REVIEW ARTICLE

CHROMATOGRAPHY AND ITS APPLICATIONS IN PHARMACY

By M. W. PARTRIDGE, B.Pharm., B.Sc., Ph.D., Ph.C.

Lecturer in Chemistry, University of Nottingham

THE extensive use of chromatography in so many unrelated fields of chemistry during the past two decades attests to its present importance as one of the most valuable of laboratory techniques. A number of comprehensive works and reviews[1,2,3,4,5,6,7,8,9,10] dealing with its history, methods and applications is now available, and it is intended here to survey only some recent developments in technique and a small selection of their applications to pharmaceutical problems.

PRINCIPLES

Chromatography is essentially a process for the fractionation of mixtures by continuous partition between two phases, one of which is moving past the other. In principle, there appears to be no restriction on the nature of the mobile and stationary phases nor on the types of equilibria involved in the partition. In practice, the most important applications have involved a liquid mobile phase and a liquid or solid stationary phase.

A solution of the mixture to be fractionated is placed on top of a column of adsorbent and slowly passed through; according to the affinity of the components of the mixture for the adsorbent, they are held in zones at the top of the column. Passage of more solvent through the column, by fractional elution and re-adsorption, develops the chromatogram by separating the zones of adsorbed material throughout the column, the more strongly adsorbed substances being nearer the top of the column. If the separate zones are readily located by their colour, in ultra-violet light or by means of a reagent streaked along the extruded column, they are separated by division of the column and eluted with a suitable solvent. As an alternative technique elution development is employed; further solvent is passed through the column in order to elute the components successively as shown by suitable chemical or physical tests. Where these methods are inapplicable, the eluate is collected in arbitrary fractions and each fraction is examined separately. In partition chromatography,[6,7,8,9,10] the column consists of an inert carrier on which is distributed a solvent immiscible with the mobile solvent. Similar principles are exploited in paper chromatography,[6,7,8,9,10] where selective adsorption on paper or partition between the mobile phase and the water present in paper, which has been equilibrated with the saturated vapours of both phases, operate to effect the fractionation.

Theoretical treatments[6,7,11] of adsorption and partition chromatography have proved of little value in experimental work, since no theory has so

far considered all the factors known to be important. The most satisfactory treatment appears to be that of Martin and Synge[11] in which distillation theory is applied to the operation of a column. Although experimental verification of theories of chromatography is available under standard conditions, the optimum conditions for a chromatographic separation are usually worked out empirically.

A useful characteristic of a given set of conditions in a chromatographic system is obtained from the ratio:

$$\frac{\text{distance moved by solute}}{\text{distance moved by solvent}} = R_F \text{ or } R$$

The R_F value is of more particular use in partition chromatography, especially on paper, since it is then related to the partition coefficient[6] which remains roughly constant over a relatively wide range of solute concentrations, whereas in adsorption chromatography continuous variation of R with concentration is usual. R_F values are, however, occasionally subject to variations due to extraneous factors and it would appear preferable to make observations of values relative to a suitable standard substance. The sequence of substances on an adsorption column is another useful descriptive and analytical character since, although the sequence may be altered by changes in the solvent and adsorbent,[9] under standardised conditions it is reproducible.

Methods

The general methods of experiment are described in the standard works.[1,2,3,4] Although there may be no clear fundamental distinction between adsorption and partition chromatography, it still remains convenient to consider them separately.

Adsorption Chromatography

Adsorbents. Amongst the following commonly used adsorbents of diminishing order of activity: alumina, magnesium oxide, calcium sulphate, calcium oxide, talc, calcium phosphate, calcium carbonate and lactose, together with those whose relative activity has not been thoroughly examined, namely silica, kieselguhr, paper, starch, clays and charcoal, there are enormous variations in adsorptive capacities and specificities which may vary further with the solvents used. A number of methods for the standardisation,[12] activation and deactivation of alumina and other adsorbents[13] are now available. Very active alumina is made by heating pure aluminium hydroxide at 380° to 400° C. for 3 hours; for the removal of alkali, commercial alumina is boiled with successive quantities of water until soluble alkali is removed, then washed with methanol and reactivated at 160° to 200° C./10 mm. Hg. pressure.[7] In choosing an adsorbent,[4] the load of mixture to be fractionated should be low so that extensive development is possible; in practice, it is frequently possible to fractionate 1 g. of a mixture on about 30 g. of adsorbent, but the column load influences the degree of fractionation. Several cases of chemical change, such as the aminolysis and oxidation of amino-acids,[14]

occurring on adsorption columns have been reported and in some instances this phenomenon has been exploited for preparative purposes.[15]

Solvents. Special purification of solvents is not usually necessary. Fractionation of mixtures of similar substances is often enhanced by elution development with a series of solvents of increasing polarity which have powers of elution in the following ascending order: light petroleum, carbon tetrachloride, *cyclo*hexane, ether, acetone, benzene, esters, chloroform, alcohols, water (at various *p*H values), pyridine and liquid organic acids. Excellent examples of this technique, known as the *durchlaufmethode*, are provided by recent work on *Strophanthus* glycosides.[16] Hydroxylic solvents may cause difficulty because of their solvent action on the adsorbent; thus alumina is appreciably soluble in methanol and in water.

Apparatus and experimental methods. The simple device described by Martin[6] is extremely efficient for the preparation of a uniformly packed column. A slurry of adsorbent and solvent of a creamy consistency is poured into the chromatogram tube and thoroughly homogenised by a few rapid strokes of a perforated disc mounted by its centre on a long, thin metal rod. The disc should fit the tube closely and the diameter of the perforations should be not more than about 1/16 in. The disc is brought to within about 1 inch of the bottom of the tube and then moved slowly downwards; this causes the solid to pack beneath it. Rapid homogenising strokes are followed by slow packing strokes until the whole column is packed. With such columns, it is often necessary to accelerate the rate of flow of developing solvent by regulated, positive pressure or suction. Even formation of the zones is facilitated by a low rate of flow, uniform pressure and the use of a long column; small temperature variations appear to have little effect. Unevenness in the development of the zones in large columns is often unavoidable, but is greatly reduced in a multiple column of 2 or 3 units with interposed mixing cells.[17,18] Extrusion of the developed column may be difficult, especially when the adsorbent has not been packed as a slurry; for this reason, the use of slightly tapering tubes has been recommended. Amongst the modifications of the usual apparatus for special purposes,[19,20] a column of filter-paper discs or strips,[21] applied hitherto mainly in the resolution of mixtures of enzymes,[22] may have extensive applications. A film of adsorbent fixed to a microscope slide by means of starch has been employed in microchromatography.[23]

Location of zones. For the detection of zones of adsorbed, colourless substances on a column, fluorescence in ultra-violet light, the formation of dark bands on a fluorescent adsorbent[24,25] or streak reagents[1,26] may be appropriate. In the location of fluorescent substances, quartz apparatus is not normally required, but quartz tubes are necessary for the observation of fluorescence-quenching on columns of fluorescent adsorbent. Such an adsorbent, suitable for the detection of substances adsorbing between 230 and 390 mμ, is prepared by mixing silicic acid with half its weight of kieselguhr ("Celite 535") and adding 2·5 per cent. each of fluorescent zinc silicate and zinc sulphide. After development, the zones are observed in ultra-violet light in a darkened room.

Streak reagents are applied with a brush as a longitudinal streak on the extruded column; sensitive reagents for aliphatic and aromatic amines, alcohols, phenols, ketones, acids, nitro compounds, nitramines, unsaturated hydrocarbons and vitamin A have recently been carefully evaluated.[26] The progress of a chromatographic fractionation may frequently be followed by a continuous recording of changes in the physical properties of the issuing eluate; for this purpose changes in conductivity,[27] refractive index[28] and ultra-violet absorbancy ratios[29] have been exploited.

When these methods for the detection of colourless substances are inapplicable, it is usual to employ elution development. Several efficient devices for the automatic collection of the eluate fractions are available.[27,30]

Partition Chromatography

The original partition column, intended for the separation of acetylated amino-acids, consisted of a column of silica gel on which the stationary phase, water, was supported; the mobile phase was a water-immiscible solvent saturated with water (chloroform with 1 per cent. of *n*-butanol). The individual partition coefficients of the components of the mixture are usually the determining factors in this type of fractionation.[7] Modifications of the stationary and mobile phases and of the support for the stationary phase have led to the application of this technique to a wide range of mixtures.

Types of partition column. It appears that to avoid displacement from the support by the mobile phase, the stationary phase must be the more polar. As a consequence water, buffers[31,32] or aqueous solutions of acids and bases[33,34,35] have been most frequently utilised as the stationary phase and a water-immiscible solvent as the mobile phase. The use of a stationary phase other than water or an aqueous solution, together with an appropriate mobile phase, has been successful in some instances[7]; thus *n*-butanol supported on cellulose acetate, nitromethane supported on silica with *n*-hexane or methanol as mobile phase and other systems[36,37] have been employed. Recently reversed-phase partition chromatographic systems, in which the less polar solvent of a given pair forms the stationary phase, have been described; these have involved the use of kieselguhr treated with dichlorodimethylsilane[38] and of powdered glass.[39] Silica,[40] kieselguhr, starch, powdered cellulose, pulped filter-paper, charcoal and powdered glass are efficient supports for the stationary phase of partition columns. Of these, selected grades of kieselguhr ("Hyflo Super Cel" and "Celite 535") are probably the most generally useful.

Apparatus and experimental methods. The apparatus and experimental techniques employed with adsorption columns are used with partition columns. After thorough trituration of the stationary phase and supporting solid by stirring with a rod in a beaker, the column is packed by Martin's method. In addition to the methods for the detection of the zones applicable to adsorption columns, suitable indicators may be incorporated in the stationary phase, particularly when mixtures of acids or bases are being examined. In elution development of a partition

column, the load of mixture undergoing fractionation is relatively low and variation in the partition coefficients with concentration may cause widening of the bands. Automatic measurement of changes in the physical properties of the eluate has frequently been employed to obtain a continuous record of the course of fractionation.[27] Adsorption on the supporting material of a partition column usually leads to a reduction in the operating efficiency of the column and may cause difficulty.[41] In some cases it may however play an important part in effecting fractionation.

