density in the region of 2 and the other in the region of 5. He had previously pointed out that although the two types of material might have similar acid absorption per g. they were often administered on a volume dose basis. It would be interesting to know whether the samples examined by Professor Brindle comprised each of these types of material or whether they were of one particular type only. If they were not of the same type, he desired to know whether the method used with the light or heavy variety showed any difference in acid absorption. The methods put forward were very elegant, but control of the material was vitiated if the actual dose consumed by the patient could vary to such an extent.

MR. D. N. GORE, commenting on Mr. Armstrong's paper, pointed out that in explaining the difference between the reactivity of aluminium hydroxide and dihydroxy aluminium aminoacetate, the author had made

the point that the greater speed of action of the latter was due to salt formation on the amino group. Whilst that was true, depending upon the *p*H of the solution, the aluminium moiety in that compound—if it were a compound—was more reactive than an equivalent weight of quite good quality dried aluminium hydroxide gel. He referred to the suggestion that pre-wetting of the dried gel increased its reactivity to acid. When determining curves for mixtures containing sodium carbonate and dried gel, in spite of the fact that the gel was wetted for some 30 to 40 minutes whilst acid was removed selectively by the sodium carbonate, it appeared to behave in a similar manner to freshly wetted gel in its reaction as soon as the carbonate was neutralised and excess of acid became available. This suggested that the presence and strength of acid was a positive factor related to the apparent reactivity of the gel.

PROFESSOR H. BRINDLE remarked that although the suggested methods differed somewhat, they were more or less on the same lines. In his own view a buffer should be present. It was not safe to judge antacid power in the absence of a buffer, and the temperature should be approximately body temperature, otherwise there would be considerable variation in the range of neutralisation when one antacid was compared with another. The end-point in the official test using an indicator was very uncertain for an antacid which had a buffer action. Very often there were 2 to 3 ml. differences in the determinations which he carried out even with considerable care being taken in trying to keep to the same *p*H. In his opinion something should be done officially in view of the difficulty in carrying out the test, subject as it was to personal variation in the choice of end-point.

MR. S. G. E. STEVENS (London) drew attention to Mr. Gore's reference to aluminium glycinate, and asked whether the N.N.R. specification of 14·5 per cent. of water was the optimum which should be aimed at, because he had found that if an attempt were made to dry very much below that, the antacid value was reduced. The purity of the aluminium glycinate used was not defined. It would seem that unless one was sure of the basic chemical nature of the compound under investigation, difficulties could be encountered. For instance, Mr. Gore stipulated that results should be read at 10-minute intervals, and it would be interesting to know the magnitude of variation to be found if the measurement were taken at, say, 9 minutes with a material which might vary by as much as 10 per cent.

MR. J. ARMSTRONG, in reply, said that the presence of sodium carbonate would not be demonstrated by their *in vitro* test. Chemical testing would be required. Some difference was shown in reactivity between heavy and light magnesium trisilicate. The heavy material gave a higher *p*H, but the general pattern was similar. Aluminium hydroxide preparations prepared by different methods gave different responses in their *in vitro* test and it was possible to prepare dried aluminium hydroxide preparations which had no antacid effect. It was agreed that the method of precipitation, conditions of reaction and washing affected the antacid properties. The results in the paper were based on commercially available

preparations of aluminium hydroxide both "liquid" and dried. It was known that aluminium compounds inactivated pepsin by forming complexes. It might be that bismuth compounds behaved similarly. It was possible to heat dried aluminium hydroxide gel with water and obtain greater reactivity. Overheating, for example at 90° C. for 5 minutes, destroyed antacid activity, while heating at 70° C. for 5 minutes gave better results than the same sample which had not been heated. All samples of dried gel were commercial samples, of B.P.C. quality, with an Al_2O_3 content of 52 to 54 per cent. Since overheating or prolonged drying could cause loss of reactivity, it was possible for a preparation dried to an Al_2O_3 content of 48 per cent. to have a higher antacid activity than a preparation further dried to contain 52 per cent. The *p*H of 1·5 was chosen as being representative of the acidity of average gastric contents. If stronger acid were used—for example *p*H 1—erroneous results might be obtained, as established by Professor Brindle's paper. The recent paper in the *Lancet* dealing with acid rebound might well cause a different approach to that problem, but further experimental evidence was necessary.

MR. D. N. GORE, in reply, said he and his co-authors had given little direct attention to methods for determining the presence of alkali in aluminium hydroxide gels. A few samples of the dried gel had shown something of the order of 1 per cent. The carbon dioxide contents were of the order of 5 to 10 per cent., which suggested the presence of basic aluminium carbonates. He agreed that the preparative history of aluminium gels to a large extent determined their properties.

The low reactivities for magnesium trisilicate were not a function of temperature. Determinations at 37° C. gave a quicker response for the sample, but the final *p*H values were the same, irrespective of temperature. He agreed that the effect of ions in aluminium hydroxide gel might have a profound effect on antacid properties. The samples of magnesium trisilicate used in the work appeared to be all of the denser type. None of the very light material had been tested. The moisture content of the aluminium glycinates typified in the paper was of the order of 5 per cent. This was determined by an infra-red moisture meter, as distinct from the prolonged heating at high temperatures described in the N.N.R. monograph. Referring to the emphasis placed by the authors of the other papers on the need for the tests to be carried out at body temperature and in the presence of buffers, he made the point that in his and his collaborators' opinion the same conclusions were reached by either method and the presence of buffer substances when testing a substance for buffer activity tended to mask the results and was of debatable value.

PROFESSOR H. BRINDLE, in reply, referring to the figures quoted from the N.F., said that an acute controversy arose with regard to the figure for bismuth carbonate, and in a weak moment he had agreed to try to settle it. He considered that one would get a good idea as to the rate of neutralisation by mixtures from consideration of the superimposed curves obtained from the constituents of the mixture. It was difficult

to see the force of the suggestion that the *p*H should be 1 rather than 1·5, for it was just as easy to obtain 1·5 as 1. In any case *p*H 1 was much lower than the average for the gastric secretion. Having examined a large number of gastric samples he had found only one to be in the neighbourhood of *p*H 1. In his view one should keep to natural acidity in testing antacids.

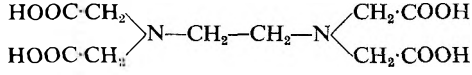
THE VOLUMETRIC DETERMINATION OF CALCIUM AND MAGNESIUM

BY R. L. STEPHENS

Received June 12, 1953

THE classical methods of determining calcium and magnesium are lengthy and not well adapted to the routine determination of these elements in batches of preparations. The British Pharmaceutical Codex does not give limits for magnesium carbonate and calcium carbonate in compound powder of magnesium carbonate probably because of the lengthy nature of these determinations. A rapid volumetric method has been worked out which enables both these elements to be determined in two titrations.

The use of the disodium salt of ethylenediamine tetra-acetic acid as a volumetric reagent for the determination of hardness in water was first described by Schwarzenbach[1] and his co-workers in 1946. The same method was applied to the determination of calcium in pharmaceutical chemicals in America by Matock and Hernandez,[2] who reported that the method was more accurate and more rapid than the official method of the United States Pharmacopeia as well as being applicable to calcium phosphate for which, they stated, the official oxalate method cannot be used. Banks[3] has applied the same method to the determination of calcium and magnesium to coal ash and ceramic materials. Banks made the suggestion that stronger solutions might be worth investigating, and in the present work this suggestion has been adopted. Knight[4] suggested the use of a screened murexide indicator to make the end-point easy to recognise.



Ethylenediamine tetra-acetic acid

The strengths of solutions of ethylenediamine tetra-acetic acid used by previous workers have been low probably because of the small quantities of materials being tested. Banks used 0·02N solutions and direct titration, Matock and Hernandez used 0·03N solutions and back titration with magnesium chloride, which avoids interference from phosphate. The present method employs 0·5N solutions and back titration with calcium or magnesium solutions as applicable. These stronger solutions give sharper end-points and permit the direct titration of weighed amounts of material without the need for preparing dilutions. The method is of general application; the only important interfering substance is iron; this must be eliminated in the ordinary way if present. Other divalent salts, such as zinc or strontium, will titrate in a similar way if present, but the absence of such salts in a pharmaceutical preparation will normally be established. One molecule of the disodium salt of ethylenediamine tetra-acetic acid combines with one atom of calcium or magnesium to form a chelate compound in which the calcium or

magnesium ions are completely sequestered. Eriochrome Black T is used as an indicator in the presence of ammonia, changing from blue to magenta on the addition of divalent ions. This gives an end-point with both calcium and magnesium. Calcium is estimated in the presence of magnesium by using murexide (ammonium purpurate) as an indicator in the presence of sodium hydroxide. Magnesium does not interfere because magnesium hydroxide is precipitated under these conditions. The colour change is from indigo to pink.

Experimental

Reagents.—0·5N Disodium ethylenediamine tetra-acetate: 146 g. of the acid was dissolved in a slight excess of 5N sodium hydroxide (220 ml.) and diluted to 2 l. This was stored in a rubber stoppered bottle to exclude carbon dioxide which precipitates the acid.

0·5N Calcium chloride: 219·1 g. of hydrated calcium chloride was dissolved in water and made up to 4 l.

0·5N Magnesium sulphate: 246·5 g. of magnesium sulphate was dissolved in water and made up to 4 l.

Calcium indicator: 0·5 g. Naphthol green, 0·2 g. murexide ground together with 100 g. sodium chloride.

Combined calcium and magnesium indicator: 0·2 g. Eriochrome Black T (Solochrome) ground with 100 g. of sodium chloride.

The solutions were standardised against pure calcium carbonate, the equivalent weight being taken as 50·04 by analogy with the acidimetric titration.

General Method.—The calcium and magnesium salts are brought into solution with the addition of the minimum amount of hydrochloric acid, in about 150 ml. of water and excess of 0·5N sodium ethylenediamine tetra-acetate added. The solution is then titrated with 0·5N calcium chloride in the following two ways to give the calcium alone and combined calcium and magnesium. Magnesium is obtained by difference.

Calcium titration.—To the solution containing excess of reagent, 4 ml. of 5N sodium hydroxide is added (more if the solution is already acid), 0·2 g. of calcium indicator is added and 0·5N calcium chloride run in slowly. The end-point is reached when a tinge of red persists in the indigo blue solution.

Calcium and magnesium titration.—To the solution containing excess of reagent 10 ml. of 5N ammonia is added and 0·2 g. of Eriochrome Black T indicator. 0·5N calcium chloride or magnesium sulphate is added until the blue of the indicator turns through violet to magenta. There is some tendency for the magenta colour first produced to fade back to violet owing to the slow sequestration; the titration must be carried to a permanent colour.

Estimation of calcium and magnesium in compound powder of magnesium carbonate.

The B.P.C. gives limits for soluble alkali and total alkali, but the proportions of calcium carbonate and magnesium carbonate can vary

widely without the powder falling outside these limits. In order to test the accuracy of the method, calcium carbonate and magnesium carbonate were assayed separately, then accurately weighed amounts were mixed together and the whole quantity dissolved in the minimum of hydrochloric acid and assayed by the general method. Table I shows the results obtained.

Estimation of calcium carbonate in compound powder of magnesium trisilicate.

It was found that the whole of the calcium carbonate dissolved when compound powder of magnesium trisilicate was boiled with dilute hydrochloric acid but that the amount of magnesium present in the solution corresponded to all the magnesium carbonate and a variable

TABLE I

DETERMINATION OF CALCIUM AND MAGNESIUM IN A MIXTURE OF CARBONATES

	By B.P. method	By titration
Calcium as $CaCO_3$ in mixture ..	0·322 g.	0·322 g.
Magnesium as MgO in mixture ..	0·108 g.	0·109 g.
$CaCO_3$ in calcium carbonate ..	98·2 per cent.	98·5 per cent.
MgO in magnesium carbonate ..	42·5 per cent.	42·1 per cent.

proportion of the magnesium present as magnesium trisilicate. It was decided therefore to adopt a method for the determination of calcium carbonate in the routine examination of batches of this preparation. Titration in the presence of the magnesium trisilicate was found unsatisfactory owing to the removal of calcium ions by the magnesium trisilicate by a base exchange mechanism at the endpoint. Filtration of the solution gave good results, the method adopted being as follows.

About 1·0 g. of compound powder of magnesium trisilicate is digested on a water bath for 20 minutes with 50 ml. of water and 5 ml. of 5N hydrochloric acid and filtered, the filter is washed with a further 50 ml. of hot water. The solution is cooled, 20 ml. of 0·5N disodium ethylenediamine tetra-acetate and 10 ml. of 5N sodium hydroxide are added and the solution titrated with 0·5N calcium chloride using the murexide indicator.

Calcium in calcium gluconate.

Gluconic acid does not interfere with this titration and since magnesium salts are not present the combined titration is suitable and either 0·5N magnesium sulphate or 0·5N calcium chloride can be used for the back titration. While either method of titration may be used for calcium in the absence of magnesium, the less specific titration in the presence of ammonia and Eriochrome Black T was usually employed since either reagent may be employed for the back titration.

About 2·0 g. of calcium gluconate (or 10 ml. of 20 per cent. solution) is dissolved in 125 ml. of water, 25 ml. of 0·5N disodium ethylenediamine tetra-acetate, 10 ml. of 5N ammonia and 0·2 g. of Eriochrome Black T indicator are added and the titration completed with 0·5N calcium

chloride or magnesium sulphate. For ampoules it is more convenient to use 2 ml. of 10 per cent. solution and titrate using 0·1N solutions.

TABLE II

CALCIUM GLUCONATE BY TITRATION

	B.P. assay per cent.	Titration per cent.
Calcium gluconate	101·2	101·1
20 per cent. solution	21·1	20·9
10 per cent. solution	9·9	9·9

Calcium or magnesium in the presence of phosphate.

Estimation of calcium in the presence of phosphate using murexide indicator was not found to be satisfactory. This was due to the rapid precipitation of calcium phosphate which effectively removed calcium ions from solution and prevented the colour change of the indicator.

In the presence of ammonia and Eriochrome Black T a good end-point was obtained if magnesium sulphate was used for the back titration instead of calcium chloride. The following method has given good results for syrup of calcium lactophosphate.

10 ml. of syrup is measured in a pipette calibrated to contain 10 ml. and transferred by washing into a titration flask. 20 ml. of 0·5N disodium ethylenediamine tetra-acetate, 10 ml. of 5N ammonia, 100 ml. of water and 0·2 g. of Eriochrome Black T indicator are added. The solution is titrated with 0·5N magnesium sulphate solution.

It was found that this method did not give a satisfactory end-point when used for calcium phosphate. It then became apparent that the sugar in the syrup of calcium lactophosphate was preventing the rapid precipitation of calcium phosphate and so producing a satisfactory end-point. Addition of syrup to the titration of calcium phosphate gave satisfactory results.

About 0·5 g. of calcium phosphate is dissolved in 100 ml. of water with the addition of 2 ml. of 5N hydrochloric acid, then 20 ml. of syrup, 20 ml. of 0·5N disodium ethylenediamine tetra-acetate, 10 ml. of 5N ammonia and 0·2 g. Eriochrome Black T indicator are added and the excess reagent titrated with 0·5N magnesium sulphate. The same method is used for calcium hypophosphite and calcium glycerophosphate.

Calcium in exsiccated calcium sulphate.

About 0·5 g. of finely ground plaster of paris with 25 ml. of 0·5N of disodium ethylenediamine tetra-acetate and 10 ml. of 5N ammonia are shaken at 40° C. for 1 hour. The whole of the calcium sulphate dissolves and the excess of reagent is titrated with 0·5N calcium chloride using 0·2 g of Eriochrome Black T indicator and about 150 ml. of water.

Influence of p*H.*

The general titration of calcium and magnesium using Eriochrome Black T indicator was found satisfactory between *p*H 9·6 and 10·4 corresponding to a wide variation in the quantity of ammonia added.

Below *p*H 9·4 the end-point became less sharp. The titration of calcium alone was satisfactory above *p*H 11·2, but at this *p*H it was not specific for calcium as magnesium ions could still bring about the colour change of the murexide. Above *p*H 12·0 the titration proceeded normally, the ionisation of magnesium hydroxide being inhibited. The addition of extra sodium hydroxide up to a final concentration of 0·5N did not affect the titration.

Effect of temperature.

Both titrations were carried out between 10° C. and 40° C. without influence on the results.

Accuracy of method.

The end-point in these titrations could be judged to within 0·05 ml. giving an accuracy on a titration of 20 ml. of ± 0·25 per cent. Satisfactory replicate titrations could be obtained with this accuracy.

Effect of traces of iron.

The presence of 0·5 mg. of iron did not interfere with the titration using Eriochrome Black T beyond changing the colour of the indicator to indigo just before the end-point. The addition of hydroxylamine hydrochloride improved the colour of the indicator only if the temperature was raised to 60° C. 1·25 mg. of iron interfered with the titration even in the presence of hydroxylamine at a raised temperature.

The titration using murexide as the indicator is somewhat more sensitive to iron. The end-point was still visible if 0·25 mg. of iron was present or 0·50 mg. in the presence of hydroxylamine. Owing to the instability of murexide this titration cannot be carried out above 50° C.

TABLE III

COMPARISON OF RESULTS WITH THOSE OBTAINED BY OTHER METHODS

Compound assayed	Found by ethylenediamine tetra-acetate titration per cent.	Comparative result per cent.	Comparative method
$CaCO_3$ in compound powder of magnesium trisilicate.	24·8	24·9	Permanganate titration of the precipitated oxalate.
$Ca(H_2PO_3)_2$ in calcium hypophosphite ..	101·5	101·7	do. do.
$CaC_3H_5(OH)_2PO_4 \cdot 2H_2O$ in calcium glycerophosphate.	100·2	100·4	do. do.
$Ca_3(PO_4)_2$ in calcium phosphate	90·2	90·2	do. do.
$CaSO_4$ in plaster of paris	92·2	92·4	Residue on ignition.
$MgSO_4 \cdot 7H_2O$ in magnesium sulphate ..	100·1	—	
MgO in magnesium oxide	99·2	99·3	By ignition.
$Mg(OH)_2$ in mixture of magnesium hydroxide.	8·62	8·62	By titration with sulphuric acid.

Stability of solutions.

The solution of disodium ethylenediamine tetra-acetate has been stored for 12 months without change in ordinary glass bottles. The amount of calcium dissolved from the glass is not enough to affect these relatively strong solutions.

0·5N magnesium sulphate is stable, 0·5N calcium chloride tends to form a slight deposit after a few months.

SUMMARY

1. A rapid method of determining calcium and magnesium by titration has been described.

2. Factors influencing the accuracy of the method have been investigated.

I wish to thank the directors of Wright Layman and Umney, Limited, for permission to publish these results.

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DISCUSSION

The paper was presented by THE AUTHOR.

DR. R. RUYSSEN (Belgium) asked whether the method could be applied to the determination of calcium and magnesium in blood.

DR. G. E. FOSTER (Dartford) mentioned a paper published recently in *Die Pharmazie*, in which were listed a number of compounds which were determined by the use of ethylenediamine tetraacetic acid. He also said that this substance was added in the United States to certain pharmaceutical preparations where traces of calcium might cause opalescence.

MR. R. L. STEPHENS, in reply, said there would be difficulties in using the method for the determination of calcium and magnesium in blood; the colour changes would be masked and the iron present would interfere. The blood would have to be ashed and its iron content removed by precipitation in alkaline solution before the calcium and magnesium content could be determined.

THE STABILITY OF ANEURINE HYDROCHLORIDE IN PHARMACEUTICAL PREPARATIONS

By H. Partington and C. E. Waterhouse

From the Research Department, Bob Martin, Ltd., Southport.

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It is well known that aneurine hydrochloride, in addition to being heat-labile, is also destroyed when maintained in media having either neutral or alkaline *p*H, with the formation of decomposition products having no vitamin B_1 activity. It has been shown by Farrer[1] that there is, for any given buffer solution, a linear relationship between the reaction velocity of the destruction of aneurine and the hydrogen ion concentration in the *p*H range 3 to 8. In all cases, the velocity of the reaction was found to increase as the *p*H rose. In a later paper Farrer[2] has shown that the concentration of buffer salts affected the rate of destruction of aneurine below *p*H 6, and that the rate of destruction is also dependent on the initial concentration of aneurine. This work was mainly concerned with thermal destruction of the vitamin in solutions, and the present paper records the results of investigations of the stability of aneurine hydrochloride in pharmaceutical powders containing either semimicro quantities or higher concentrations, and also in tablets, when these are stored at normal temperatures over a period of years. It would be expected that vitamin decomposition in a relatively dry, non-hygroscopic powder would be very much less than in a solution under comparable conditions of acidity, etc. This has, in general, been found to be the case, although significant loss of vitamin occurred even in these relatively dry powders in the least acid media. The observations were carried out on samples taken from routine production and usually involving fairly large batches of material. Large numbers of routine determinations have been carried out and some 110 samples of the various formulations were selected for special study.

Analytical Methods

The analytical method used for the determination of aneurine hydrochloride throughout the series followed that laid down in the British Pharmacopœia, 1948, Appendix XIII. With the exception of certain of the earlier results, which are referred to subsequently, the photoelectric method was used employing the Spekker Fluorimeter, the only deviation from this method being that where necessary, when dealing with buffered materials, the proportion of hydrochloric acid employed to dissolve the sample prior to oxidation was somewhat increased. The increase was carried out only to the extent necessary to give a residual *p*H of approximately 2 prior to the oxidation, and at this stage the solution was therefore comparable to the B.P. solution employed when assaying the pure aneurine hydrochloride. The thiochrome method has been the subject of numerous papers and has been criticised, especially for use with larger concentrations

of aneurine, but it has been our experience that this method yields results, at the concentrations dealt with, well within the precision of ± 5 per cent. quoted by Adamson and Handisyde[3], who preferred a gravimetric method for the estimation of higher concentrations to avoid large dilutions.

In examining pharmaceutical preparations such as capsules, syrups and elixirs, Elvidge[4] found it necessary to use an adsorption technique on a synthetic zeolite, but he was able to use the direct acid extraction method for tablets. As indicated above, we have also used the direct extraction method for all the powders and tablets under study.

Where a figure for the *p*H of the samples is quoted, this refers to the *p*H, determined electrometrically, of a 2 per cent. w/v solution, or suspension, of the sample in distilled water, the determination being carried out after a stable value had been reached. This is, of course, an arbitrary figure, and does not necessarily represent the conditions obtaining in the relatively dry powder, which is the environment in which any decomposition during storage has actually occurred. The *p*H figures quoted are useful, however, in classifying the various samples examined.

Experimental Results

All the samples tested had been stored under the following conditions, and for the period of time indicated in the tables below. The powders or tablets were in glass containers, corked, but not hermetically sealed and not full: there was, therefore, a limited amount of atmospheric oxygen in contact with the sample. They were stored almost the whole time in the dark, at a temperature which varied between 60° and 70° F., and in relatively dry external atmospheres with relative humidity not exceeding 65 per cent.

Group 1. Formula: aneurine hydrochloride 15 I.U. per 5 grains, in a base of lactose, in presence of calcium phosphate B.P., ferric phosphate, manganese lactate, copper sulphate.

The initial analyses were carried out by the visual method for the fluorimetric assay of aneurine hydrochloride, described in the Appendix XIII of the B.P. 1948. It will be seen by comparison with later results for the initial analyses, that although the standard deviation is much greater than in groups where the photoelectric method was employed, the analysis is none the less remarkably near to the correct figure, having in mind the limitations of the older visual method. There is, of course, a somewhat large standard deviation in the results for the final analysis but this includes not only assay variations but also the effect of a slightly variable rate of vitamin decomposition among the individual samples in the group under study.

Group 2. Formula: aneurine hydrochloride 15 I.U. per 5 grains, in a lactose base, in presence of ingredients as for Group 1, with the addition of calcium gluconate B.P.

In this case also half the initial determinations of aneurine content were carried out by the visual method and half by the photoelectric method, the latter method being commenced in October, 1948, and continued throughout all the remaining determinations. The smaller standard deviation in

the results for the final analysis is indicative of a more uniform rate of vitamin loss in the samples of this group.

Group 3. Formula: aneurine hydrochloride 31 I.U. per 5 grains, in a lactose base, in presence of nicotinic acid, calcium phosphate, calcium gluconate, ferric phosphate, copper sulphate, cobalt lactate.

This formulation, having a *p*H value of 4·5 and being fairly highly buffered, gives, as would be expected, greater vitamin stability. It is interesting to note, however, that the average decrease in vitamin content is more or less the same for materials stored for either 1, 2, or 3 years. This would seem to indicate an initial small loss of vitamin activity within a fairly short period of manufacture, followed by little further loss when stored for longer periods.

TABLE I

POWDERS CONTAINING LOW CONCENTRATION OF ANEURINE HYDROCHLORIDE

Group	Date of preparation of material	Initial aneurine content by analysis	Storage period	Aneurine content after storage	Average decrease in aneurine content per cent.	*p*H of 2 per cent. suspension
1	From September, 1947, to August, 1948.	14·6 I.U. Standard Deviation ± 2·57	4½ years	9·8 I.U. Standard Deviation ± 2·04	33	6·5
2	From September, 1948, to July, 1949.	14·5 I.U. Standard Deviation ± 2·05	3½ years	11·6 I.U. Standard Deviation ± 1·04	19·7	5·5
3 (*a*)	From August, 1949, to August, 1950.	30·9 I.U. Standard Deviation ± 0·224	3 years	28·4 I.U. Standard Deviation ± 1·4	8·2	4·5
3 (*b*)	From September, 1950, to August, 1951.	31·8 I.U. Standard Deviation ± 1·22	2 years	28·4 I.U. Standard Deviation ± 0·81	10·8	4·5
3 (c)	From September, 1951, to August, 1952.	31·7 I.U. Standard Deviation ± 1·47	1 year	29·3 I.U. Standard Deviation ± 1·44	7·5	4·5
4 (*a*)	From January, 1948, to March, 1949.	34·3 I.U. Standard Deviation ± 2·56	2 years	31·1 I.U. Standard Deviation ± 1·83	9	6·0
4 (*b*)	From January, 1948, to March, 1949.	34·3 I.U. Standard Deviation ± 2·56	4 years	29·5 I.U. Standard Deviation ± 2·07	14	6·0

Group 4. Formula: aneurine hydrochloride 35 I.U. per 5 grains, in a lactose base, in presence of calcium gluconate, calcium phosphate, ferric phosphate.

In this group an observation of the aneurine content of each sample under study was made at the end of 2 years, in addition to the recent analysis after 4 years. The average decrease at 9 per cent. in the first 2-year period, compared with 5 per cent. in the second 2-year period, is of considerable interest and suggests that a detailed graph of vitamin loss under these conditions would be exponential in form. Another point of interest here is that in the absence of traces of any heavy metals, such as copper, the average rate of vitamin loss, even at *p*H 6·0, under these conditions is less than the average loss at *p*H 5·5 over a similar period in the presence of these metals.

Group 5. Formula: A simple mixture of aneurine hydrochloride with Lactose.

From these determinations it is interesting to note that in simple

aneurine mixtures at higher concentrations, it is possible to obtain analytical results within quite narrow limits, as evidenced by the low standard deviation. It is interesting also to note the relatively small average decrease in aneurine content at this *p*H, and a number of the figures are reproduced in Table II to show that loss of vitamin content does not appreciably increase with increased age of sample at this concentration and under these conditions.

TABLE II

POWDERS CONTAINING HIGHER CONCENTRATION OF ANEURINE HYDROCHLORIDE

Group	Date of preparation of material	Initial aneurine content per cent.	Aneurine content, at January, 1953 per cent.
5 (*a*)	Batch No. 8, January, 1950	0·81	0·71
5 (*b*)	Batch No. 14, June, 1950.	0·81	0·78
5 (*c*)	Batch No. 18, September, 1950 ..	0·80	0·76
5 (*d*)	Batch No. 22, December, 1950 ..	0·81	0·78
5 (*e*)	Batch No. 27, June, 1951	0·79	0·76
5 (*f*)	Batch No. 31, August, 1951	0·79	0·78
5 (*g*)	Batch No. 35, January, 1952.. ..	0·82	0·76
5 (*h*)	Batch No. 39, July, 1952	0·78	0·79
5 (*j*)	Batch No. 42, October, 1952 ..	0·79	0·73

Average initial aneurine content: 0·80 per cent.; Standard Deviation 0·013.
Average final aneurine content: 0·76 per cent.; Standard Deviation 0·027.
Average decrease in aneurine content: 5 per cent.
*p*H of 2 per cent. solution: 3·8.

Group 6. Formula: aneurine hydrochloride 15 I.U. per 3 grains, in a lactose base, in presence of calcium and ferric phosphates, heavy magnesium carbonate, starch, acacia, talc.

In the majority of samples, re-analysis after 6 years showed no vitamin detectable at the limit of sensitivity of the fluorimetric assay. One sample gave approximately 2·5 I.U. per tablet remaining, and another approximately 1 I.U. per tablet. The complete decomposition of the vitamin in a medium in which the moisture content does not exceed 2 per cent. shows that the observation of Farrer[1] and others on the decomposition of aneurine in alkaline solutions applies also to alkaline powders and tablets. The loss of vitamin potency might not be expected in relatively dry media where the total moisture content does not exceed 2 per cent., and where the alkali present is normally regarded as "water insoluble." It is evident that aneurine is very sensitive to traces of alkali.

Group 7. Formula: aneurine hydrochloride 15 I.U. per 3 grains, in a lactose base, in presence of calcium and ferric phosphates, starch, acacia, talc.

This formula is similar to that of Group 6, but without the mild alkalinity contributed by the magnesium carbonate.

Group 8. Formula: aneurine hydrochloride 15 I.U. per 3 grains, in a lactose base, in presence of calcium gluconate, ferric phosphate, starch, acacia, talc.

There was small difference in formulation between the two groups quoted. Batches manufactured from July, 1948, to December, 1948, contained 10 per cent. of calcium gluconate, and those from January, 1949, to July, 1949, contained 20 per cent. of calcium gluconate. Although the *p*H of the suspension in both groups was 5·5, the presence of double the

quantity of calcium gluconate in the latter group may well have been responsible for the much smaller decrease in vitamin content compared with the first series.

Group 9. Formula: aneurine hydrochloride 34 I.U. per 3 grains, in a lactose base, in presence of calcium gluconate, ferric phosphate, nicotinic acid, starch, acacia, talc, and small quantities of cobalt lactate and copper sulphate.

Due to the presence of 0·5 per cent. of nicotinic acid in this formula, the resulting *p*H of the suspension has moved down to 5·0. The average

TABLE III

TABLETS CONTAINING LOW CONCENTRATION OF ANEURINE HYDROCHLORIDE

Group	Date of preparation of material	Initial aneurine content by analysis	Storage period	Aneurine content after storage	Average decrease in aneurine content per cent.	*p*H of 2 per cent. suspension
6	From October, 1946, to June, 1947.	15 I.U.	7 years	2·5 I.U. 1·0 I.U.	88	8·5
7	From October, 1947, to May, 1948.	15 I.U.	5 years	12·9 I.U. Standard Deviation ± 0·77	14	7·0
8 (*a*)	From July, 1948, to December, 1948.	16·3 I.U. Standard Deviation ± 1·31	4 years	13·25 I.U. Standard Deviation ± 0·375	18·7	5·5
8 (*b*)	From January, 1949, to July, 1949.	15·5 I.U. Standard Deviation ± 0·57	3½ years	14·5 I.U. Standard Deviation ± 0·515	7·2	5·5
9	From July, 1949, to October, 1950.	33·1 I.U. Standard Deviation ± 2·4	2½ years	29·9 I.U. Standard Deviation ± 1·05	9·6	5·0

decrease in aneurine content is, however, similar to that in the second series of tablets considered under Group 8, where the *p*H was 5·5. The vitamin loss at these *p*H values is like those recorded in Group 3 for powders—approximately 10 per cent. over a fairly wide range of storage periods.

It is evident that to produce a preparation with virtually no loss over long storage periods, a *p*H of tablet in the region of 4, comparable with the powders quoted in Group 5, must be aimed at.

The Influence of Copper on the Rate of Aneurine Destruction

The effect of copper in buffer solutions at various *p*H when heated at 100° C. was studied by Farrer[5]. He used copper sulphate giving Cu concentrations of 2 and 20 p.p.m. in the solutions under test. In phosphate and phosphate/phthalate solutions copper accelerated the rate of destruction of aneurine at all *p*H values studied, greater acceleration being obtained with the greater concentration of copper. The effect was modified where copper was in the presence of radicals with which it could form complex ions, such as tartrate or citrate.

The tablets to the formulation quoted in Group 9 contained copper sulphate giving a Cu concentration of 87 p.p.m. It will be seen that even in the presence of this relatively high concentration of copper there was an average vitamin loss of only 9·6 per cent. after 2½ years

storage. The loss of 9 per cent. of the vitamin content in presence of so much copper, and at *p*H 5·0, compares with a similar loss of 9 per cent. recorded after 2 years in the formula under Group 4. This formula contains no copper and has a similar base of gluconate/phosphate, but no nicotinic acid.