Paper Chromatography

Partition chromatography on paper[40] has developed rapidly and there is now an extensive literature devoted to the subject.[3,10] In this process, a small spot of solution containing 1 μg. to several mg. of the mixture is placed near the top of a strip of filter-paper, the end of the paper near the spot is inserted in a trough containing a solvent saturated with water, and the whole is suspended in a suitable chamber whose atmosphere is saturated with the vapours of water and of the solvent. When the solvent has flowed a suitable distance down the paper, the position of the solvent front is marked and the paper is dried. The components of the fractionated mixture are then treated with an appropriate reagent, usually applied by spraying, and finally characterised by comparison of their R_F values with the simultaneously determined values for authentic materials. Caution must be exercised in the characterisation of compounds solely on the basis of their R_F values.

In developing a two-dimensional paper chromatogram, the spot of solution is placed near one corner of a square sheet of filter-paper and one edge is inserted in the solvent trough. After the solvent has flowed nearly to the opposite edge of the sheet, the paper is removed, dried and developed at 90° to the direction of flow of the first solvent with a second solvent in the trough. By using two solvents, the degree of fractionation is greatly enhanced. The relative importance of partition and adsorption in effecting fractionation in paper chromatography is doubtful in many cases[8,42] and ion-exchange may have some significance in this connection.[43]

Apparatus and experimental methods. Of the various grades of filter-paper, Whatman No. 1 to 5 appear to be most satisfactory[44]; for larger scale work, the use of thicker paper, made from Whatman No. 1 and blotting paper, has been described.[45] The scope of paper chromatography may be extended by impregnation of filter-paper with various substances to modify the properties of the stationary phase; amongst the substances applied in this way are phosphate buffer,[46] alumina,[47,48] silica,[49] rubber latex[50] and resin.[51] The solvents employed are usually partially miscible with water and of relatively low volatility, such as *n*-butanol, collidine, lutidine, piperidine, furfuryl alcohol, liquefied phenol, although all types of solvent have been used and good results have been obtained with water-miscible solvents.[42,52]

Many ingenious modifications of the original experimental techniques have been evolved.[53] The most important general modification involves

development by capillary ascent,[54] a device which has the merit of greater simplicity in operation; simple forms of apparatus for this purpose,[55] especially for exploratory experiments,[56] have been described. Amongst the modifications in detail of the original procedure are convenient forms of solvent troughs,[57] chambers[58] and methods for the simultaneous development of a number of paper strips[54,59] and sheets.[60] A rapid method employing discs of filter-paper is available[61] and for substances which require prolonged development a pad of cellulose may be attached to the end of the paper strip.[62] Since after fractionation on paper the separated materials can usually be eluted without loss,[63] quantitative work on paper depends largely on the availability of analytical methods sufficiently sensitive for the examination of the eluted materials.

Location of zones. For the detection of colourless substances on a paper chromatogram, a sensitive test revealing a number of different substances is most valuable. Ninhydrin is most commonly employed for amino-acids on paper strips and sheets[64]; iodine in ethanolic solution or as vapour may be used for the location of amines, amino-acids, amino-alcohols, guanidines, purines, pyrimidines and alkaloids[65]; carbohydrates can be detected by ammoniacal silver nitrate,[7,66] by an acidified ethanolic solution of β-naphthylamine containing a trace of ferric sulphate[67] and by solutions of phenols in acidified *n*-butanol[68]; fluorescence in ultra-violet light or the quenching of fluorescence[69] have been exploited for purines and pyrimidines,[70] pterins,[71] amino-acids and peptides[72] and porphyrins.[6] By heating the paper to incipient charring many substances can be located.[61] Certain substances, such as antibiotics and growth factors, can be detected by their biological effect when the paper is incubated in contact with agar seeded with a selected organism.[73,74] Compounds containing radioiodine,[75,77] radiocarbon,[78] radiosulphur[79] and radiophosphorus[80] are detected by means of a Geiger counter or by development of an autoradiograph formed when the paper is placed in contact with X-ray film. Light transmittancy,[81] refractive indices of a liquid in contact with the paper[82] and X-ray and electron diffraction patterns of the substances after leaching[83] should be capable of wide application. In paper chromatography salts may separate into ions and cause difficulty in the detection of other substances.

Method of Tiselius

In the special techniques elaborated by Tiselius,[84] a solution of the mixture is forced upwards through a column of adsorbent and the course of fractionation is followed by a continuous record of the refractive index of the liquid leaving the column. When the process is carried out as a *frontal analysis*,[85] as with fatty acids, no developing solvent is used and only the first fraction is likely to be pure; for this reason, *elution analysis*, employing a developing solvent, or *displacement analysis*,[7,86,87] employing a substance of greater affinity for the adsorbent, are to be preferred for preparative purposes. Displacement development has been applied in the fractionation of sugars, amino-acids and peptides on charcoal columns.[86]

Structural Differences and Chromatographic Separations of Organic Compounds

Adsorption chromatography is usually the most selective technique for the fractionation of mixtures of weakly polar molecules; partition chromatography is applicable to substances which can be distributed between immiscible solvents. No general relationship has been deduced between molecular architecture and the ease of separation of organic compounds,[7,9] because of the complexity of the attractive forces in chromatographic systems, of variations in the state of molecules in different chromatographic systems and of the frequently unexpected effects of polar groups.[88] The information summarised in Table I[9] indicates that the degree of separation depends on the functional groups and is least when molecules differ in features which produce least difference in the respective attractions between the solute and the two phases.

TABLE I

Difference in structure	Ease of separation
Isomerism of acyclic compounds	poor to fair
Isomerism of acyclic and cyclic compounds	poor to fair
Position of centres of unsaturation	poor to fair
Isomerism of conjugated and unconjugated compounds	fair to good
Number of centres of unsaturation	good
Geometrical isomerism	fair to good
Optical isomerism[89]	poor
Molecular dimensions	poor to fair
Number of polar groups	good
Polarity of functional groups	good

Applications

A comparison of chromatography with other important general methods for the separation of mixtures such as distillation, sublimation, fractional crystallisation, partition and chemical separations shows that it possesses two extremely valuable features; it is applicable to small quantities of material and the conditions of operation normally cause no change in the components of the mixture being separated. Its applications to pharmacy are now so extensive that no more than a general indication of its value in pharmaceutical analysis and as a preparative and analytical method in a number of groups of substances can be given here.

Pharmaceutical Analysis

When the necessary experimental procedures have been studied in detail, chromatographic methods are of particular value in dealing with three types of analytical problems; (1) tests for homogeneity of substances liable to contamination with chemically similar substances; (2) the identification of pharmaceutical substances and preparations; (3) the determination of the individual components of complex mixtures or of substances in dilute solution. Rigorous standardisation of all the possible variables is of the greatest importance because of the frequently unpredictable effects of minor variations in chromatographic processes.

Tests for homogeneity are of particular value in the standardisation of substances obtained from natural sources, such as alkaloids, glycosides

steroids and lipoids. The presence of ergotaminine in ergotamine may be demonstrated by the appearance of two fluorescent bands on an alumina column using chloroform as developing solvent[90]; contamination of ergometrine with ergometrinine is readily shown by their separation on a paper strip.[91] Similar examples are afforded by the chromatographic detection of photolytic decomposition products of riboflavine[92] and of the homogeneity of cardiac glycosides.[93,94]

The identification of pharmaceutical substances and preparations depends upon the determination of R_F values or on observations of the sequence of the distribution of the components of a complex mixture on a column. In one of the methods for the identification of a sulphonamide, the sodium salt, after development on paper with aqueous methanol, is located by spraying with copper sulphate solution and characterised by the colour of the stain and its R_F value.[95] The recognition of the individual alkaloids of ergot has hitherto depended upon careful measurements of a series of physical constants, particularly optical rotation in different solvents. A simple solution to this problem is provided by paper chromatography of the amino-acids liberated on hydrolysis of the alkaloids.[91] The characterisation of galenical preparations[96,97,98,99] and crude drugs[100] by their behaviour on columns is a reliable and simple procedure when the conditions have been carefully standardised; some of the observed variations, as for example with extracts of male fern,[101] may be due to the preparations themselves. The scope of such methods is found empirically; thus whereas certain varieties of jalap can be distinguished,[102] attempts to identify aloe from different sources in this way were unsuccessful.[103]

In quantitative analysis, chromatography is employed in the isolation of the desired material in a form suitable for its determination by a standard chemical, physical or biological method. *Datura stramonium* has been assayed by adsorption on an alumina column of the alkaloids in an ethereal extract of the alkalinised drug, followed by elution with ethanol, evaporation to remove volatile bases and titration of the remaining total alkaloids.[104] This method yields results in agreement with those obtained by the usual procedure and is simpler in operation. The individual alkaloids of solanaceous drugs can be isolated quantitatively from partition chromatogram columns.[32,105] In the control of purity of salts of physiologically active bases, it is desirable to determine the base. For this purpose, solvent extraction can be conveniently replaced by chromatography on appropriately standardised alumina.[106]

Anthraquinone-containing drugs, for which analytical procedures have been hitherto unsatisfactory, can be standardised by isolation of the hydroxyanthraquinones on a magnesium oxide–kieselguhr column; these are then eluted and determined spectrophotometrically.[103] Further examples of quantitative processes are mentioned below and it appears probable that these methods are capable of extension to such problems as the determination of thyroxine in thyroid and the assay of injections and lamellæ.

Naturally occurring pigments. Chromatography has been extensively

used in the isolation, characterisation and determination of the 60 or so carotenoids at present known and continues as the method of choice in the examination of new materials for carotenoids.[107] Examination of urinary and fæcal porphyrins[108] and bile pigments[109] by chromatographic methods are now diagnostic aids.[110]

Steroids. The *durchlaufmethode* has been most frequently employed in the fractionation of colourless steroids on adsorption columns.[111,112,113] An example of the use of coloured derivatives[114] is afforded by the separation of the 2:4-dinitrophenylhydrazones of androsterone, testosterone, œstrone and progesterone on alumina, which is developed first with benzene, then with acetone and light petroleum and finally with chloroform. Separations on paper [115,116] have been effected by conversion of steroids to water-soluble derivatives, for example by reaction with Girard's reagent.[117] In the assay of progesterone in oily solution, the ketosteroid is isolated on a paper strip, located by the *m*-dinitrobenzene–potassium hydroxide reaction, extracted and determined spectrophotometrically.[118]

Amino-acids, peptides and proteins. The applications of chromatography to amino-acids, peptides and proteins has formed the subject of several reviews.[6,7,8,9,10,119] Recent developments in work on this group of compounds has included extension of the qualitative and quantitative methods for amino-acids[85,120,121,122,123]; the examination of peptides,[124] proteins[125] and enzymes,[126,127] insulin,[128,129] liver fractions,[7,130,131] of urinary excretion of amino-acids[132]; nitrogen metabolism of the thyroid,[75,79] of micro-organisms[133] and of enzyme systems[134] and studies of adrenocorticotrophins.[135]

Carbohydrates. The value of chromatographic methods in carbohydrate chemistry[3,4,5,6,7,8,9,10,136] provides an excellent illustration of their capabilities in the separation of stereoisomers. By means of adsorption[137,138] and partition[139] columns and paper strips[140,141] numerous separations of monosaccharides, polysaccharides and their derivatives have been effected. In the quantitative determination of sugars[142,143,144] and their derivatives[145] on paper, the mixture is developed with butanol on a broad strip and the positions of the zones are located by spraying a narrow strip cut from the side of the broad strip. By matching the sprayed strip with the remainder, the unsprayed zones can be marked and cut out; each sugar is then extracted with water and determined by an oxidation titration.

Glycosides. Alumina columns have recently been applied in a systematic study of a number of glycosides, such as those of *Strophanthus*,[16,146] *Adenium multiflorum*[147] and *Acokanthera venenata*[148]; two cardiac glycosides have similarly been obtained from *Adenium Hougel.*[149] A digitalis extract, mixed with methanol and chloroform, afforded on an alumina column three zones which by repetition of the process were eventually fractionated into gitoxigenin, digitoxigenin, gitoxin, digitoxin, purpurea glycosides A and B and four new glucosides.[150,151] Similar separations can be effected on paper strips.[152] Rutin can be separated quantitatively from other flavanol-3-glycosides in quantities of 10 to

40 μg. by paper chromatography, using *n*-butanol, acetic acid and water as developing solvent.[153]

Vitamins. Adsorption columns are applied in the estimation of fat-soluble vitamins for their purification prior to spectrophotometric or colorimetric determination.[154,155,156] Precautions must be taken against the decomposition of the vitamins, especially on alumina. Vitamin A in fish-liver oil is converted to the alcohol, fatty acids are removed and the vitamin is purified, with less than 2 per cent. loss, on a dicalcium phosphate column, using ether–light petroleum as developing solvent, and determined spectrophotometrically after elution.[157] Vitamin D is separated from other steroids, vitamin A and carotenoids on a column of magnesium oxide and kieselguhr.[158] A method for the determination of the vitamins B_{12} in fermentation liquors and liver extracts involves resolution of the vitamins on paper strips and a microbiological assay[73,159]; paper chromatography is being used extensively in structural studies of these vitamins.[160] Chromatographic methods continue to yield valuable results in the isolation, characterisation and determination of other vitamins and allied substances.[74,161,162,163,164,165,166]

Antibiotics. Chromatography has been exploited in the isolation of many antibiotics, both on the laboratory and industrial scales.[34,167,168,169] Paper methods have proved especially successful for their characterisation and assay[7,35,46] and in structural studies.[170,171] In one method for the assay of penicillins,[172] filter-paper is soaked in 30 per cent. potassium phosphate buffer of *p*H 6 to 7 and dried in air; a spot of solution of the mixed sodium salts is then developed on the strip with wet ether and the zones are located by placing the strip on agar inoculated with *Bacillus subtilis*; mixtures of pure penicillins are used as reference standards in the quantitative interpretation of the results. By suitable modification, this type of process is made applicable to the examination of penicillin culture filtrates,[173] streptomycin[73] and other antibiotics.[174,175] *Streptomyces* cultures have been investigated[176] using paper impregnated with toluene-*p*-sulphonic acid, followed by a photographic procedure[177] to obtain a permanent record of the results of a biological test. Paper chromatography has resulted in important advances in the elucidation of the structures of the bacterial antibiotics which are peptides.[178]

Alkaloids. As work on less readily accessible plant material extends, adsorption,[179,180,181,182,183,184,185,186] partition[41,187,188,189] and paper[190,191] chromatographic methods are being increasingly applied to the isolation, characterisation and estimation of alkaloids. A method which appears to be capable of wide application is illustrated by the fractionation of the Reineckates of alkaloids of calabash curare on an alumina column.[192] Several alkaloidal assay processes involving the use of adsorption columns have been described.[104,106,193,194] For the isolation of pomegranate alkaloids a partition column consisting of phosphate buffer distributed on kieselguhr is used.[41] Morphine is quantitatively separated from the ψ-morphine and coloured degradation products formed in injections of morphine by elution with ethanol from an alumina column.[188] The scope of these methods in alkaloidal chemistry is indicated by their use

in the separation of cinchona alkaloids,[195] in the identification of curare alkaloids,[196] in the degradation of germitrine on alumina,[197] in the quantitative isolation of morphine, codeine and diamorphine from viscera[198] and in the isolation of new bases from *Colchicum autumnale*.[199]

Anthraquinone derivatives. Cascara[200] and aloe[201] have been assayed by isolation of certain of their anthraquinone derivatives on adsorption columns; similar columns have been used in the examination of *Frangula* extract.[202] For paper chromatograms, an acetone solution of the anthraquinone derivatives is developed with light petroleum saturated with methanol and the spots are located by heating after spraying with methanolic magnesium acetate.[203] A comprehensive method for the determination of emodins in drugs is based on similar principles.[204]

Comment

Chromatography has further important applications in several large groups of substances, such as organic acids, lipoids and inorganic compounds, and in numerous separative problems which do not correspond to the simple classification adopted here. Mere consideration of the complexity of the mixtures and analytical problems to which it has been successfully applied does not adequately demonstrate its remarkable usefulness. Its true value becomes more evident from an appraisal of the deductions which have been made from the results of chromatographic experiments; recent developments in knowledge of the structures of polysaccharides, proteins and nucleic acids provide appropriate illustrations.

References

1. Zechmeister and Cholnoky, *Principles and Practice of Chromatography*, trans. Bacharach and Robinson, 1943.
2. Strain, *Chromatographic Adsorption Analysis*, 1942.
3. Zechmeister, *Progress in Chromatography*, 1938–47, 1950.
4. Williams, *An Introduction to Chromatography*, 1946.
5. Cassidy, *Adsorption and Chromatography*, 1951.
6. Biochemical Society Symposia No. 3, *Partition Chromatography*, 1949.
7. Discussions of the Faraday Society No. 7, *Chromatographic Analysis*, 1949.
8. Martin, *Ann. Rep. chem. Soc.*, 1948, **45**, 267.
9. Strain, *Anal. Chem.*, 1949, **21**, 75; 1950, **22**, 41; 1951, **23**, 25.
10. Clegg, *ibid.*, 1950, **22**, 48.
11. Martin and Synge, *Biochem. J.*, 1941, **35**, 1358.
12. Monaghan, Suter and Le Rosen, *Anal. Chem.*, 1950, **22**, 811.
13. Trueblood and Malmberg, *ibid.*, 1949, **21**, 1055.
14. Wachtel and Cassidy, *J. Amer. chem. Soc.*, 1943, **65**, 665.
15. Wald, *J. Gen. Physiol.*, 1948, **31**, 489.
16. Euw and Reichstein, *Helv. chim. Acta*, 1950, **33**, 522, 544, 666, 1006, 1551, 2153.
17. Claesson, *Arkiv Kemi Mineral. Geol.*, 1947, *A***24**, No. 16.
18. Hagdahl, *Acta chem. scand.*, 1948, **2**, 574.
19. Miller and Kirchner, *Anal. Chem.*, 1951, **23**, 428.
20. Gault and Ronez, *Bull. Soc. chim., France*, 1950, V, **17**, 597.
21. Porter, *Anal. Chem.*, 1951, **23**, 412.
22. Zechmeister, *Science*, 1951, **113**, 35.
23. Meinhard and Hall, *Anal. Chem.*, 1949, **21**, 185; 1950, **22**, 344.
24. Sease, *J. Amer. chem. Soc.*, 1947, **69**, 2242; 1948, **70**, 3630.
25. Brockmann and Beyer, *Angew. Chem.*, 1951, **63**, 133.
26. Le Rosen, Monaghan, Rivet, Smith and Suter, *Anal. Chem.*, 1950, **22**, 809.
27. Randall and Martin, *Biochem. J.*, 1949, **44**, *Proc.* ii.