The powders to the formulation recorded in Group 3 contained an even higher proportion of copper sulphate, giving a Cu concentration of 202 p.p.m. This formulation, having a *p*H value of 4·5, shows a vitamin loss of only 8 to 10 per cent. after as long as 3 years. This concentration of copper is 10 times as great as the higher of the two concentrations used by Farrer. It is evident, therefore, that the effect of copper is greatly diminished in dry powders containing phosphate/gluconate buffers, when stored at room temperatures over long periods.

Summary

1. Compound powders and uncoated tablets containing various concentrations of aneurine hydrochloride have been re-assayed after storage for a number of years.

2. Data are given to show the range of vitamin contents for the various formulæ as originally manufactured and after storage. The results indicate that in these relatively "dry" media aneurine is quite stable for long periods at *p*H 4. In formulæ of *p*H 4·0 to 5·0 there is a loss of the order of 10 per cent., most of which occurs relatively soon after manufacture. In media of *p*H greater than 5 there is correspondingly greater loss, until at *p*H 6·5 there may be a loss, after a period of years, of up to 33 per cent. At higher *p*H values, practically the whole of the vitamin content eventually disappears. These losses follow the pattern of the behaviour of aneurine in solution and during thermal decomposition, but in media of low moisture content decomposition tends to be less rapid.

3. The effect of the presence of proportions of heavy metals, such as copper and cobalt, has been examined in the light of their known effect on solutions containing the vitamin. It has been found that with relatively high concentrations of copper there appears to be little effect due to these metals on the vitamin loss under the conditions studied. It appears, therefore, that the effect of copper is greatly diminished in dry powders when stored at room temperatures over long periods.

We should like to acknowledge the assistance of Miss M. Hardaker in carrying out certain of the analyses.

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1. Farrer, *Biochem. J.*, 1945, **39**, 128.
2. Farrer, *ibid.*, 1947, **41**, 167.
3. Adamson and Handisyde, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 370, 423.
4 Elvidge, *ibid.*, 1947, **20**, 257.
5. Farrer, *Biochem. J.*, 1947, **41**, 162.

DISCUSSION

The paper was presented by MR. C. E. WATERHOUSE.

MR. N. L. ALLPORT (London) said that he had analysed tablets containing exsiccated ferrous sulphate and calcium carbonate to which aneurine hydrochloride had been added, and had found that the content of aneurine hydrochloride was much below that claimed. He was surprised to find that the authors were apparently satisfied that so long as the components were dry, the iron present did not interfere. The authors did not specifically mention exsiccated ferrous sulphate but they did refer to ferric phosphate, and he wondered whether calcium carbonate mixed with ferric phosphate would cause a serious deterioration in the aneurine content.

DR. K. BULLOCK (Manchester) quoted results of his own experiments using a spray dried powder of ascorbic acid containing traces of copper. The catalytic activity of the copper was inhibited while the powder remained dry, but in solution the ascorbic acid was oxidised rapidly. He had found that moisture had considerable effect and asked the authors for more details about the moisture content of their powders and how it was determined. In powders which contained starch or hydrated salts the question of whether the moisture was free or bound would arise. He presumed that it was the free water which would take part in the various reactions. In the summary the authors pointed out that the results indicated that in relatively dry media aneurine was quite stable for long periods at *p*H 4. He had given considerable thought as to what should be meant by the *p*H of dry powders. The normal meaning of the term *p*H was, of course, based on dilute solutions, and what was meant by *p*H in powder seemed to be difficult to decide. In order to compare figures for storage, it was necessary to have some concept of how the rate of decomposition varied with time, and he wondered whether the authors had any figures to show whether the decomposition rate was linear, logarithmic or followed some other law.

DR. R. E. STUCKEY (London) said that he also was interested to note that the authors found most of the tablets to be stable even though in almost every case they had an appreciable iron content. He had found that in the presence of ionised ferric iron, aneurine was distinctly unstable. Could the authors state whether ferric ions would be present when their tablets which contained ferric phosphate and various calcium salts were taken up in water? In tablet form ferrous sulphate reacted with calcium carbonate forming calcium sulphate which set hard, like plaster of paris, and the water released would probably catalyse decomposition.

MR. T. D. WHITTET (London) said that he had made injection of aneurine hydrochloride B.P. 1953 but after 6 to 12 months' storage a distinct precipitate had formed. When the samples were assayed they gave full theoretical values and it would be interesting to know whether the authors had had any similar experience.

DR. F. WOKES (King's Langley) referred to the stability of different forms of aneurine, and said there was a good deal of evidence to suggest

that aneurine in its natural complexes was considerably more stable than synthetic aneurine hydrochloride. During the last 10 or 11 years he had been responsible for standardising the aneurine content of preparations containing partly natural aneurine, no doubt in complex, and partly added synthetic aneurine. He had found stabilities in those preparations tested over periods of years to be higher than those recorded in the paper. The moisture contents in some cases were between 1 and 2 per cent., but there were also preparations containing about 20 per cent. moisture which were of comparable stability. The *p*H was between 4·8 and 5 and iron was present.

MR. C. E. WATERHOUSE, in reply, said that trouble had been experienced with powders which contained magnesium carbonate, another insoluble and alkaline material. He felt that in Mr. Allport's case the trouble might be due more to the calcium carbonate than the iron present. In passing, he observed that on examining a commercial powder stated to contain 50 I.U./g. of aneurine, and magnesium carbonate, it was found to contain only 15 I.U./g. The references to the depression of the catalytic activity of copper in low moisture systems were interesting. It was well known that copper in solution caused fairly rapid loss of the vitamin. The moisture content of the materials under discussion was of the order of 0·2 to 0·5 per cent. for powders and 1·5 to 2 per cent. for tablets. The meaning of the term *p*H in reference to dry powders was stated in the paper. On the question of storage, he explained that the data set out had only been accumulated as parts of other investigations and routine tests over the years, and the paper was a record of the results obtained. The iron present was in the form of ferric phosphate and was, as far as one could say, in an insoluble form. It was, therefore, quite different in its effect from ferrous sulphate mixed with calcium salts. He was unable to comment on the point raised by Mr. Whittet. He agreed that natural aneurine might be more stable than synthetic, but he had shown that the synthetic vitamin was very stable over a long period of time provided that the formulation and the ultimate *p*H of the substances present were carefully studied.

CYANOCOBALAMIN AND HYDROXOCOBALAMIN IN VITAMIN B_{12} INJECTIONS

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Hydroxocobalamin, also termed vitamin B_{12b}, has been isolated from liver[1,2,3] and from *Streptomyces* cultures[3], the two main commercial sources of vitamin B_{12}, in which it apparently may form a considerable proportion of the total vitamin B_{12} potency. Its physiological activity, microbiological and clinical, appears to be the same as that of cyanocobalamin[4]. It is, however, much less stable to ascorbic acid[5], and to certain other biological agents, than cyanocobalamin. Vitamin B_{12} has been officially defined as cyanocobalamin, and characterised by spectrophotometric data which clearly distinguish between the two pure substances. Such data are applied, however, not only to crystalline cyanocobalamin but also to solutions of it used as injections. In such solutions cyanocobalamin can be converted to hydroxocobalamin by exposure to light under suitable conditions[6]. Therefore vitamin B_{12} injections, even though made originally from pure cyanocobalamin, may contain cyanocobalamin and hydroxocobalamin in varying proportions, according to storage conditions.

Our interest in this problem arose during an investigation[7] into the cyanide and thiocyanate metabolism in man, in which injections of vitamin B_{12} were being administered, and it was desired to know how much cyanocobalamin these were providing. A method was needed for the determination of cyanocobalamin, mixed with different proportions of hydroxocobalamin, in the amounts found in vitamin B_{12} injections. Microbiological methods are rather tedious and usually do not differentiate between cyanocobalamin and hydroxocobalamin in such mixtures. Such differentiation can be effected by applying a suitable microbiological method to the mixtures before and after destruction of the hydroxocobalamin with ascorbic acid under suitable conditions[8], but this is not effective in the presence of iron and therefore is not applicable to liver extracts. It has been used by two of us (F.W.N. and S.J.G.F.) in the present investigation as an independent check on a limited number of samples. A less tedious method is based on phasic separation with benzyl alcohol[9], the cyanocobalamin being determined spectrophotometrically after removal of the hydroxocobalamin. This method, however, requires more material than might be available if only one or two ampoules of low potency were being examined. Therefore we made a spectroscopic study[10] of the effect of light on vitamin B_{12}, and the spectrophotometric determination of cyanocobalamin mixed with known proportions of the irradiation product

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containing hydroxocobalamin thus obtained. This provided the basis for the present investigation.

Materials and Methods

Materials

Several hundred vitamin B_{12} ampoules from 66 different batches made by 7 different manufacturers were obtained from hospital pharmacists in 18 centres in the United Kingdom, who kindly provided also details of the storage conditions. Many of the ampoules contained rather more than 1 ml. (e.g., 1·1 to 1·2 ml.), but the results on these are quoted as the content per ml., on the assumption that only 1 ml. would be injected.

Vitamin B_{12} satisfying the U.S.P. XIV requirements was obtained from 3 different batches purchased in the open market, and dried at 105° C. *in vacuo* over phosphorus pentoxide to constant weight before spectrophotometric determinations were made. Similar determinations were made on hydroxocobalamin prepared by illumination of this cyanocobalamin, also on a specimen of crystalline hydroxocobalamin kindly provided by Dr. J. G. Heathcote of the Distillers Co. (Biochemicals) Ltd. and used as received.

Spectrophotometric data were obtained on a Beckman DU photoelectric spectrophotometer, the method of Morton and Stubbs[11] being used for calculation of the actual cyanocobalamin content. A 1 cm. cell was used throughout, as specified in the B.P. 1953. This, however, gave rather low readings with some of the weaker injections. We therefore recommend the procedure of B.P.C. Supplement 1952, which permits the use of cells of different lengths. *p*H measurements were made on a Cambridge portable *p*H meter.

Microbiological assays

(a) *Cyanocobalamin plus hydroxocobalamin*

The method of Cooperman, Drucker and Tabenkin[12] was used. This employs *Lactobacillus lactis* Dorner (ATCC 8000), and the only modification introduced by us lay in the preparation of the medium for inoculum which was prepared according to Hendlin and Soars[13]. Assays were carried out at two levels only (in view of the small amount of material available) but in complete duplicate (different inocula, sets of tubes, etc.). The response was measured in terms of 0·05N sodium hydroxide and smooth curves were drawn with mechanical aid. Results were calculated by direct reference from sample to standard curve by inspection. There is a linear relation between response and logarithm of dose. This was used in one or two instances, but gave results so closely in agreement with those obtained by direct reference that the more lengthy calculation was abandoned.

(b) *Cyanocobalamin only*

Hydroxocobalamin was destroyed by ascorbic acid[8] and residual cyanocobalamin determined microbiologically as before.

In these microbiological assays the dilutions used in first assays on each

sample were all 1 in 10^6. In samples 2 and 3 the content of cyanocobalamin was so much reduced, that less diluted solutions were used in the 2nd assays (see footnote to Table II).

RESULTS

Microbiological and spectrophotometric results for cyanocobalamin plus hydroxocobalamin

The microbiological method was first tested on 3 solutions of known content, prepared from (1) crystalline cyanocobalamin in distilled water, (2) crystalline cyanocobalamin converted to hydroxocobalamin by illumination, (3) mixture of equal volumes of (1) and (2).

TABLE I

MICROBIOLOGICAL AND SPECTROPHOTOMETRIC ASSAYS OF CYANOCOBALAMIN PLUS HYDROXOCOBALAMIN IN VITAMIN B_{12} SOLUTIONS

Sample	Cyanocobalamin + hydroxocobalamin (μg./ml.)			
	Microbiological			
	Mean results in		Final mean	Spectrophotometric*
	1st assay	2nd assay		
Solutions:				
1	105	90	98	90
2	104	86	95	90
3	98	90	94	90
Injections:				
4	12·6	10·9	11·8	20·3
5	19·8	19·6	19·7	26·3
6	18·0	19·0	18·5	30·2
7	23·9	22·3	23·1	21·8
8	22·2	22·3	22·3	23·7
9	17·7	21·0	19·3	26·4
10	25·2	24·9	25·1	31·4
11	16·2	16·7	16·5	17·4

* In calculating these results allowance has been made for $E\,\frac{1\ \text{per cent.}}{1\ \text{cm.}}$ at 361 mμ for hydroxocobalamin being lower than that for cyanocobalamin, as indicated in Figure 1.

The difference in results between the 1st and 2nd assays (Table I) must be ascribed to experimental error. The coefficient of variation observed in these assays was, however, never greater than ±10 per cent. and often much less. The mean microbiological results in these assays ranged from 104 to 109, and averaged 106 as a percentage of the mean spectrophotometric results. The two methods were next compared on samples of 8 different batches of injections. The mean microbiological results ranged from 58 to 106 and averaged 80 per cent. of the mean spectrophotometric results. However, the former had been determined several weeks after the latter, and may have been lower partly because of losses during storage of the diluted injections even although this was in complete darkness in the refrigerator.

Microbiological and spectrophotometric results for cyanocobalamin only

On the above 3 solutions excellent agreement was again obtained between the results by the two methods (see Table II). On the 8 injections

the mean microbiological results were again lower than the mean spectrophotometric results, ranging from 71 to 97 and averaging 86 per cent. of the latter.

Proportion of cyanocobalamin in total cobalamins

Determination of this proportion by the microbiological method gave mean results ranging from 76 to 115 and averaging 95 per cent. of the mean spectrophotometric results (see Table III).

The comparison of the two methods on this limited number of samples having indicated a reasonable degree of agreement, the spectrophotometric method was applied to a much larger number of samples, providing data for a comprehensive survey which would have taken very much longer to complete by microbiological methods.

TABLE II

ASSAYS OF CYANOCOBALAMIN IN VITAMIN B_{12} SOLUTIONS BY MICROBIOLOGICAL METHOD AFTER DESTROYING HYDROXOCOBALAMIN WITH ASCORBIC ACID AND BY SPECTROPHOTOMETRIC METHOD

	Cyanocobalamin μg./ml.			
	Microbiological			
	Mean results in		Final mean	Spectrophotometric
Sample	1st assay	2nd assay		
Solutions:				
1	89	100	94·5	90
2	nil	(*a*) 0·03 (*b*) 0·03	0·03	nil
3	43	49	46	45
Injections:				
4	10·4	10·3	10·4	14·6
5	16·9	18·3	17·6	18·5
6	11·1	12·3	11·7	15·5
7	19·6	20·4	20·0	21·8
8	20·4	21·9	21·2	23·7
9	22·6	21·9	22·3	26·4
10	26·4	27·5	27·0	31·4
11	17·1	15·8	16·5	17·4

Dilutions used in microbiological assay: $1:10^6$ in all cases except
2nd assay Sample 2 (*a*)—1:2500
" " " 2 (*b*)—1:1250
" " " 3 —1:667,000

TABLE III

CYANOCOBALAMIN CONTENT AS PERCENTAGE OF TOTAL COBALAMIN CONTENT* IN SOLUTIONS AND INJECTIONS

Sample	Microbiological	Spectrophotometric	Spectrophotometric as percentage of microbiological
1	97	100	103
2	49	50	102
3	nil	nil	100
4	89	68	76
5	89	71	80
6	63	52	83
7	87	100	115
8	95	100	105
9	115	100	87
10	108	100	93
11	100	100	100
	Mean of all		95·0

* i.e., cyanocobalamin plus hydroxocobalamin as in Table I.

Spectrophotometric assay of vitamin B_{12} injections by official methods

U.S.P. XIV, B.P.C. Supplement 1952 and B.P. 1953 all assay these injections by their extinction at 361 mμ. From the extinction readings is calculated the cyanocobalamin content, using 207 as $E_{1\,cm.}^{1\,per\,cent.}$ However, hydroxocobalamin also produces a definite extinction at 361 mμ (see Fig. 1). Hence the results obtained by these official methods, although purporting to measure only the cyanocobalamin, to a certain degree also measure any hydroxocobalamin which may be present. The extinction at 361 mμ produced by a given concentration of hydroxocobalamin is only about half that given by the same concentration of cyanocobalamin. We have determined the actual cyanocobalamin content in its mixtures with hydroxocobalamin by means of Morton and Stubbs[11] method, taking readings at 355, 361 and 366 mμ for this purpose. Our data indicate that E 355 mμ for cyanocobalamin = E 366 mμ, and 355 mμ is an isosbestic point in such mixtures[10]. The approximate hydrocobalamin content can be calculated by doubling the difference between the cyanocobalamin content, determined as described above, and the official "vitamin B_{12} content" calculated on E 361 mμ.

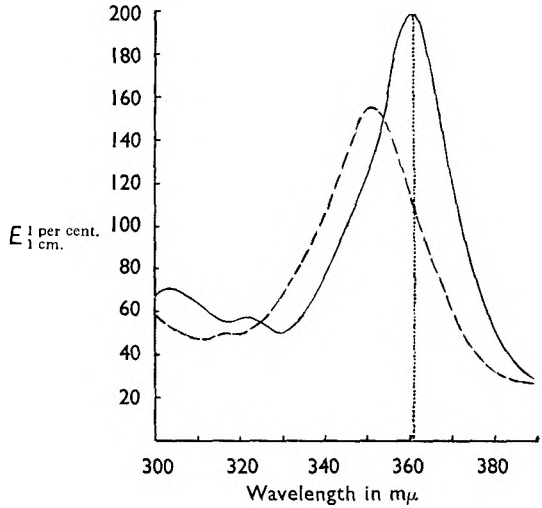

FIG. 1. Absorption curves for cyanocobalamin and hydroxocobalamin. Dotted line indicates wavelength at which extinction is measured in official spectrophotometric assays.

—— Cyanocobalamin.
– – Hydroxocobalamin.

Official identification tests for vitamin B_{12}

U.S.P. XIV specifies a maximum extinction at 361 ± 1 mμ for vitamin B_{12} injections. B.P. 1953 requires extinctions to be taken at 361 mμ for assay of these injections, without specifying that this is a maximum. However, in the B.P. monograph for crystalline vitamin B_{12} the maximum is required to lie between 360 and 362 mμ, giving the same tolerance as in U.S.P. XIV.

Now, in mixtures of cyanocobalamin and hydroxocobalamin, as the proportion of the latter increases the position of the maximum gradually shifts from 361 to 351 mμ. Figure 2 summarises data taken from a number of our experiments[7] on such mixtures, showing that the maximum

does not drop definitely below 360 mμ until the cyanocobalamin content falls to about 75 per cent. of the total cobalamin content.

U.S.P. XIV also specifies a maximum at 278 ± 1 mμ for vitamin B_{12} injections. The presence of bacteriostatics in such injections may interfere with readings at 278 mμ, or even with those at 361 mμ, and B.P. 1953 makes provision for this by stating that if the presence of a bacteriostatic causes an error exceeding 2 per cent. in the assay based on 361 mμ, this assay is not to be used. Presumably readings would then be taken at 548 mμ, as in U.S.P. XIV and B.P.C. Supplement 1952. The tolerances allowed in the actual position of the peak would again permit the presence of a considerable proportion of hydroxocobalamin.

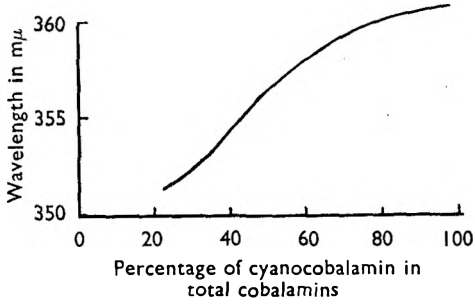

FIG. 2. Shift in 361 mμ max. of cyanocobalamin when mixed with different proportion of hydroxycobalamin.

U.S.P. XIV and B.P.C. Supplement 1952 also give as an identification test for vitamin B_{12} injections a specified range within which the ratio E 361 mμ/E 548 mμ must lie. A similar range is specified in the B.P. 1953 monograph for crystalline vitamin B_{12}. This test, however, does not exclude the presence of hydroxocobalamin in any proportion up to 100 per cent.

Since none of these identification tests satisfactorily excludes hydroxocobalamin, the vitamin B_{12} injections in general use may contain considerable proportions of this cobalamin in the mixture of total cobalamins, although still complying with the official requirements.

"Vitamin B_{12} content" of vitamin B_{12} injections by official methods

These methods purport to measure the content of anhydrous cyanocobalamin but in fact they determine the cyanocobalamin plus about half the hydroxocobalamin. This is the official "vitamin B_{12} content." This content is required in U.S.P. XIV to lie between 90 and 115 per cent., in B.P.C. Supplement 1952 between 90 and 110 per cent., and in B.P. 1953 between 79·5 and 96·5 per cent. of the claim.

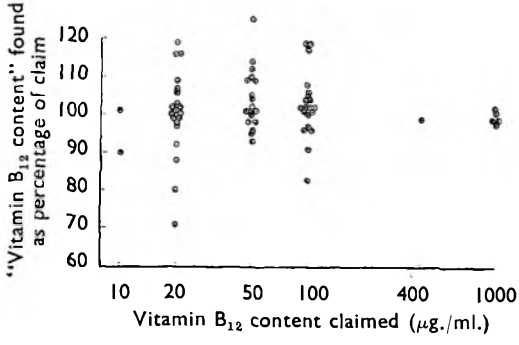

FIG. 3. "Vitamin B_{12} content" of vitamin B_{12} injections as determined by the official spectrophotometric method.

Our results on 66 batches of injections showed their official

"vitamin B_{12} content" to range from 70 to 125 and average 100·3 per cent. of the claim. As will be seen from Figure 3 the variations were similar over the whole range of strengths of the injections, from 10 to 1000 μg./ml. (plotted on logarithmic scale to save space). Hence it seems justifiable to combine all the results in a distribution diagram, as in Figure 4. This shows

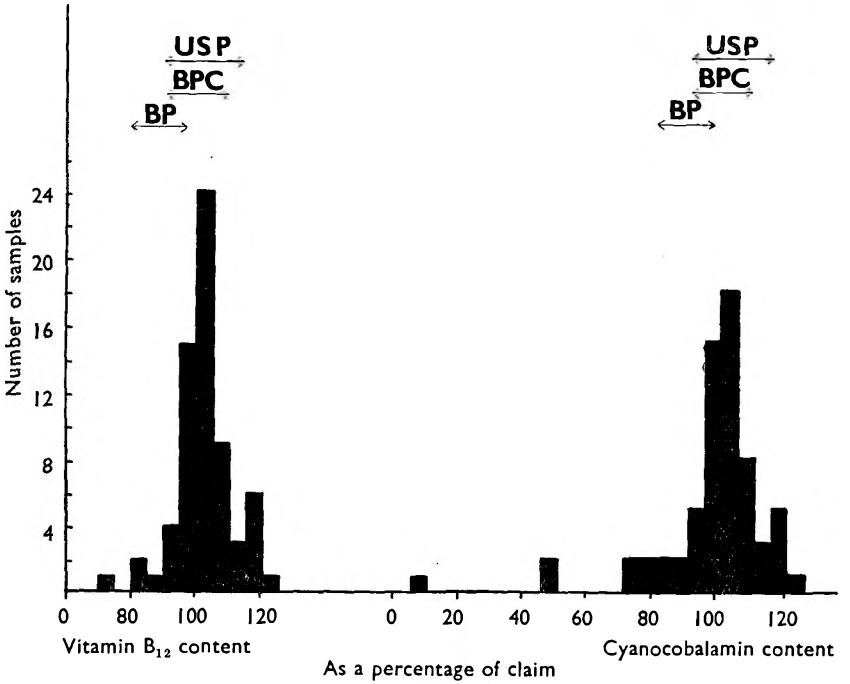

FIG. 4. Distribution diagrams for "vitamin B_{12} content" and cyanocobalamin content of vitamin B_{12} injections, expressed as a percentage of claim. The arrows indicate ranges of this percentage permitted by U.S.P. XIV, B.P.C. supplement 1952 and B.P. 1953.

that 55 out of 66, or 83 per cent. of the batches, satisfied the U.S.P. XIV requirements, and 53 out of 66 or 80 per cent. of the batches, satisfied the requirements of the B.P.C. Supplement 1952. The injections had been manufactured to meet these requirements, and even after storage for some time by far the greater part of them succeeded in doing so.

When the injections were checked against the requirements of B.P. 1953 there was a very different story. Only 11 out of 66 or 17 per cent. of the batches, satisfied these requirements. 54 of the batches, or 82 per cent. gave too high results. Our results in this survey suggest that most of the vitamin B_{12} injections at present in use in this country would not satisfy the Pharmacopœial requirements. In order to avoid wastage of expensive material, it might be advisable to raise the maximum permitted level in B.P. 1953.

Cyanocobalamin content of vitamin B_{12} injections

The true cyanocobalamin content of the injections, determined by

applying the Morton-Stubbs correction to the above figures, showed a rather different distribution, ranging from 9 to 125 per cent. of the claim (see *Fig.* 4). In 6 batches this content was above 115 per cent. of the claim, thus exceeding the U.S.P. requirements. In 11 batches it was less than 90 per cent. of the claim, and in 49 batches it lay between 90 and 115 per cent. of the claim. For the B.P.C. requirements the distribution was similar, 9 batches being above the requirements, 46 satisfying them and 11 being too low. Only 11 of the injections complied with the B.P. requirements. 48 batches had too high contents and 7 batches too low. In these latter the hydroxocobalamin content was quite considerable, exceeding the corresponding cyanocobalamin content in 3 of the batches.

Comparison of cyanocobalamin and official "vitamin B_{12} content"

Since exposure to light gradually converts cyanocobalamin to hydroxocobalamin[3,14,15] the proportion of cyanocobalamin in the official "vitamin B_{12} content" might be expected to be lower in injections stored for longer periods of time, if they had been exposed to light. In Figure 5 this proportion is plotted against storage time, for each of the batches for which details of storage conditions were available. The results show that whilst the proportion of cyanocobalamin seems to remain constant in some batches, in others it tends to fall during prolonged storage, even though the ampoules were in cardboard boxes not exposed to direct daylight. There was also some indication that, when other factors are equal, the cyanocobalamin content falls rather more rapidly in low potency injections (e.g., 20 μg./ml.) than in high potency injections (e.g., 100 μg./ml. or higher).

Effect of pH

Previous workers[6] have found that a *p*H lower than 6 in cyanocobalamin solutions favours its conversion by daylight to hydroxocobalamin. We carried out some experiments on solutions in which the concentrations were similar to those in the above injections, and found that after $1\frac{1}{2}$ hours exposure at *p*H 4 in white glass stoppered test tubes to direct sunlight on the open roof, there was no cyanocobalamin left (see Table IV). *E* 361 mμ fell to little more than half the initial value. A parallel experiment at *p*H 5 again showed complete destruction of the cyanocobalamin, though the reduction in *E* 361 mμ was not quite so great. At *p*H 6·2, 45 per cent. and at *p*H 6·9, 24 per cent. of the cyanocobalamin was destroyed. When the solutions were stored in a dark cupboard or even in diffused light in the laboratory there was no loss of cyanocobalamin. These results

TABLE IV

ACTION OF DAYLIGHT ($1\frac{1}{2}$ HOURS) ON VITAMIN B_{12} SOLUTIONS AT DIFFERENT *p*H

	Cobalamin (µg./ml.) after storage in			
	Dark		Direct sunlight	
pH	"Total"	Cyano	"Total"	Cyano
4·0	23·2	23·2	13·2	0
5·0	23·1	23·1	16·2	0
6·2	23·2	23·2	19·8	12·8
6·9	23·2	23·2	21·2	17·7

show that the stability of cyanocobalamin solutions to light can be greatly increased by raising their *p*H from 5 to 7.

No requirements for the *p*H of vitamin B_{12} injections are given in U.S.P. XIV. The B.P.C. Supplement 1952 specifies a *p*H between 4·5 and 6·5 and B.P. 1953 a *p*H between 3·5 and 5·5. None of the injections we examined had a *p*H above 6·3, and the *p*H of most lay between 5 and 6. Hence their *p*H was not a limiting factor.

Protection against light

Ampoules from a batch of vitamin B_{12} injections containing 20·0 μg./ml. by the official method and 18·6 μg./ml. of cyanocobalamin, at *p*H 5·9, were stored under different conditions, and samples examined at intervals. The results, summarised in Table V, showed no loss of cyanocobalamin

TABLE V

EFFECT OF DAYLIGHT ON VITAMIN B_{12} INJECTIONS

	In dark at				In open air in bottle of			
	Room temperature		37° C.		Clear glass		Brown glass	
Days	"Total"	Cyano	"Total"	Cyano	"Total"	Cyano	"Total"	Cyano
0	20·0	18·6	—	—	—	—	—	—
5	20·0	18·6	20·1	18·4	14·1	2·6	15·7	4·4
12	—	—	—	—	10·6	0	—	—

after 5 days in the dark at room temperature, and only 1 per cent. loss in the dark at 37° C. After storage of the ampoules in a white glass bottle in the open air on the roof 86 per cent. of the cyanocobalamin had been lost in 5 days, and 100 per cent. in 12 days. A parallel experiment in which the ampoules were stored in the open air in an amber glass bottle alongside the white glass bottle showed a loss of 76 per cent. of cyanocobalamin in the same 5 days. Thus the amber glass had provided very little protection against the photochemical action of the light.

However, this light was much more intense than the diffused light in our laboratory. Thus, in some 405 μg./ml. ampoules stored for 6 months

TABLE VI

EFFECT OF STORAGE CONDITIONS ON VITAMIN B_{12} INJECTIONS ORIGINALLY CONTAINING 405 μg./ml.

	Contents as percentage of initial content	
	"Total"	Cyano
After 6 months' storage:		
(*a*) in dark at 37° C.	100	100
(*b*) in diffused light at 0° C.	99	99
(*c*) in diffused light at room temperature	98	98

(Dec. to May) in a beaker on a shelf in the laboratory facing north west we found only 2 per cent. loss of cyanocobalamin (see Table VI). There was only 1 per cent. loss in similar ampoules stored in a cardboard box in comparative darkness in the refrigerator, and no loss after storage in the dark at 37° C.

The greater stability towards light of these injections may have been partly due to their high potency. This would fall in line with our general findings on the whole series of injections. We did in fact experience in one batch of ampoules with an initial content of 104 μg./ml. (by official method) a loss of about 10 per cent. of cyanocobalamin after 5 months storage (from August to December) in diffused light in the laboratory, under similar conditions.

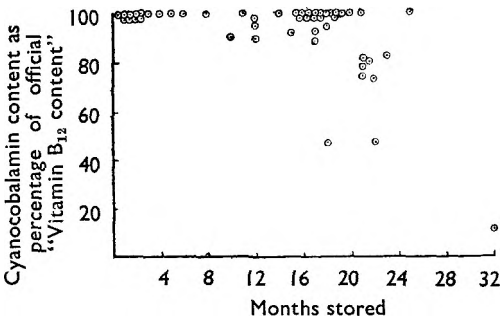

FIG. 5. Effect of storage on cyanocobalamin content (expressed as a percentage of the official "vitamin B_{12} content") of vitamin B_{12} injections.

Reversal effects

It has been stated[6] that if a solution of cyanocobalamin, in which partial conversion to hydroxocobalamin has been produced by the action of light, is then stored in the dark, there is gradual reversion to cyanocobalamin. We tested this effect by taking some of the above 20 μg./ml. injections in which after exposure to direct daylight for 5 days only 24 per cent. of the original cyanocobalamin content remained. On putting them in the dark for another 13 days our assays then showed the cyanocobalamin content to have increased to 96 per cent. of the initial value.

When cyanocobalamin solutions were aerated during illumination to remove the cyanide thus liberated and subsequently stored in the dark, no reversion to cyanocobalamin took place. Thus the reversal effect depends on the hydroxocobalamin which has been formed by the action of the light being able to take up again the cyanide left in the solution, from which it cannot escape because of storage in a sealed container such as an ampoule.

TABLE VII

CYANOCOBALAMIN CONTENT (μg./ml.) OF VITAMIN B_{12} INJECTIONS FROM U.S.A.

Claimed	Found	Found as percentage of claim
30	30·4	101·3
60	60·4	100·7
100	99·3	99·3
1000	1027	102·7
2000	1985	99·3

When this paper was being completed we received from U.S.A. samples of vitamin B_{12} injections in use in that country. Our spectrophotometric assays on these samples showed them all to comply with U.S.P. XIV requirements. No hydroxocobalamin was detected. The cyanocobalamin content ranged from 99·3 to 102·7 and averaged 100·7 per cent. of the claim (see Table VII).

We are indebted to Mr. F. W. Adams, Secretary of the Pharmaceutical Society, for putting us in touch with hospital pharmacists and group pharmacists who kindly supplied numerous samples with details of their storage conditions.