28. Hellström and Borgiel, *Acta chem. scand.*, 1949, **3**, 401.
29. Beroza, *Anal. Chem.*, 1950, **22**, 1507.
30. Crook and Datta, *Chem. Ind.*, 1951, 718.
31. Levi, *Biochem. J.*, 1943, **43**, 257.
32. Evans and Partridge, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 126.
33. Fischback, Eble and Mundell, *J. Amer. pharm. Ass., Sci. Ed.*, 1947, **36**, 220.
34. Dobson and Stroud, *Biochem. J.*, 1949, **45**, *Proc.* i.
35. Higuchi and Peterson, *Anal. Chem.*, 1949, **21**, 659.
36. Partridge and Swain, *Nature, Lond.*, 1950, **166**, 272.
37. Boldingh, *Rec. Trav. chim. Pays-Bas*, 1950, **69**, 247.
38. Howard and Martin, *Biochem. J.*, 1950, **46**, 532.
39. Partridge and Chilton, *Nature, Lond.*, 1951, **167**, 79.
40. Gordon, Martin and Synge, *Biochem. J.*, 1943, **37**, 79; 1944, **38**, 65.
41. Chilton and Partridge, *J. Pharm. Pharmacol.*, 1950, **2**, 784.
42. Bentley and Whitehead, *Biochem. J.*, 1950, **46**, 341.
43. Lambert, *Industr. Engng. Chem.*, 1950, **42**, 1349.
44. Kowkabany and Cassidy, *Anal. Chem.*, 1950, **22**, 817.
45. Mueller, *Science*, 1950, **112**, 405.
46. Karnovsky and Johnson, *Anal. Chem.*, 1949, **21**, 1125.
47. Datta, Overell and Stack-Dunne, *Nature, Lond.*, 1949, **164**, 673.
48. Bush, *ibid.*, 1950, **166**, 445.
49. Kirchner and Keller, *J. Amer. chem. Soc.*, 1950, **72**, 1867.
50. Boldingh, *Experientia*, 1948, **4**, 270.
51. Kressmann, *Nature, Lond.*, 1950, **165**, 568.
52. Bender, *Biochem. J.*, 1951, **48**, *Proc.* xv.
53. Müller and Clegg, *Anal. Chem.*, 1951, **23**, 396.
54. Longenecker, *ibid.*, 1949, **21**, 1402.
55. Wolfson, Cohn and Devanay, *Science*, 1949, **109**, 541.
56. Gage, Douglass and Wender, *J. chem. Educ.*, 1950, **27**, 159.
57. Atkinson, *Nature, Lond.*, 1948, **162**, 858.
58. Kawerau, *Biochem. J.*, 1950, **47**, *Proc.* xxiii.
59. Singer and Kenner, *Anal. Chem.*, 1951, **23**, 387.
60. Datta, Dent and Harris, *Science*, 1950, **112**, 621; *Biochem. J.*, 1950, **46**, *Proc.* xlii.
61. Rutter, *Analyst*, 1950, **75**, 37.
62. Miettinen and Virtanen, *Acta chem. scand.*, 1949, **3**, 459.
63. Laidlaw and Reid, *Nature, Lond.*, 1950, **166**, 476.
64. Atkinson, Stuart and Stuckey, *Analyst*, 1950, **75**, 447.
65. Brante, *Nature, Lond.*, 1949, **163**, 651.
66. Partridge, *ibid.*, 1946, **158**, 270; 1949, **164**, 442.
67. Novellie, *ibid.*, 1950, **166**, 745.
68. Bryson and Mitchell, *ibid.*, 1951, **167**, 864.
69. Pollard, McOmie and Elbeih, *ibid.*, 1949, **163**, 292.
70. Holiday and Johnson, *ibid.*, 1949, **163**, 216.
71. Renfrew and Piatt, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 657.
72. Woiwod, *Nature, Lond.*, 1950, **166**, 272.
73. Winsten and Eigen, *J. Amer. chem. Soc.*, 1948, **70**, 3333; *J. biol. Chem.*, 1949, **181**, 109.
74. Harrison, *Analyst*, 1951, **76**, 77.
75. Lemmon, Tarpey and Scott, *J. Amer. chem. Soc.*, 1950, **72**, 758.
76. Gross, Lebond, Franklin and Quastel, *Science*, 1950, **111**, 605.
77. Clayton, Free, Page, Somers and Woollett, *Biochem. J.*, 1950, **46**, 598.
78. Borsook, Deasy, Haagen-Smit, Keighley and Lowy, *J. biol. Chem.*, 1948, **173**, 423; **174**, 1041; **176**, 1383.
79. Keston, Udenfriend and Levy, *J. Amer. chem. Soc.*, 1950, **72**, 748.
80. Boursnell, *Nature, Lond.*, 1950, **165**, 399.
81. Müller and Clegg, *Anal. Chem.*, 1949, **21**, 192, 1123.
82. Claesson, *Nature, Lond.*, 1947, **159**, 708.
83. Christ, Burton and Botty, *Science*, 1948, **108**, 91.
84. Tiselius, *Advances in Protein Chemistry*, 1947, **3**, 67.
85. Hurd, Thomas and Frost, *J. Amer. chem. Soc.*, 1950, **72**, 3733.
86. Tiselius and Hagdahl, *Acta chem. scand.*, 1950, **4**, 394.
87. Holman and Hagdahl, *J. Amer. chem. Soc.*, 1950, **72**, 701; *J. biol. Chem.*, 1950, **182**, 421.
88. Strain, *Industr. Engng. Chem.*, 1950, **42**, 1307.
89. Vonino and Carassito, *Nature, Lond.*, 1951, **167**, 569.

90. Stoll and Hofmann, F.P. 788,812.
91. Foster, Macdonald and Jones, *J. Pharm. Pharmacol.*, 1949, **1**, 802.
92. Hais and Pecáková, *Nature, Lond.*, 1949, **163**, 768.
93. Jaminet, *J. Pharm. Belg.*, 1950, **50**, 297.
94. Mesnard and Devze, *Bull. Soc. Pharm., Bordeaux*, 1950, **88**, 109, 114.
95. Takagi, Suzuki and Imaedo, *Japan. J. Pharm. Chem.*, 1950, **22**, 145.
96. Ulrix, *Bull. Soc. Roy. Sci., Liége*, 1943, **12**, 108.
97. Christensen and Jensen, *Dansk. Tidsskr. Farm.*, 1947, **21**, 68.
98. di Bacco, *Boll. chim.-farm.*, 1948, **87**, 124.
99. de la Vega, *Galenica Acta*, 1949, **2**, 85.
100. MacMorran, *J. Pharm. Pharmacol.*, 1950, **2**, 773.
101. Fichler, *Pharm. Acta Helvet.*, 1938, **13**, 123.
102. Shellard, *J. Pharm. Pharmacol.*, 1951, **3**, 286.
103. Brody, Voigt and Maher, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 666.
104. Rubin and Harris, *ibid.*, 1950, **39**, 477.
105. Evans and Partridge, *J. Pharm. Pharmacol.*, 1949, **1**, 593.
106. Van Os, *ibid.*, 1949, **1**, 55, 350.
107. Karrer and Jucker, *Carotenoids*, 1950.
108. Nicholas and Rimington, *Biochem. J.*, 1951, **48**, 306, 309.
109. With, *Z. physiol. Chem.*, 1942, **275**, 166.
110. McSwiney, Nicholas and Prunty, *Biochem. J.*, 1950, **46**, 147.
111. Trickey, *J. Amer. chem. Soc.*, 1950, **72**, 3474.
112. Haslewood and Wootton, *Biochem. J.*, 1950, **47**, 584.
113. Mason and Schneider, *J. biol. Chem.*, 1950, **184**, 593.
114. Umberger and Curtis, *ibid.*, 1949, **178**, 265, 275.
115. Heftmann, *Science*, 1950, **111**, 571; *J. Amer. chem. Soc.*, 1951, **73**, 851.
116. Zaffaroni, Hechter and Pincus, *ibid.*, 1951, **73**, 1390.
117. Zaffaroni, Burton and Keutmann, *J. biol. Chem.*, 1949, **177**, 109; *Science*, 1950, **111**, 6; cf. *J. biol. Chem.*, 1951, **188**, 763.
118. Haskins, Sherman and Allen, *J. biol. Chem.*, 1950, **182**, 429.
119. Synge, *Chem. and Ind.*, 1950, 339.
120. Brush, Boutwell, Barton and Heidelberger, *Science*, 1951, **113**, 4.
121. McFarren, *Anal. Chem.*, 1951, **23**, 168.
122. Landua, Fuerst and Awapara, *ibid.*, 1951, **23**, 162.
123. Blackburn and Lowther, *Biochem. J.*, 1951, **48**, 126.
124. Brenner, Müller and Pfister, *Helv. chim. Acta*, 1950, **33**, 568.
125. Swingle and Tiselius, *Biochem. J.*, 1951, **48**, 171.
126. Hanes, Hird and Isherwood, *Nature, Lond.*, 1950, **166**, 288.
127. Green and Schroeder, *J. Amer. chem. Soc.*, 1951, **73**, 1385.
128. Archer, Fromageot and Jutisz, *Biochim. Biophys. Acta*, 1950, **5**, 81.
129. Fromageot, Jutisz, Meyer and Pénasse, *C.R. Acad. Sci., Paris*, 1950, **250**, 1905.
130. Shaw, *J. Pharm. Pharmacol.*, 1949, **1**, 701.
131. Campbell, *Biochem. J.*, 1951, **48**, *Proc.* xix.
132. Berry and Cain, *Arch. Biochem.*, 1949, **24**, 179.
133. Dagley, Dawes and Morrison, *Nature, Lond.*, 1950, **165**, 437.
134. Baltzley, Berger and Rothstein, *J. Amer. chem. Soc.*, 1950, **72**, 4149.
135. Brink, Meisinger and Folkers, *ibid.*, 1950, **72**, 1040.
136. Isherwood and Jermyn, *Biochem. J.*, 1951, **48**, 515.
137. Fischer, *Dansk. Tidsskr. Farm.*, 1950, **24**, 1.
138. Wolfrom, Bower and Maher, *J. Amer. chem. Soc.*, 1951, **73**, 875.
139. Bell and Palmer, *Nature, Lond.*, 1949, **163**, 846.
140. Hough and Jones, *J. chem. Soc.*, 1950, 1199.
141. Jeanes, Wise and Dimler, *Anal. Chem.*, 1951, **23**, 415.
142. Albon and Gross, *Analyst*, 1950, **75**, 454.
143. Bartlett, Hough and Jones, *Chem. and Ind.*, 1951, 76.
144. Hough, Jones and Wadman, *J. chem. Soc.*, 1950, 1702.
145. Jermyn and Isherwood, *Biochem. J.*, 1949, **44**, 402.
146. Schindler and Reichstein, *Helv. chim. Acta*, 1951, **34**, 108, 608.
147. Hunger and Reichstein, *ibid.*, 1950, **33**, 1993.
148. Euw and Reichstein, *ibid.*, 1950, **33**, 485.
149. Hunger and Reichstein, *ibid.*, 1950, **33**, 76.
150. Ulrix, *J. Pharm. Belg.*, 1948, **3**, 2.
151. Petit, Pesez, Bellet and Amard, *Bull. Soc. chim. France*, 1950, *V*, **17**, 288.
152. Svendsen and Jensen, *Pharm. Acta Helvet.*, 1950, **25**, 241.
153. Gage and Wender, *Anal. Chem.*, 1950, **22**, 708.
154. Shantz, *Science*, 1948, **108**, 417; *J. biol. Chem.*, 1950, **182**, 515.

155. Eden and Sellars, *Biochem. J.*, 1950, **46**, 261.
156. Wendler, Slates, Trenner and Tishler, *J. Amer. chem. Soc.*, 1951, **73**, 719.
157. Hjarde, *Acta chem. scand.*, 1950, **4**, 628.
158. De Witt and Sullivan, *Industr. Engng. Chem., Anal. Ed.*, 1946, **18**, 117.
159. Woodruff and Foster, *J. biol. Chem.*, 1950, **183**, 569.
160. Buchanan, Johnson, Mills and Todd, *J. chem. Soc.*, 1950, 2845.
161. Keleman, Tanos and Halmagyi, *Biochem. J.*, 1950, **47**, 138.
162. Lyons and Wheeler, *Analyst*, 1950, **75**, 49.
163. Schaefer, *J. Ass. off. agric. Chem., Wash.*, 1950, **33**, 615.
164. Huebner, *Nature, Lond.*, 1951, **167**, 119.
165. Jackson, Whitfield, De Vries, Nelson and Evans, *J. Amer. chem. Soc.*, 1951, **73**, 337.
166. Analytical Methods Committee, *Analyst*, 1950, **75**, 568.
167. Regna and Murphy, *J. Amer. chem. Soc.*, 1950, **72**, 1045.
168. Benedict, Stodola, Shotwell, Borud and Lindenfelser, *Science*, 1950, **112**, 77.
169. Glazko, Dill and Rebstock, *J. biol. Chem.*, 1950, **183**, 679.
170. Solomans and Regna, *J. Amer. chem. Soc.*, 1950, **72**, 2974.
171. Winsten, Jarowski, Murphy and Lazier, *ibid.*, 1950, **72**, 3969.
172. Glister and Grainger, *Analyst*, 1950, **75**, 310.
173. Albans and Dobson, *ibid.*, 1950, **75**, 657.
174. Grundy, Schenck, Clark, Hargie, Richards and Sylvester, *Arch. Biochem.*, 1950, **28**, 150.
175. Dutcher, Hosansky, Donin and Wintersteiner, *J. Amer. chem. Soc.*, 1951, **73**, 1384.
176. Peterson and Reineke, *ibid.*, 1950, **72**, 3598.
177. Drake, *ibid.*, 1950, **72**, 3803.
178. Hinman, Caron and Christensen, *ibid.*, 1950, **72**, 1620.
179. Adams and Govindachari, *ibid.*, 1949, **71**, 1956.
180. Trautner and Roberts, *Analyst*, 1948, **73**, 140.
181. Mendez, Kirch and Voigt, *J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 538.
182. Stolman and Stewart, *Analyst*, 1949, **74**, 543.
183. Bower, Anderson and Titus, *Anal. Chem.*, 1950, **22**, 1056.
184. Leonard and Beyler, *J. Amer. chem. Soc.*, 1950, **72**, 1316.
185. Stoll, Rutschmann and Schlientz, *Helv. chim. Acta*, 1950, **33**, 375.
186. Fischer and Buchegger, *Pharm. Zentralh.*, 1950, **89**, 146, 185.
187. Tappi, *Ann. Chim., Roma*, 1950, **40**, 345.
188. Girard, *Ann. pharm. franc.*, 1950, **8**, 571.
189. Jensen and Svendsen, *Pharm. Acta Helvet.*, 1950, **25**, 31.
190. Munier and Macheboeuf, *Bull. Soc. chim. biol.*, 1949, **31**, 1144; 1950, **32**, 192, 904; *C.R. Acad. Sci., Paris*, 1950, **230**, 1177.
191. Schute, *Pharm. Weekbl.*, 1951, **86**, 201.
192. Karrer and Schmid, *Helv. chim. Acta*, 1946, **29**, 1853; 1947, **30**, 1162, 2081.
193. Lasslo and Webster, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 193.
194. Kellett and Cadman, *J. Pharm. Pharmacol.*, 1950, **2**, 732.
195. Lussman, Kirch and Webster, *J. Amer. pharm. Ass., Sci. Ed.*, 1951, **39**, 368.
196. Schmid and Karrer, *Helv. chim. Acta*, 1950, **33**, 512.
197. Fried, White and Wintersteiner, *J. Amer. chem. Soc.*, 1950, **72**, 4621.
198. Stolman and Stewart, *Analyst*, 1949, **74**, 536.
199. Santavy and Reichstein, *Helv. chim. Acta*, 1950, **33**, 1606.
200. Gibson and Schwarting, *J. Amer. pharm. Ass., Sci. Ed.*, 1947, **36**, 269; 1948, **37**, 206.
201. Svendsen and Jensen, *Scientia Pharm.*, 1949, **17**, 118.
202. Jørgensen, *Dansk. Tidsskr. Farm.*, 1940, **14**, 169; 1950, **24**, 111.
203. Shibata, Takito and Tanaka, *J. Amer. chem. Soc.*, 1950, **72**, 2789.
204. Fischer and Buchegger, *Pharm. Zentralh.*, 1950, **89**, 261.

RESEARCH PAPERS

SOME OBSERVATIONS ON THE INHIBITION OF THE ACTION OF HYALURONIDASE ON HYALURONIC ACID BY GENTISIC ACID AND ITS OXIDATION PRODUCTS

By J. Forrest, B. G. Overell, V. Petrow and O. Stephenson

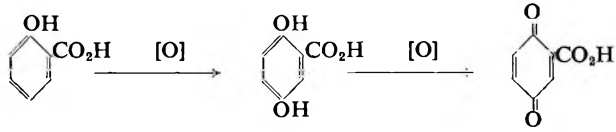
*From The Research Laboratories, The British Drug Houses, Ltd., London, N.*1

Received January 9, 1952

The spreading factor, hyaluronidase, plays an important part in health and in disease. Its biological significance lies in its ability to depolymerise hyaluronic acid, which is present in many animal tissues and which appears to play a part in binding water in interstitial spaces. In synovial fluid, for example, hyaluronic acid holds cells together in a jelly-like matrix which serves as a lubricant and shock absorber in joints. Balance between hyaluronidase and hyaluronic acid is maintained by an enzyme inhibitor linked with the pseudoglobulin fraction of the serum. Hyaluronidase inhibition is also exercised by heparin, chondroitinsulphuric acid and gastric mucin.

Certain rheumatic conditions are characterised by destruction or disorganisation of the synovial membranes, a process in which hyaluronidase is thought to play a part. Thus Guerra[1] observed that intradermal injection of 1 per cent. hyaluronidase solution containing 1 per cent. of Evans Blue into patients suffering from rheumatic fever gave unique reactions characterised by enormous diffusion of the dye. Guerra interpreted these results as indicating increased hyaluronidase activity during the disease process. He made the further observation that administration of sodium salicylate decreased the severity of these reactions. He concluded that salicylates inhibit the "spreading effect" of hyaluronidase, an action to which he attributed, in part, the beneficial results of salicylate therapy in disorders of this character.

Following these observations attempts were made by a number of workers to substantiate Guerra's claims by *in vitro* studies. The method employed consisted in measurement of the degree of depolymerisation of a hyaluronic acid solution by a standardised enzyme preparation. The results obtained, however, showed clearly that salicylates had no effect upon the hyaluronidase-hyaluronic acid system except in relatively enormous concentrations.[2,3,4,5] The observations recorded by Guerra were, therefore, ascribed to the formation of salicylate metabolites, which were considered to be the true inhibitors of the action of hyaluronidase upon hyaluronic acid.



(I) (II) (III)

The metabolic changes undergone by salicylates (I) *in vivo* were studied by Meyer and Ragan,[6] who isolated gentisic acid (II) and gentisuric acid from the urine of patients on salicylate therapy. In their hands gentisic acid was found to exercise a markedly inhibiting effect on the depolymerisation of hyaluronic acid by hyaluronidase after short incubation with the enzyme. Clinical studies,[7] too, appeared to show that gentisates were equivalent, and in some respects superior, to salicylates in the treatment of rheumatic fever.

The observations recorded by the foregoing authors on the hyaluronidase inhibiting activity of gentisates were not supported by the independent studies of Lowenthal and Gagnon,[8] who found that both (I) and (II) were devoid of activity *in vitro*. Gentisoquinone (III), however, which may be regarded as a simple oxidation product of (II), proved, in contrast, to have very marked activity. No evidence was obtained that the latter compound was formed from (II) *in vivo*.

Studies reported by Roseman, Pearson and Dorfman[9] supplied an explanation for these apparently conflicting results. The hyaluronidase inhibiting activity previously ascribed to gentisic acid was traced to the presence of impurities in the material. Highly purified (II) was devoid of activity, but could be made active by shaking its alkaline solution in air. Roseman *et al.* concluded, on this evidence, that the hyaluronidase inhibiting activity of gentisic acid was due to an impurity which was probably an oxidation product.

Our own interest in derivatives of salicylates and gentisates having hyaluronidase inhibiting activity arose from the view that such compounds might prove of value in certain rheumatic disorders in which disorganisation of the hyaluronic acid matrix of synovial fluid by hyaluronidase is thought to occur. Indirect evidence supporting this viewpoint has recently been provided by a number of workers, who have shown that the increased permeability of the synovial membrane to dyestuffs resulting from hyaluronidase action may be antagonised by both cortisone and adrenocorticotrophic hormone.[10,11] We, therefore, undertook a study of certain oxidation products of (I) and (II), hoping thereby to obtain a product worthy of biological study *in vivo* (cf. the publication by Hetzel and Hins[12] which appeared after completion of our studies).

Our own work has fully confirmed the earlier views expressed by Roseman *et al.* (*loc. cit.*). Crude gentisic acid does, in fact, contain a small quantity of a highly active inhibitor, the identification of which formed our first objective. For this purpose we required an adequate supply of the crude acid, which was obtained by oxidising 10 kg. of salicylic acid with ammonium persulphate.[13] Purification of this material gave 2 kg. of "sludge" which showed marked activity and was, therefore, submitted to exhaustive fractionation, the progress of which was followed by hyaluronidase inhibiting activity evaluation at every stage.

A small fraction (1·5 per cent; *Compound A*) was ultimately obtained which had an inhibitory index of 10,000 units/g. (cf. pure gentisic acid; 58 units/g.) (see *Experimental* for definition of units employed). The material was nearly black. It was almost insoluble in water, chloroform,

and ether, but was somewhat more soluble in ethyl acetate and in the lower alcohols. Its acidic character was indicated by its solubility in alkalis, in which it readily dissolved to give a dark brown solution. Combustion analysis indicated a formula roughly approximating to $(C_6H_4O_3)_n$. The physical and chemical properties of *Compound A* were thus identical with those attributed by Eller and Koch[14] to the "humic acids" which are formed by oxidation of phenols with persulphate or with air in alkaline solution. Its formation from salicylic acid must, therefore, proceed through an hydroxyquinone type of intermediate (*vide infra*) by a mechanism involving polymerisation.[15] Some evidence supporting this view was obtained by parallel experiments in which humic acids were obtained from gentisic acid by the aerial oxidation procedure already referred to (Eller and Koch, *loc. cit.*).