Discussion

Our findings indicate that whilst most vitamin B_{12} injections, as they are used in hospitals in this country, have a cyanocobalamin content lying between 115 and 90 per cent. of the claim, in a certain number of batches this content is well below the claim. Much of this deficiency is probably due to change of cyanocobalamin to hydroxocobalamin, caused by exposure to daylight, even although this has to pass through a cardboard container. The possibility thus arises that patients given vitamin B_{12} injections may sometimes receive appreciable amounts of hydroxocobalamin. We are not aware of any ill effects thus being produced. Pure hydroxocobalamin has been tested clinically and found[16] to have the hæmopoietic activity of cyanocobalamin with no unpleasant side effects. In the course of our investigation into the cyanide and thiocyanate metabolism in man, injections of cyanocobalamin containing up to 50 μg. of hydroxocobalamin have been given without untoward effects.

The present official requirements for vitamin B_{12} injections permit the presence of considerable proportions of hydroxocobalamin. If this is felt to be undesirable, the simplest way of determining the amount present seems to lie in a spectrophotometric method such as we have used. It could provide a more satisfactory check on the efficacy of procedures, especially protection from light, aimed at preventing change of cyanocobalamin to hydroxocobalamin in vitamin B_{12} injections.

Summary

1. A study of 66 different batches of vitamin B_{12} injections made by 7 different manufacturers and obtained from hospital pharmacists in 18 centres in the U.K., has shown that they may contain considerable proportions of hydroxocobalamin, sometimes as much as half of the total cobalamin present.

2. Although vitamin B_{12} is defined in U.S.P. XIV, B.P.C. Supplement 1952 and B.P. 1953 as cyanocobalamin, the official spectrophotometric assays and identification tests for vitamin B_{12} injections do not satisfactorily measure any hydroxocobalamin present, and do not detect it unless it forms at least 25 per cent. of the total cobalamin content.

3. Published methods of determining cyanocobalamin in presence of appreciable proportions of hydroxocobalamin are tedious, and need larger quantities of material than might be available when examining 2 or 3 ampoules of vitamin B_{12} injection. However, one of these methods, based on microbiological assays of vitamin B_{12} activity before and after destruction of hydroxocobalamin with ascorbic acid under given conditions, has been used to check on 11 samples of vitamin B_{12} solutions and injections a spectrophotometric method of determining cyanocobalamin, involving a Morton-Stubbs correction based on extinctions at 355, 361 and 366 mμ. The agreement between results obtained by the two methods was sufficiently satisfactory to justify applying the spectrophotometric method to the 66 different batches.

4. A survey of these has shown that, whilst most vitamin B_{12} injections have a cyanocobalamin content lying between 90 and 110 per cent. of the

claim, in some batches this content is well below the claim. This is probably due to conversion of cyanocobalamin to hydroxocobalamin by the action of light. This conversion takes place readily in the *p*H range of the injections (3·5 to 6·5) and can be prevented by storage in the dark. The cardboard boxes normally used to contain ampoules do not appear to provide sufficient protection. However, when ampoules containing vitamin B_{12} injections in which hydroxocobalamin has been formed by the action of light are subsequently stored in the dark, the hydroxocobalamin may revert to cyanocobalamin.

5. The cyanocobalamin content of most of the vitamin B_{12} injections at present in use in the U.K., when related to the claimed content, appears to be higher than is permitted in B.P. 1953. It is suggested that the present upper limit of 96·5 per cent. of the claim should be increased a little, in order to avoid loss of much valuable material.

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DISCUSSION

The paper was presented by MISS N. BAXTER.

MR. G. SYKES (Nottingham) said he would like to see more evidence for the assertion that cardboard boxes were not light proof. With regard to the method of assay, he wondered whether the authors had used *Lactobacillus leichmannii*, *Bacterium coli* or even the later technique of Ford using an *Ochromonas* culture, because some of the results quoted in Table I were subject to considerable variation. The range of ratios of microbiological assays to spectrophotometric assays appeared to be rather wider than could be normally attributed to microbiological assay errors. The suggestion that it might be due to storage, the microbiological assays having been conducted at different times from the spectrophotometric assays, did not seem to be supported by his impression that cyanocobalamin solutions were much more stable than was indicated in the paper.

MR. D. C. ADAMSON (London) referred to the official identification tests for vitamin B_{12} mentioned in the paper, and said that while he agreed that the ratio of extinctions at 550 and 361 mμ was not very different for hydroxocobalamin and cyanocobalamin, if the wavelengths 278 and 361 mμ were chosen the ratios were of significant difference. The B.P. limits for the latter ratio excluded more than about 10 per cent. of hydroxocobalamin. In his laboratories the position of the maxima and the ratios as determined on the completed solution for injection were taken into consideration. The results quoted for the American samples were somewhat disturbing. The authors inferred that American manufacturers had a more stable form of vitamin B_{12} or that their method of preparation of the solution gave greater protection than did British methods. He wondered whether there was not some other explanation. It would be interesting to know if the American samples were obtained from hospital stocks after some storage period or if they were obtained direct from manufacturers. In the latter case it would manifestly be unfair to compare them with samples taken from hospitals in England. In the early days it was general practice to include a small overage as well as to standardise on the anhydrous cyanocobalamin content. Some of the high results given in the paper might well be due to that. It was well known that light could cause the change from cyanocobalamin to hydroxocobalamin and that the reaction was readily reversible. The authors had not given any experimental evidence that light could affect an ampouled cyanocobalamin solution when protected by a cardboard box. It was difficult to believe that sufficient light could pass through a box to cause even a temporary change in the solution. He had found no evidence of any batch of cyanocobalamin solution being less stable than others. It was well known that cyanocobalmin was fairly readily broken down by both acids and alkalis. Acid conditions were unlikely to develop in an ampouled solution, but it might well be that occasionally the glass ampoules yielded sufficient alkali to account for some of the authors' rather low results.

DR. J. G. HEATHCOTE (Speke) said that whilst there might be some justification for the criticisms which had been levelled at the method described by the authors and possibly at some of the results obtained, it should be pointed out that it was the first serious attempt to obtain a discriminatory method for the determination of cyanocobalamin in the presence of hydroxocobalamin apart from the microbiological method to which reference had been made. He hoped to publish shortly a method for determining various forms of vitamin B_{12}.

MR. H. GRAINGER (London) referred to the storage of vitamin B_{12} injections and said he had found it convenient to obtain solutions of vitamin B_{12} in high concentration and to dilute them as required. It was not clear from the paper whether there was any difference in the rate of change reported by the authors between highly concentrated and dilute solutions.

MR. W. H. C. SHAW (London) said he was particularly interested in the application of the Morton and Stubbs correction for determining

cyanocobalamin in the presence of hydroxocobalamin. That well-known correction made the basic assumption that relative absorption over a range of wavelengths was linear. If hydroxocobalamin were the only absorbing substance other than cyanocobalamin over the wavelength selected, it seemed a reasonable assumption. But if there were any other decomposition products present, it might not be valid. In addition, the Morton and Stubbs correction was not very satisfactory for substances such as cyanocobalamin having absorption curves showing very sharp maxima. Very small errors in the wavelengths, particularly at subsidiary points which were on steep portions of the absorption curve, affected the results considerably. The assays for cyanocobalamin and hydroxocobalamin might have been carried out by utilising the extinction at 361 mμ before and after the addition of cyanide to convert any hydroxocobalamin into cyanocobalamin, and he wondered whether the authors had carried out any assays along those lines.

DR. F. WOKES, in reply, said that the cardboard boxes used were those in which the samples were received. A number were sealed up and therefore it was a fair assumption to make that the samples had been stored in those boxes since manufacture. As some of the ampoules showed losses it was assumed that such losses could occur on storage in cardboard boxes. As a result of subsequent investigation, he had found that in direct sunlight losses in potency of ampoules stored in cardboard boxes were greater than those of ampoules stored in cardboard boxes protected by black photographic paper. The only organism used in the microbiological assays so far had been *Lactobacillus lactis* Dorner. With regard to the question of stability of solutions of different concentrations, the strength of the injections was in the range from 10 to 1000 μg/ml. and no differences had been noted. In tests carried out since the paper was completed he had found that in still higher concentrations vitamin B_{12} was more stable. Some of the ampoules had obviously been given overages to allow for possible losses, and the results were recorded as found. *p*H determinations had been made on most of the injections tested and it had been found that the *p*H lay within the range quoted, so it was not, in his view, a disturbing factor in any of the results. Measurements had been taken at 3 peaks, 278, 361 and 548 mμ. After consideration it was decided to base the findings on 361 mμ first of all because it was sharper and higher than the others, and also because peak 278 mμ was not often available due to the presence of antiseptics in injections. In his view the results in respect of the Morton and Stubbs correction were satisfactory. The American samples were obtained direct from the manufacturers but they were some months old.

THE COLORIMETRIC ESTIMATION OF MORPHINE IN CAMPHORATED TINCTURE OF OPIUM

By G. Tunstall and Mary P. Taylor

From the Pharmaceutical Research and Development Unit, Evans Medical Supplies Ltd.

Received July 8, 1953

The official assay for morphine in camphorated tincture of opium utilises the measurement of the yellowish-brown colour produced by the reaction of morphine with a nitrite in acid solution, followed by the addition of excess of alkali. This colour reaction was first reported by Radulescu[1], and later used as a quantitative method by Fabinyi[2] while van Arkel[3] made the production of the colour more reliable by controlling the acidity and time of reaction, but claimed no great accuracy. Allport and Jones[4] evolved a rapid estimation using a tintometer and claimed an accuracy of ± 10 per cent.

Adamson and Handisyde[5] investigating the method by use of a Spekker absorptiometer reported that the length of time for the reaction in acid solution had a marked effect upon the depth of colour produced. The same authors[6] later critically reviewed the conditions for the production of the colour as applied to the official method, and recommended a time for reaction of exactly 15 minutes, in a specified volume of 0·1 N hydrochloric acid. Under these conditions they gave the method an accuracy of ± 2 per cent. Stephens[7] has given further information on the reaction, particularly with regard to the effect of temperature.

As to the specificity of the reaction when applied to the estimation of morphine, Radulescu[8] examined some 150 vegetable extracts, but found only one of doubtful origin which gave the same reaction. Adamson and Handisyde[5] reported that narcotine, papaverine and codeine do not give a reaction, but the same authors[6] stated that it is given generally by other phenols and substances containing a phenolic group. It is interesting to note in this connection that Nicholls[9] found that Mannich's method for the estimation of morphine, which utilises the precipitation of morphine as its dinitrophenyl ester, was not specific when applied to opium and its products owing to excessive interference from other phenolic alkaloids present.

The official method now in use consists essentially of the evaporation of a portion of the tincture, extraction of the residue with lime water, followed by extraction of the lime water extract with ether. After reduction of the *p*H of the aqueous solution with ammonium sulphate the morphine is extracted with ethanol and chloroform, the mixed solvents are removed, and the residue dissolved in acid. The acid solution is diluted to a definite volume to render it 0·1 N and a portion is used for the development of the colour with nitrite and ammonia, which is then measured by comparison with a standard morphine-nitrite reagent.

It has been noticed in this laboratory, that the official assay of

camphorated tincture of opium gives results significantly higher than expected. Allowing no error in manufacture and assuming the official assay for tincture of opium to be correct, camphorated tincture of opium which contains 1/20th of its volume of tincture of opium should contain between 0·0475 to 0·0525 per cent. of morphine. The official limits are 0·045 to 0·055 per cent. Some results showing discrepancies ranging between + 6 and + 20 per cent. are given in Table I. The quantity of morphine calculated to be present in each batch of camphorated tincture of opium is derived from the strength of the particular batch

TABLE I

Tincture of Opium		Camphorated tincture of Opium		
Batch	Morphine by Assay	Batch	Morphine calculated	Morphine by Assay
	per cent.		per cent.	per cent.
61	0·97	260	0·049	0·055
62	0·95	266	0·048	0·058
63	1·05	273	0·053	0·056
64	0·98	278	0·049	0·058
65	0·97	280	0·049	0·059

of tincture of opium used in its preparation shown on the same line in the table. It had been noted frequently too that if the final solution in 0·1 N hydrochloric acid were boiled, a red colour was produced. It was decided to examine the official method to see if an interfering substance, possibly associated with the red colour, could be isolated and subsequently removed.

In the following experiments the optical density of the colours produced was measured on a Spekker absorptiometer in a 4 cm. cell using OBI glass filters, and the equivalent quantity of morphine estimated by reference to a standard curve prepared under the same conditions. In all cases a "blank" containing the same quantity of the extracted morphine in 0·1 N acid solution, with the ammonia added before the nitrite, was used for comparison.

Experimental

Effect of Camphor, benzoic acid and oil of anise. Samples were prepared—(1) As the official preparation, (2) As the official preparation omitting camphor, benzoic acid and anise oil, (3) As the official preparation substituting an equivalent quantity of morphine for the tincture of opium, and (4) As the official preparation omitting the tincture of opium. They were made to contain (where applicable) a calculated content of 0·050 per cent. of morphine.

The official assay was performed on each sample, and it was found that there was no interference due to camphor, benzioc acid or anise oil. In the samples containing tincture of opium as the source of morphine, results 10 to 12 per cent. higher than theory were obtained, whilst in sample (3), the recovery of pure morphine was complete and reproducible to ± 2 per cent. Where no source of morphine was included in sample 4, there was no recovery of "apparent" morphine. These results are given in Table II.

The ether extractions were examined to see if they contained a substance which gave Radulescu's reaction and if this were the case, to attempt further ether extractions under these conditions, to effect its complete removal. The ether layers were evaporated and dissolved in 0·1 N hydrochloric acid, but it was found that these solutions gave no colour with nitrite and ammonia. However it was found in the case of sample 1 and 2, that if the acid solution was first boiled, no red colour was produced, but the solution then gave a reaction with nitrite and ammonia. The colour was equivalent to about 8 per cent. of the morphine content of the samples. Further ether extraction yielded no colour after evaporation and boiling with 0·1 N acid. It would appear that there is a substance originating in the tincture of opium which gives Radulescu's reaction after boiling with 0·1 N acid, but no red colour, and is completely removed by the two extractions with ether.

TABLE II

(1) Official preparation	(2) Official preparation omitting camphor, etc.	(3) Preparation using pure morphine	(4) Official preparation omitting tincture of opium
per cent.	per cent.	per cent.	per cent.
0·056	0·056	0·050	0·000
0·056	0·055	0·049	0·000
0·055	0·056	0·050	0·000
0·056	0·056	0·050	0·000

Extraction with ether at reduced p*H.* Garratt[10] in an examination of the colorimetric estimation of morphine in aromatic powder of chalk with opium, stated that phenolic and non-phenolic substances, present in the aromatic portion of the preparation gave a colour but that they may be removed by extraction with 3 quantities of ether after the addition of ammonium sulphate. This method of extraction was tried, in addition to the preliminary ether extractions, which, as mentioned above, extracted one type of interfering substance. It was found that a further substance which gave Radulescu's reaction and also a red colour on boiling with dilute hydrochloric acid was extracted. The conditions were varied but it was found that if the interfering substance (as indicated by the red colour given with hot dilute hydrochloric acid) were to be extracted completely, some of the true morphine content was also extracted. If the conditions were arranged so that an insignificant quantity of morphine was extracted, some of the interfering substance was left behind. The two sets of conditions were—(1) For complete extraction of the interfering substance as indicated by the red colour with hot dilute hydrochloric acid. Extract with 3 quantities of ether, each of 10 ml., mix the extracts and wash with 5 ml. of water, discard the ether and extract the mixed aqueous layers with chloroform and ethanol mixture. (2) For the removal of an insignificant quantity of morphine. Extract with 3 quantities, each of 5 ml., mix the extracts of ether, and wash with 2 separate 5 ml. quantities of water. Discard the ether layer and extract the mixed aqueous layer with chloroform and ethanol mixture. Table

III gives the results obtained by the two methods on samples containing an equivalent morphine content of 0·050 per cent.

TABLE III

First method of extraction				Second method of extraction			
Official preparation		Preparation containing pure morphine		Official preparation		Preparation containing pure morphine	
Morphine by assay	Equivalent morphine in ether layer	Morphine by assay	Equivalent morphine in ether layer	Morphine by assay	Equivalent morphine in ether layer	Morphine by assay	Equivalent morphine in ether layer
per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.
0·048	0·0086	0·047	0·0025	0·052	0·0041	0·049	0·0009
0·047	0·0089	0·047	0·0025	0·052	0·0043	0·049	0·0008
0·048	0·0084	0·046	0·0027	0·053	0·0040	0·048	0·0012
0·048	0·0086	0·047	0·0026	0·053	0·0039	0·049	0·0009
0·048	0·0086	0·047	0·0027	0·052	0·0041	0·049	0·0010

Extraction with Benzene. As the method of extraction at reduced *p*H was effective in removing the interfering substance and inaccurate only because of the solubility of morphine in ether, a search was made for another organic solvent in which morphine was less soluble. Tickle[11] gives the partition of morphine in aqueous solution with a number of organic solvents. Of those mentioned, benzene was considered the best to use as the solubility of morphine in this solvent is minimal. It was found that benzene was effective in extracting the substance which gave Radulescu's reaction and also a red colour on boiling with 0·1 N hydrochloric acid. Two extractions with 10 ml. quantities of benzene were found to be sufficient, but troublesome emulsification took place. It was found that the use of 2 quantities, each of 25 ml. of benzene eliminated the emulsification and removed less than 1 per cent. of the morphine. Subsequently a single extraction with 25 ml. of benzene was found to remove the interfering substance effectively. The results obtained using one 25 ml. benzene extraction, which was washed with 5 ml. of water, are given in Table IV. The samples were made to contain 0·050 per cent. of morphine.

TABLE IV

Official preparation		Preparation containing pure morphine	
Morphine by assay	Equivalent morphine in benzene layer	Morphine by assay	Equivalent morphine in benzene layer
per cent.	per cent.	per cent.	per cent.
0·049	0·0064	0·050	0·0003
0·050	0·0062	0·049	0·0003
0·050	0·0062	0·050	0·0002
0·050	0·0062	0·050	0·0002
0·049	0·0064	0·050	0·0002

As a simplification, direct estimation of the morphine after the ether and benzene washings without the chloroform-ethanolic extraction was attempted, but it was found that the results obtained were some 10 to 15 per cent. higher than was theoretically predicted. This was confirmed by an examination of the aqueous layer after extraction with chloroform-ethanol which yielded no morphine on further extraction but which itself

when acidified, gave a brown colour with nitrite and ammonia deeper than the very slight colour already present.

Recommended modification. The interfering substance may be removed by inserting in the official method for the assay of camphorated tincture of opium: after the addition of 0·15 g. of ammonium sulphate ". . . extract the aqueous layer with 25 ml. of benzene, separate, wash the benzene with 5 ml. of water and combine the aqueous layers. Discard the benzene and extract the aqueous layer (which now measures 30 ml.) with a mixture of 30 ml. of chloroform and 30 ml. of alcohol. . . ." Results obtained by this modified method for a number of production batches are given in Table V. Samples marked with an asterisk are from other sources of manufacture.

TABLE V

Sample	Official assay	Modified assay	Calculated
	per cent.	per cent.	per cent.
1	0·058	0·050 0·050 0·049	0·050
2	0·063	0·050 0·050 0·050	0·049
3	0·056	0·049 0·048 0·049	0·0485
4*	0·057	0·053	—
5*	0·053	0·048	—
6*	0·055	0·049	—
7*	0·065	0·055	—

Examination of interfering substance.—(1) The morphine-nitrite colour is yellowish-brown, whereas the nitrite colour of the interfering substance may best be described as being yellow. (2) On boiling a hydrochloric acid solution of the interfering substance a red colour is produced and the depth of colour produced with nitrite and ammonia is increased. (3) Morphine may be completely removed from solution in ethanol by a column of ionic exchange resin amberlite IRA400 (OH) but it was found that an ethanolic solution of the interfering substance was not affected.

Even though it has not been proved as being the interfering substance, an alkaloid present in opium which gives a red colour on boiling with dilute acid is rhœadine. The substance extracted by the preliminary ether extractions may be thebaine which is converted into thebanine (having phenolic groups) by the action of dilute hydrochloric acid.

Summary

(1) A substance, other than morphine, which gives Radulescu's reaction and interferes with the colorimetric method of assay for camphorated tincture of opium was detected.

(2) A method which effectively removes the substance without significant loss of morphine, and with the minimum of alteration to the official method is given.

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DISCUSSION

The paper was presented by MR. G. TUNSTALL.

MR. R. L. STEPHENS (Brighton) asked whether the authors had applied their method to the determination of morphine in Tinct. Chlorof. et Morph.

DR. G. E. FOSTER (Dartford) said that the variations reported by the authors and others in the morphine content of camphorated tincture of opium compared with that of the tincture of opium from which it is made were due to the assays for the two preparations being conducted by different methods. If the authors had succeeded in solving the problem by extraction with benzene, they would earn the gratitude of a great number of analysts.

MR. G. TUNSTALL, in reply, said that he had not applied the method to the determination of morphine in Tinct. Chlorof. et Morph. The method had been used to determine morphine in tincture of opium and results were within $\pm$ 1 per cent. of those obtained using the official assay.

ELEVATED TEMPERATURE AS AN ARTIFICIAL BREAKDOWN STRESS IN THE EVALUATION OF EMULSION STABILITY

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Since emulsion stability is dependent upon the mechanical strength of the interface, the stability may be estimated by the rate of coalescence of the dispersed globules. Many factors can lead to low stability, but they are effective only in so far as they modify the properties of the adsorbed film at the oil/water interface. When a pharmaceutical, cosmetic, or confectionery emulsion is prepared on a large scale in industry, the formulation has often been developed as the result of long storage tests involving examinations for deterioration at intervals of weeks or months. These emulsions must have a relatively high degree of stability, hence the breakdown is slow. Attempts have been made to find some means of evaluating stability rapidly. The storage period should be reduced considerably. This condition would require an artificial breakdown stress which should have a relationship to shelf storage. The addition of electrolytes, acids, or alkalis usually increases the breakdown rate, but this fact is not reliable as a basis for tests. The addition of these substances changes the constitution of the preparation, and we should in fact be examining a product which varies from the original. Cockton and Wynn[1] have used centrifugal force to apply a standard artificial breakdown stress, although as they pointed out, there does not appear to be any simple relationship between the behaviour of an emulsion in a centrifuge and under normal storage conditions. Centrifuging exaggerates the value of "g" in Stokes equation. Thus, a stable emulsion whose two phases have dissimilar densities may seem inferior to one that is less stable, but which has a smaller variation in the density between the two phases. It was decided in the work described here, to make an initial attack on the problem of assessing the value of elevated temperature as an artificial breakdown stress. Provided the constituents are stable to heat, heating merely accelerates the normal mechanism of breakdown, and may therefore, be regarded as artificial ageing. Since emulsion degradation is a thermodynamic process, it may well be that the rate of decrease of the total area of the interface is directly related not only to time, but also to temperature. The first of these relationships has been shown by King and Mukherjee[2] to be linear. A further argument in favour of the use of elevated temperature for artificial breakdown is the fact that pharmaceutical emulsions should have as one of their criteria of suitability for large scale production, the ability to withstand a rise or fall in temperature within limits.

The term "stability of an emulsion" when used in this paper refers to the type of stability with respect to "cracking" and ignores the stability with regard to "creaming."

Much may be learned about the state of an emulsion at any given time

by performing a size frequency analysis, i.e., an examination of the relative proportions of globules falling into different size groups. This involves the counting of a large number of globules[3,4]. Size frequency analyses are, however, not simple determinations as they involve the counting and measurement of a large number of globules. Curves may be obtained by measuring about 400 globules, but the accuracy of the results increases if the number of globules measured is larger. In the past, this method has involved the use of microprojection apparatus. In the present work a simpler apparatus is described. A less tedious method than size frequency analyses proposed by Smith and Grinling[5] involves a direct count of a smaller number of globules, and eliminates the necessity for measurement. An important factor concerning this globule counting method, discussed by Cooper[6], is the fact that in certain emulsions a portion of the dispersed phase may be "solubilised," or the globules may be so small that they may not be detected when examined microscopically. If this method were to be applied to such an emulsion, it would appear to be less stable than it actually is. In view of the fact, however, that Cockton and Wynn[1] have recently used this method with modifications to obtain reproducible results, it was decided to divide the present work into two parts, one making use of the globule counting method and the other in obtaining size frequency analyses.

Experimental Methods

(1) *Preparation of Emulsions*

(*a*) *Materials*

Internal phase. Heavy Liquid Petrolatum U.S.P. was used in each emulsion, the same sample being used throughout the experiments. The concentration of the dispersed phase was arbitrarily fixed at 25 per cent. v/v for all the experiments, except the one in which cetyltrimethylammonium bromide was used, when the concentration was 50 per cent. v/v. It is hoped to extend this investigation at a later date to emulsions prepared with a vegetable oil, thereby giving data for two types of oils commonly encountered in pharmaceuticals and cosmetics.

External phase. Distilled water was used. Each sample was examined for freedom from microscopically suspended material.

Emulsifying agents. The following were used, the concentrations varying with different emulsions examined:—

(1) Polyethylene glycol 400 monostearate U.S.P., 1 and 2 per cent. w/v.
(2) Acacia, 5 and 6·25 per cent. w/v.
(3) Polysorbate 80 U.S.P., 0·1 per cent. w/v.
(4) Castile soap N.F. (amend white powder), 0·5 and 1·0 per cent. w/v.
(5) Cetyltrimethylammonium bromide, 2 per cent. w/v.
(6) Sodium lauryl sulphate, 0·5 per cent. w/v.

The emulsifying agents were selected so as to provide examples of different types (anionic, cationic, non-ionic, and a natural gum).

(*b*) *Method of preparation* (Quantities of 1000 ml.).

Equipment

(1) *Waring blender.* This is a cylindrically-shaped blender of approximately 1500 ml. capacity. Agitation of the liquids is brought about by rapidly rotating steel blades assembled in the base of the container. The speed of rotation is controlled by means of a variable resistance.

(2) *Club aluminium hand homogeniser.* This is a simple hand-operated lever-type of machine. The pumping action forces the emulsion from a bowl, through a fine orifice under high pressure. At the same time the movement of the handle lever agitates the liquid in the bowl by means of a beater.

Method

In all cases the required weight of the emulgent was dissolved in, or mixed with water and then made up to the calculated volume and then added to the oil. The liquids were premixed in the Waring blender at approximately 3000 r.p.m. for 10 minutes. The emulsion so formed was then passed through the homogeniser once at a temperature of 21° to 23° C. This method gave some degree of standardisation. Since the primary object of this work was to study the breakdown of various emulsions, and not to compare stabilities, a higher degree of standardisation was not considered necessary.

(2) *Storage of Emulsions*

(*a*) *Equipment.* A refrigeration unit, incubators, and hot air ovens were used to give a range of temperatures usually varying from 4° to 85° C.

(*b*) *Storage.* It was observed that at high temperatures small volumes (approx. 10 ml.) of an emulsion cracked before larger volumes (approx. 100 ml.) of the same emulsion. Hence, it was decided that each sample was to be 100 ml. in volume. This volume of the emulsion was distributed to each of a number of wide-mouth jars which were firmly closed by means of screw-caps. A sample of each emulsion was stored at different temperatures for the stated periods of time.

(3) *Examination of Emulsions*

(A) *Method of globule counting*

Equipment

(*a*) *Hæmacytometer cell.* The depth of the cell was 0·1 mm. The diluted emulsion was run in by capillarity after the cover-glass had been fixed in position. Errors due to unequal sedimentation were reduced by this quick method of filling. A hæmacytometer cell was preferred to a Helber counting cell which is 0·02 mm. in depth. It was believed that the latter type of cell would introduced a certain amount of selective sedimentation, i.e., the larger globules would tend to settle against the bottom of the cell or against the cover-glass, and not be drawn in with the rush of liquid at the same rate as the smaller globules. An additional objection to the Helber cell is that globules of a diameter greater than 20μ are encountered. This is greater than the depth of the Helber cell, which would then cause distortion and filtration of the larger globules. The Helber cell would,

however, permit thorough searching of the ruled area with less focussing than would a hæmacytometer. The difficulty of focussing was overcome by using a diluting fluid of high specific gravity. The oil globules would, in the course of 10 minutes, rise to the top of the cell, and would then lie in the same plane. When the microscope was focussed on this plane, it meant that the hæmacytometer rulings were out of focus. In order to compensate for this, a ruled ocular disc was placed in the eyepiece. These rulings were in the form of a block of 16 squares corresponding exactly to the 16 small squares of the hæmacytometer rulings. This procedure obviated the use of the hæmacytometer rulings altogether, the cell being used simply as a cell of convenient depth.

Method

The emulsion was first diluted to such an extent that when the cell was filled, a countable number of globules (10 to 20) was contained in each small square of the eye-disc. The microscope used was a binocular fitted with a × 20 objective and a × 10 eyepiece. Generally the degree of dilution varied between 1 in 500 and 1 in 1000. Sampling was carried out after gently rotating the container to obtain an even dispersion. 1 ml. of the emulsion was withdrawn from the centre of the sample by means of a 1 ml. pipette, and transferred to a sufficient quantity of 50 per cent. aqueous propylene glycol to produce one-half the final volume, after which a further quantity of the aqueous propylene glycol was added with thorough stirring to produce the final volume. The aqueous propylene glycol was selected because it possessed the following advantages at the concentration used: (1) it prevents Brownian movement of the globules; (2) it allows the globules of oil to rise to the top of the cell in about 10 minutes; (3) it is chemically stable and has a standard viscosity; (4) it has no intrinsic emulsifying action and thus eliminates the risk of additional emulsification of the oil; (5) it was shown experimentally that the risk of breakdown of the emulsions upon dilution was absent; an exception was the case of the emulsion using cetyltrimethylammonium bromide as the emulsifier, but this emulsion was only examined by the size frequency analysis method.

The reasons discussed under (2), (3), and (4) clearly show the value of this diluting fluid over acacia or gelatin solutions which have been used in the past. For the emulsion prepared with cetyltrimethylammonium bromide, a 40 per cent. aqueous glycerin solution was used as the diluting fluid.

After the counting chamber containing the dilution of the appropriate strength had been prepared, it was allowed to stand for 10 minutes before being examined to ensure that the distribution of the globules was uniform. If not, the chamber was cleaned and refilled. When the distribution was satisfactory, the globules contained in blocks of 16 small squares in 5 different fields (total of 80 small squares) were counted. In order to avoid counting the same globules twice, the count for each square included all those globules which lay on or touched the top and left-hand side of the square, and excluded all those which lay on or touched the bottom and

right-hand side. After making each count, the chamber and the cover-glass were washed with sodium lauryl sulphate solution, rinsed with distilled water, and dried with a soft tissue.

In the light of the experience gained in the present work the authors are of the opinion that the squares counted should be done so in blocks rather than by selecting them at random. Since each determination is one of a series, the approximate number of globules that each square should contain is known to the worker. If the first portion of the squares do not conform to this approximation, there is a risk during random selection of unwittingly selecting squares to compensate for irregular counts. The number of globules per square was found to conform to a normal frequency distribution pattern.

(B) *Size frequency analyses*

Equipment

(*a*) *Hæmacytometer cell.* This cell was used again in order to give a cell of suitable depth. The rulings were ignored. The cell was filled with the same precautions as were exercised in Method A.

(*b*) *Camera lucida.* A Bausch and Lomb camera lucida was used. This accessory is fitted with assemblies for adjusting illumination from both the image and the drawing paper. The microscope was fitted with a $\times$ 90 oil-immersion objective and a $\times$ 20 eyepiece. This combination was adjusted so that 1μ in the image corresponded to 2 mm. on the paper.

Method

The emulsion was diluted to such an extent that when the cell was filled and the globules allowed to rise, the individual globules could be seen with a reasonable amount of space between them. The degree of dilution was far less than that necessary in Method A. It was not required to prepare the dilutions with any great degree of accuracy as was necessary in the former method. In practice the dilution was 1 in 10 or 1 in 20. Sampling was carried out with the same precautions that were exercised in the first method, and a period of 10 minutes was again allowed to elapse between filling the cell and the next stage in the procedure. The angle of the camera lucida was adjusted to avoid distortion on the paper. The outlines of the globules were traced on to sheets of paper. The outlines of all the globules in a given area were traced. The field was varied on a number of occasions for each sample. This procedure was repeated until a large number of outlines were so traced, the number varying from 400 to 800 per determination. After some practice this operation could be performed in less than 45 minutes. Measurements were made upon the diameter of each outline traced, and were recorded in groups, viz., under 1 mm., 1 to 2 mm., 2 to 3 mm., etc.

RESULTS

(A) *Method of globule counting*

Calculation. From the total number of globules counted in a given number of squares, the following values can be calculated:—

(*a*) "H" which expressed the number of millions of globules into which

1 cu. mm. of oil has been subdivided. If the number of small squares counted is 80, then the volume under 80 small squares in the hæmacytometer is $(80 \times 1/10 \times 1/20 \times 1/20) = 1/50$ cu. mm.

If N is the number of globules counted in this volume of a 1 in Z dilution of an emulsion containing 25 per cent. v/v of oil then,

$$\text{"H"} = N \times 50 \times Z \times \frac{100}{25} \times 10^{-6}$$

(*b*) "S" is the total area of the interface given by 1 ml. of the emulsified oil. This is a value related to "specific interface." The latter expression is defined as the total area of interface given by 1 g. of the internal phase.

The volume of a globule (assumed spherical) $= 4\pi r^3/3$

$$= \frac{\pi d^3}{6}$$

$$= \frac{1}{\text{"H"} \times 10^6}$$

$$\text{Therefore d (in mm.)} = \sqrt[3]{\frac{6}{\text{"H"} \times 10^6 \times \pi}}$$

The surface of 1 droplet $= \pi d^2$ sq.cm.

Therefore "S" $= \pi d^2 \times$ "H" $\times 10^3$.

Tabulated data. A typical series of globule counts is given in Table I, using an emulsion prepared from liquid petrolatum and polysorbate 80 U.S.P. "S"values after various periods of storage at various temperatures are given for four additional emulsions in Table II.

TABLE I

EMULSION OF LIQUID PETROLATUM WITH 0·1 PER CENT. OF POLYSORBATE 80 U.S.P.
(By Globule Count Method)

	3 days			6 days		
Temperature °C.	N	"H"	"S"	N	"H"	"S"
30	332	66·4	19,590	311	62·2	19,150
37	332	66·4	19,590	301	60·2	18,950
45	326	65·2	19,450	164	32·8	15,400
50	308	61·6	19,080	128	25·6	14,100
55	285	57·0	18,600	126	25·2	14,030
60	238	47·6	17,500	115	23·0	13,600

N = Number of globules counted in 80 squares in a dilution of 1:1000.

(B) *Size frequency analyses*

(*a*) *The variation of distribution of sizes.* This was calculated for each sample examined as follows: The recordings of the measurements were expressed as actual diameters of the globules and the average size for each group was calculated. Thus, the average for the group falling under 0·5μ was taken as 0·25 μ, and the average of the group falling between 0·5 and 1 μ was taken as 0·75 μ. In the same way each of the successive size groups were expressed as the average of their actual size. The very large globules were assumed to have an arbitrarily fixed diameter of 15 μ. This assumption was made because large globules occur in small numbers, and

TABLE II

TOTAL AREA OF INTERFACE 'IN SQ. CM./CU. MM.' OF LIQUID PETROLATUM IN 4 EMULSIONS AFTER VARYING PERIODS OF STORAGE AT VARIOUS TEMPERATURES
(By Globule Count Method)*

Emulsion	Emulsion A			Emulsion B		Emulsion C	Emulsion D				
Period of storage	1 day	2 days	3 days	1 day	4 days	4 days	1 day	1·5 days	2 days	2·5 days	3·5 days
Temperature °C.											
4	—	—	—	—	—	—	19,950	18,270	16,200	14,900	12,650
30	23,100	22,690	21,980	17,940	19,750	28,800	19,700	17,800	16,090	14,830	13,880
37	22,130	21,520	21,230	19,680	19,130	28,800	18,930	17,600	17,690	17,050	17,260
45	22,120	21,170	20,500	19,250	18,430	25,250	18,260	17,600	15,590	15,000	—
50	21,770	20,750	20,420	18,050	18,950	24,900	—	—	—	—	—
55	20,570	20,500	20,100	19,100	17,830	24,950	17,830	17,200	15,480	14,560	13,000
60	20,250	20,200	19,780	17,700	17,330	24,850	—	—	—	—	—
65	—	—	—	—	—	—	17,350	—	14,560	13,900	—
75	—	—	—	—	—	—	—	14,210	13,990	14,300	—
85	—	—	—	—	—	—	14,380	13,770	14,490	14,370	12,930
"S" before storage	23,320			20,000		28,000	22,530				

Emulsion A. 6·25 per cent. of acacia as emulsifier.
" B. 1 per cent. of polyethylene glycol monostearate 400 U.S.P. as emulsifier.
" C. 2 per cent. of polyethylene glycol monostearate 400 U.S.P. as emulsifier.
" D. 0·5 per cent. of castile soap N.F. (amend white powder) as emulsifier.

* The values given are the results of a single determination or an average of 2 determinations.

the frequency of their occurrence is not statistically reliable. The total number of globules in each size-group was determined and expressed as a percentage of the total number of globules.

(*b*) *Average diameter.* The arithmetical mean was calculated from the formula, $d = \frac{\Sigma m,}{n}$

where m = the diameter of each globule,
and n = the number of globules measured.

(*c*) *Calculation of the value for "S".* "S" is the total area of the interface given by 1 ml. of the emulsified oil.

The volume of a globule (assumed spherical) $= \frac{\pi d^3.}{6}$

Therefore the number of globules per cm.^{3} $= \frac{6}{\pi d^3.}$

The surface of a globule $= \pi d^2$

Therefore "S" $= \pi d^2 \times \frac{6}{\pi d^3}$

$= \frac{6}{d}$

(*d*) *Tabulated data.* A typical series of size frequency analyses is given in Table III, using an emulsion prepared from liquid petrolatum and 0·5 per cent. w/v of castile soap N.F. "S" values calculated from various size frequency analyses are given in Table IV. Figures 1, 2, 3 and 4 show

size frequency curves obtained for various emulsions after storage at different temperatures.

CONCLUSIONS

1. The techniques described are capable of detecting relatively small degrees of deterioration in emulsions. These changes should provide a useful method of predicting changes on a macroscopic scale. Subsequent storage tests have roughly followed the pattern shown by the short-term tests.

TABLE III

EMULSION OF LIQUID PETROLATUM WITH 0·5 PER CENT. OF SODIUM LAURYL SULPHATE SIZE FREQUENCY ANALYSES BEFORE STORAGE AND AFTER 10 DAYS' STORAGE AT VARIOUS TEMPERATURES

	Before storage	4° C.	30° C.	37° C.	45° C.	55° C.	65° C.	75° C.	85° C.
Average diameter μ	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.
0·25	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·00	
0·75	0·68	0·30	1·59	1·60	0·22	0·00	0·00	0·00	
1·25	9·10	3·85	9·10	2·40	3·26	4·65	10·18	3·24	
1·75	31·14	28·00	31·60	24·00	17·82	18·40	24·69	21·27	
2·25	25·91	25·65	24·90	27·49	28·28	26·46	29·28	27·15	
2·75	11·58	16·00	11·37	15·48	16·09	16·08	10·43	16·39	
3·25	6·14	6·82	6·36	8·54	10·87	11·78	3·31	7·29	
3·75	4·55	5·63	3·64	4·54	6·74	5·00	4·07	3·85	
4·25	2·73	3·26	2·73	5·07	3·26	4·65	3·82	3·85	
4·75	2·27	2·22	1·82	3·21	2·49	2·50	0·76	1·82	
5·25	1·82	2·22	1·36	1·60	2·61	2·50	1·27	1·82	
5·75	1·59	1·33	1·14	0·53	1·74	1·07	1·02	1·62	
6·25	0·68	0·89	0·68	0·53	1·96	1·25	0·51	0·81	
6·75	0·23	0·59	0·68	1·33	0·65	1·43	1·27	1·23	
7·25	0·45	0·59	0·45	0·27	0·65	0·89	1·02	1·23	
7·75	0·23	0·74	0·45	0·80	0·87	0·89	0·76	1·01	
8·25	0·23	0·59	0·23	0·27	0·43	0·36	0·51	1·01	
8·75	0·23	0·30	0·23	0·27	0·22	0·00	0·51	0·40	
9·25	0·00	0·15	0·00	0·53	0·22	0·18	1·02	0·61	
9·75	0·23	0·15	0·00	0·27	0·00	0·18	1·02	0·81	
10·25	0·00	0·15	0·45	0·27	0·43	0·18	0·25	0·81	
10·75	0·00	0·30	0·23	0·27	0·00	0·00	0·51	0·81	
11·25	0·00	0·00	0·45	0·00	0·22	0·18	0·51	0·40	
11·75	0·00	0·00	0·00	0·00	0·00	0·36	0·76	0·40	
12·25	0·00	0·00	0·00	0·00	0·00	0·00	1·02	0·40	
12·75	0·00	0·00	0·00	0·00	0·22	0·36	0·51	0·40	
15·00*	0·23	0·30	1·59	0·80	0·87	0·72	1·02	1·23	
Total number of globules measured	440	675	441	375	460	563	393	494	
Average diameter μ	2·522	2·817	2·763	2·936	3·111	3·084	3·215	3·390	
"S"	24,350	21,630	22,510	20,900	19,720	19,800	19,000	17,910	

Oil Separated

* All globules larger in size than 13 μ were placed in this group.

2. In accord with the findings of previous workers, this work has shown that finer dispersions usually are more stable, although this cannot be stated as a general rule.

3. The degree of dispersion in an emulsion, when examined immediately after preparation, gives some indication of the emulsifying power of the emulgent used, if the method of preparation is standardised. Thus, 1 per cent. castile soap N.F. has better emulsifying power than 0·5 per cent. sodium lauryl sulphate in liquid petrolatum-water emulsions. See Tables III and IV. From Table IV, it will be seen that the emulgents

TABLE IV

TOTAL AREA OF INTERFACE (IN SQ. CM. CU. MM.) OF LIQUID PETROLATUM IN EMULSIONS AFTER VARYING PERIODS OF STORAGE AT VARIOUS TEMPERATURES CALCULATED FROM SIZE FREQUENCY ANAYLSES

Emulsion	E		F		G			
Period of storage	10 days		15 days		15 days		40 days	
Temperature °C.	N	"S"	N	"S"	N	"S"	N	"S"
4	565	24,550	380	18,100	410	21,200	230	19,900
30	360	24,040	475	19,920	826	26,480	350	22,600
37	525	24,680	416	20,710	741	26,750	350	19,900
45	460	23,750	410	18,350	818	25,590	472	18,610
55	555	22,910	405	18,020	490	21,200	608	17,300
65	630	21,600	336	17,860	709	19,800	C	—
75	540	21,590	478	16,800	C	—	C	—
85	C	—	C	—	C	—	C	—
"S" before storage	630	25,100	—	—	—	—	—	—

C—Oil separated. N—Number of globules measured.
Emulsion E—1·0 per cent. castile soap N.F. (amend white powder) as emulsifier.
Emulsion F—2·0 per cent. cetyltrimethylammonium bromide as emulsifier. This emulsion contains 50 per cent. liquid petrolatum.
Emulsion G—5·0 per cent. of acacia as emulsifier.

may be arranged in the following decreasing order of emulsifying power, relative to liquid petrolatum-water emulsions: (*a*) polyethylene glycol 400 monostearate U.S.P., 2 per cent.; (*b*) acacia, 6·25 per cent.; (*c*) castile soap N.F., 0·5 per cent.; (*d*) polyethylene glycol 400 monostearate U.S.P., 1 per cent.

4. The results obtained by both methods show that above 40° C., the rate of decrease of interfacial area increases with a rise in temperature.

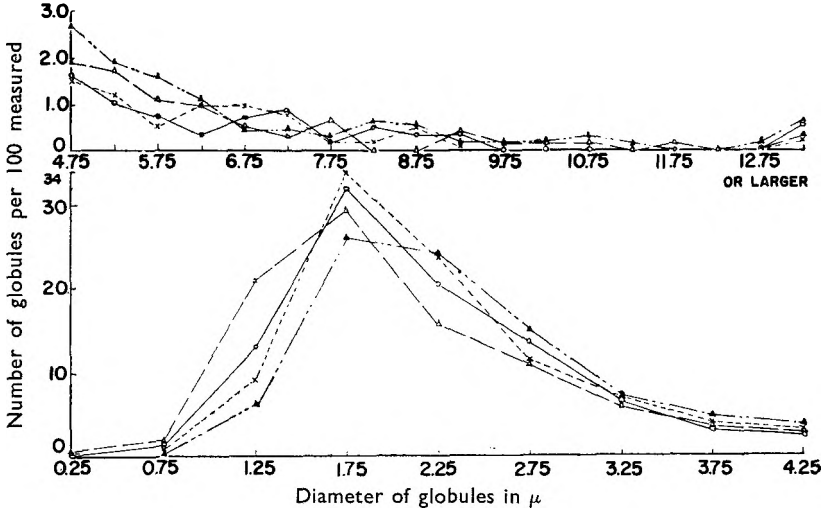

FIG. 1. Size frequency analyses. Liquid petrolatum emulsion with 1·0 per cent. w/v castile soap N.F. as emulsifier, before storage and after 10 days.

— — △ — — Before storage.
——○—— 4° C.
- - - -×- - - - 37° C.
— - - ▲ - - — 65° C.

Within the range of about 30° to 45° C., temperature has a less marked influence upon stability, but below a temperature of about 30° C., the stability usually decreases as the temperature falls to 4° C. This implies a

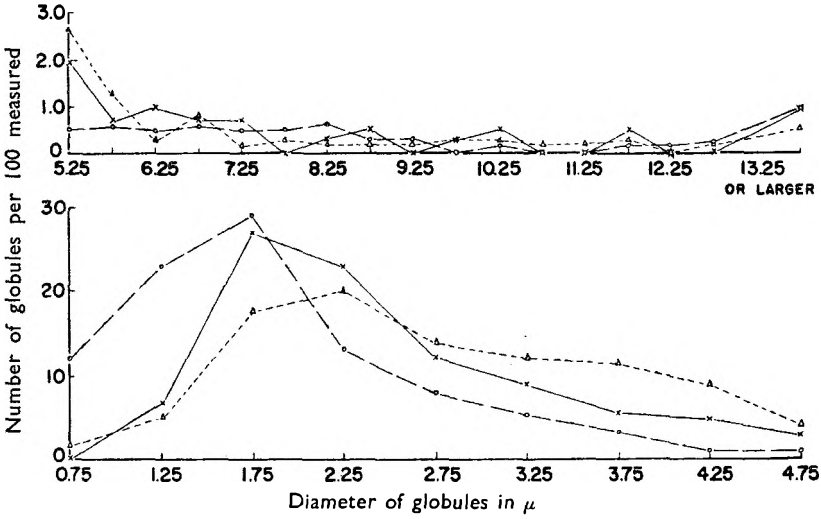

FIG. 2. Size frequency analyses. Liquid petrolatum emulsion with 5·0 per cent. w/v acacia as emulsifier. After 15 days storage.

——×—— 4° C.
— — ○ — — 37° C.
- - - - △ - - - - 65° C.

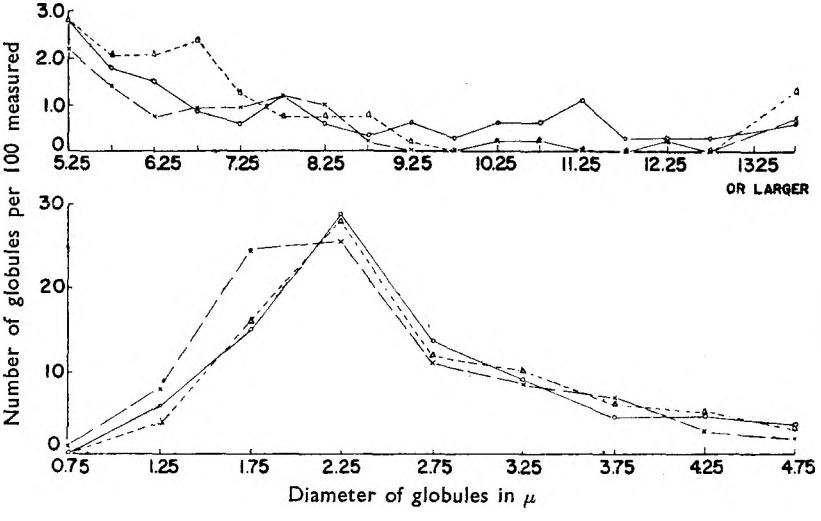

FIG. 3. Size frequency analyses. Liquid petrolatum emulsion with 2·0 per cent. w/v cetyltrimethylammonium bromide. After 15 days storage. This emulsion contains 50 per cent. v/v of dispersed phase.

- - - - △ - - - - 4° C.
— — × — — 37° C.
—— ○ —— 65° C.

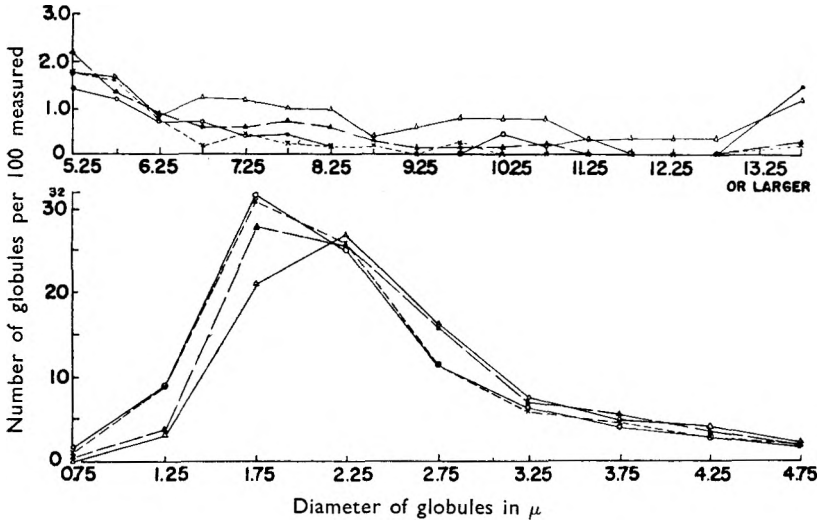

FIG. 4. Size frequency analyses. Liquid petrolatum emulsion with 0·5 per cent. w/v sodium lauryl sulphate, before storage and after 10 days.

× Before storage
▲ 4° C.
○ 30° C.
△ 75° C.

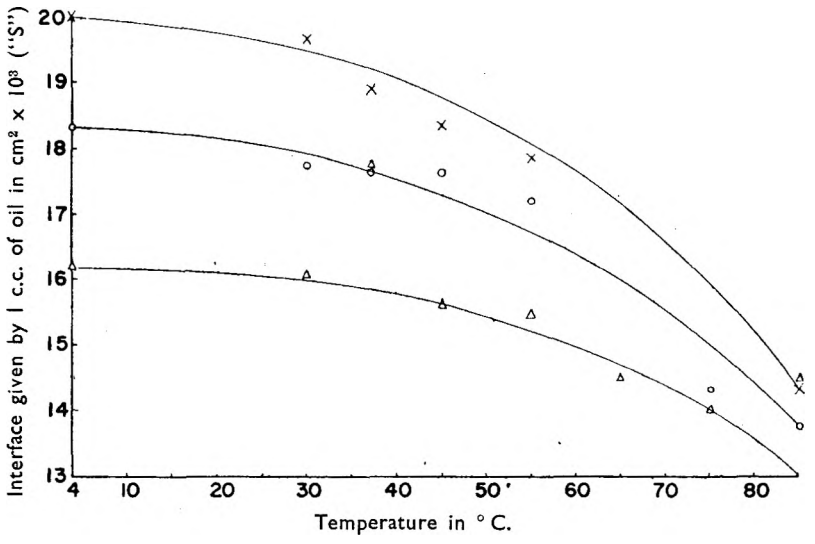

FIG. 5. Relationship of "S" to temperature of storage. Liquid petrolatum emulsion with 0·5 per cent. w/v castile soap N.F. as emulsifier, by method of globule counting.

× After 1 day's storage.
○ After 1·5 day's storage.
△ After 2 day's storage.

temperature of optimum stability varying with the emulsion examined. The emulsions containing castile soap and cetyltrimethylammonium bromide that were examined, have their optimum stability at about 37° C., while the remainder have their optimum stability at lower temperatures.

5. Previous workers have defined emulsion stability on the basis of the rate of decrease of specific interface. This work indicates that in doing so, a temperature should be defined. It is recommended that a temperature of 30° C. be selected, because the emulsions examined in this work show an optimum stability at about this temperature.

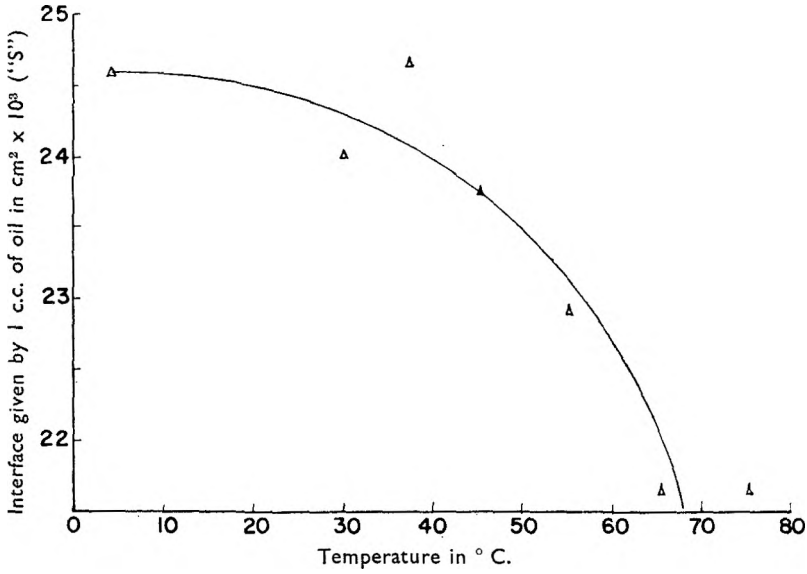

FIG. 6. Relationship of "S" to temperature of storage. Liquid petrolatum emulsion with 1·0 per cent. w/v castile soap N.F. as emulsifier. Calculated from size frequency analyses after 10 day's storage.

6. The size frequency analyses gave normal frequency distribution curves. These curves possess a "skewness" which becomes more pronounced as the emulsion breaks down. The peak of these curves is at a globule size of about 1·75μ. There is a shift of the peak to a larger globule size as the temperature becomes further removed from the optimum. The percentage of large size globules was small, but this figure increased with a rise in temperature. At the same time there was a reduction in the smaller size globules. It should be noted that the average globule size does not correspond to the peak of the curve.

7. "S" was selected as the function related to breakdown on which to base the curves, because this value was shown by King and Mukherjee to be linearly related to time of storage. The curves obtained in the present work relating "S" to temperature, were sensibly similar in each case. This fact indicates that short-term stability tests performed at elevated temperatures will give some reliable information as to the comparative behaviour of emulsions under normal storage conditions.

Discussion

The problem of assessing emulsion stability is complex. Storage tests, in general, are unsatisfactory because many months may be required to elapse before their results may be evaluated, and then only approximately, if examined visually. The experimental methods described in this paper have yielded some information about the behaviour of emulsions under different conditions of storage. The results obtained by the two methods are similar, although it is believed that the results given by the size frequency analyses method are more reliable than those given by simple globule counts. The rate of decrease of the interfacial area gives a measure of emulsion stability, i.e., the greater the rate of decrease, the lower the stability. Elevated temperature may be used to accelerate the rate of decrease, and stabilities of individual emulsions may be compared at various temperatures. The results obtained in this work in no way offer a complete assessment of the problem. It is hoped that modifications of these techniques will prove of value to workers formulating or testing emulsions.

Summary

1. The breakdown of emulsions was studied at different temperatures varying from 4° to 85° C.
2. Two methods were employed: size frequency analyses and a method of globule counting.
3. It is considered that size frequency analyses yield a more complete and reliable picture of the internal state of the emulsion.
4. Elevated temperature is proposed as an artificial breakdown stress for the evaluation of emulsion stability.

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DISCUSSION

The paper was presented by Mr. H. P. Levius.

Mr. E. Adams (Plymouth) expressed the view that while the idea was a good one, the results were rather disappointing. It was apparent from the graphs in Figure 1 that the increase in globule size was not really very much, and he asked whether it could be increased by yet further prolonging the period of heat treatment. At the same time, the authors might consider whether the separation of oil (shown in Table IV) would not be a better end-point. A warning should also be issued that certain emulsifying agents—some of those prepared by the condensation of ethylene oxide—were liable to a change in type, that is to say, to change from oil-in-water to water-in-oil emulsifiers on a rise in temperature.

MR. J. H. OAKLEY (London) said that he had been under the impression that evenness of particle size with as small a diameter as possible was a criterion of stability, in fact, the ultimate aim; but he was not now convinced that it was always true. It might be that evenness of particle size and close packing produced a strain in the emulsion causing a tendency to instability and increased viscosity, whereas variation in the particle size, by allowing the globules to fit better into the inter-globular spaces, lessened the strain thus increasing the stability. He asked whether the authors had noticed any evidence that evenness of size decreased stability.

MR. VAN ABBE (Loughborough) said that the method involved confusion between the effects of temperature on viscosity and on actual emulsification. In practice, a test must take account of viscosity effects in emulsions, and from that point of view the centrifugal method carried out at the temperature of storage might be expected to be more reliable. A storage test at elevated temperature might be an accelerated test for storage under temperate conditions; but it was not an accelerated test for tropical conditions.

MR. J. ARMSTRONG (Nottingham) asked the authors whether any correlation between stability and viscosity existed. He had found that emulsions stored at 20° C. were far more stable than those stored at 37° C. It would be of interest to know whether creaming had occurred in the authors' emulsions. While that was not quite so important as breakdown, it was, nevertheless, an important feature of pharmaceutical emulsions that they should not cream on storage. Was any correlation noted between creaming and general stability of the emulsions?

MR. H. P. LEVIUS, in reply, said that separation of oil had been tried as a criterion but had been found impracticable because the deterioration was gradual and it was impossible to fix a definite end-point. He agreed that small globule size was not invariably indicative of emulsion stability, although in general, small size increased stability. It was, however, something to be sought after because it enhanced the appearance of the emulsion. The work had not been concerned with the causes of instability. In fact, some of the emulsions were purposely made rather unstable in order that deterioration might be studied without studying its causes. In general, however, increase in viscosity increased stability, but no definite rule could be laid down. He agreed that variation in particle size might increase stability. He also agreed that the fact that emulsions showed up well at elevated temperatures did not mean that they would also show up well under normal conditions and vice versa. The temperature at which maximum stability was obtained varied a great deal for different emulsions. Creaming had been experienced, but their emulsions had been agitated carefully. He could not say whether there was any correlation between tendency to cream and tendency to crack.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART V. COMPARISON OF TESTS FOR STERILITY OF OILS

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INTRODUCTION

THE degree of bacterial contamination of pharmaceutical preparations may be investigated by means of two types of test:—(1) The *in vivo* type of test, in which the preparations are injected into animals and the occurrence of any symptoms noted. This type of test detects pathogenic organisms but cannot prove sterility since non-pathogens will not be detected. (2) The *in vitro* type of test, in which the preparations are mixed with suitable nutrient media and the resultant growth or absence of growth noted. This type of test detects "common saprophytic contaminants, and the pyogenic cocci and spore-bearing bacteria pathogenic to man," but would fail to detect some of the more delicate pathogens.

It will be seen, therefore, that there can never be absolute proof of the sterility of a bulk of material. Firstly, the tests used will only detect certain types of organisms, and secondly it is, in the nature of things, only possible to test a fraction of the material because the bulk must be used although, on account of the thorough mixing involved in manufacture, it can perhaps be assumed that any contamination is evenly distributed throughout the bulk.

It is contamination of the type detected by *in vivo* tests which it is most important to avoid in pharmaceutical preparations but such tests are expensive, difficult to carry out, and, unless large numbers of animals are used, the results are inexact. The "Tests for Sterility" of the pharmacist are usually tests of the *in vitro* type. This is justified only because, under the special conditions of manufacture, contamination with robust saprophytes, cocci and spores is more likely than contamination with delicate pathogens. It is for the same reasons that it is not illogical to evaluate the quality of "Tests for Sterility" by determining their ability to detect slight contamination with such organisms as *Bacterium coli*, the spores of *Bacillus subtilis*, *Streptococcus fæcalis*, etc. Sterility is an absolute term allowing of no comparative degrees, it involves the complete absence of living organisms. It should be clearly understood that, on the other hand, "Tests for Sterility," in conjunction with a statistical analysis of the results obtained, cannot do more than give a probability of the absence of more than a certain degree of contamination. This is of course quite sufficient. Since the B.P. test and other such tests are in fact *tests for contamination*, which effectively limit but do not completely exclude the possibility of contamination in the bulk of the material, it might be better to replace the phrase "complies with the tests for sterility" by the more accurate phrase "does not respond to the tests for contamination."

"Testing for Sterility" in oily preparations presents difficulties of its own. As previously shown in this Department[1], while spores remain viable in oils for long periods of time they do not germinate and do not multiply; vegetative organisms do not multiply and tend to die off. To detect bacteria in oils by an *in vitro* "Test for Sterility" it is therefore necessary first to cause the bacteria to be transferred from the oil phase to the aqueous phase of the nutrient medium.

It has sometimes been urged that a particular "Test for Sterility" must be satisfactory because it has been used for many years to control, on a large scale, a variety of preparations for parenteral use which have proved to be satisfactory in practice. All that such experience proves is that material failing to give rise to growth in *in vitro* tests also fails to give rise to infection when used parenterally. It indicates nothing about the ability of the test to detect minimal contamination of a type or degree which will not give rise to infection. Indeed it might well be that in a well managed works, even after many years use, the test might never have been tried on contaminated samples. The difficulty is not overcome by introducing a little contaminated dust into the preparations and then showing that the test indicates contamination.

To examine a "Test for Sterility" of oils it is first necessary to prepare samples of oils containing micro-organisms, preferably of known species, evenly distributed, in very low, but approximately known, concentration. Suitable quantities of such oils can then be examined by the particular test and the results submitted to statistical examination to see whether the proportion of samples showing growth, to those showing absence of growth, is consistent with the known degree of contamination of the oil. It is important that the concentration of the organisms in the oil should be known, at least approximately, because it might well be that only a fraction of the organisms present pass from the oil phase to the aqueous phase in a particular test. Under such conditions, with an oil containing on an average one or more organisms per sample quantity, some, or even all, of the particular samples might fail to give rise to growth.

For the above reasons the work described in this paper is divided into the following sections—(1) A description of the materials and methods used including a description of the three "Tests for Sterility" examined, namely, the test of the British Pharmacopœia, the Filtration Test of Davies and Fishburn[2], and a modified limiting viable count test described in this paper. (2) An account of the preparation of oils lightly contaminated with spores of *B. subtilis* suitable for use in evaluating "Tests for Sterility." (3) The results obtained by submitting the prepared oils to the three "Tests for Sterility" and a statistical examination of the results.

Materials and Methods

The *test organisms* used were the spores, prepared as previously described[3], of *B. subtilis*, Marburg, National Collection of Type Cultures No. 3610.

The *oil* used was arachis oil B.P.

The *peptone* used was Evans bacteriological peptone.

Solutions of peptone powder were made with sterile glass-distilled water, which was also used for diluting them; a separate investigation[3] having shown that glass-distilled water was not likely to be harmful to spores of *B. subtilis* during the relatively short time of exposure in the dilutions.

The *oil solvent* used to remove the oil from suspensions in oil was commercial light petroleum b.pt. 40° to 60° C. once redistilled and passed through a Seitz filter.

The *media* used were nutrient agar, prepared as previously described[3], and broth containing 2 per cent. of peptone, 1 per cent. of Hepamino and 0·5 per cent. of sodium chloride.

All *incubations* were carried out at 37° C.

Since one type of ærobic test organism only was used in the work described in this paper only those portions of the B.P. "Tests for Sterility" relative to ærobes are considered. Any conclusions expressed about the three "Tests for Sterility" considered, refer only to those tests as applied to ærobes.

The B.P. "Test for Sterility." In the test as described in the B.P. 1948 there was no reference to oily injections. By inference, the oily material was to be added to an unspecified quantity of medium (provided that the ratio of medium to oil was sufficient to annul the effects of any antiseptic present). No mention was made of the extent of the oil-water interface nor of intermixture of the two phases by shaking. These latter factors influence not only the transfer of the organisms from the oil phase to the aqueous phase but also the availability of oxygen since oxygen does not easily diffuse through an oil layer.

The B.P. 1953 remedied these omissions to some extent by stating "When oily solutions or suspensions are being tested, they are distributed as uniformly as practicable throughout the media, and the medium is shaken at intervals during cultivation." The importance of the extent of the oil-water interface and the effect of shaking had been investigated in experiments carried out prior to the publication of the B.P. 1953.

Quantities of 1 ml. of an oil containing approximately 4 spores in 10 g. were introduced into 10 ml. quantities of broth, (*a*) in bottles of 2 cm. internal diameter, so that the oil formed a visible continuous layer on the broth, and (*b*) in jars of 6·5 cm. internal diameter so that the oil was insufficient to form a visible continuous layer on the broth. Of the 60 samples in each type of container, 30 were shaken daily (50 up and down movements on one occasion each day) and 30 were not shaken, all for a period of 5 days incubation. During a further 5 day period of incubation all the containers were shaken daily. Growth or its absence was recorded at 5 and 10 days, the results being shown in Table I. A statistical analysis of the results is shown in Table II from which it can be concluded that using a 5 day incubation period the test is satisfactory using wide jars, whether shaken or not, and in narrow bottles, if these are shaken. It is not satisfactory to use the narrow bottles if they are not shaken. Thus the B.P. 1953 test is satisfactory in this respect. In further work described in this paper 2 cm. internal diameter bottles shaken daily were used. A

sample was reported as negative if there was absence of growth and positive if growth occurred.