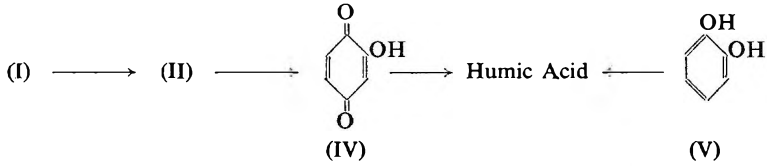
Aerial oxidation of a solution of gentisic acid containing 3 equivalents of alkali for a period of 40 hours gave a black solution which liberated carbon dioxide on acidification. Extraction with ether removed unchanged material (*ca.* 50 per cent.). Further extraction with ethyl acetate and with *n*-butanol gave a series of fractions ranging from dark semi-solids to black tars which showed low activity (300 to 900 units/g.) and thus differed sharply from *Compound A*. The results were nevertheless sufficiently encouraging to warrant further extension.

The facility with which decarboxylation apparently occurred during the aerial oxidation of gentisic acid appeared to be highly significant in view of the proposed formulation of *Compound A* as a humic acid. We were, therefore, led to examine the alkaline aeration of an equimolar mixture of gentisic acid and hydroquinone. Although in this instance results similar to the foregoing oxidation were obtained, a trace of a black compound of inhibitory index *ca.* 3000 units was nevertheless isolated. Pursuing this line of reasoning further we next studied the oxidation of an equimolar mixture of gentisic acid and benzoquinone in a solution containing 3 equivalents of alkali. The result proved satisfactory. A nearly black sparingly soluble humic acid, *Compound B*, was obtained, which strongly resembled *Compound A* in appearance, in physical and chemical properties, and in inhibitory index. Variation in the proportions of gentisic acid to benzoquinone caused only slight change in the activity of the product, although the analytical figures of the humic acids thus obtained varied somewhat over a range of values.

In view of these results the oxidation of benzoquinone itself was studied. Aeration of its solution in the presence of one equivalent of alkali gave high yields of black insoluble products which, after washing well with water and drying at 95° C., had inhibitory indices of *ca.* 10,000 units, i.e., were only slightly less active than *Compounds A* or *B*. Benzoquinone itself, it should be added, reacted exothermically with aqueous alkali, to yield, without aeration, a product having activity *ca.* 3000 units/g.

It seems reasonable to conclude from the above evidence that the development of hyaluronidase-inhibiting properties by solutions of salicylates and gentisates (cf. Roseman *et al.*[9]) is due to the production of compounds of the humic acid type. This view is further strengthened

by the observation that the naturally-occurring humic acids of soil and peat show definite hyaluronidase-inhibiting properties (cf. Table II). On this basis the formation of humic acids from salicylates (I) and gentisates (II) would presumably occur through intermediate formation of hydroxyquinones (cf. IV[15]), which would then take part in a polymeric process in which other suitably constituted phenolic components could participate.

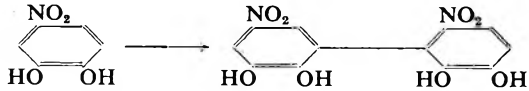


(IV) (V)

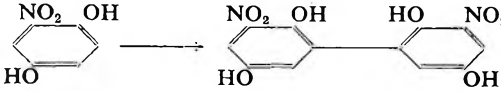
The formation of humic acids from (I) or (II), however, is accompanied by complex side reactions, as gentisic acid, for example, absorbs up to *ca.* 2 moles. oxygen on shaking in alkaline solutions (see Experimental, Table I), a value greatly in excess of that required to convert (II) into (IV). The extent of oxidation of phenols by air in alkaline solution appears, in fact, to depend largely upon the amount of alkali present (cf. Table I), which likewise determines the degree of oxidation of the resulting humic acids. Expressions purporting to represent the empirical formulae of humic acids are thus meaningless, agreement between analytical figures and formulae such as $(C_6H_4O_3)_n$ proposed by Eller and Koch[14] being purely fortuitous.

Some evidence supporting the view that the hydroxyquinone (IV) forms an important link in the chain of reactions which lead to the humic acids is also furnished by observations on the oxidation of catechol (V). This compound contains preformed an ortho-dihydroxy-group corresponding to that present in (IV), and gives a product (*Compound C*) of high activity (13,800 units/g.) on alkaline oxidation. 1:2:4-Trihydroxybenzene, the reduction product of (IV), likewise gives material of high activity, though in somewhat lower yield.

The marked activity exhibited by the catechol oxidation product (*Compound C*) led us to examine the aerial oxidation of a number of compounds containing the *ortho*-dihydroxybenzene residue. These are listed in Table II, from which it can be seen that products of activity 10,000 to 20,000 units/g. may be obtained from several different starting materials.



(VI) (VII)



(VIII) (IX)

The oxidation of 4-nitrocatechol (VI) and nitrohydroquinone (VIII), it will be noted, led to the formation of crystalline tetrahydroxy-dinitrodiphenyl derivatives, which we tentatively formulate as (VII) and (IX), respectively. Of these, the latter showed pronounced activity (8000 units/g.). All the other oxidation products listed in Table II formed dark infusible solids of the typical humic acid type.

Biological evaluation of *Compounds A*, *B* and *C* was kindly undertaken by Dr. S. W. F. Underhill and his staff (Physiological Research Laboratories). All three compounds exercised inhibition on the spread of hæmoglobin co-injected with hyaluronidase intradermally into the backs of rabbits, but the effects were accompanied by physiological actions of undesirable character.

Experimental

Melting points are uncorrected.

Hyaluronidase was prepared from bull testes by the method of Madinaveita.[16] Hyaluronic acid was prepared from umbilical cords by ethanol precipitation as described by McClean,[17] or by the ammonium sulphate fractionation procedure of Hadidian and Pirie.[18]

Hyaluronidase assay. The method employed closely followed the viscometric assay developed by Swyer and Emmens[13] in which the reduction in viscosity of a standard hyaluronic acid solution is measured after 20 minutes incubation with the enzyme. An Ostwald type viscometer of 5 ml. capacity and flow time 10 seconds was employed throughout the investigation. The concentration of the hyaluronic acid solution was adjusted to give a flow time of *ca.* 30 seconds at 25° C.

4 ml. of hyaluronic acid solution was pipetted into a test-tube containing 1 ml. of M/60 *p*H7 buffer (McIlvaine's buffer containing disodium hydrogen phosphate, citric acid, with added sodium chloride[20]), and the mixture equilibrated in a water bath thermostatically maintained at 25 ± 0·1° C. 1 ml. of hyaluronidase solution in 0·5 per cent. gum arabic (added to preserve potency), which had also been equilibrated, was then run in, a stop-watch was started, and the two solutions mixed with a warm pipette. 5 ml. of the mixture was then pipetted into the temperature-equilibrated viscometer and, after exactly 20 minutes incubation, the flow time (f_e) measured. The flow times of the substrate and solvents without enzyme (f_s) and solvents alone (f_o) were measured in the same way. A measure of the enzyme activity was then given by calculation of the flow-time index (F.T.I.):

$$\text{F.T.I.} = 1000 \left(\frac{f_s - f_e}{f_s - f_o} \right)$$

Inhibitor Assay. Most of the inhibitors studied were soluble in McIlvaine's buffer. The inhibitors could, therefore, be introduced directly into the system. 0·06 g. of the inhibitor was weighed out, dissolved in buffer by warming on the steam bath and made up to 10 ml. with buffer. 1 ml. of this 0·6 per cent. solution was added to 4 ml. of hyaluronic acid solution and the assay procedure followed as before. The final

concentration of the inhibitor in the viscometer under these conditions was 0·1 per cent. w/v. If complete inhibition of the enzyme occurred at this concentration, the inhibitor solution was diluted 1 in 10 with buffer and again assayed. The dilution procedure was continued until a reading (f_i) was obtained which showed between 25 and 75 per cent. inhibition of the enzyme. Outside these limits of inhibition the experimental error became too large, for reasons which are discussed below.

The percentage inhibition of the enzyme was calculated from the flow-time index:

$$\text{Per cent. Inhibition} = \frac{\text{F.T.I.}_{\text{enzyme}} - \text{F.T.I.}_{\text{inhibitor}}}{\text{F.T.I.}_{\text{enzyme}}} \times 100$$

$$= \left(\frac{f_i - f_e}{f_s - f_e}\right) \times 100$$

Scale of Units. The expression of inhibitory activity in terms of the concentration of inhibitor required to produce a certain percentage of enzyme inhibition has been employed by, *inter alia*, Meyer and Ragan[6] and Lowenthal and Gagnon.[8] It is not entirely satisfactory, however, as percentage inhibition does not vary with concentration of inhibitor in linear fashion (*vide infra*). Thus, the concentration of inhibitor which causes partial inhibition varies with concentration of the enzyme and the temperature and may, in addition, be affected by extraneous protein or by decomposed enzyme. Furthermore, the activity of a solid enzyme preparation decreases on storage over a period of months at a somewhat greater rate than does its inhibitor-binding power. Finally, the preparation of successive batches of enzyme possessing reasonably constant activity is difficult, whilst the maintenance of a single standard enzyme preparation of constant activity over the period of time occupied by the investigation was clearly impossible. In these circumstances it was necessary to evolve some method whereby the results obtained with successive enzyme preparations could be correlated and compared.

By plotting (percentage inhibition) against — log [inhibitor] (where [inhibitor] represent the concentration of inhibitor in g./100 ml.), sigmoid curves of the type shown in Figure 1 are obtained for different enzyme preparations but with the same inhibitor.

Examination of these curves reveals (i) that the relationship between the two functions varies approximately in linear fashion over the inhibition range of 25 to 75 per cent. and (ii) that various enzyme preparations (A, B, C) give approximately parallel curves in this region of inhibition. It is thus possible to adopt a single typical curve AA′ to express the relationship in question and to equate the values for a particular enzyme preparation with those of the "standard" curve AA′ by introducing a simple arithmetical correction into the value of — log [inhibitor] which will correspond to $\frac{XA''}{XB''}$ for enzyme B, $\frac{XA''}{XC''}$ for enzyme C, etc. Calibration of successive enzyme preparations in terms of the standard preparation and inhibitor may thus be effected.