The Filtration "Test for Sterility" of Davies and Fishburn. The test was carried out exactly as described by the authors[2] except that the light petroleum had a boiling point of 40° to 60° C. instead of 80° to 100° C.[4]

A sample was reported as negative if there was absence of growth and positive if growth occurred.

TABLE I

RESULTS OF B.P. TESTS ON OIL ST3

Type of container	After 5 days at 37° C.	After a further 5 days at 37° C. all samples shaken daily
Narrow bottles shaken daily	15	16
Wide jars shaken daily ..	17	17
Narrow bottles not shaken ..	1	8
Wide jars not shaken ..	12	15

Figures give the number of 1 ml. samples out of 30 showing growth.

The Limiting Viable Count Technique as such and modified as a "Test for Sterility." The most desirable "Test for Sterility" for oils would incorporate a viable count technique so that if the oil were not sterile, not only would this fact be shown, but also the degree of contamination would be indicated. A technique satisfactory for use in carrying out viable counts on heavily contaminated oils has already been described[4].

TABLE II

STATISTICAL ANALYSIS OF THE RESULTS GIVEN IN TABLE I

Period of incubation	Comparison	χ^2	P
5 days	Wide jars shaken compared with wide jars not shaken	1·67	0·1 to 0·2
	Narrow bottles shaken compared with narrow bottles not shaken	16·7	<0·001
	Wide jars shaken compared with narrow bottles shaken	0·268	0·5 to 0·7
	Wide jars shaken compared with narrow bottles	11·9	<0·001
Further 5 days all samples shaken daily	Narrow bottles not shaken during first five days' incubation compared with all other samples	6·43	0·01 to 0·02

P Is the probability of obtaining by chance as big a value of χ^2 as the one shown if there is no difference between the results compared.

Two difficulties were encountered in applying this technique to oils containing only a few spores per g. To appreciate the first difficulty it is necessary to bear in mind the fact that the oil samples examined either already contained some spray-dried peptone, or, for the reason described below, some sterile spray-dried peptone was mixed into the oil. The particles of a spray-dried powder have the form of whole or broken shells. Some of these hollow particles retain air and on centrifuging a suspension in oil and light petroleum a few of the particles, carrying with them adherent spores rise to the surface, instead of sinking to the bottom of the

liquid, and are thus lost on decantation. This error is relatively small for heavily contaminated oils but may be appreciable when only a few spores are present. Breaking down the air-retaining particles by trituration in a pestle and mortar was unsatisfactory because at the same time an appreciable number of spores were found to be killed (up to 50 per cent. in 15 minutes). This is in accordance with the findings of Curran and Evans[5] and King and Alexander[6].

The difficulty was finally overcome by milling the original powder with glass beads in a closed jar; a technique previously successfully used by Bullock, Keepe and Rawlins[3] for obtaining an even distribution of *Bact. lactis ærogenes* in spray-dried powders.

The second difficulty in performing viable counts on only slightly contaminated oils is that under natural conditions, i.e., unless the organisms have been introduced in a large bulk of powder, on mixing the oil with light petroleum and centrifuging, only a negligible deposit is obtained; far too small a quantity to use for a viable count after washing with more light petroleum. To overcome this difficulty the oil to be examined was always mixed with 5 per cent. of sterile, spray-dried, milled, peptone powder before diluting with solvent and centrifuging. This peptone carried the spores down with it and formed a workable deposit. The small but definite improvement in the recovery of spores from oil, resulting from the use of additional peptone powder is shown in Table III. The percentage recovery figures are discussed later.

TABLE III

PERCENTAGE RECOVERY OF SPORES FROM OIL

Treatment	Spores added per g. of oil	Spores recovered per g. of oil	Percentage recovery	t	P
No sterile peptone added to oil ..	3848	3480	90·4	3·808	<0·001
Unmilled sterile spray-dried peptone lightly triturated with the oil	3848	3620	94·1	2·752	0·001 to 0·01
Milled sterile spray-dried peptone shaken with the oil	3848	3740	97·2	1·290	0·2 to 0·3
Milled sterile spray-dried peptone lightly triturated with the oil ..	3848	3860	100·3	0·246	0·8 to 0·9

P is the probability of obtaining by chance as big a value of t as the one shown if there is no difference between the results compared.

Incorporating these two modifications, the limiting viable count technique was carried out according to the following directions.

Accurately weigh about 5 g. of oil into which exactly 5 per cent. of sterile, milled peptone has been introduced by light trituration. Add 5 ml. of light petroleum, mix, centrifuge and discard the clear supernatant liquid; repeat this process once. Remove the last traces of solvent from the deposit under reduced pressure. Take up the deposit in water in the exact proportion of 10 ml. of water for every 5 g. of oil sampled and perform a roll-tube count on the solution using 5 quantities, each of 1 ml., of the aqueous suspension to give quintuplicate tubing.

The total number of colonies in the 5 tubes gives the number

of spores present in 2·5 g. of the oil sampled. So far this constitutes the limiting viable count technique. To compare this test with the other two "Tests for Sterility," a sample was reported as negative if all 5 roll-tubes showed no colonies and as positive if one or more colonies occurred in one or more tubes. This constitutes the modified limiting viable count technique "Test for Sterility."

If it were at any time considered that the limiting viable count technique should be recommended as a simple "Test for Sterility" and not as a method of obtaining a viable count to assess other "Tests for Sterility," it would be advisable to take up in broth the whole washed residue centrifuged out of the oil and light petroleum mixture and to record absence of growth as negative and growth as positive as is usual in "Tests for Sterility."

The Preparation of Oils Suitable for Use in Evaluating "Tests for Sterility"

3 procedures for the preparation of the oils contaminated with spores of *B. subtilis* were available—

(1) Contamination of the oil with peptone powder obtained by spray-drying a solution of peptone containing only a small number of spores per g. of peptone. This procedure was discarded because it involved submitting a relatively small sample of spores to the drying process and might therefore result in the use of a selected sample.

(2) Contamination of the oil with a lightly contaminated peptone powder obtained by serial dilution of a heavily contaminated spray-dried powder with sterile spray-dried peptone. This procedure was tried and found to be satisfactory provided that the dilutions were mixed thoroughly by milling as previously described[3]. The method was, however, not adopted because the long milling periods required were time consuming.

(3) Contamination of the oil with a quantity of heavily contaminated, spray-dried, milled, peptone powder and serial dilution of the mixture with sterile oil. This method was found to be satisfactory, and was adopted.

2 spray-dried peptone powders containing spores of *B. subtilis* were used in the preparation of the oils. The first powder (P1) contained approximately 3200 spores per g. It was mixed with sterile oil to give a contaminated oil (Oil P1) containing approximately 260 spores per g. of oil. A portion of Oil P1 was diluted with a further quantity of sterile oil to give an "Oil for Sterility Test" (Oil ST1) containing approximately 2 spores per g. of oil.

The second powder (P2) contained approximately 96,000 spores per g. and was mixed with sterile oil to give Oil P2 containing approximately 1700 spores per g. This latter was again diluted with sterile oil to obtain Oil P2′ containing approximately 20 spores per g.

Two quantities of Oil P2′ were diluted separately with sterile oil to give two "Oils for Sterility Test," Oil ST2 and Oil ST3 both containing approximately 4 spores in 10 g. of oil.

The next problem was to form an estimate of the number of spores per

g. in the oil, and to show whether that number could satisfactorily be recovered by the limiting viable count technique. The estimate was made by carrying out a viable count on the original powder when, knowing the proportion of powder added to the oil, the number of spores per g. of the latter was calculated. A limiting viable count technique on the oil was then performed and the recovery calculated as a percentage. These were the methods used to obtain the percentage recovery figures in Table IV.

TABLE IV

RECOVERY OF SPORES FROM THE OILS BY THE LIMITING VIABLE COUNT TECHNIQUE

Material examined	Number of spores added per 10 g. of oil	Number of spores recovered per 10 g. of oil	Percentage recovery	t	P
Powder Pl		32020			
Oil Pl	2595	2605	100·4	0·272	0·7 to 0·8
Oil STl	19·98	20·48	102·5	0·205	0·8 to 0·9
Powder 2		962000			
Oil P2 ..	17590	17410	98·98	0·461	0·6 to 0·7
Oil P2' ..	207	204	98·54	0·385	0·6 to 0·7
Oil ST2 ..	4·101	4·160	101·5	0·0967	>0·9
Oil ST3 ..	4·028	4·0	99·31	0·064	>0·9

P is the probability of obtaining by chance as big a value of t as the one shown if there is no difference betweeen th results compared.

This treatment is inexact because the counts per g. for both powder and oil are means derived by the use of dilution factors from the mean counts of batches of roll-tubes. In the above elementary treatment no indication is given of the possible errors of these mean counts of batches of roll-tubes so no definite degree of significance can be given to the figures representing the percentage recovery. These defects can be remedied by the following statistical treatment.

Let $\bar{x}$ be the mean roll-tube count of the contaminated oil, $\bar{y}$ be the mean roll-tube count of the powder used to contaminate the oil. It must be emphasised that $\bar{x}$ and $\bar{y}$ are the means of the counts of the number of colonies in roll-tubes, the roll-tubes having been obtained by suitable serial dilutions of solutions of either the contaminating powder or the powder recovered from a sample of oil.

The extent of these serial dilutions is taken into account by means of a constant r which incorporates three factors. (1) A factor representing the ratio of the volume of water used to reconstitute the sample of contaminating powder to the weight of this powder sample; (2) a factor representing the ratio of the weight of oil contaminated to the weight of powder used to contaminate it; (3) a factor representing the ratio of the volume of water used to reconstitute the powder recovered from the sample of oil, to the weight of this oil sample.

If the recovery is satisfactory we have

$$\bar{y} = r\bar{x}$$
$$\therefore r\bar{x} - \bar{y} = 0$$

To ascertain if $r\bar{x} - \bar{y}$ differed significantly from zero, use was made of the expression

$$\frac{rx - \bar{y}}{\sqrt{\left[\frac{r^2}{n_1}\left\{\frac{\Sigma(x-\bar{x})^2}{n_1-1}\right\} + \frac{1}{n_2}\left\{\frac{\Sigma(y-\bar{y})^2}{n_2-1}\right\}\right]}}$$

in which the symbols have their usual meaning. If n_1 and n_2 are large, the significance of this expression can be assessed by reference to the table of the t distribution with ∞ degrees of freedom. The results obtained by this procedure are shown in columns 5 and 6, Table IV.

If an oil is to be used to assess the effectiveness of "Tests for Sterility" it is essential that there should be evidence that the organisms in it are evenly distributed. Such evidence was obtained by submitting the results obtained from a number of samples subjected to the limiting viable count technique to one of four different mathematical treatments according to the degree of contamination of the oil as follows. (1) If the oil contained 20 or more spores per g. an analysis of variance was carried out as previously described[3]. (2) With oils containing between 4 and 20 spores per g. the transformation $y = \sqrt{x + 0{\cdot}375}$, where x represented an individual roll-tube count, was used prior to carrying out an analysis of variance on the y values in the usual way. (3) If the oil contained between 8 and 40 spores in 10 g. a similar transformation $y = \sqrt{T + 0{\cdot}375}$ was used where T represents the sum of the counts of the five roll-tubes obtained by the quintuplicate tubings from one sample of oil. The mean square was compared with the theoretical value of 0·25 to obtain a variance ratio and probability value. (4) If the oil contained less than 8 spores in 10 g. yet another treatment was necessary.

Quintuplicate counts were made on 50 samples of oil. For the 5 tubes representing each sample two figures were recorded—the number of tubes free from colonies, and the number of tubes containing one or more colonies. A frequency table was constructed from which a value of χ^2 could be calculated according to the usual formula. Provided the number of samples is large, χ^2 can be taken to have a normal distribution, whose mean and variance can be calculated.

The value of the ratio—

$$\frac{\chi^2 - \text{mean}}{\sqrt{\text{variance}}}$$

was therefore referred to the Normal Distribution Table to obtain a normal deviate and probability value.

The data in Tables IV and V shows that Oils ST1, ST2 and ST3 contain the spores in even distribution and that approximately 100 per cent. of the spores can be recovered by the limiting viability count technique. The oils are thus suitable for use in assessing the efficiency of "Tests for Sterility."

It should be mentioned that the probability values for Oils ST2 and ST3 given in the last column of Table V are slightly lower than the normally accepted 5 per cent. level but this was not considered to be unsatisfactory

in view of the extremely low counts of these oils and the fact that the approximations made in the calculation might emphasise any tendency to give a low value to P.

Comparison and Evaluation of the "Tests for Sterility"

Two series of tests were carried out. In the first series Oil ST1 containing approximately 2 spores per g. was used as the test oil. 50 approximately 5 g. samples of this oil were examined by the modified limiting viable count technique. Since the spores recovered from each sample of oil were suspended in 10 ml. of water and 5 × 1 ml. quantities of this were roll-tubed the total number of colonies counted in the 5 tubes corresponded to the number of spores in 2·5 g. of oil. All 50 samples gave one or more colonies in one or more of the 5 roll-tubes, i.e. 50 positives out of 50 samples were obtained. 50 approximately 1 ml. samples of Oil ST1 examined by the B.P. test all gave rise to growth in the broth. Similarly 50 approximately 5 ml. samples submitted to the Filtration Test all gave rise to growth in the broth. It can thus be concluded that all 3 "Tests for Sterility" can be relied on to show contamination in oils containing 2 or more spores per g. of oil.

TABLE V

Evenness of distribution of spores in the oils

Material examined	Method of calculation of variance ratio (see text)	Variance ratio	P
Powder P1	1	2·077	0·5 to 0·1
Oil P1	1	1·174	>0·2
Oil ST1	3	1·308	0·1 to 0·2
Powder P2	1	2·525	0·05 to 0·1
Oil P2	1	1·142	>0·2
Oil P2′	1	1·584	0·1 to 0·2
Oil ST2	4	*2·262	0·024
Oil ST3	4	*2·098	0·036

* Normal deviate.

P is the probability of obtaining by chance as big a value of variance ratio as shown if the spores are evenly distributed in the oils.

The second series of tests was more exacting since the test oil was Oil ST2 containing approximately 4 spores in 10 g. Considering a positive result to be growth in the broth in the case of the B.P. and Filtration tests and one or more colonies in one or more tubes of the limiting viable count technique test, the results are given in Table VI.

TABLE VI

Results of submitting 50 samples of oil ST2 to each of the 3 tests

Modified limiting viable count technique	Filtration test	B.P. test (1 ml. samples)	B.P. test (5 ml. samples)
42	31	25	43
(41·6)	(30·2)	(25·0)	(43·0)

Figures give the number of samples out of 50 showing growth.

The B.P. test is carried out on 1 ml. samples of oil. In order to make the results more comparable with the results of the other two tests the B.P. test was repeated using 5 ml. samples of oil.

The results for the three possible pairs of the three "Tests for Sterility"

have been compared by obtaining the corresponding values of χ^2 from a 2×2 contingency table. These values are given in Table VII, together with the figures representing the probability of obtaining by chance as large a value of χ^2 assuming that there was no difference between the two tests compared. The results obtained by the Filtration Test differ significantly from those obtained by the other two tests which themselves give concordant results.

TABLE VII

STATISTICAL ANALYSIS OF THE RESULTS GIVEN IN TABLE VI

Comparison	χ^2	P
Modified limiting viable count technique compared with the filtration test	6·42	0·01 to 0·02
Modified limiting viable count technique compared with the B.P. test (5 ml. samples)	0·142	0·5 to 0·7
Filtration test compared with the B.P. test (5 ml. samples)	8·352	0·001 to 0·01

P is the probability of obtaining by chance as big a value of χ^2 as the one shown if there is no difference between the results compared.

In all work with "Tests for Sterility" it is assumed that all manipulations will be carried out aseptically. In practice a low proportion of accidental contaminations does occur. Whenever in the above experiments a sample of an "Oil for Sterility Test" was examined an identical test was carried out on a sample of sterile oil. In these controls for the B.P. test no accidental contamination occurred. The proportion of controls accidentally contaminated was 4·0 per cent. for the Filtration Test and 4·7 per cent. for the modified limiting viable count technique. It is to be expected that these figures will vary with the worker and with the laboratory but they should always be low. With a skilled worker accidental contamination does not appreciably alter the results obtained by the 3 "Tests for Sterility." This can be seen by comparing in Table VI the figures not in brackets which were obtained by not allowing for accidental contamination with those in brackets which were obtained from the same results but making an allowance for accidental contamination.

DISCUSSION

Of the "Tests for Sterility" of oils examined, two ensure the transfer of the organisms from the oil phase to the aqueous phase of the nutrient media by dissolving out the oil in a volatile solvent and removing and washing mechanically the organisms by filtration or by centrifugal force before taking up in water or broth. In the B.P. "Test for Sterility" simple shaking of the oil and broth is relied upon to effect the transfer. At first sight this latter method does not appear to be very effective and indeed it is possible that the effectiveness may vary with the nature of the organism, particularly it may vary according as whether the organism is, or is not, acid-fast. That the B.P. test is satisfactory in this respect for the spores of *B. subtilis* is indicated even in the preliminary results shown in Table I and analysed in Table II. Provided that the containers are shaken daily for 5 days it was found that for an oil containing approximately 4 spores in 10 g. about half the 1 ml. samples examined showed growth in the broth.

This preliminary work could not be accepted as proof of the reliability of the B.P. test however, because it did not take into account the limits of error involved in the estimate of approximately 4 spores per 10 g. of oil. Further, to use the B.P. test in this way to confirm a count amounts to "counting by dilution to extinction," a method involving considerable uncertainty[7].

3 oils suitable for use in assessing the sensitivity of "Tests for Sterility" of oils, Oils ST1, ST2 and ST3 were prepared by introducing into sterile oil a known proportion of a contaminated powder. In this way a calculated figure could be obtained for the number of spores per g. of oil. A modification of the viable count technique for oils as previously described[4] was then used to obtain an experimental figure for the number of spores per g. of oil. An inspection of the agreement between the calculated and experimental figures given in Table IV indicated the reliability of the modified technique which, to avoid confusion has been called "the limiting viable count technique." This indication was confirmed by a statistical analysis of the figures, the results of which are given in the last two columns of Table IV. It is clear that the probability values indicate that there is no significant difference between the calculated and experimental figures for the number of spores per g. of the oils examined. It was shown that the spores in the oils were evenly distributed by a different mathematical treatment (Table V) of the same experimental results. It can be seen from the probability values that the spores are evenly distributed in the oils. At this point it had been established that the limiting viable count technique was reliable and that the spores in the S.T. oils were evenly distributed in approximately known concentrations.

The Filtration and B.P. "Tests for Sterility" were then critically examined in two ways. (1) By ascertaining by inspection whether the results obtained by them were consistent with the known concentrations of the spores in the oils; (2) by comparing statistically the results obtained by them with the results obtained by using the limiting viable count technique as a "Test for Sterility," i.e., by recording only growth or no growth from each sample instead of recording the number of colonies obtained from each sample.

It was first shown that with an oil containing 2 spores per g. all 3 tests consistently indicated contamination in all samples.

As shown in Table VI the B.P. test gave 25 positives out of 50 $\times$ 1 ml. samples of an oil containing approximately 4 spores in 10 g. Such a result would give rise to considerable confidence in the B.P. test. According to the other results quoted in Table VI the most sensitive "Test for Sterility" was the modified limiting viable count technique since with 50 samples 42 positives were recorded. Although 5 g. samples were taken for this test it will be recalled that only half of the powder recovered from the oil in each case was tubed and examined for growth; thus the actual results correspond to 2·5 g. samples of the oil, which on the average could be expected to contain only one spore each. The B.P. test came next with 43 positives out of 50 $\times$ 5 ml. samples, each sample liable to contain on an average 2 spores. The Filtration Test gave a lower recovery of 31

positives out of 50 × 5 ml. samples. These results indicate that all 3 tests as applied to oils are capable of giving a positive result with a sample of oil containing only one spore.

The above conclusions, drawn from a simple inspection of the figures are supported by the statistical analysis presented in Table VII. It is here shown that the limiting viable count technique relating to 2·5 g. samples of Oil ST3 gave similar results to the B.P. test using 5 ml. samples while the results obtained by the Filtration Test were significantly different but only slightly less satisfactory.

It should be pointed out that these conclusions by no means show that the Filtration Test is not satisfactory although in our hands it has proved to be somewhat less sensitive than the two other tests in the absence of antiseptics. One of the claims made for the Filtration Test was that in it oil soluble antiseptics could be extracted from the organisms rendering them capable of multiplying in broth when otherwise they would not have multiplied even in a suitable nutrient medium or, presumably, if introduced into animal tissues.

In the experiments reported in this paper only the spores of *B. subtilis* have been used as test organisms. It should be remembered in this respect that there is evidence that vegetative bacteria tend to die off in oils and other systems of low moisture content[1]. Work is indeed at present in hand using *Str. fæcalis* as test organism and it is hoped later to use anærobic bacteria and spores. Meanwhile it was thought that the results obtained so far were of sufficient interest to warrant publication.

Summary

1. The preparation of oils containing as few as 4 spores in 10 g. in even distribution has been described. Such oils are suitable for use in evaluating "Tests for Sterility."

2. A limiting viable count technique has been elaborated by means of which the number of spores in such oils can be counted with reasonable accuracy.

3. The limiting viable count technique was then modified so as to serve as a "Test for Sterility" of oil. This and the B.P. "Test for Sterility" as applied to oils and the Filtration Test of Davies and Fishburn have been compared and evaluated. Although all 3 tests were found to be reasonably satisfactory the above order was that of decreasing sensitivity.

It is a pleasure to express our thanks to Mr. A. M. Walker, B.A., for suggestions and advice concerning the statistical treatment of the results reported in this paper.

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DISCUSSION

The paper was presented by MR. N. H. BOOTH.

MR. E. ADAMS (Plymouth) suggested the use of a tube of 8 mm. internal diameter by 25 mm. long, open at both ends, half immersed inside one of the bottles. He considered that perhaps the insertion of such a tube, into which the oil was transferred, brought the oil into contact with the broth and allowed the organisms to diffuse downwards and into the surrounding medium which was in contact with the air, thus obviating the necessity for shaking both the narrow bottles and the wide jars.

MR. G. SYKES (Nottingham) said there was little doubt that the B.P. method of shaking the mixture of oil and broth daily was a great improvement over leaving it to stand for 5 days. In connection with work which he had done on filtration of oils, an endeavour had been made to estimate the number of organisms in the oils, and one of the first observations made was that vegetative organisms died out very rapidly. An attempt to recover bacterial spores in a similar way was not quite so successful as that of Dr. Bullock and his co-workers. It was suspected that organisms were being lost somehow, and having made the suspension of organisms in the oil, shaking them once with water or with broth gave only a partial recovery—something of the order of 40 to 60 per cent.—of the anticipated number of organisms. Similarly, in an aqueous suspension of bacterial spores, when shaken with oil the organisms disappeared from the aqueous phase. In terms of the filtration test, he was less fortunate in the choice of organisms than Dr. Bullock and his team in that the treatment of the organisms with light petroleum always gave a significant kill. In order to avoid the use of the word "sterile" on labels, a more true statement of scientific fact would be to say "sterilised" or "Passed the test for sterility."

DR. G. E. FOSTER (Dartford) said that although the paper was a valuable contribution, it could only be regarded as a beginning, because in carrying out tests for sterility a great number of factors were involved. From experience he appreciated that the composition of the culture medium was important.

MR. A. ROYCE (Nottingham) said that he had some experience in testing oils for sterility. He agreed that narrow tubes were not very good and that the wide tube gave much better results. A sloppy agar (0·2 to 0·25 per cent.) was used, and it was found that the recoveries from a lightly contaminated sample of oil were improved by as much as 50 per cent. by shaking with this medium instead of plain broth. It would be interesting to know whether the authors had tried varying the culture medium.

MR. J. W. LIGHTBOWN (Mill Hill) expressed the view that the method of contaminating the oils should be carefully examined. The oil was being contaminated, not with bacterial cells or spores, but with spray dried peptone particles in which the organisms were embedded. The size of the spray dried particles (something of the order of 20 to 50 μ) was larger than that of a spore. Also the surface of the particles must be quite different from that of the spore. Those two factors would

have an influence in assessing the value of the B.P. test. In the results described where the B.P. test was carried out with a continuous layer of oil over the surface of a tube, without shaking, no growth was obtained. It was likely that the spores had gone into the broth. That would be worth investigating using an oil with a fairly high concentration of peptone and examining microscopically. It was quite likely that growth was obtained on shaking because of aeration. Another factor which should be considered in the B.P. test was the presence of unsaturated fatty acids. Those were markedly toxic in small concentrations to some organisms. Obviously the strain of organism being used did not appear to be sensitive, but a number of strains of sporing ærobic organisms would not germinate in the presence of small traces of these acids. The claim that trituration caused 50 per cent. mortality of spores was interesting. Was it trituration of the spray dried powder with oil or trituration of the spray dried powder alone? It was difficult to believe that conditions were much less drastic than shaking with glass beads or particles of sand. He wondered why the authors did not use another technique of preparing the contaminated oils, that is, spray drying spores in some material which would be soluble in oil so that it would dissolve and leave the spores completely free.

MR. G. SYKES (Nottingham) raised the further point that peptone, being hygroscopic, might offer encouragement to the organisms to pass from the oily phase into the water phase.

DR. K. BULLOCK referring to the difference between his results and those obtained by Mr. Sykes, said that in the work done by Mr. Sykes there was some moisture present whereas in his own work there was not. He had been able to show in another connection that, in the absence of moisture, enzyme systems in general were much more resistant to all toxic agents which he had tried, particularly volatile solvents. In his view the difference in results might be due to the amount of moisture present.

MR. N. H. BOOTH, in reply, said that Mr. Adams's suggestion of putting an open tube into the bottle of broth was interesting, but he doubted whether it would work. With such a narrow tube there would probably be a smaller interface between the oil and broth, and better results could in those circumstances hardly be expected. With regard to the killing of organisms with solvent, he had made extensive tests with various solvents using *Bacillus subtilis* as the test organism and had found that light petroleum (b.pt. 40 to 60° C.) was quite satisfactory and did not give any kill. Previous work substantiated that vegetative organisms died off very rapidly in oils. He agreed that the culture medium used for the test had an effect on the recovery. Oil-soluble material to aid contamination of the oil had already been tried using stearin, and the use of such material had been considered. The trituration which resulted in 50 per cent. kill was of powders without oil present and carried out with a pestle and mortar. With regard to the relationship between peptone particles and spore size, milled powder had been used for contamination and the particles of peptone had been broken up as much as possible without actually killing any of the spores present. Therefore the particles

of peptone present in the oils were very much smaller than 20 to 50 μ. The point concerning aeration in the B.P. was quite valid. A sloppy agar medium was used for shaking out oils and found to be quite satisfactory.

ALKALOID BIOGENESIS

PART II. CHANGES IN THE ONTOGENETIC PRODUCTION OF ALKALOIDS IN *Atropa* AND *Datura*

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IN the course of a programme of experimental work on the biogenesis of the alkaloids of the tropane group, information was required on changes in the proportions of the individual alkaloids typical of the species during the life cycle of the plant. This work has afforded evidence from which may be made certain speculations on the problem of alkaloid biogenesis. Moreover, the results have some relevance to the medicinal use of these plants. Fluctuations in the proportions of the individual alkaloids, although not detected by the usual methods for the standardisation of these drugs, could be expected to lead to fluctuations in the physiological effects of galenical preparations of the drugs.

EXPERIMENTAL

The plants studied were *Atropa belladonna* and *Datura tatula* var. *inermis*. The results of a limited number of experiments on *Datura ferox* are also discussed. Plant material from specimens, cultivated in the usual manner and dried at 60° to 65° C., was analysed by the procedure we have described previously[1]. The identity of the alkaloids was checked in a number of cases by the preparation of crystalline derivatives. Collection of the aerial parts of *A. belladonna* was continued each year until damage due to frost occurred. The roots and rootstock of third-year plants were so large as to render sampling difficult; the material analysed was a representative sample from 4 plants.

Analytical results are recorded in Tables I and II and, for clarity, certain of the results are presented graphically in Figures 1 and 2.

DISCUSSION

In the interpretation of the results, it is recognised that information on the percentage of alkaloid in a dried sample of plant material may in many instances be of little use in making deductions relating to the process of alkaloid synthesis in the plant. Difficulties in expressing the results in a significant manner are illustrated by the values recorded in Table III. In 2 sets of samples selected for approximate constancy of the average weight of their roots, the percentage of alkaloids and the absolute weight per root are roughly equivalent. The average weights of the shoots growing on these roots differed widely and whereas the percentage of alkaloid decreased during the month's growth, the absolute weight of alkaloid increased. However, certain useful deductions may be made when a given alkaloid is found not to be formed or when changes occur in the relative proportions of the individual alkaloids. The re-location of the alkaloids said to occur

TABLE I

HYOSCINE AND HYOSCYAMINE IN *A. belladonna*

Sample	Date of collection	Hyoscine		Hyoscyamine	
		In dried sample per cent.	Proportion of total alkaloids per cent.	In dried sample per cent.	Proportion of total alkaloids per cent.
First year plants—					
Small seedlings	1.6.51	0·040	89	0·005	11
Young plants beginning to flower	8.8.51	0·062	15	0·34	85
Flowering plants with young fruits:—					
Aerial parts	27.8.51	0·030	10	0·27	90
Old leaves from base of plants	27.8.51	0·004	5	0·072	95
Tops of flowering branches	27.8.51	0·070	15	0·40	85
Young shoots from decapitated plants	11.9.51	0·15	26	0·42	74
Fruiting plants:—					
Aerial parts	26.9.51	0·00	0	0·21	100
Tops of branches bearing leaves	26.9.51	0·031	6	0·51	94
Roots and rootstock	26.9.51	0·022	6	0·32	94
Shoots from decapitated plants	3.11.51	0·00	0	0·11	100
Second year plants—					
Plants with unexpanded shoots, 3 in. tall:—					
Aerial parts	8.3.52	0·052	8	0·60	92
Roots and rootstock	8.3.52	0·020	4	0·49	96
Plants 6 in. to 9 in. tall:—					
Aerial parts	15.4.52	0·028	6	0·44	94
Roots and rootstock	15.4.52	0·015	2	0·61	98
Plants 3 ft. tall beginning to flower:—					
Aerial parts	12.5.52	0·002	1	0·28	99
Roots and rootstock	12.5.52	0·001	0·5	0·33	99·5
Flowering plants:—					
Aerial parts	13.6.52	0·006	3	0·19	97
Tops of flowering branches	13.6.52	0·018	4	0·39	96
Roots and rootstock	13.6.52	0·001	0·5	0·27	99·5
Flowering plants with young fruits:—					
Aerial parts	15.7.52	0·001	0·5	0·18	99·5
Tops of flowering branches	15.7.52	0·022	4	0·59	96
Unripe fruits	15.7.52	0·005	2	0·31	98
Roots and rootstock	15.7.52	0·003	1	0·32	99
Fruiting plants with immature fruits:—					
Aerial parts	14.8.52	0·00	0	0·21	100
Tops of flowering and fruiting branches	14.8.52	0·00	0	0·36	100
Roots and rootstock	14.8.52	0·007	2	0·27	98
Fruiting plants with mature fruits:—					
Aerial parts	19.9.52	0·00	0	0·095	100
Tops of fruiting branches	19.9.52	0·003	1	0·21	99
Fruits	19.9.52	0·00	0	0·12	100
Roots and rootstock	19.9.52	0·00	0	0·31	100
Shoots from decapitated plants	19.9.52	0·00	0	0·67	100
Roots and rootstock	20.10.52	0·002	0·5	0·32	99·5
Roots and rootstock	15.12.52	0·005	1	0·50	99
Underground buds	15.12.52	0·00	0	0·64	100
Third year plants—					
Roots and rootstock	12.1.53	0·00	0	0·40	100
Underground shoots	12.1.53	0·005	1	0·70	99
Seeds (viable)	23.1.53*	0·003	1	0·29	99
Emerging shoots	17.2.53	0·013	4	0·32	96
Roots and rootstock	17.2.53	0·004	1	0·36	99
Plants 2 in. to 3 in. tall:—					
Aerial parts	16.3.53	0·008	1	0·70	99
Roots and rootstock	16.3.53	0·00	0	0·49	100
Plants 3 in. to 4 in. tall:—					
Aerial parts	27.3.53	0·00	0	0·59	100
Roots and rootstock	27.3.53	0·00	0	0·53	110

* Date of analysis.

during drying[2] can have no effect upon the results since each part was dried separately before analysis.

Changes in the total alkaloid content of the aerial parts of *A. belladonna* shown in Figure 1 are in agreement with the observations of other investigators[3,4,5,6] and demonstrate that during the first year, the concentration rises to a maximum at the beginning of flowering and then declines during fruiting. Young shoots arising during the period of the late stages of flowering on decapitated plants are considerably richer in alkaloids than similar shoots which develop later in the season.