In order to calculate the activity of a particular inhibitor, the value

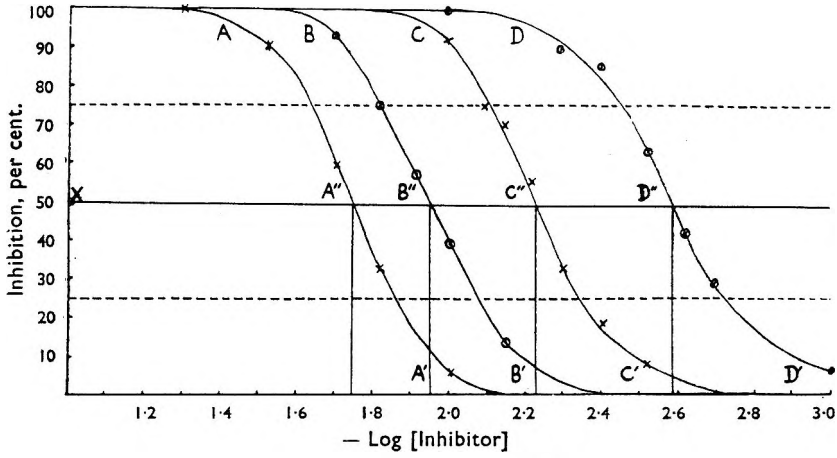


FIG. 1.

Variation of percentage hyaluronidase inhibition with the negative logarithm of the concentration of inhibitor.

Curve AA′; enzyme preparation (2) with inhibitor (a).
Curve BB′; enzyme preparation (2) with inhibitor (b).
Curve CC′; enzyme preparation (1) with inhibitor (a).
Curve DD′; enzyme preparation (1) with inhibitor (b).

Enzyme preparation (1) contained 100 mg./100 ml. organic solids.
Enzyme preparation (2) contained 400 mg./100 ml. organic solids.

$$\frac{XA''}{XB''} = 0{\cdot}89; \qquad \frac{XC''}{XD''} = 0{\cdot}86$$

of — log [inhibitor] required to bring about an inhibition in the region 25 to 75 per cent. is determined. This value is corrected for the enzyme preparation which is being used, and the corrected value employed to determine the — log [inhibitor] required to produce 50 per cent. inhibition by reference to curve AA′. The antilog of this value gives the reciprocal of the concentration producing 50 per cent. inhibition, which is defined as the hyaluronidase inhibiting activity. This in units/g. thus becomes $\frac{1}{x}$, where x = [inhibitor] required to produce 50 per cent. inhibition. The scale of units is, of course, purely arbitrary, and is related only to the original activity of the enzyme preparation used for developing the technique.

The success of the method clearly depends upon accurate calibration of each enzyme sample for, whereas the reproducibility of values with the same enzyme is to within ± 5 per cent., the reproduction of results obtained with different enzyme preparations may only be to within ± 20 per cent. Nevertheless, the values obtained for the different inhibitors over a period of 8 months would appear to be comparable.

Isolation of Compound A. Oxidation of 10 kg. of salicylic acid with persulphate as described by Forrest and Petrow[13] gave 2 kg. of sludge. The dried material was finely ground to a sternutatory powder in a ball mill in a draught chamber, and the resulting product extracted with

chloroform (10 l.) under a reflux condenser for 1 hour, followed by immediate filtration of the boiling solution. The extraction process was repeated a further 9 times, the amount of material extracted decreasing with successive operations from >350 g. on the first extraction to less than 40 g. at the 10th extraction. The material recovered from the chloroform (*ca.* 1·6 kg.) had very low activity and was largely salicylic acid admixed with a small quantity of gentisic acid. The chloroform-insoluble dark brown residue (*ca.* 120 g., m.pt. 170° to 190° C.) was extracted 3 times with 1 l. quantities of ether to give:—

fraction	(i):	66 g., m.pt. 188° to 190° C.;	activity	< 200
,,	(ii):	12·3 g., m.pt. 177° to 183° C.;	,,	= 1200
,,	(iii):	1·5 g., m.pt. 166° to 178° C.;	,,	= 7500

The marked decrease in the quantity of material extracted, combined with the decrease in m.pt. and increase in activity in passing from fraction (i) to (iii) appeared to indicate the presence, in the insoluble residues of a chloroform/ether insoluble or sparingly soluble material of high activity. The residues from the ether extractions were, therefore, dissolved in absolute ethanol (250 ml.) at 50° C., filtered from *ca.* 5 g. of inorganic matter, the solvent removed under reduced pressure, and the residue (32 g.; m.pt. 230° to 240° C. (decomp.); activity (*ca.* 11,000 units/g.) extracted with hot water (150 ml., at 80° to 85° C.) for 30 minutes. Collection of the insoluble material, followed by washing with water and drying, gave *Compound A* as a black amorphous solid (16 g.), m.pt. *ca.* 240° C. (decomp.). Found: C, 57·1; H, 3·9; $C_6H_4O_3$ requires C, 58·1; H, 3·2 per cent.; hyaluronidase inhibiting activity 11,500 units/g., apparently unchanged by further extraction or precipitation procedures.

The red-brown aqueous extracts from *Compound A* gave a permanent violet colour with ferric chloride. Evaporation yielded a red-brown hygroscopic solid of negligible activity.

Aerial Oxidation of Gentisic Acid in Alkaline Solution. Methyl gentisate is readily accessible in a state of high purity and was, therefore, employed in many of the experiments in preference to the free acid. It undergoes facile and rapid hydrolysis in the presence of caustic alkalis. Numerous oxidation experiments were performed in the course of the investigation. Only typical data for each group of reactants and conditions has been recorded for reasons of brevity.

Methyl gentisate (100 g.) in water (400 ml.) was treated with potassium hydroxide (100 g.) and the mixture cooled to room temperature. A rapid stream of carbon dioxide free air was then aspirated through the solution for 40 hours. The black mixture was acidified with concentrated hydrochloric acid (150 ml.) when evolution of carbon dioxide occurred. The dark crystalline material which separated (38 g., m.pt. 197° to 202° C. (decomp.), activity 76 units/g.) was collected and identified as unchanged gentisic acid. The aqueous liquors were then extracted 4 times with ether (300 ml. per extraction) yielding a further quantity of crude gentisic acid (34 g., m.pt. 195° to 200° C. (decomp.), activity 74 units/g.). The aqueous mother liquors were then extracted with ethyl acetate

(3 × 300 ml.) to yield a pitch-like black solid (4 g., activity 933 units/g.) and finally with *n*-butanol to give a black solid of activity 501 units/g.

Aerial Oxidation of Gentisic Acid + Hydroquinone in Alkaline Solution. Methyl gentisate (50·4 g.) and hydroquinone (33 g., 1 equiv.) in water (250 ml.) and potassium hydroxide (3 equivs.) was oxidised by rapid aspiration of air for 16 hours. Acidification with concentrated hydrochloric acid (100 ml.) gave a small quantity of a black solid (2 g., activity 3,150 units/g.) which was collected. Ether extraction of the mother liquors gave crude gentisic acid (56 g., activity 102 units/g.). Subsequent extractions with (i) ethyl acetate gave nearly black material (10 g.) of activity 379 units/g.; (ii) *n*-butanol gave a similar product (10 g.) activity 83 units/g.

Aerial Oxidation of Gentisic Acid + Benzoquinone in Alkaline Solution. Methyl gentisate (33·4 g.) and benzoquinone (21·6 g., 1 equiv.) in water (200 ml.) containing 3 equivalents of potassium hydroxide were oxidised as above for 24 hours. Addition of concentrated hydrochloric acid precipitated a black, infusible solid, *Compound B* (26·5 g.) of activity 11,000 units/g. Ether extraction of the mother liquors gave crude gentisic acid (19 g. activity 65 units/g.). Subsequent butanol extraction gave a smaller quantity (4 g.) of less active black material (activity 1075 units/g.). Variation in the gentisic acid/benzoquinone ratio gave materials of comparable activity.

Aerial Oxidation of Catechol. Aerial oxidation of catechol (55 g.) in aqueous solution in the presence of 3 equivalents of alkali for 30 hours, followed by acidification as before, gave a black infusible solid (30 g.), *Compound C.* Found: C, 52·5; H, 3·5 per cent., activity 13,800 units/g.

Aerial Oxidation of 1 : 2 : 4-*Triacetoxy Benzene.* Aerial oxidation for 24 hours of 1 : 2 : 4-triacetoxybenzene (50·4 g.) in aqueous solution in the presence of 4 equivalents of potassium hydroxide, followed by acidification of the mixture, gave a black infusible humic acid (8 g.). Found: C, 50·0; H, 3·5 per cent., activity 9550 units/g.

Experiments with Benzoquinone. Benzoquinone (130 g. of moist, freshly steam-distilled material) in water (600 ml.) and sodium hydroxide (60 g.) was heated on the steam bath with shaking for 1 hour, when solution was complete. A portion (260 ml.) of the resulting solution was acidified with concentrated hydrochloric acid (40 ml.) and the black, infusible precipitate (20 g.) collected, washed thoroughly, and dried. Found: C, 60·1; H, 4·1 per cent. activity 4920 units/g.

The remainder of the solution (460 ml.) was aspirated with air for 24 hours. Acidification (150 ml. of hydrochloric acid) was accompanied by liberation of carbon dioxide and precipitation of a dark brown infusible humic acid (60 g.). Found: C, 55·9; H, 2·8 per cent. activity 10,470 units/g.

Absorption of Oxygen by Alkaline Solutions of Gentisic Acid. 0·25 Molar solutions of gentisic acid containing sodium hydroxide were shaken in the presence of oxygen in a Warburg type apparatus at 22° C. until equilibrium had been reached. The results obtained are shown in Table I.

TABLE I

Sodium hydroxide mols./mol. of genisic acid	Total oxygen absorbed (mols.)
1·00	No absorption
1·25	0·507
1·50	0·859
1·75	1·216
2·00	1·387
2·25	1·662
2·5	1·810
2·75	1·947
3·00	2·092
3·5	2·157

Miscellaneous Experiments. The results obtained by the aerial oxidation of a variety of compounds, in aqueous alkaline solution, are summarised in Table II. Unless otherwise stated, the products obtained formed black infusible solids.