TABLE II

HYOSCINE AND HYOSCYAMINE IN *D. tatula*

Sample	Date of collection	Hyoscine		Hyoscyamine	
		In dried sample per cent.	Proportion of total alkaloids per cent.	In dried sample per cent.	Proportion of total alkaloids per cent.
Seeds (viable)	20.9.51	0·045	24	0·14	76
Young plants, 2 in. to 3 in. tall	27.6.52	0·17	82	0·038	18
Plants beginning to flower	15.7.52	0·15	65	0·080	35
Flowering plants with immature fruits	27.7.52	0·14	52	0·13	48
Flowering plants with immature fruits	15.8.52	0·075	45	0·093	55
Fruiting plants:—					
Aerial parts	10.9.52	0·053	32	0·11	68
Leaves and tops	10.9.52	0·065	32	0·14	68
Fruiting plants	7.10.52	0·045	29	0·11	71
Seeds (dried)	7.10.52	0·058	29	0·14	71
Seeds (viable)	7.10.52*	0·090	33	0·18	67

* Date of analysis 16.2.53.

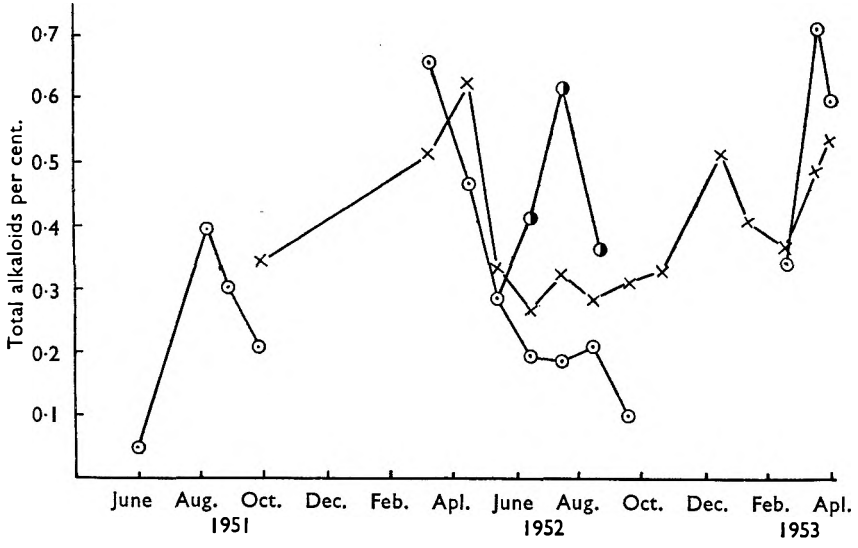

FIG. 1. Variations in total alkaloids of the aerial parts, roots and rootstock and tops of flowering branches of *Atropa belladonna*.

⊙——⊙ Aerial parts.
×——× Root and rootstock.
◑——◑ Tops of flowering branches.

Alkaloid accumulation in the aerial parts during the second year follows a somewhat different course. The concentration decreases rapidly during growth until flowering begins, remains steady during flowering and declines during fruiting. The changes in concentration of total alkaloids in the young tops resemble those in the aerial parts of the first-year plants; a

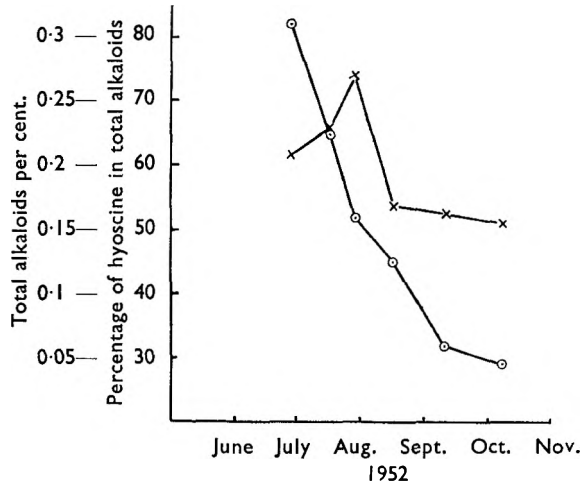

FIG. 2. Variation in the total alkaloids and the percentage of hyoscine in the total alkaloids of *Datura tatula*.

⊙——⊙ Percentage of hyoscine in total alkaloids.

×——× Total alkaloids.

maximum concentration is attained at the period of flowering. It would therefore appear that by excluding the young tops from the aerial parts of the second-year plants, a steady decline in alkaloid concentration would be observed. This implies that there is some parallelism between total

TABLE III

TOTAL ALKALOID PRODUCTION DURING ONE MONTH'S GROWTH OF *A. belladonna*

Date of collection	Average weight of dried part studied		Total alkaloids in dried material		Weight of total alkaloids per individual part	
	Root	Shoot	Root	Shoot	Root	Shoot
	g.	g.	per cent.	per cent.	g.	g.
15.4.52	18	10	0·63	0·47	0·113	0·048
12.5.52	22	67	0·33	0·28	0·072	0·187

alkaloid production and overall growth of the young parts of the plant. In agreement with this, shoots developing on decapitated plants during the period of decline in alkaloid concentration in the normal plant contain roughly 3 times the concentration of alkaloid found in the tops of fruiting branches of the intact plant. The early stages of the third-year plants

resemble those observed during the second year; the concentration falls markedly during about 1 inch growth in 10 days in March.

Changes in alkaloid concentration in the roots were followed during about 18 months from the end of the first season and were found to follow roughly the same pattern as those recorded for the aerial parts.

The ratio of hyoscine to hyoscyamine in *A. belladonna* is not simply related to the quantity of total alkaloids. Seeds in which hyoscine forms only 1 per cent. of the total alkaloids produce seedlings in which hyoscine is the predominant alkaloid. This condition is reversed by the time the young plants have begun to flower and thereafter the proportion of hyoscine in the total alkaloids declines until little or none is detectable in the second- and third-year plants. It is probable that the capacity to produce hyoscine does not disappear completely at any time, since, under the stimulation of new growth produced during the normal growing season, significant quantities are usually produced; this is particularly well demonstrated by the quantity found in the young shoots formed on decapitated plants. These findings provide corroboration of the histochemical evidence for the production of alkaloids in meristematic tissues[4]. The production of hyoscine in the root apparently declines with age.

The changes in the total alkaloids of *D. tatula* var. *inermis* summarised in Table II and Figure 2 show that the concentration rises to a maximum at the time of flowering. In a short series of experiments, similar changes were observed in *D. ferox*.

Changes in the relative proportions of hyoscine and hyoscyamine in *D. tatula* appear to have no simple relationship to the concentration of total alkaloids. From seeds in which hyoscine forms 24 per cent. of the total alkaloids, there are produced seedlings in which this proportion has risen to 82 per cent. From Figure 2, it can be seen that in the latter stages of growth up to fruiting the proportion of hyoscine gradually drops to about 30 per cent. of the total alkaloids and then remains almost steady. Hegnauer[6] has recorded qualitative evidence which agrees with these results.

In *D. ferox* the total alkaloids in the seeds contain 95 per cent. of hyoscine and no meteloidine. On germination, the proportion of hyoscine falls to 50 per cent. and that of meteloidine rises to 26 per cent. of the total alkaloids; in the later stages of growth up to flowering the proportion of hyoscine rises gradually to 85 per cent., whereas meteloidine then forms only 7 per cent. of the total alkaloids. Unidentified bases, other than hyoscine and meteloidine, make up the proportions of total alkaloids not accounted for.

In these 3 solanaceous plants, each of which produces hyoscine, the pattern of changes in its ratio to the other alkaloids differs in each case, although in *A. belladonna* and *D. tatula* there exists the quantitative similiarity of a decrease in its proportion during growth. However, in all cases, changes in the rate of production of alkaloids oxygenated at the 6 and 7 positions of the tropane ring appear to be related to the processes occurring in the plant at times of great metabolic activity.

In *A. belladonna*, where the production of an oxygenated tropane

alkaloid is very small, the site of alkaloid biogenesis is the roots[7]. The aerial parts of *D. tatula* and *D. ferox* are capable of synthesising hyoscyamine and meteloidine respectively and it is probable that hyoscine is formed in the aerial parts of *D. tatula* and in both the roots and aerial parts of *D. ferox*[8].

In the absence of further biological evidence, an interpretation of these results in terms of the various speculations on chemical mechanisms for the biosynthesis of tropane alkaloids[9,10,11,12] is not possible. The results recorded here appear to provide some indication that the product of synthesis is related to the site of synthesis. When synthesis occurs in the aerial parts significant quantities of the oxygenated alkaloids, hyoscine and meteloidine, are produced, whereas in the roots the tendency is towards the production of hyoscyamine with the reduced tropane ring. Our earlier findings[8] on the site of biogenesis of the alkaloids of *D. innoxia* are, however, in direct contradiction to these deductions, since in this plant hyoscine is formed in the roots and hyoscyamine in the aerial parts. Unfortunately no information is available on changes in the ontogenetic production of the alkaloids in this plant. As biological evidence accumulates, it is becoming increasingly apparent that alkaloid synthesis is unlikely to be simply related to the general metabolic processes of the plant.

Summary

1. Changes in the ontogenetic production of the alkaloids of *A. belladonna* and *D. tatula* have been studied.

2. The greatest changes in the ratio of hyoscine to hyoscyamine usually occur at times of greatest meristematic activity. Similar features are to be observed in changes in the ratio of hyoscine and meteloidine in *D. ferox*.

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ALKALOID BIOGENESIS IN TETRAPLOID STRAMONIUM

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Investigations on the biosynthesis of alkaloids in plants have directed attention towards the site in which the synthetic processes take place. In the examination of this problem plants belonging to the Solanaceæ, and particularly those which produce tropane alkaloids, have been extensively employed, and a great deal of information has been obtained by the use of intergeneric grafts. A large number of different graft combinations have been employed and the results have been fully reviewed[1,2].

These experiments have directed attention towards the root as the locus of synthesis. Thus, when grafts are prepared in which *D. stramonium* or *D. tatula* are grown as scions on non-alkaloid-producing stocks, no alkaloid can be detected in any part of the plant; conversely when *D. stramonium* or *D. tatula* are used as the stock and non-alkaloid-producing plants used as the scions alkaloids are present in all parts of the plant in amounts comparable with those found in normal plants of *Datura*. Such experiments have been carried out by a number of workers[3–9], and their results have been confirmed by our own results. Heike[10] grafted *D. stramonium* on to *Nicotiana rustica* and found nicotine in the scion but no tropane alkaloids, as indicated by no mydriatic action. Some conflicting evidence has, however, been reported; Hegnauer[11] found small amounts of alkaloid in all parts of the plant when *D. tatula* was grafted on tomato; similar results are reported by Mothes and Romeike[12] using *D. stramonium* on tomato. A possible explanation of these results may be that different grafting methods were employed, and that the small amounts of alkaloid reported by these workers were produced in the scions while growing on their own roots before being severed from them and connected to the root of the stock.

The weight of evidence, therefore, does appear to suggest that in *D. stramonium* and *D. tatula* the root is the site of alkaloid synthesis; further evidence in support of this hypothesis is given by the exudation of tropane alkaloids from roots of *D. stramonium* grown *in vitro* after the aerial parts have been removed[7].

Investigations on the alkaloid content of species of *Datura* have shown that a large number of factors may influence the amount of total alkaloid present in the plant. Among these factors the influence of polyploidy on the alkaloid content in species of *Atropa*, *Datura* and *Hyoscyamus* was investigated by one of us (J. M. R.)[13] and it was shown that in tetraploid plants considerable increases in alkaloid content are found in the aerial parts when compared with diploid controls. This fact has been confirmed for *D. stramonium* and *D. tatula* by later workers[14,15]. When this work was commenced in October 1950, no information was available

on the influence of polyploidy on the alkaloid content of the roots of plants of any of these genera; however, Steinegger[16] has recently (December 1952) published a value, viz. 0·127 per cent., for the alkaloid content of the tetraploid root of *Datura stramonium*.

In view of the evidence suggesting the root as the locus of synthesis in *D. stramonium* and *D. tatula* it seemed of interest to investigate the relationship between the alkaloid content in the root and in the aerial parts in diploid and tetraploid plants of these species, and to compare the results of analyses of alkaloid content of root and aerial parts between individual diploid and tetraploid plants, and to confirm the conclusions by making graft experiments. It was hoped that in this way further information could be obtained on the locus of synthesis of the alkaloids and that this could be related to the increased alkaloid content of the aerial parts of tetraploid plants.

Experimental

The experiments were designed along two main lines:

(*a*) To compare the amount of total alkaloid present in the roots and in the aerial parts of diploid and tetraploid plants of *Datura stramonium* and *D. tatula* for individual plants grown under identical conditions.

(*b*) To produce series of reciprocal grafts between diploid and tetraploid plants of Stramonium 2ns/4ns, 4ns/2ns; also to produce grafts between diploid and tetraploid plants of the two species and diploid and tetraploid tomato plants as follows:—

1. Diploid tomato scion on diploid *D. stramonium* (or *D. tatula*) stock (2nt/2ns).
2. Diploid tomato scion on tetraploid *D. stramonium* (or *D. tatula*) stock (2nt/4ns).
3. Tetraploid tomato scion on diploid *D. stramonium* (or *D. tatula*) stock (4nt/2ns).
4. Tetraploid tomato scion on tetraploid *D. stramonium* (or *D. tatula*) stock (4nt/4ns).

thus to compare the amount of total alkaloid present in the roots and in the aerial parts (i.e., stocks and scions) of each graft with the results obtained in (*a*). In addition, a number of grafts of diploid stramonium scions on diploid tomato stocks were prepared (2ns/2nt).

On harvesting the grafts of diploid tomato scion on diploid stramonium root in 1952 it was found that two of the tomato scions had rooted at the graft union, so that two active root systems were present. These were collected and analysed separately.

In the production of the tetraploid plants of the *Datura* species 3 plants were obtained which on examination were found to be branch chimeras, i.e., tetraploid plants with diploid branches. These plants were allowed to grow to maturity, when the tetraploid and diploid branches were collected and analysed separately.

5 samples of *Datura stramonium* seed and 3 samples of *Datura tatula* seed used in these experiments were obtained from different geographical

sources. Different samples are indicated by the letters A, B, C, D, E for the *D. stramonium* seed, and Q, R, S, for the *D. tatula* seed. One sample of tomato seed, var. Sunrise, was used throughout. For all the samples the mature diploid plants conformed to the type descriptions of the species.

Production of tetraploids. In 1951 and 1952 tetraploidy was induced in seeds of both species of *Datura* by soaking in 0·8 per cent. aqueous solution of colchicine for 4 days, the seeds having previously been soaked in water for about 12 hours. In the treatment of the tomato seeds the same concentration of colchicine solution was used, but the seeds were allowed to remain in the solution until germination, after which they were removed from the solution and sown in the usual manner; it was found that a greater number of tetraploids could be obtained by this method.

After colchicine treatment and subsequent germination of the seeds, seedlings were selected which showed swelling in the region of the hypocotyl; in most cases these were later found to be tetraploid.

In addition to the use of colchicine-treated seed, in 1952 the filial generation (F_1), was grown from seed set by tetraploids produced in 1951. Similarly, seed was collected from the diploid plants in 1951 and used for some of the 1952 sowings. Tetraploidy was proved in the maturing plants by measurements of stomatal size, and confirmed in the majority of the plants by measurements of the pollen grain diameter. Previous workers[17] have shown that increase in the size of the stomata and of the pollen grains when compared with diploid control plants are good criteria of tetraploidy. Similar methods were used to confirm the tetraploid nature of tetraploid scions when grown on diploid stocks. In the reciprocal grafts, however, the aerial parts of the tetraploid stocks were cut off before sufficiently mature for measurement of stomatal sizes to be possible; for these grafts, therefore, chromosome counts were performed on root tip preparations obtained from the stock roots a short time before harvesting of the graft, using the aceto-lacmoid squash technique.

All the tetraploid plants grew well and apart from an occasional stunted plant, were as vigorous as the corresponding diploid plants. The average weight of dried material obtained per plant was approximately equal to that obtained from the diploid controls.

Method of Grafting. The grafts were carried out on young seedlings just past the cotyledon stage, i.e., with the first pair of leaves apparent but not fully expanded. The method known as side grafting was employed. The seedling to be used as the scion was cut off about 1 to 2 cm. below the growing point, and the stem shaped to form a wedge. An oblique cut penetrating to about the centre was made in the hypocotyl of the seedling to be used as the stock at a point about 1 cm. below the cotyledons. The scion shoot was inserted in this cut and the two stems firmly bound together with wet bast using 2 or 3 separate ties. The area was wrapped round loosely with more wet bast and left for 1 to 2 weeks. At the end of this period the aerial shoot of the stock was cut off just above the graft union, and new bast ties were applied as necessary to prevent constrictions in the expanding stem. After about a month

complete union had in most cases been effected, and no further ties were necessary.

In both growing seasons the majority of the grafted plants grew well, although a number of the tetraploid scions remained somewhat stunted and produced very few flowers.

TABLE I

Datura stramonium 2n AND 4n

STOMATAL SIZES AND ALKALOIDAL CONTENTS (per cent. dry weight)

Plant sample	Diploid control			Tetraploid		
		Alkaloidal content			Alkaloidal content	
	Stomatal size μ	Aerial parts	Root	Stomatal size μ	Aerial parts	Root
1951.						
DSC 1	38 × 27	0·34	0·13	49 × 35	0·49	0·20
2	38 × 23	0·32	0·15	56 × 35	0·40	0·32
3	33 × 22	0·38	0·18	47 × 35	0·27	0·29
4	37 × 24	0·34	0·13	49 × 32	0·39	0·17
5	35 × 24	0·18	0·06	48 × 32	0·45	0·16
6	36 × 28	0·13	0·12	52 × 35	0·42	0·20
	—	—	—	55 × 36	0·32	0·39
	—	—	—	50 × 32	0·46	0·18
DTR 1	37 × 25	0·47	0·18	49 × 37	0·76	0·24
2	35 × 24	—	—	45 × 31	0·66	0·18
1952.						
DSBI 1	29 × 22	0·14	0·05	41 × 28	0·25	0·11
(F_1) 2	37 × 24	0·12	0·04	45 × 35	0·38	0·20
3	28 × 21	0·20	0·06	43 × 30	0·30	0·13
4	34 × 23	0·16	0·08	41 × 27	0·33	0·14
5	—	—	—	43 × 28	0·23	0·13
6	—	—	—	46 × 28	0·27	0·18
7	—	—	—	44 × 28	0·28	0·09
8	—	—	—	49 × 33	0·23	0·15
9	—	—	—	51 × 32	0·23	0·12
10	—	—	—	49 × 29	0·25	0·09
DTR 1	34 × 23	0·32	0·14	40 × 31	0·59	0·14
(F_1) 2	35 × 24	0·29	0·16	48 × 31	0·45	0·15
3	33 × 22	0·35	0·11	48 × 32	0·55	0·18
4	31 × 22	0·35	0·14	49 × 32	0·38	0·19
5	33 × 22	0·33	0·15	47 × 34	0·65	0·26
6	35 × 23	0·30	0·11	49 × 32	0·55	0·22
1952–53.						
DSB 1	37 × 25	0·14	0·12	45 × 28	0·17	0·16
2	34 × 23	0·16	0·08	47 × 30	0·21	0·10
3	33 × 22	0·15	0·09	43 × 29	0·20	0·21
4	32 × 22	0·15	0·09	41 × 28	0·20	0·08
5	—	—	—	46 × 29	0·18	0·08

Cultural details. Colchicine-treated and untreated seeds of all the samples of *D. stramonium*, *D. tatula* and tomato were sown in March–April in John-Innes seed compost in a cool greenhouse. The seedlings were pricked off into boxes and planted out in the open in May–June. During the 1951 season the grafted plants were not planted out in the open but were potted on in John-Innes potting compost and kept in a cool greenhouse; some ungrafted tetraploid and diploid plants from the same seed sample were also kept in the greenhouse throughout to act as controls. Owing to the somewhat inconsistent results obtained from the analyses of

the tetraploid plants grown throughout in the greenhouse (see Table II) it was decided in the 1952 season to transplant all the plants into the open, the grafted plants being planted out as soon as the union was complete and bast ties were no longer necessary.

In both seasons 1951 and 1952 the complete set of experiments was carried out at two sites, one in London and the other in Derbyshire; at both sites the beds to which the plants were transplanted had received only the normal routine manuring treatment. Tetraploid and diploid samples, together with the grafts, were grown side by side in the same beds.

TABLE II

Datura stramonium 2n AND 4n
RANGE OF INDIVIDUAL PLANT ESTIMATIONS (cf. Table I)
ALKALOIDAL CONTENTS
(Per Cent. Dry Weight)

Plants	Aerial parts		Roots	
	Control	4n	Control	4n
1951.				
DSA plot ..	0·23–0·34–0·47	0·22–0·40–0·68*	0·10–0·14–0·17	0·10–0·14–0·19
DSA greenhouse	0·10–0·17–0·25	0·14–0·24–0·30*	0·06–0·11–0·17	0·08–0·09–0·11
DSB	0·26–0·31–0·34	0·19–0·30–0·45	0·11–0·14–0·20	0·12–0·15–0·21
DSC plot ..	0·13–0·28–0·38	0·26–0·39–0·49*	0·06–0·13–0·18	0·16–0·22–0·39*
DSC greenhouse	0·19–0·23–0·31	0·18–0·22–0·29	0·07–0·10–0·13	0·05–0·10–0·13
DSE plot ..	0·19–0·26–0·33	0·23–0·27–0·36	0·07–0·08–0·10	0·11–0·16–0·24*
DSE greenhouse	0·27–0·29–0·33	0·76*	0·09–0·12–0·15	0·20*
DTR	0·47	0·65–0·70–0·76*	0·18	0·18–0·21–0·24*
1952.				
DSB	0·13–0·17–0·20	0·09–0·22–0·37*	0·03–0·06–0·08	0·04–0·08–0·11
DSB I (F_1) ..	0·12–0·15–0·20	0·17–0·27–0·38*	0·04–0·06–0·08	0·09–0·13–0·20*
DSB II (F_1) ..	—	0·22–0·28–0·36*	—	0·10–0·12–0·13*
DSE	—	0·18–0·19–0·20	—	0·08
DTQ	0·34–0·39–0·41	0·12–0·20–0·26	0·11–0·13–0·16	0·11–0·22–0·32*
DTR (F_1) ..	0·29–0·32–0·35	0·38–0·53–0·65*	0·11–0·13–0·16	0·14–0·19–0·26*
DTS (F_1) ..	0·16–0·23–0·32	0·31–0·32–0·33*	0·08–0·11–0·14	0·20–0·21–0·22*
1952–53.				
DSB	0·14–0·15–0·16	0·17–0·19–0·21*	0·08–0·10–0·12	0·10–0·16–0·21*
DTR (F_1) ..	0·19–0·22–0·24	0·63*	0·09–0·10–0·11	0·14*

* Indicates significant increase compared with 2n control.

The 1952–53 experiment was commenced late in the season of 1952 and consequently the plants could not be transplanted to the open. All the plants were kept in a cool-warm greenhouse for the whole period of their growth, and artificial illumination was supplied, giving a daily light period of 10 hours. This experiment was carried out in London only.

All plants were harvested when fully grown and still in flower. The whole plant was dug up and separated into root and aerial parts; the more mature fruits and the thicker parts of the stems were discarded. In harvesting the grafted plants the stock and scion were separated at the graft union and the small portion of hypocotyl remaining at the top of the root was discarded. The tomato on stramonium grafts in which rooting of the tomato scion had occurred from the graft union were dug up and the scion roots carefully separated from those of the stock; similarly the tetraploid and diploid branches of the branch chimeras were carefully

separated from one another. All the root samples were washed free from soil, and together with the aerial parts, dried in a current of air at 55° C. Immediately after drying, each sample was reduced to a moderately coarse powder and stored in a well-closed container from which moisture was excluded by silica-gel. Roots and aerial parts of individual plants were kept separate throughout.

Method of estimation. The colorimetric method of Allport and Wilson[18] with slight modifications, was employed throughout this work to determine the amount of total solanaceous alkaloids present in the samples. A Unicam quartz spectrophotometer was used to measure the colour intensity; 1 cm. cells were used throughout. Preliminary experiments showed that the optimum wavelength for measurement of the colour was 555 mμ. Using a sample of pure atropine alkaloid, the relation between colour intensity and concentration of solanaceous alkaloid was obtained for a series of concentrations ranging from 0·2 to 1·6 mg. per cent. The graph of these results was a straight line (Fig. 1), showing that between these concentrations the reaction obeys the Beer-Lambert Law, K (1 mg. per cent. solanaceous alkaloid) = 0·652.

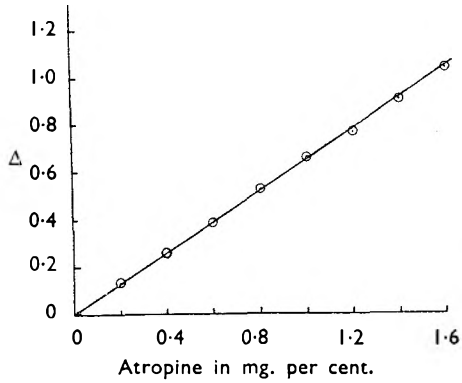

FIG. 1.

In the analyses of the plant samples certain modifications in the quantities of reagents used were necessary to allow for the wide variations in the alkaloid content. These variations from the standard method were shown, by estimation of known mixtures of atropine alkaloid with an inert substance (powdered grass) and by comparison with results obtained by the Pharmacopœial method of assay, not to affect the accuracy of the results.

RESULTS

The results are set out in Tables I to VII. Under the heading "plant sample" the initials DS are used to indicate *Datura stramonium* and DT to indicate *Datura tatula;* the third letter in each case represents the sample of seed used. The symbol (F_1) indicates plants raised from seed obtained from diploid and tetraploid plants grown in the previous year; hence when the symbol (F_1) is not given the tetraploid plants were obtained by direct colchicine treatment of the seeds. The samples DSBI (F_1) and DSBII (F_1) were grown in 1952 from seed collected in 1951 from 2 diploid and 2 tetraploid plants of DSB.

Table I gives examples of stomatal sizes and total alkaloid content of aerial parts and roots of individual diploid and tetraploid plants grown in 3 different seasons. Similar results have been summarised in Table II;

in the 1951 crop comparative analyses are given for teploitraploid and did plants from the same samples transplanted to the open (plot) and kept in the greenhouse. Table III gives the analyses of the 3 plants which had

TABLE III

4n DATURAS WITH 2n BRANCHES
ALKALOIDAL CONTENT
(Per Cent. Dry Weight)

	Aerial parts 2n branch	Aerial parts 4n branch	Roots (4n)
1951 DSC ..	0·24	0·34	0·18
1951 DSD ..	0·33	0·46	0·11
1952 DTQ ..	0·17	0·26	0·12

TABLE IV

Datura stramonium GRAFTS I
Scion = 2n plants. Stock = 4n plants
ALKALOIDAL CONTENT
(Per Cent. Dry Weight)

Plant sample	Aerial parts	Roots
1951.		
DSC Control 2n plants ..	0·19–0·23–0·31	0·07–0·10–0·13
Graft 1	0·22	0·06
" 2	0·19	0·07
" 3	0·17	0·13
DSE Control 2n plants ..	0·27–0·29–0·33	0·09–0·12–0·15
Graft 1	0·36*	0·11
" 2	0·43*	0·18*
1952.		
DSA Control 2n plants ..	0·30–0·48–0·59	0·05–0·12–0·16
Graft 1	0·19	0·19*
DSBI Control 2n plants ..	0·12–0·15–0·20	0·04–0·06–0·08
(F_1) Graft 1	0·24*	0·04
" 2	0·23*	0·05
" 3	0·18	0·06
DTS Control 2n plants ..	0·16–0·23–0·32	0·08–0·11–0·14
(F_1) Graft 1	0·39*	0·13
" 2	0·29*	0·13
" 3	0·36*	0·12
" 4	0·32*	0·17*
1952–53.		
DSB Control 2n plants ..	0·14–0·15–0·16	0·08–0·10–0·12
Graft 1	0·42*	0·16*
" 2	0·19*	0·11
DTR Control 2n plants ..	0·19–0·22–0·24	0·09–0·10–0·11
(F_2) Graft 1	0·41*	0·13*

* Indicates significant increase compared with 2n control.

diploid and tetraploid branches. The results of the graft experiments are set out in Tables IV to VII. The grafts on tomato stocks are not included as in every instance no trace of solanaceous alkaloid was found in either the tomato stock or the *Datura* scion. Analyses of the tomato roots from the 2 control grafts (diploid tomato scions on diploid stramonium stocks)

which had rooted at the graft union showed a small amount of solanaceous alkaloids (about 0·02 per cent. dry weight) to be present in each sample.

Discussion of Results

The results given in Tables I and II, which are obtained from analyses of more than 200 individual plants, grown in 3 successive years, support previous work showing that in general there is an increase in alkaloid content in the aerial parts of tetraploid plants of *Datura stramonium* and

TABLE V

Datura stramonium GRAFTS II

Scion = 4n plants. Stock = 2n plants

ALKALOIDAL CONTENT

(Per Cent. Dry Weight)

Plant sample	Aerial parts	Roots
1951.		
DSA Control 2n plants ..	0·10–0·17–0·25	0·06–0·11–0·17
Grafts 1–8	0·14–0·17–0·20	0·06–0·09–0·13
DSC Control 2n plants ..	0·19–0·23–0·31	0·07–0·10–0·13
Graft 1	0·33*	0·37*
" 2–8	0·17–0·21–0·25	0·08–0·12–0·16
DSE Control 2n plants ..	0·27–0·29–0·33	0·09–0·12–0·15
Graft 1	0·34*	0·15
" 2	0·38*	0·21*
" 3	0·22	0·10
" 4	0·21	0·11
1952.		
DSA Control 2n plants ..	0·30–0·48–0·59	0·05–0·12–0·16
Graft 1	0·67*	0·16
" 2–6	0·35–0·46–0·50	0·12–0·14–0·17
DSBI Control 2n plants ..	0·12–0·15–0·20	0·04–0·06–0·08
(F_1) Graft 1	0·28*	0·04
" 2	0·18	0·06
DTS Control 2n plants ..	0·16–0·23–0·32	0·08–0·11–0·14
(F_1) Graft 1	0·14	0·16*
1952–53.		
DSB Control 2n plants ..	0·14–0·15–0·16	0·08–0·10–0·12
Graft 1	0·16	0·11
" 2	0·26*	0·14*
" 3	0·33*	0·08
" 4	0·32*	0·13*
DTR Control 2n plants ..	0·19–0·22–0·24	0·09–0·10–0·11
(F_1) Graft 1	0·23	0·09

* Indicates significant increase compared with 2n control.

D. tatula when compared with diploid controls; this increase was found to occur both in tetraploid plants produced by colchicine treatment of the seed, and in the tetraploid plants of the first filial generation (F_1). Occasional abnormal behaviour was observed; for example, very little increase in alkaloid content was found in the aerial parts of any of the induced tetraploid plants of the DSB series grown in 1951, although significant increases occurred in 1952 and 1952–53, and the F_1 generations from two tetraploid plants (not analysed in 1951) when grown in 1952 showed considerable increases (Tables I and II). The DSE series in 1951 showed

no alkaloid increase in tetraploid plants grown on the plot, but a single plant grown in the greenhouse showed considerable increase; no increases occurred in induced tetraploid plants of the same series in 1952 (Table II). Similarly, induced tetraploid plants of the DTQ series grown in 1952 showed no alkaloid increase in the aerial parts. Differences between the behaviour of plants grown on the plot and those grown in the greenhouse have already been indicated for the DSE series, and Table II gives comparable results for the DSA and DSC series also. Thus while the DSA

TABLE VI

TOMATO: STRAMONIUM GRAFTS I

Control Grafts:—Scion = 2n tomato, Stock = 2n stramonium
Test Grafts Scion = 4n tomato, Stock = 2n stramonium

ALKALOIDAL CONTENT
(Per Cent. Dry Weight)

Plant sample	Aerial parts	Roots
1951.		
DSA Control 2n stramonium plants	0·10–0·17–0·25	0·06–0·11–0·17
Control grafts 1–5	0·17–0·22–0·28	0·05–0·07–0·08
Test graft 1	0·36*	0·12*
" " 2	0·17	0·11*
" " 3	0·17	0·08
DSC Control 2n stramonium plants	0·19–0·23–0·31	0·07–0·10–0·13
Control grafts 1–6	0·21–0·27–0·35	0·08–0·10–0·15
Test graft 1	0·44*	0·20*
DSE Control 2n stramonium plants	0·27–0·29–0·33	0·09–0·12–0·15
Control grafts 1–3	0·18–0·22–0·24	0·15–0·17–0·18
1952.		
DSA Control 2n stramonium plants	0·30–0·48–0·59	0·05–0·12–0·16
Control grafts 1–4	0·20–0·21–0·23	0·09–0·12–0·14
Test graft 1–2	0·14–0·16–0·18	0·12
DSC Control 2n stramonium plants	0·23–0·32–0·39	0·09–0·11–0·15
Control grafts 1–4	0·20–0·23–0·25	0·06–0·09–0·12
Test graft 1–2	0·10–0·12–0·14	0·06–0·07–0·08
1952–53.		
DSB Control 2n stramonium plants	0·14–0·15–0·16	0·08–0·10–0·11
Control grafts 1–2	0·10	0·08
Test graft 1	0·16*	0·12*
" " 2	0·21*	0·09

* Indicates significant increase compared with 2n control.

plants showed normal increases in alkaloid content in the aerial parts of tetraploid plants grown both on the plot and in the greenhouse when compared with diploid controls, tetraploid plants of the DSC series showed no increases when grown in the greenhouse, but significant increases when grown on the plot.

The results obtained by analyses of the scions and stocks of the reciprocal grafts between diploid stramonium and diploid tomato plants support those already reported by other workers[5–9]. Alkaloids were present in all the tomato scions and in the majority of them the amount was approximately equal to that found in normal diploid *Datura* plants (Tables VI and VII). This, and the absence of alkaloids in any of the *Datura* scions

grown on tomato stocks, further support the hypothesis that the alkaloids are synthesised in the root in these species.

Comparison of the total alkaloid content of the roots of tetraploid and diploid plants shows that in general the roots of tetraploid plants were considerably richer in alkaloids than the corresponding diploid controls (Tables I and II). Among the tetraploid plants which showed no increase in alkaloid content in the aerial parts some likewise showed no increase

TABLE VII

TOMATO: STRAMONIUM GRAFTS II

Control Grafts:—Scion = 2n tomato, stock = 2n stramonium
2:4 Test Grafts:—Scion = 2n tomato, stock = 4n stramonium
4:4 Test Grafts:—Scion = 4n tomato, stock = 4n stramonium

ALKALOIDAL CONTENT
(Per Cent. Dry Weight)

Plant sample	Aerial parts	Roots
1951.		
DSA Control 2n stramonium plants	0·10–0·17–0·25	0·06–0·11–0·17
Control grafts 1–5	0·17–0·22–0·29	0·05–0·07–0·08
2:4 Test graft 1	0·22	0·10*
4:4 Test graft 1	0·16	0·18*
DSC Control 2n stramonium plants	0·19–0·23–0·31	0·07–0·10–0·13
Control grafts 1–6	0·21–0·27–0·35	0·08–0·10–0·15
2:4 Test grafts 1–2	0·21	0·07
1952.		
DSA Control 2n stramonium plants	0·30–0·48–0·59	0·05–0·12–0·16
Control grafts 1–4	0·20–0·21–0·23	0·09–0·12–0·14
2:4 Test grafts 1–2	0·12–0·17–0·21	0·11–0·13–0·14
DTS Control 2n stramonium plants	0·16–0·23–0·32	0·08–0·11–0·14
(F_1) Control grafts 1–5	0·09–0·15–0·32	0·11–0·14–0·17
2:4 Test grafts 1–2	0·11	0·09
4:4 Test graft 1	0·29*	0·16*
1952–53.		
DSB Control 2n stramonium plants	0·14–0·15–0·16	0·08–0·10–0·12
Control grafts 1–2 ..	0·10	0·08
2:4 Test graft 1	0·19*	0·15*
DTR Control 2n stramonium plants	0·19–0·22–0·24	0·09–0·10–0·11
(F_1) Control grafts 1–2	0·05	0·07–0·08–0·09
2:4 Test grafts 1	0·05	0·07
2	0·11*	0·05*
4:4 Test graft 1	0·13*	0·12*

* Indicates significant increase compared with 2n control.

in the roots (DSB 1951, DSC greenhouse, Table II); others showed considerable increases (DSE plot, 1951, DTQ 1952, Table II). Conversely some of the tetraploid plants which were richer in alkaloid in the aerial parts did not show a corresponding increase in the roots (DSA plot, 1951, DSB 1952, Table II).

On the hypothesis that the root is the site of alkaloid synthesis it would appear, therefore, that in tetraploid plants there is increased alkaloid production by the tetraploid roots; this is accompanied by an increase in the alkaloid content of the aerial parts. Examination of the graft experiments in which tetraploid stramonium plants were used as stocks shows that in the 2ns/4ns series, 11 out of the 16 diploid scions had increases in

alkaloid content when compared with control plants grown on diploid roots (Table IV); these increases would appear to be due to the influence of the tetraploid stocks. A smaller number of the stocks showed definite increases in alkaloid content when compared with diploid controls, but the amounts present are comparable in all cases with those found in normal tetraploid plants (cf. Tables I and II). In the tomato on stramonium grafts, 2 tomato scions of the 2nt/4ns series were somewhat richer in alkaloid content than those of the control grafts 2nt/2ns (Table VII); one of these showed an increase also in the alkaloid content of the tetraploid stock. The remaining 7 grafts showed no increase in either the scions or stocks. These results suggest, therefore, that a diploid tomato scion when grown on a tetraploid stramonium stock has an alkaloid content equivalent to that found when grown on a diploid stramonium stock and that in addition the diploid tomato scion has a depressant effect on the alkaloid content of the tetraploid stramonium stock. This contrasts markedly with the results obtained in the 2ns/4ns series. Analyses of the 4nt/4ns grafts (Table VII) showed some increase in alkaloid content in 2 of the scions and in all the stocks; hence it appears that a tetraploid tomato scion does not depress the alkaloid content of a tetraploid stramonium stock to the same extent as does a diploid tomato scion.

The results of the graft experiments in which diploid stramonium plants were used as stocks are given in Tables V and VI. In the 4ns/2ns series (Table V) 25 grafts showed alkaloid contents in both scions and stocks equal to those found in normal diploid plants; one graft (DSC 1) showed remarkable increase in alkaloid content in the stock and some increase in the scion; 7 other grafts showed increases in the scions, and 4 of these had corresponding increases in the stocks; one other had alkaloid enrichment in the stock but not in the scion. Among the 4nt/2ns grafts (Table VI), 5 had alkaloid contents equivalent to diploid controls in both scions and stocks; 4 showed alkaloid enrichment in the scions and 3 of these also showed increases in the stocks; one other had increased alkaloid content in the stock but not in the scion. The general inference from these results, therefore is that when diploid stramonium plants are used as stocks the alkaloid content in both scions and stocks is equivalent to that of normal diploid plants although there is some indication of an influence by tetraploid scions, resulting in increased alkaloid content in both scions and stocks in some of the grafts.

The results of the analyses of the branch chimeras, given in Table III, further suggest that the tetraploid or diploid nature of the aerial parts influences the alkaloid content; in all 3 plants in which tetraploid and diploid branches were growing on the same root system the tetraploid branch was found to have a higher alkaloid content than the diploid branch. This is possibly due to an increased storage capacity by the tetraploid branch, although it does not preclude the possibility of differential rates of synthesis occurring in the branches.

Recent work by Evans and Partridge[19] using reciprocal grafts between *Datura tatula* and *D. ferox* has indicated that the aerial parts of *D. tatula* exhibit some faculty for the synthesis of hyoscine and hyoscyamine. While

the results of our graft experiments strongly suggest that the main alkaloid synthesis does take place in the roots of *D. stramonium* and *D. tatula*, the capacity of the aerial parts to synthesise may be an explanation of the scion influence if it is assumed that this capacity is increased by tetraploidy. It was shown by Warren-Wilson[9] that downward translocation of alkaloids does occur in *Atropa belladonna*; the presence of alkaloids in the tomato roots growing from the graft union on the 2nt/2ns grafts shows that downwards translocation also occurs in the *Datura* species. Thus the alkaloid content of the root system must be regarded as a balance between rate of synthesis therein, removal upwards in the transpiration stream, and downward translocation with other elaborated storage products. Similarly the leaf content of alkaloid is a balance between alkaloid received in the transpiration stream and that removed by downward translocation, with the possibility, not yet proved, of some alkaloid synthesis in the leaf.

Summary

1. Previous work showing a considerable increase in alkaloid content in the aerial parts of tetraploid plants of *Datura stramonium* and *D. tatula* compared with diploid controls has been confirmed, a maximum increase of 286 per cent. above 2n mean being recorded in an F_1 plant.

2. When diploid *D. stramonium* scions were side-grafted on diploid tomato stocks no trace of solanaceous alkaloid was found in either the scions or stocks. In the reciprocal grafts alkaloids were present in both stocks and scions in amounts equivalent to those found in normal diploid *Datura* plants.

3. Comparison of the total alkaloid content of the roots of tetraploid and diploid plants of *D. stramonium* and *D. tatula* showed that the roots of tetraploid plants were considerably richer in alkaloids than the corresponding diploid controls, the increase being up to threefold.

4. Reciprocal grafts between tetraploid and diploid *D. stramonium* suggest that the 4n or 2n nature of the stock determines the alkaloid content of the scion and stock. Thus in 2ns/4ns grafts the alkaloid content of scion and stock was equivalent to that found in normal 4n plants; in 4ns/2ns the alkaloid content was equivalent to that found in normal 2n plants. Some divergent results were obtained indicating possible influence by the scion on alkaloid content of both stock and scion.

5. In grafts 2nt/4ns the tomato scion appears to have a depressant effect on the alkaloid content of the 4n stramonium stock; this effect is less marked when 4n tomato is used as the scion.

6. In branch chimeras the 4n branch is richer in alkaloid than the 2n branch growing on the same root.

7. The possible capacity of the aerial parts to synthesise alkaloids is discussed.

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F. L. C. Blackwall their thanks are due for continued horticultural assistance.

This communication forms part of the subject-matter of a thesis to be presented by one of us (B. P. J.) for the degree of Doctor of Philosophy in the Univeristy of London.

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DISCUSSION

The two papers on Alkaloid Biogenesis were taken together, the first being presented by DR. W. C. EVANS and the second by MISS B. P. JACKSON.

DR. W. MITCHELL (London) pointed out that in their concluding sentence the authors of the first paper suggested that alkaloid formation was probably not related simply to the metabolic processes of the plant. Was it, then, their opinion that alkaloids were waste products, as had so long been the general view? Presumably if the relation were not direct they were not waste products but more probably were utilised in some way in the metabolic cycle. The fact that the alkaloid content often reached a maximum at flowering suggested that they played an active part, especially since the content thereafter usually diminished. Again, why should solanaceous plants pack their seeds with alkaloids if they were waste products? He asked the age of the belladonna seedlings when it was found that 89 per cent. of the total alkaloids was hyoscine, and at what stage the drop in alkaloidal content took place. The quantities of hyoscine in *Atropa belladonna* at all stages were very small and they appeared to have been determined by titrimetric methods. Could the authors be certain that that material really was hyoscine? In particular, had they been able to collect enough to prepare a crystalline derivative such as the aurichloride? Only thus could one be really satisfied as to the identity of the alleged hyoscine. In the last sentences of the second paper, reference was made to alkaloids being translocated to the root amongst other "storage products." Could it be inferred

that these authors also did not class vegetable alkaloids as waste products in all cases?

DR. K. BULLOCK (Manchester) asked whether the authors of the second of the papers had yet reached the stage of being able to compare the ordinary and tetraploid plants in terms of yield of alkaloid per acre. Secondly, had they ever tried expressing their results in terms of alkaloidal nitrogen as a percentage of total nitrogen, and, if so, had that yielded any interesting results?

DR. T. E. WALLIS (London) said that the authors confirmed the customary method of collecting belladonna when flowering commenced. Possibly the plant made some use of the alkaloids in its metabolism, and in that connection he noticed how differently *D. tatula* and *D. erox* behaved when they were germinating. *D. tatula* seemed to accumulate hyoscine during germination, whereas in *D. ferox* the hyoscine decreased. That looked as though one of the plants was using hyoscyamine to do something in its metabolism and the other was using hyoscine for a similar purpose. Apparently the authors of the first paper found that *Atropa belladonna* on the whole produced its alkaloid in the roots, whereas the *D. tatula* plants produced their alkaloid both in the stems and the root. He wondered whether the authors had considered the fact that belladonna was a perennial plant and *D. tatula* and *D. ferox* were annual plants.

DR. R. RUYSSEN (Belgium) said that one of his workers had discovered that the rate of biosynthesis of the alkaloids of cinchona in leaves of young plants was almost parallel to that of carbohydrates, which were studied using ^{14}C, under the same conditions of light, temperature and season.

DR. W. C. EVANS, commenting on the other paper, said that the problem of the site of the biogenesis of alkaloids was associated with difficulties in experimentation as well as in interpreting the results. In a previous publication they (Evans and Partridge) had shown that *D. ferox* scions grafted on to *D. tatula* roots accumulated hyoscyamine in the leaves, and that had been taken as direct evidence for the synthesis of hyoscyamine in *D. tatula* leaves. He considered that the paper under discussion afforded considerable evidence of the formation of alkaloids in the leaves of *D. tatula* and *D. stramonium*. In that respect the branch chimeras were very interesting plants. Here there were plants which had both $4n$ and $2n$ branches on $4n$ stocks. From the analysis of those plants the authors suggested that increased storage capacity was possibly the reason for the increase in alkaloid content, but he did not agree because solanaceous leaves were capable of storing large quantities of injected hyoscine or hyoscyamine without any ill effects on the plant itself. Tables VI and VII served to emphasise the necessity for extreme caution in interpreting results based on grafts between plants of different genera and plants which normally produced alkaloids and those which did not. In fact, all the results in the two tables could be used as evidence that it was the tomato leaves which synthesised the alkaloids as a result of precursors or stimulation from the stramonium stock, and that would

be particularly well borne out by the fact that 4*n* tomato scions when grafted on to 4*n* stocks produced more alkaloid than when the 2*n* scions were grafted on to the same stocks. This hypothesis was not very probable, but it showed that reliable information on the site of synthesis could not be obtained from somewhat unnatural material. Turning to the experimental procedure, he considered it a great pity that the authors did not determine both the hyoscine and the hyoscyamine present in the plants rather than just total alkaloid, because the crucial point was that it had not yet been proved that both hyoscine and hyoscyamine were in fact synthesised in the same organ in the stramonium plants. Their previous work had shown that in *D. tatula* different alkaloids were synthesised in different organs, and therefore estimations of total alkaloids were liable to give confusing results. That could be one reason for the rather numerous inexplicable results which the authors had reported in their paper.

MR. H. B. WOODHEAD (Manchester) said that it would be interesting to investigate the transpiration streams to find out whether the alkaloids or their precursors were transported in the plant.

DR. W. C. EVANS in reply said that so far as solanaceous alkaloids were concerned it was not known whether they played any part in the general metabolism or were merely waste products. They might exist in equilibrium with other substances, and as soon as they were formed perhaps they might be changed into something else, in which case, of course, they were important so far as the metabolism of the plant was concerned. The age of the belladonna seedlings when the alkaloidal content consisted of 89 per cent. hyoscine was about 6 or 8 weeks. They had no doubt that the alkaloid expressed as hyoscine was, in fact, hyoscine. In a fair number of cases this had been proved by the preparation of the aurichloride or picrate. Dr. Wallis's point was very interesting and had engaged the attention of the authors. They were aware of the fact that *D. tatula* seemed to build up hyoscine whereas in *D. ferox* the amount of alkaloid tended to decrease. It had to be borne in mind in recording such results that the percentage of alkaloids expressed as dry weight might give misleading results from the point of view of the amount of alkaloid which was actually in the plant. He doubted whether reliable conclusions could be drawn from analyses of the alkaloids found in the transpiration stream.

MISS B. P. JACKSON, in reply, said they had not themselves determined the yield per acre of the diploid and tetraploid plants, but this was being investigated by other workers and there was little doubt that the yield of alkaloid per acre was considerably higher in tetraploid plants than in diploid plants. The alkaloidal nitrogen had not been determined as a percentage of total nitrogen. Evans and Partridge in an earlier paper stated that hyoscyamine was synthesised in the leaves of *D. tatula*, but examination of their results showed that the quantity of hyoscyamine found in the grafts of *D. tatula* grown on *D. ferox* stocks was very small in two cases and moderate in the third. It seemed possible that the hyoscyamine had been carried over during the process of grafting, as

she had noted that in the method used the scions were allowed to remain on their own roots for a period of 5 days before being severed. She agreed that the results with the branch chimeras were indicative of there being a differential rate of synthesis of the alkaloids in the aerial parts. However, the results of the grafts using tomato seemed to provide overwhelming evidence of synthesis in the roots, and the complete absence of alkaloid in *D. stramonium* when grown on tomato root did, in her view, suggest that a *D. stramonium* top was incapable by itself of producing alkaloid.

DR. J. M. ROWSON pointed out that he and Miss Jackson had made use of interspecific and intergeneric grafts, whereas Dr. Evans had omitted in his earlier paper to include any intergeneric grafts. It would be interesting to investigate the effect of grafting *D. ferox* on a non-alkaloid producing plant.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Ascorbic Acid, Determination of. G. di Bacco. (*Boll. chim.-farm.*, 1953, **92**, 115.) The usual method of determining ascorbic acid with iodine cannot be used in the presence of vitamin B_1 and the author recommends the reduction of picric acid to picramic acid in alkaline solution and estimating the amount of the latter colorimetrically. 1 g. of ascorbic acid will reduce 0·43 g. of picric acid to produce 0·3768 g. of picramic acid. The reagents required are:—a 1 in 1000 solution of picric acid, a 2 per cent. solution of sodium hydroxide, and a standard solution of 0·235 g. of picramic acid in 400 ml. of hot water with 25 ml. of sodium hydroxide solution, cooled and made up to 1 l. This solution keeps well in dark glass bottles. Weigh exactly about 50 mg. of ascorbic acid and dissolve in sufficient water to make 50 ml. Place 5 ml. of this solution, 3 ml. of picric acid solution and 2 ml. of sodium hydroxide solution in a 10 ml. graduated flask. Place 8 ml. of picramic acid solution, 1·8 ml. of sodium hydroxide solution and 0·2 ml. of water in another 10 ml. flask. Heat the two flasks for exactly 3 minutes in a boiling water bath, cool in running water, adjust to 10 ml. and compare the two solutions in a colorimeter. 5 mg. of ascorbic acid produce 1·884 mg. of picramic acid, which is the quantity contained in the 8 ml. of standard solution used. When testing solutions of ascorbic acid they should be suitably diluted; the presence of sulphites or bisulphites does not interfere. For tablets, a number corresponding to about 100 mg. is powdered in a mortar with 30 ml. of a 2 per cent. solution of metaphosphoric acid in boiled and cooled distilled water. After a minute the solution is decanted, the residues treated with another 30 ml. of acid solution, and then with distilled water and the united solutions neutralised carefully and made up to 100 ml.; 5 ml. are used for the estimation. If lactose or glucose are present the method is not applicable. If vitamin B_1 is present this can be precipitated with picric acid. For example, with a solution containing 50 mg. of ascorbic acid and 5 mg. of aneurine per ml., 1 ml. is mixed with 5 ml. of saturated picric acid solution, allowed to stand for 10 minutes and then centrifuged for 10 minutes. 3 ml. of the clear liquid is diluted to 25 ml., 5 ml. of this solution, 0·4 ml. of distilled water, 2·60 ml. of picric acid solution and 2 ml. of sodium hydroxide solution are mixed and treated as for pure ascorbic acid. H. D.

Digitalis, Chemical Assay of. D. H. E. Tattje and F. H. L. van Os. (*Pharm. Weekbl.*, 1953, **88**, 237.) By determinations carried out before and after enzymatic hydrolysis of the glycosides, it is possible to determine separately the primary glycosides, secondary glycosides and aglycones in digitalis leaf. For the final determinations both the Baljet reaction and the Lindewald reaction are used. The former gives all the aglycones, free and combined, and the colour is measured at 498 mμ. With the second reaction only the aglycones combined with digitoxose (i.e. glycosides) are determined, the colour being determined at 580 mμ. In both cases a standard of pure digitoxin is used. Details are as follows: 0·5 g. of dried leaf is allowed to stand for 15 minutes with water, and made up to 50·5 g. with water. After shaking for 1 hour, 5 g. of lead acetate solution (15 per cent.) is added, and the mixture is filtered: 36·67 g. of the filtrate

is shaken 3 times for 1 minute with 20 ml. quantities of chloroform. The chloroformic solution is dried, filtered and the filter is washed with 3 quantities, each of 5 ml. chloroform. The chloroformic solution is divided into two portions, the chloroform is distilled off and the residues are used for the two colorimetric determinations, giving the two values B_1h and L_1h. Another 0·5 g. of the leaf is treated with water and 10 drops of an ethanolic solution of methyl *p*-hydroxybenzoate (25 per cent.) and made up to 50·5 g. After standing for 3 days at 30° C. the mixture is shaken for 1 hour and the determination is continued as above, giving the values B_2d and L_3d. The results are calculated as follows: percentage of primary glycosides (purpurea glycosides A and B) $= x =$ 1·96 ($B_3d - B_1h$) or 1.59 ($L_3d - L_1h$). Percentage of secondary glycosides (digitoxin + gitoxin) $= L_3d - 0{\cdot}825 \times$ Percentage of aglycones (digitoxigenin + gitoxigenin $= 0{\cdot}4\,(B_3d - L_3d)$. In the case of stabilised leaf it is necessary to add 0·5 g. of an enzyme powder, the weight being made up to 51·0 g., and the mixture macerated for 6 days at 40° C. This enzyme preparation is prepared by a modification of the method of Stoll and Kreis: 3 kg. of finely-cut fresh leaf is shaken for 2 hours with 4·5 l. of ethanol, and the mixture is pressed out. The pressed cake is cut fine and transferred to 7 l. of ethanol (50 per cent.). After shaking for 1 hour the mixture is again pressed out and the residue is broken up and dried carefully at 35° C. It is then converted to a coarse powder and the extraction is repeated as before. This process is repeated again. After careful drying the final product is brought to a B 30 powder. G. M.

Morphine in Poppies, Determination of. H. Baggesgaard-Rasmussen. (*Ann. pharm. franç.*, 1952, **10**, 693.) A simple, rapid method for the determination of morphine in whole plants, leaves, stems and capsules, giving reproducible results with an error of less than 5 per cent. on samples containing as little as 0·1 mg. of morphine, was required. Assays depending on the formation of nitrosomorphine were investigated because they can be used with small samples and are unaffected by the presence of other alkaloids of opium with the exception of laudinine and codamine which occur in negligible proportions in poppy plants. The 2-nitrosomorphine formed may be determined colorimetrically in the visible or ultra-violet, or polarographically. The following method was used. To 5 ml. of a solution containing 0·005 to 0·1 per cent. of morphine in N hydrochloric acid add 2 ml. of M potassium nitrite. Allow to stand for exactly 5 minutes and add 3 ml. of a 20 per cent. solution of methylcellulose. Pass nitrogen through the solution to remove dissolved oxygen and determine the nitrosomorphine polarographically, using a standard curve prepared with known quantities of morphine similarly treated at the same temperature. Potassium nitrite and hydroxide gave better results than the corresponding sodium salts by providing a better parallelism between residual and diffusion currents. Methylcellulose was employed to adjust the surface tension to a suitable value. The temperature had to be carefully controlled, because the diffusion current rises about 1·6 per cent. per ° C. The assays indicated that the morphine yield of poppy plants is greatly affected by climatic conditions at the time of harvest, and exposure of the harvested plants to rain removes some of the morphine. Poppies yielded 2 to 3 per cent. of morphine, rising to 5 to 6 per cent. when conditions of cultivation were very good. Figures are given for the morphine content of leaves, stem and capsules of the plant during growth as well as for the various parts of the ripe capsules. G. B.

***iso*Nicotinic Acid, Determination of.** E. F. G. Herington. (*Analyst*, 1953, **78**, 174.) A rapid colorimetric method of analysis is described which permits the estimation of *iso*nicotinic acid in samples of mixed pyridine carboxylic acids containing nicotinic, picolinic and dipicolinic acids. A solution containing trisodium pentacyanoamminoferrate, glycerol and acetic acid is used for the determination; the reagent solution is green, whereas *iso*nicotinic acid produces a reddish colour, and hence it is necessary to use a photoelectric absorptiometer with Ilford No. 604 filters to measure the depth of colour. The colour reaction is sensitive to hydrogen-ion concentration and is therefore carried out in the presence of a large controlled excess of acetic acid, glycerol being added to the reagent both to intensify the colour and to keep the complexes in solution. The method of preparation of the trisodium pentacyanoamminoferrate is given and results of the analyses of synthetic mixtures are quoted; picolinic acid does not interfere with the determinations. R. E. S.

Organic Acids, Circular Paper Chromatography of. J. W. Airan, G. V. Joshi, J. Barnabas and R. W. P. Master. (*Analyt. Chem.*, 1953, **25**, 659.) The method described makes use of circular paper chromatography for the identification of 5 organic acids. An airtight glass tank, 30 cm. in diameter, was used with, inside, a filter paper disc supported 2 cm. from the bottom arranged over a 10 ml. circular Petri dish. At the centre of the filter paper a 2 cm. radius circle was drawn and 5 μl. of 1 per cent. aqueous solutions of tartaric, citric, malic, malonic, and succinic acids were separately spotted at 5 points on this circle. The spots were air-dried, and through a slit made at the centre of the disc a small strip of paper was inserted to take up the solvent onto the paper. Within 3 hours the solvent front had reached the edge of the disc and, after air-drying, the chromatogram was sprayed with bromophenol blue reagent, yellow bands developing against the blue background. A 0·1 per cent. ethanolic solution of mercurochrome was also used as a spray reagent, white bands developing against a pink background. R. E. S.

Oxalate and Calcium, Determination of. F. Burriel-Martí, J. Ramirez-Munoz and E. Fernandez-Caldas. (*Analyt. Chem.*, 1953, **25**, 583.) Attempts were made to apply the decrease in optical density when ferric salicylate is treated with oxalate, to the determination of oxalate ion by indirect colorimetry. Measurements must be done in an acetic acid medium, the unknown solutions added to the coloured reagent being neutral, weakly acid with acetic acid, or slightly alkaline with ammonium hydroxide; errors of ± 4 per cent. encountered in recovery experiments, but the method was reliable in the concentration range $2{\cdot}5 \times 10^{-6}$ to $2{\cdot}5 \times 10^{-5}$ mole of oxalate. While tartrate did not interfere even when in greater concentration than oxalate, citrate gave positive errors by decrease in the optical density, which reached approximately 7 per cent. when the concentration of citrate is 1·6 times that of oxalate. The method was extended to the determination of calcium by precipitation as oxalate followed by indirect colorimetric determination of the oxalate which remains in the solution. Calcium in the presence of magnesium could also be determined by this procedure the amount of oxalate being increased owing to the slow precipitation of calcium due to the interaction of the magnesium and calcium ions which increases the solubility of the calcium oxalate. The results compared satisfactorily with those obtained by volumetric methods in the range 1·2 to 2·2 mg. of calcium. R. E. S.

Particle Size Distributions, Determination of. J. S. Smith and R. Gardenier. (*Analyt. Chem.*, 1953, **25**, 577.) A simple apparatus for the determination of particle size distributions as functions of the Stokes diameter in liquid media is described. The data provided by sedimentation experiments using any apparatus of this sort give the weight fraction of the particles which have settled out from the suspension as a function of the time. A graphical method due to Oden gives the cumulative weight fractions oversize as the intercepts on the weight fraction axis of tangents drawn to the curve of weight fraction versus time at times equivalent to the appropriate Stokes diameters. It is suggested in this paper that in many cases the cumulative fraction oversize as a function of the Stokes diameter is not the most cogent information obtained from the experiment. It is also suggested that the true distribution plot of fraction per unit diameter versus the diameter obtained from the cumulative curve is also not the best information, as these distribution curves imply that a great deal more information is available than is justified by the precision of the measurement. Details of the type of apparatus used are given together with the mathematical theory involved. The liquid chosen was the 2-ethylhexyl monoether of ethylene glycol. Tables are given of the results of 6 measurements of a powder of unknown distribution. R. E. S.

Quinidine Sulphate and Strychnine Nitrate, Assay of. D. Köszegi and E. Salgo. (*Pharm. Zentralh.*, 1953, **92**, 157.) Potassium mercuritetrathiocyanate, which has the formula $K_2Hg(SCN)_4$, contains only two titratable thiocyanate groups. Since it precipitates alkaloids, it may be used for the assay of alkaloidal salts by a titration procedure. The reagent solution is prepared by rubbing down 21·6 g. of yellow mercuric oxide with 39 g. of potassium thiocyanate and 200 g. of water, adding dilute nitric acid until the mercuric oxide has almost completely dissolved, and making up to 1 l. The reaction must remain somewhat alkaline and should be checked with litmus paper. For the assay, about 0·25 g. of quinidine sulphate or strychnine nitrate is dissolved, with warming, in 20 ml. of water. After cooling, an excess (25 ml.) of reagent is added, the mixture is made up to 50 ml. and filtered: 25 ml. of the filtrate is treated with 20 ml. of 0·05N silver nitrate solution, and the excess of silver is determined, after acidification, by titration with 0·05N thiocyanate using ferric alum as indicator. The weight of alkaloidal salt is then equal to $[a - (b - 2c)]f$, where a = number of ml. of 0·05N mercurithiocyanate solution, b = number of ml. of 0·05N silver nitrate solution, c = ml. of 0·05N thiocyanate solution, and f = amount of alkaloidal salt equivalent to 1 ml. of reagent (0·01987 g. of strychnine nitrate or 0·019562 g. of quinidine sulphate). G. M.

Theobromine in Cocoa Residues, Determination of. K. W. Gerritsma and J. Koers. (*Analyst*, 1953, **78**, 201.) A study has been made of the methods available for the determination of theobromine in cocoa residues and a new method is proposed. The residues are shaken for 5 minutes with chloroform in an ammoniacal medium; after dehydration with anhydrous sodium sulphate, the chloroform solution is filtered and the residue and filter are washed with chloroform. The chloroform is removed from the filtrate by distillation and the residue is dissolved in water, after which silver nitrate is added and the liberated nitric acid is titrated with alkali. Details of the method are given together with the results obtained in a comparison with Wadsworth's method (*Analyst*, 1921, **46**, 32); good agreement was shown between the two methods and in 20 replicate determinations, the results obtained by the new method lay between 3·00 and 3·02 per cent. R. E. S.

FIXED OILS, FATS AND WAXES

Oils and Fats, Preservation of. B. Siegfried and R. Schneider. (*Pharm. Acta Helvet.*, 1953, **28**, 131.) Three antioxidants, propyl gallate, nordihydroguaieretic acid and ascorbyl palmitate, were tested on samples of almond and olive oils, and lard, which had already undergone a certain amount of oxidation and showed peroxide values of 4 to 8. For vegetable oils, which contain a certain amount of natural antioxidants, the effect of the additions was not great, best results being attained with ascorbyl palmitate (0·05 per cent.). In lard only the phenolic antioxidants were effective, an addition of 0·05 per cent. of propyl gallate or of nordihydroguaieretic acid being sufficient to preserve the material for 6 months. G. M.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Borate Complexes of Sugars and Related Compounds, Separation of, by Ion Exchange Chromatography. L. P. Zill, J. X. Khym and G. E. Cheniae. (*J. Amer. chem. Soc.*, 1953, **75**, 1339.) Further studies were made on the separation of sugars and related compounds by the ion exchange chromatography of their borate complexes on strong-base anion exchangers. The sugars which are known to occur in complex mixtures, such as those obtained from plants, were studied with regard to their elution characteristics. The following compounds were investigated; raffinose, rhamnose, stachyose, sorbose, gentiobiose, melibiose, melezitose, turanose, sedoheptulose, sedoheptulosan, dulcitol, sorbitol and mannitol. A number of separations are reported. The borate from the isolated sugar-borate complexes was removed by distillation as volatile methyl borate. The structure of the sugars in relation to the affinity of the sugar-borate complexes for the ion exchange resin is discussed. A. H. B.

Starches from Peas, Fractionation of. A. L. Potter, V. Silveira, R. M. McCready and H. S. Owens. (*J. Amer. chem. Soc.*, 1953, **75**, 1335.) Smooth-seeded Alaska and wrinkled-seeded Perfection pea starches were isolated by sedimentations from their aqueous suspensions and then fractionated with amyl alcohol and *n*-butanol into amylose and amylopectin. These starches have 35 and 66 per cent. amylose content respectively. The following properties of the separated amyloses and amylopectins were then examined: (*a*) molecular weights by osmotic pressure measurements, (*b*) end-group assays by periodate oxidation, (*c*) iodine affinities and blue values, and (*d*) limiting viscosity measurements. The average molecular weight of Alaska pea amylose was found to be 125,000 and Perfection pea amylose to be 100,000, while the molecular weights of the corresponding amylopectins were 2,000,000 and 140,000. From periodate oxidation and molecular weight data, the degree of branching of these pea-starch amyloses is low and similar to those of other amyloses prepared by similar methods from other plant sources. Alaska pea amylopectin has an average of 25 glucose residues per terminal non-reducing glucose unit, while Perfection pea amylopectin has 36. Besides the difference in molecular weight and end-group assay of these amylopectins, the iodine potentiometric titration curve of Perfection pea amylopectin is different from that of other amylopectins. The results of the investigation show that starch from smooth-seeded Alaska peas is similar to cereal and root starches, while that from wrinkled-seeded Perfection peas is different. Besides having a higher amylose content, the amylopectin fraction of the wrinkled pea starch has a much smaller molecular weight and a smaller degree of branching than amylopectins from other plant sources. A. H. B.

ORGANIC CHEMISTRY

Urea and Thiourea, "Insertion" Compounds of. W. Schlenk. (*Chim. et Industr.*, 1953, **69**, 454.) X-ray studies show that when certain hydrocarbons of the urea or thiourea crystal enlarges, forming a honeycomb structure. The react with urea or thiourea to form addition compounds the original lattice hydrocarbon molecules are accommodated in the canals of the lattice. Straight-chain hydrocarbons, benzene, *cyclo*hexane and aliphatic compounds with 1 or 2 lateral methyl groups may thus be distinguished from branched chains and tetra-substituted carbon compounds which do not form addition compounds with urea or thiourea. Certain very thin molecules are incapable of causing enlargement of the urea or thiourea lattice, but form compounds when wider molecules are present in addition. Mixtures of paraffins, fatty acids, etc., may be fractionated by filtration through urea or thiourea and the method is applicable to the separation of isomers such as *n*- and *iso*octane. Separation is not always complete, depending on the equilibrium between the addition compound and the liquid phase. When this method was applied to the analysis of ozokerite the material was divided into 4 fractions (1) forming an addition product with urea, (2) forming an addition product with thiourea, (3) with both and (4) with neither. Examination of the physical constants of the fractions enabled the following composition to be attributed to the ozokerite: paraffins, average about C_{35}, the greater unbranched, 60 per cent., *cyclo*paraffins, 20 to 30 per cent.; condensed cyclic systems, not more than 20 per cent. and hydrocarbons having lateral groups longer than $-CH_3$, not more than 20 per cent. G. B.

PLANT ANALYSIS

Growth Substances in Plant Extracts, Chromatography of. T. A. Bennett-Clark and N. P. Kefford. (*Nature, Lond.*, 1953, **171**, 645.) Plant extracts from a variety of etiolated seedlings and roots were studied including pea (*Pisum sativum*) shoots and roots, broad bean (*Vicia faba*) shoots and roots, sunflower (*Helianthus annuus*) shoots, maize (*Zea mays*) roots, potato tubers and shoots, and *Ægopodium* rhizomes. Macerated plant tissues were extracted with ethanol for 24 hours at $-5°$ C., the extract being centrifuged, concentrated under reduced pressure, and the aqueous residue extracted with ether at *p*H 3. After purification the ether-soluble acidic substances were suitably concentrated and applied to the starting line of a chromatogram as a spot or a strip, from a dropping pipette in a stream of nitrogen. The chromatograms were developed in *iso*propanol : water : : 10 : 1, with ammonia in the base of the tank; indole-3-acetic acid and indol-3-acetonitrile were run simultaneously and their positions detected at the conclusions of the development by spraying with a ferric chloride–perchloric acid mixture and nitrous acid–nitric acid mixture respectively. Biological testing, using subapical sections of *Avena* coleoptiles and sections from the top of the pea, was performed on developed chromatograms. On all the chromatograms of the acidic fractions of ether extracts of shoot and root material which were bioassayed, 3 active areas were clearly detected. The central active area (R_F = approx. 0·3) corresponded with indole-3-acetic acid; the term accelerator-α was suggested for the substance with R_F value less than that for indole-3-acetic acid and the term inhibitor-β for that with R_F value greater. All shoot and root materials studied contained hormones α and β and indole-3-acetic acid, although no information is available at present on the nature of either α or β is available.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Œstrone and β-Œstradiol, Biosynthesis of, in the Perfused Ovary. N. T. Werthessen, E. Schwenk and C. Baker. (*Science*, 1953, **117**, 380.) In order to investigate whether the ovary may synthesise œstrone or β-œstradiol from acetate, some ovaries were perfused with sodium acetate labelled with ^{14}C in the carboxyl group. Two perfusions of long duration were carried out with ovaries obtained from one pregnant and one non-pregnant animal. Immediately after the perfusion was terminated, the mixture of ovaries and perfusion liquid was converted into a mash, and extracted. The details of the method of isolation of the radioactive œstrone, β-œstradiol and cholesterol are recorded. Thus isolated surviving sow ovaries, when perfused with sodium acetate labelled in the carboxyl group, produce labelled œstrone, β-œstradiol and cholesterol. The experiments described do not allow any conclusion as to whether the hormones are derived from the cholesterol, or whether cholesterol and the hormones are produced from a common precursor. A. H. B.

Procaine and Succinylcholine Diiodide, Substrate Competition between, for Plasma Cholinesterase. F. F. Foldes, P. G. McNall, D. L. Davis, C. H. Ellis and A. L. Wnuck. (*Science*, 1953, **117**, 383.) Because both procaine and succinylcholine are hydrolysed by the same enzyme it was considered possible that the additive effect of the simultaneous administration of these two substances on respiratory depth might be due to substrate competition between the two agents for the plasma cholinesterase. *In vitro* hydrolysis studies were carried out for the quantitative determination of this substrate competition. Aliquot portions of heparinised human plasma were treated with (*a*) procaine in different concentrations; (*b*) the same procaine concentrations as in (*a*) with succinylcholine diiodide added, and (*c*) constant procaine concentration with varying succinylcholine diiodide concentrations. All plasma samples were incubated at 37° C. for 10 minutes and then the procaine and *p*-aminobenzoic acid concentrations determined. The results show that increasing the procaine concentration from 50 to 400 μg./ml. had very little effect on the quantity of procaine hydrolysed. However, when 100 μg./ml. of succinylcholine diiodide was added before incubation, the quantities of procaine hydrolysed increased with increasing procaine concentrations. Similarly, when the procaine concentration was kept constant at 100 μg./ml. and the succinylcholine diiodide concentration was increased, the quantity of procaine hydrolysed decreased with increasing succinylcholine diiodide concentrations. Other experiments using Warburg's micromanometric technique and Ting's method for the determination of procaine showed that procaine hydrochloride inhibits the enzymatic hydrolysis of succinylcholine dichloride and succinylcholine inhibits the enzymatic hydrolysis of procaine hydrochloride. The inhibitory effect of procaine hydrochloride on the hydrolysis of succinylcholine dichloride was shown to be greater than that of succinylcholine dichloride on the enzymatic hydrolysis of procaine hydrochloride, which indicates that, although the hydrolysis rate of succinylcholine dichloride is greater than that of procaine hydrochloride, the affinity of the latter to the enzyme is considerably greater than that of the succinylcholine dichloride. The additive effects of succinylcholine and procaine were also demonstrated with mammalian sciatic-gastrocnemius preparations of dogs and cats. The practical importance of the observation that competition exists between procaine and succinylcholine for the plasma cholinesterase lies in the fact that they might be employed simultaneously in anæsthetised patients.

A. H. B.

BIOCHEMICAL ANALYSIS

Cholesterol in Serum, Determination of. A. Zlatkis, B. Zak and A. J. Boyle. (*J. Lab. clin. Med.*, 1953, **41**, 486.) A method is described for the direct estimation of cholesterol in serum which is more sensitive and less time consuming than previous methods. It consists of adding a fixed volume of concentrated sulphuric acid, glacial acetic acid and ferric chloride solution to 0·1 ml. of serum in 3 ml. of glacial acetic acid. A purple colour is formed in 1 minute, the absorption being measured in a spectrophotometer at 560 mμ. The concentration is read from a predetermined response curve using standard cholesterol solutions. The colour obeys Beer's law and remains stable over several hours. Comparisons were made with the other methods of Kingsley-Schaffert and Schoenheimer-Sperry procedures and the results were in good agreement. G. F. S.

Corticosteroids, Paper Chromatography of. E. H. Sakal and E. M. Merrill. (*Science*, 1953, **117**, 451.) The simple procedure described is applicable to both descending and ascending paper chromatography, and the application to the latter method is outlined. A sheet of Whatman No. 1 filter paper (43 × 43 cm.) is folded into a cylinder in the manner indicated by Wolfson *et al.* (*Science*, 1949, **109**, 541). Small droplets of the solutions to be chromatographed are applied to the paper on the starting line and the paper then placed in a cylindrical glass jar (15 cm. diameter and 46 cm. high) containing a one-phase solvent mixture of xylene (225 ml.) and absolute methanol (75 ml.), and the jar then closed by a gas-tight cover. The solvent is allowed to ascend on the paper to a distance of about 25 cm. from the starting line (a period of about 2½ hours required). The paper is then removed and air-dried and the locations of the various steroids found by normal procedures. Advantages of the above procedure over those reported in the literature are as follows. A one-phase solvent system is used, rendering unnecessary the equilibration of the paper in the solvent vapour prior to development; pretreatment of the paper with any solvent is unnecessary; lateral diffusion of steroid spots during development is quite limited, thus rendering unnecessary the precutting of the paper into a pattern of separated strips; the development period required for the resolution of mixtures of corticosteroids is not greater than 2 to 3 hours; air-drying of the paper afterwards is completed in less than 30 minutes. A. H. B.

Ethanol in Biological Fluids, Determination of. I. Sunshine and R. Nenad. (*Analyt. Chem.*, 1953, **25**, 653.) A modification of the determination of ethanol in blood by oxidation with potassium dichromate is described. The method uses potassium dichromate in the central wall of a Conway cell, complete diffusion (evaporation) taking 20 minutes. The amount of ethanol present is determined colorimetrically, the contents of the centre well being compared with prepared standards, or the optical density being measured photoelectrically. Known amounts of ethanol were added to blood and urine and the samples were assayed for ethanol; results indicated that the recovery of ethanol was within ±0·02 per cent. of the amount added. R. E. S.

Mannosidostreptomycin and Dihydromannosidostreptomycin, Determination of. J. Levine, G. Selzer and W. W. Wright. (*Analyt. Chem.*, 1953, **25**, 671.) The method proposed depends on the methanolysis of the sample to give

methyl streptobiosaminide dimethyl acetal or methyl dihydrostreptobiosamide, streptidine and methyl mannoside. Methanolysis was effected by refluxing in absolute methanolic sulphuric acid for 2 hours; the presence of water resulted in separation of streptidine sulphate, discolouration of the solution, and destruction of carbohydrate. Of the products of methanolysis, all except methyl-mannoside were removed by ion exchange; passage of the solution over a mixture of anion and cation exchangers removed cations completely; eluates prepared from methanolysed mannosido-free streptomycin being free from carbohydrate. Removal of intact streptomycin for the determination of the free sugar blank was accomplished by using mixed cation and anion exchange resins in which the anion exchange portion had been converted to the chloride form; use of the anion exchange resin in the usual hydroxide form resulted in the loss of free sugar. It was found that mannose reacted quantitatively in acid solution with a small excess of 2·4-dinitrophenylhydrazine when the solution was evaporated to complete dryness at a slow rate; the residue from evaporation dissolved in aqueous ethanolic alkali to give a stable purple solution having an absorption maximum at 556 mμ and this colour was used for spectrophotometric estimation. R. E. S.

Urea, Determination of. H. S. Friedman. (*Analyt. Chem.*, 1953, **25**, 662.) A modification of the quantitative Fearon reaction for urea in blood and other body fluids is presented in which fuming hydrochloric acid is eliminated. Attempts to reproduce a standard curve using potassium persulphate to develop the colour were unsuccessful owing to the photo-sensitivity of the reaction. A procedure was developed using a solution of arsenic in 1:1 sulphuric acid; details of the method are given together with a specimen calibration curve.
R. E. S.

CHEMOTHERAPY

***N*-Naphthylmethyl-2-haloethylamine Derivatives, Antihistamine and Anti-adrenaline Properties of.** J. D. P. Graham and G. P. Lewis. (*Brit. J. Pharmacol.*, 1953, **8**, 54.) A series of *N*-naphthylmethyl-*N*-(aryl or alkyl)-2-haloethylamines have been examined for their antihistamine, anti-adrenaline, anti-acetylcholine and antipituitrin actions. None of the compounds antagonised the depressor response to acetylcholine or the pressor response to pituitrin. Most of the compounds antagonised the pressor response to adrenaline and its excitatory actions on the cat's pregnant uterus, rabbit uterus and cat nictitating membrane, but did not affect the inhibitory actions of adrenaline on the cat non-pregnant uterus, rabbit intestine or the effects of adrenaline on the heart. In small doses the haloethylamines potentiated the pressor response to adrenaline and stimulated isolated perfused rabbit hearts. While these compounds reversed the pressor effects of adrenaline they were much less active against sympathetic nerve stimulation. Some of the compounds competitively antagonised the effects of histamine, and structural configurations which favoured anti-adrenaline action also favoured antihistamine action. The presence of 1-naphthylmethyl in the molecule was favourable, while the 2-naphthylmethyl lowered activity. If the halogen atom in the haloethyl side-chain was fluorine the molecule was inactive while the presence of bromine conferred the greatest activity. G. F. S.

PHARMACY

NOTES AND FORMULÆ

Oxytetracycline (Terramycin). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1953, **151**, 1291.) Oxytetracycline is an antibiotic produced by the growth of the actinomycete *Streptomyces rimosus* on suitable media. It is the dihydrate of 4-dimethylamino-1:4:4a:5:5a:6:11:12a-octahydro-3:5:6:10:12:12a-hexahydroxy-6-methyl-1:11-dioxo-2-naphthacenecarboxamide and occurs as a dull yellow, odourless, slightly bitter, crystalline powder, m.p. 179·0° to 182·0° C., with decomposition. It is soluble in acids and alkalis, very slightly soluble in acetone, ethanol, chloroform and water, and practically insoluble in ether. It gives a dark brown colour with ferric chloride, a precipitate of copper when warmed with Fehling's solution, and a red colour with sodium hydroxide and diazobenzenesulphonic acid. When a solution in diluted hydrochloric acid is treated with an ethanolic solution of α-naphthol and superimposed on sulphuric acid, a reddish brown colour appears at the interface. The specific rotation of a 1 per cent. solution of the anhydrous substance in 0·1 N hydrochloric acid is −208° to −216°. A 0·00125 per cent. solution of the anhydrous substance buffered at *p*H 2·0 exhibits ultra-violet absorption maxima at about 269 mμ, and 353 mμ ($E^{1\%}_{1\,cm.}$, 300 to 312), minima at about 232 and 299 mμ, and a slight inflection at about 313 mμ; the ratio of the absorptions at 269 and 353 mμ is 1·32 to 1·38. Oxytetracycline contains not more than 25 p.p.m. of heavy metals and yields not more than 0·6 per cent. of sulphated ash. The loss of weight on drying in a vacuum oven at 75° C. for 6 hours is not more than 7·75 per cent. It is assayed spectrophotometrically by measuring the absorption at 353 mμ of a 0·00125 per cent. solution buffered at *p*H 2·0, and contains 96·0 to 104·0 per cent. of oxytetracycline. G. R. K.

Potassium Penicillin O (Cer-O-Cillin Potassium). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1953, **151**, 1491.) Penicillin O is allylmercaptomethyl penicillin and is obtained by growing the mould in a medium containing allylmercaptoacetic acid. It is stable in the dry form at room temperature for at least 3 years, and does not need refrigeration. Solutions may be kept for 3 days in a refrigerator without significant loss of potency. Clinically, penicillin O has been shown to be as effective as benzylpenicillin (penicillin G) and less likely to cause sensitivity or allergic reactions. It has a similar range of antibacterial activity, and is particularly useful as a substitute in the treatment of patients sensitive to benzylpenicillin. Allergic reactions to penicillin O have been observed in less than 1 per cent. of patients who have no history of previous allergic reactions to benzylpenicillin, while about 90 per cent. of patients sensitive to benzylpenicillin tolerate therapeutic doses of penicillin O without the development of allergic phenomena. Some patients may lose their sensitivity to benzylpenicillin during a short course of penicillin O. When given by mouth, penicillin O may produce an onion-like odour of the breath, which subsides shortly after the drug is discontinued. It produces blood levels comparable to those obtained with benzylpenicillin administered by similar routes. Potassium penicillin is given by mouth or by intramuscular injection (intermittent or continuous infusion). In general the dosage is the same as that recommended for benzylpenicillin. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Amœbicides, Evaluation of. J. S. Vegas. (*J. Amer. med. Ass.*, 1953, **151**, 1059.) This study involves 264 cases of amœbiasis with follow-up physical and stool examinations ranging from 6 to 24 months. In order to evaluate the effectiveness of the amœbicides to be investigated the series was studied under 8 treatment groups, as follows:—bismuth glycolylarsanilate, aureomycin, aureomycin and bismuth glycolylarsanilate, chloramphenicol, chloramphenicol and bismuth glycolylarsanilate, oxytetracycline, oxytetracycline and bismuth glycolylarsanilate, and chloroquine and bismuth glycolylarsanilate. Of these treatments the combination of bismuth glycolylarsanilate and chloroquine was found the most effective; of 102 patients treated, 91 (89·2 per cent.) finally became free of cysts. Treatment consisted in the administration of 2 tablets daily, spaced at 12-hourly intervals, each tablet containing 500 mg. of bismuth glycolylarsanilate and 150 mg. of chloroquine, the course lasting 15 days. The few side-effects occasionally observed (nausea, vomiting, pruritus ani, lower left quadrant pain and diarrhœa) do not necessitate interruption of treatment; side-effects may be further reduced by administration of vitamin B complex. The combination of the non-absorbable arsenical, bismuth glycolylarsanilate, with the highly absorbable chloroquine is a logical and effective treatment for amœbiasis, which should be considered as a systemic and not a purely intestinal disease. S. L. W.

Carbimazole, Antithyroid Activity of. A. G. Macgregor and H. Miller. (*Lancet*, 1953, **264**, 881.) The antithyroid activity of this drug (neomercazole) and methimazole were compared by the technique of Stanley and Astwood (*Endocrinology*, 1947, **41**, 66), a dose of the antithyroid drug being given after administration of a tracer dose of radioactive iodine, and the effect on the normal curve of accumulation of iodine in the thyroid gland observed. In doses of 10 mg. both drugs caused complete inhibition of uptake of radioactive iodine by the thyroid gland during the whole period of observation and a similar effect was observed with doses of 2·5 mg. of each drug. The effect is comparable to 200 to 300 mg. of methylthiouracil. In doses below this level carbimazole appears to be consistently rather more potent than methimazole. Clinical experience suggests that both drugs are effective antithyroid agents, but considerable caution should be exercised in the transfer of the results of potency trials of this type to the clinical field. These tests suggest a potency possibly 50 times greater than that of methylthouracil, but this does not imply that the therapeutic dose is correspondingly low, since it is now agreed that, weight for weight, methimazole is probably only about 10 times as active clinically as methylthiouracil. S. L. W.

Carbimazole (Neomercazole), Treatment of Thyrotoxicosis with. H. Poate. (*Lancet*, 1953, **264**, 879.) Experience in the treatment of 9 cases suggests that this drug is superior to any of the thiouracil compounds, not only for control of thyrotoxicosis but also for reducing vascularity of the gland before operation. The usual dosage employed was 10 mg. thrice daily until thyrotoxicosis was controlled, after which it was reduced to 10 mg. twice daily, or even 5 mg. twice daily. It reduces the basal metabolic rate more slowly than the thiouracils but does not induce or increase thyroid hyperplasia and does not interfere with the leucocytes. It therefore appears to be a safer remedy and there is reason to think that once control of a primary thyrotoxicosis is obtained a comparatively short period of maintenance therapy will result in a complete cure. S. L. W.

Carbimazole, Treatment of Thyrotoxicosis with. D. Doniach. (*Lancet*, 1953, **264**, 873.) The author presents a clinical evaluation of this antithyroid

compound (neomercazole, 2-carbethoxythio-1-methylglyoxaline) in the treatment of 120 thyrotoxic patients, 30 of whom received the drug pre-operatively and 90 as curative treatment. The patients were followed for periods of up to 21 months. Of the 120 patients 93 became euthyroid within 2 to 8 weeks, and 27 responded more slowly. Of the latter 9 were operated on and the rest were controlled in 3 to 6 months. The optimum initial dosages were 15, 30 and 45 mg. daily by mouth for mild, average and severe cases respectively. After 4 to 12 weeks this dosage could be reduced in early remission to two-thirds or half, and after 6 months' treatment 5 to 10 mg. a day sufficed for maintenance in average cases; mild cases were maintained on 2 mg./day within 3 months. Treatment was stopped when the patient was able to remain euthyroid on 2 to 5 mg. daily for about 6 months; to date, about 12 months' treatment has been found necessary. Goitrogenic reactions were avoided, in all except 7 cases, by careful control of the maintenance dose; no toxic reactions were seen. Weight for weight carbimazole seems to be at least as effective as, and very much less toxic than, methimazole. It is probably hydrolysed to methimazole before it exerts its antithyroid action, and its increased efficiency must be due to the maintenance of a steadier blood level produced by the slow release of methimazole. S. L. W.

Cortisone, Effect of, on Experimental Corneal Tuberculous Lesions. H. Greenburgh, J. M. Robson and D. R. C. Willcox. (*Brit. J. Pharmacol.*, 1953, **8**, 120.) The effects of intravitreous injections of cortisone have been studied against intracorneal infection of rabbits with *Mycobacterium tuberculosis*. Cortisone depressed the severity of the lesions during its administration, but after discontinuance of therapy there followed a rapid increase in the severity of the lesions. Promethazine, given subcutaneously, had no effect on the development of the corneal lesions, although it penetrated the anterior chamber. It is suggested that cortisone may act by rendering the cornea resistant to enzymatic action. G. F. S.

Diphenan in the Treatment of Oxyuriasis. L. M. Dowsett and A. E. Brown. (*Lancet*, 1953, **264**, 1070.) An investigation into the efficacy of diphenan (*p*-benzylphenylcarbamate) as a vermifuge was carried out on 174 school children aged from 3 to 10 years. The presence of thread worms was demonstrated in these cases by the application of a strip of cellophane tape to the skin in the anal region by means of a wooden applicator. The cellophane was then stuck on a microscope slide and examined under a low power objective, when the ova could be seen. Cellophane swabs were examined daily for 9 days before treatment and if any of these were positive, diphenan was given for 10 days; swabs were again examined for 9 days after treatment. The dosage of diphenan was from 0·5 to 1 g. according to age thrice daily and was far in excess of that recommended by the manufacturers. Only 35 (20 per cent.) of the children were subsequently free of infestation; 44 per cent. were improved in the sense that they gave fewer positive swabs after treatment than before, 18 per cent. were worse and 18 per cent. gave the same number of positive swabs before and after treatment. All 35 "cures" had been only lightly infested, 31 of them having given only one, two or three positive swabs out of the nine taken for diagnosis. Of 15 children who gave 9 diagnostic negatives, only 6 remained negative after receiving the same dosage of diphenan. No toxic reactions were noted. H. T. B.

Ethanol, Distribution of, in the Human Body. H. Handovsky, W. Van Hecke and F. Thomas. (*Acta pharm. tox. Kbh.*, 1952, **9**, 18.) Ethanol determinations were made on a series of organs and body fluids from 93 healthy

persons killed by accident when under the influence of ethanol. Blood samples were taken from the femoral vein and all analyses were carried out by Widmark's method. The ethanol concentrations found in the organ or body fluid were plotted against the ethanol concentration in blood from the same individual, and the ratios are shown in the following table, together with S, the estimated value of the standard deviation of a single observation.

Organ or body fluid	Total No. ratios	Mean ratio and standard deviation of mean	S = standard deviation of a single observation
Urine	80	0·77	±0·17
Cerebrospinal fluid	25	0·79	±0·20
Bile	55	0·89	±0·15
Testes	43	1·18	±0·33
Brain	48	1·48	±0·35
Cardiac muscle	51	1·30	±0·25
Skeletal muscle	57	1·28	±0·31
Spleen	33	1·50	±0·32
Kidney	60	1·32	±0·27
Liver	57	1·92	—

From these figures it is seen that the body fluids have a higher ethanol concentration and the organs a lower ethanol concentration than the blood. From the ratios it is possible to calculate the concentration of ethanol in the blood from the ethanol concentrations in organs (except the liver) and body fluids, and *vice versa*. S. L. W.

Hyaluronidase, Bioassay on Rabbits. V. M. Venturi. (*Acta pharm. tox., Kbh.*, 1953, **9**, 93.) A cannula was inserted into the subcutaneous tissue of the dorsal area of the forelegs of unanæsthetised adult albino rabbits and a 0·9 per cent. sodium chloride solution infused through it under constant pressure from a 100 or 200 ml. burette, the pressure being adjusted to an infusion rate of about 1 ml./minute. The hyaluronidase preparation used contained 2·5 V.R. units per ampoule: the contents of an ampoule were dissolved in 2·5 ml. of 0·9 per sodium chloride solution. Solutions of the contents of not less than 2 ampoules were mixed and the mixture diluted to 1:10 or 1:100. Thus 3 different hyaluronidase solutions were obtained containing 1·0, 0·1 and 0·01 V.R. units/ml. For control purposes 0·9 per cent. sodium chloride solution without hyaluronidase was used. After the infusion rate had been adjusted to about 1 ml./minute the average infusion rate was calculated for several periods of 10 minutes each. The rate was expressed in ml./minute. After the infusion rate had been constant over several periods hyaluronidase was injected and the average infusion rate calculated for a series of 10 minute periods extending over one or more hours. The value measured before the enzyme injection was in each case taken as 1·0 and the values measured after the injection expressed in terms of this; these are therefore referred to as infusion rate (relative values). In the following table are set out means, their standard deviation and fiducial limits (P = 0·05) of the infusion rate (relative values) 20 minutes after injecting different amounts of hyaluronidase or control physiological saline.

Injected solution	Means	Fiducial limits
Physiological saline	0·738 ± 0·096	0·953 — 0·523
Hyaluronidase 0·01 V.R. units	1·136 ± 0·290	1·792 — 0·480
Hyaluronidase 0·10 V.R. units	1·825 ± 0·133	2·118 — 1·523
Hyaluronidase 1·00 V.R. units	3·010 ± 0·182	3·417 — 2·603

S. L. W.

Local Anæsthetics, Specificity of. S. Wiedling. (*Acta pharm. tox., Kbh.*, 1953, **9**, 75.) The local anæsthetic and spasmolytic activities against acetylcholine, histamine, and barium-induced spasms were determined for some 20 commercially available compounds of the general type aromatic residue-intermediate chain-amino group. The compounds are used clinically as local anæsthetics, antispasmodics, antihistaminics, and analgesics, and include such substances as syntropan, procaine, amethocaine, lidocaine, cinchocaine, diethazine, promethazine, diphenhydramine, methadone, adiphenine, antistine, antergan, mepyramine and caramiphen. Determinations of local anæsthetic activity were carried out by a comparative method on the rabbit's cornea using 2 per cent. lidocaine solution as standard. Determinations of activity against spasms produced by acetylcholine, histamine or barium were carried out by a comparative "curative" method on isolated guinea-pig small intestine, using diphenhydramine as the standard substance. A high degree of specificity was shown to be exhibited only by certain compounds, such as cinchocaine and lidocaine, used clinically as local anæsthetics. The least specific substances are certain antihistamines, such as diphenhydramine, antergan and promethazine, and certain antispasmodics, such as diethazine. While compounds of the general type aromatic residue-intermediate chain-amino group show very different properties, local anæsthetic activity of some degree is common to all of them. Some of them also exert a spasmolytic effect against acetylcholine, histamine and barium-induced spasms. There is, however, no connection between the acetylcholine potency and the local anæsthetic action; the antihistamine effect is also independent of the local anæsthetic action, and no general connection between this action and the antibarium effect can be established. There would appear to be a certain parallelism between the antiacetylcholine and the antibarium activities. S. L. W.

Primidone in Treatment of Refractory Epilepsy. B. H. Smith and F. L. McNaughton. (*Canad. med. Ass. J.*, 1953, **68**, 464.) Primidone (mysoline, 5-ethyl-5-phenylhexahydropyramidine-4:6-dione) was given to a series of 66 patients, 23 females and 43 males ranging in age from 4 to 63 years, all of whom had been treated for a considerable time with hitherto available drugs, singly or in combination, with little success. No particular type of epilepsy was studied, the chosen cases including 17 with idiopathic epilepsy, 35 with focal seizures and 14 with unlocalised cerebral seizures. More than half had had seizures for more than 10 years, 10 of them for over 30 years. Primidone was administered in 0·25 g. tablets, the dosage being 1 or 2 tablets daily according to age; this treatment was given in addition to previous medication. After 4 days the dosage was increased by one tablet, the other medicament being correspondingly reduced, and thereafter at weekly intervals this was continued. No patient received more than 8 tablets a day, some showing good response on less. When possible, primidone was given alone in order to assess its value, but where a good result was obtained on a combined treatment of, say, primidone and sodium diphenylhydantoinate further alteration was not tried. In 14 successful cases, primidone was being used alone in dosages of 4 to 8 tablets daily. The side effects produced by the drug included transient drowsiness and dizziness as the most common; vertigo, nausea, ataxia and skin rashes occurred in some cases, but no serious toxic effects were noted. Of the 61 cases treated for more than 4 months, 23 (35 per cent.) had the number of their attacks reduced by half or more. 16 of these patients had been on primidone for more than a year. All types of epilepsy appear to derive benefit, cases of major convulsion, petit mal and automatism being included among those benefited. H. T. B.

1-Phenyl-1-*cyclo*pentyl-3-piperidino-1-propanol Hydrochloride in the Treatment of Parkinsonism. D. W. Mulder. (*Proc. Mayo Clin.*, 1953, **28**, 210.) This compound (designated compound 08958) is chemically related to benzhexol. Pharmacological studies indicate that it is an active antispasmodic with fewer side-effects than atropine. It is slightly more toxic than atropine in mice and rats by either oral or intravenous administration. Of 102 patients with parkinsonism treated with the compound and observed originally for a 3 to 5-day period, 75 showed sufficient objective and subjective improvement to warrant continuation of the drug. The improvement included increased rapidity of finger movements, diminution of tremor, improvement of gait and of handwriting, and increased ability to perform well-learned motor patterns. The evidence of improvement continued to be demonstrated over follow-up periods of from 6 months to 2 years. Patients with oculogyric crises were particularly benefited. The remaining 27 said that their previous medication seemed more satisfactory or the side-effects were more distasteful than with other medicaments. Gastro-intestinal symptoms, including transient nausea, with associated anorexia, and occasionally dryness and soreness of the mouth, occurred in 21 patients; mental symptoms (lightheadedness, stupidity or confusion) occurred in 22 patients, and were most marked in older patients, particularly those with arteriosclerotic changes. The initial dose of the drug is 1·25 mg. thrice daily, gradually increased over a period of several days to 2·5 mg. thrice daily. The optimal dose for most patients is from 7·5 to 15·0 mg. daily. This preliminary clinical trial suggests that the effectiveness of the compound is approximately equal to that of benzhexol and that it may be employed for the partial relief of parkinsonism. S. L. W.

1-Propyl-3-ethyl-6-aminothiouracil; Clinical Trial of Diuretic Properties. A. G. Spencer and H. G. Lloyd-Thomas. (*Brit. med. J.*, 1953, **1**, 957.) This oral diuretic (designated Compound S.C. 2614) was tested 57 times in 36 subjects. The compound was given by mouth in 125 mg. tablets, 2 every 3 hours until 1250 mg. had been taken. In normal subjects there was a satisfactory diuresis in 9 out of 10 tests. In 22 tests on 10 œdematous patients with heart disease there was a good diuretic response in 19. The compound usually failed to produce a satisfactory diuresis in any other group of patients; there were 19 failures in 25 tests on 17 patients. No useful diuresis occurred in cardiac patients without pitting œdema, in patients with a low plasma sodium, a serum albumin below 2 g./100 ml. or a blood urea over 150 mg./100 ml. In renal disease there were a few useful diuretic responses, but the evidence was insufficient to assess value. The diuretic action of the compound was seriously offset by the high incidence of gastro-intestinal disturbances (epigastric discomfort, anorexia, nausea and vomiting). Though rarely severe, these were sufficient to elicit a spontaneous complaint from 23 per cent. of the test subjects. The authors conclude that the results of this experiment do not justify extensive trials of the compound at the present time. S. L. W.

Strychnine, Effect of Vitamin B_2 Deficiency on the Toxicity of. C. F. Poe and J. F. Suchy. (*Arch. int. pharmacodyn.*, 1953, **93**, 244.) It has been previously shown that severe vitamin B_1 deficient rats are more susceptible to strychnine than normal rats. This paper reports that vitamin B_2 deficient rats are not more susceptible and confirms a previous observation that strychnine is more toxic for female rats than for male rats. G. F. S.

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