TABLE II

Compound	Equivalents of of potassium hydroxide	Hours Aeration	Hyaluronidase inhibiting activity of product units/g.
Toluquinone	1	22	4600
2:5-Dihydroxybenzoquinone	—	—	70
2:5-Dihydroxybenzoquinone	4	25	5030
2:5-Di*tert.*-butylbenzoquinone (*a*)	2	5·5	210
2:4:5:2′:4′:5′-Hexahydroxydiphenyl	3	24	16,250
Chlorohydroquinone	0·5	24	3730
Nitrohydroquinone	2	11	7800 (*b*)
Purpurogallin	3	30	3200
1:2:4:1′:2′:4′-Hexahydroxydiphenyl methane	6	10	17,020
Protocatechuic acid	4	18	12,070
9-Phenyl-2:3:7:4′-tetrahydroxyfluorene (*c*)	9	20	430
Tetrahydroxyxanthylium chloride	5	20	19,000
Alizarin	2	20	370
β-Methylæsculetin	2	20	360
4-Nitrocatechol	—	—	<10
4-Nitrocatechol	2	24	870 (*b*)
Dihydroxyphenyl-hydroxybenzoquinone (*d*)	—	—	38,000
Soil humic acid	—	—	3100; 2800 (*e*)
Peat humic acid	—	—	3800; 3200 (*e*)

(*a*) Calesnick and Beutner,[21] report that 2:5-di*tert.*-butyl-benzohydroquinone shows very high hyaluronidase inhibiting activity. This observation could not be confirmed as the material proved to be too insoluble for examination by the technique employed in this work.
(*b*) Oxidation product obtained crystalline, see Experimental.
(*c*) Prepared by the method of Liebermann and Lindenbaum.[22]
(*d*) Prepared in very low yield by allowing *o*-benzoquinone to decompose spontaneously in chloroform solution (Jackson and Koch).[23]
(*e*) Determined on sample dried at 90° C.

Preparation of 2:4:5:2′:4′:5′-Hexahydroxydiphenyl.[24] Triacetoxybenzene (50·4 g.) was hydrolysed by heating for 15 minutes with 10 per cent. sulphuric acid (300 ml.). The mixture was then cooled and added to a suspension of benzoquinone (10·8 g.) in 10 per cent. sulphuric acid (200 ml.). After stirring for 6 hours the light blue-grey solid was collected and dried, m.pt. 277° to 280° C. Found: C, 57·2; H, 4·0. $C_{12}H_{10}O_6$ requires C, 57·6; H, 4·0 per cent. Yield 21 g.

Oxidation of Nitrohydroquinone. Nitrohydroquinone (15·5 g.) in water (100 ml.) containing sodium hydroxide (8 g.) was aspirated with

air for 11 hours. The mixture was acidified with concentrated hydrochloric acid (13 ml.) when marked frothing occurred, together with evolution of some nitrous fumes. The precipitated solid (3·5 g.; m.pt. 250° to 260° C. was collected and crystallised from ethyl acetate-light petroleum to give 2:5:2′:5′-tetrahydroxy-3:3′-dinitrodiphenyl, small bright red needles, m.pt. 240° C. (decomp.). Found: N, 9·1; $C_{12}H_8O_8N_2$ requires N, 9·1 per cent.

Acetylation in pyridine solution with acetic anhydride for 1 hour on the water-bath gave *tetra-acetoxydinitro-diphenyl*, yellow shining plates from acetic acid, m.pt. 191° C. Found: C, 50·2; H, 3·4; N, 6·2; $C_{25}H_{16}O_{12}N_2$ requires C, 50·4; H, 3·4; N, 5·9 per cent.

Oxidation of Nitrocatechol. 4-Nitrocatechol (10 g.) in water (150 ml.) containing sodium hydroxide (5·6 g., 2 equivs.) was aspirated with air for 24 hours. Acidification precipitated a brown microcrystalline material (6 g.; m.pt. > 300° C.) which was purified by recrystallisation from ethyl acetate-light petroleum. 2:3:2′:3′-*Tetrahydroxy*-5:5′-*dinitrodiphenyl* formed brown microcrystals, m.pt. > 300° C. (decomp.). Found: C, 46·7; H, 2·5; N, 9·5; $C_{12}H_8O_8N_2$ requires C, 46·8; H, 2·6; N, 9·1 per cent.).

Acetylation with acetic anhydride under reflux for 2 hours gave 2:3:2′:3′-*tetraacetoxy*-5:5′-*dinitrodiphenyl*, small brown needles from aqueous acetic acid, m.pt. 170° C. Found: C, 50·0; H, 3·7; N, 5·8; $C_{20}H_{16}O_{12}N_2$ requires C, 50·4; H, 3·4; N, 5·9 per cent.

Preparation of soil humic acid.[25] The soil was shaken with cold, dilute hydrochloric acid to decompose "humates" and carbonates and then washed free from acid. The residue was extracted with cold 2 per cent. sodium hydroxide in a closed bottle for several days, and the dark extract decanted off and centrifuged free from suspended particles. The "bright" aqueous extract was acidified with hydrochloric acid and the precipitate centrifuged off and shaken with successive portions of cold, 96 per cent. ethanol, until no further coloured material dissolved. The residual humic acid was divided into two portions, which were dried at room temperature and at 90° C. respectively.

Preparation of peat humic acid. Peat was dissolved in cold, 20 per cent. sodium hydroxide and the solution centrifuged free from insoluble matter. The "bright" extract was acidified with hydrochloric acid and the humic acid centrifuged off and washed with water. It was collected and divided into two portions, which were dried at room temperature and at 90° C. respectively.

The humic acids from soil and peat were only partly soluble in the buffer solutions employed for assay.

Summary

1. Evidence is presented to show that the appearance, in solutions of salicylates and gentisates, of inhibiting properties on the depolymerisation of hyaluronic acid by hyaluronidase, may be due to production of compounds of the humic acid type.

2. This view is strengthened by the observation that the naturally-occurring humic acids of soil and peat inhibit the action of the enzyme upon the mucopolysaccharide acid.

3. The humic acids derived from salicylic acid, gentisic acid, and catechol, show high *in vitro* activity as inhibitors.

The authors thank the Directors of The British Drug Houses, Ltd., for permission to publish these results.

References

1. Guerra, *J. Pharmacol*, 1946, **87**, 193; *Science*, 1946, **103**, 686.
2. Meyer, *Physiol. Rev.*, 1947, **27**, 335.
3. Dorfman, Reimers, and Ott, *Proc. Soc. exp. Biol. N.Y.*, 1947, **64**, 357.
4. Peke, *Science*, 1947, **105**, 391.
5. Swyer, *Biochem. J.*, 1948, **42**, 32.
6. Meyer and Ragan, *Fed. Proc.*, 1948, **7**, 173.
7. Meyer and Ragan, *Science*, 1948, **108**, 281.
8. Lowenthal and Gagnon, *Canad. J. Res.*, 1948, **26**E, 200.
9. Roseman, Pearson and Dorfman, *Fed. Proc.*, 1949, **8**, 245.
10. Seifter, Baeder and Begany, *Proc. Soc. exp. Biol., N.Y.*, 1949, **72**, 277.
11. Benditt, Schieler, Wong and Dorfman, *ibid*, 1950, **75**, 782.
12. Hetzel and Hins, *Lancet*, 1951, **261**, 94.
13. Forrest and Petrow, *J.chem. Soc.*, 1950, 2304.
14. Eller and Koch, *Ber. dtsch. chem. Ges.*, 1920, **53**, 1469.
15. Erdtmann, *Proc. roy. Soc.*, 1933, **143**A, 177.
16. Madinaveita, *Biochem. J.*, 1941, **35**, 447.
17. McClean, *ibid.*, 1943, **37**, 169.
18. Hadidian and Pirie, *ibid.*, 1948, **42**, 260.
19. Swyer and Emmens, *ibid.*, 1947, **41**, 29.
20. McIlvaine, *J. biol. Chem.*, 1921, **49**, 183.
21. Calesnick and Beutner, *Proc. Soc. exp. Biol., N.Y.*, 1949, **72**, 629.
22. Liebermann and Lindenbaum, *Ber. dtsch. chem. Ges.*, 1904, **37**, 1171, 2728.
23. Jackson and Koch, *Amer. chem. J.*, 1901, **26**, 13.
24. Brizina, *Monatsh.*, 1901, **22**, 594.
25. Du Toit and Page, *J. agric. Sci.*, 1932, **22**, 116.

THE STRUCTURE OF THE FLOWERS OF *DATURA STRAMONIUM* L. AND *D. TATULA* L.

By T. E. Wallis and S. Rohatgi

From the Museum of the Pharmaceutical Society of Great Britain

Received January 2, 1952

Introduction

Linnaeus, in his *Species Plantarum*, first edition[1], 1753, gives a very brief diagnosis of the plant *D. stramonium* to which are added a few words about the leaves in the second edition, but this does not include any description of the flowers. A description of the gross morphology of the flower appears in *Medicinal Plants*, by Bentley and Trimen,[2] 1880. The histology of the entire plant, including that of the flower, has been studied in considerable detail by Fischer[3] in 1937, but the few drawings and photographs used to illustrate his work are unsatisfactory. It seemed desirable, therefore, to make a fresh examination of the flower and to write a new description of its gross morphology and histology and to illustrate it by adequate drawings.

Material

The specimens used for this study were collected from the following sources:

1. Flowers collected from the Chelsea Physic Garden in June, 1947.
2. Three specimens of dried flowers from the Museum of the Pharmaceutical Society of Great Britain, one of them labelled; Mr. Stewart, Glasgow, 1938.
3. Flowers from a commercial sample of the crude drug from Messrs. British Drug Houses, London, purchased, 1949.

Gross Morphology

The inflorescence is described as a dichasial cyme in which each bract is displaced by adnation to the shoot up to the point where the next branches arise. The flowers are solitary, shortly pedicellate, erect and arising at the fork of the stems.

Calyx (Fig. 2A). *Sepals*, forming a pale-green tubular structure, broadening slightly towards the base, with 5 sharply projecting ridges over the midribs; the *tube* measuring about 2 to 3 cm. long; *lobes* acutely triangular and varying somewhat in size, measuring about 10 mm. long; *æstivation* valvate; calyx fugaceous, the abscission taking place along a circumferential line near the base, leaving a small, saucer-shaped persistent part.

Corolla (Fig. 4A). *Petals*, white, delicate and funnel-shaped, the long tubular part being 4 to 5 cm. long and projecting beyond the calyx and then increasing in diameter as it approaches the lobes which are rounded-triangular and spreading, each about 6 to 10 mm. long and having

a thin, acuminated, folded, almost filiform tip; *æstivation* plicate and twisted; corolla fugaceous.

Andrœcium (Fig. 4A). *Stamens*, the free part of the filaments about 17 to 25 mm. long, the adherent part, which is about 20 to 25 mm. long,

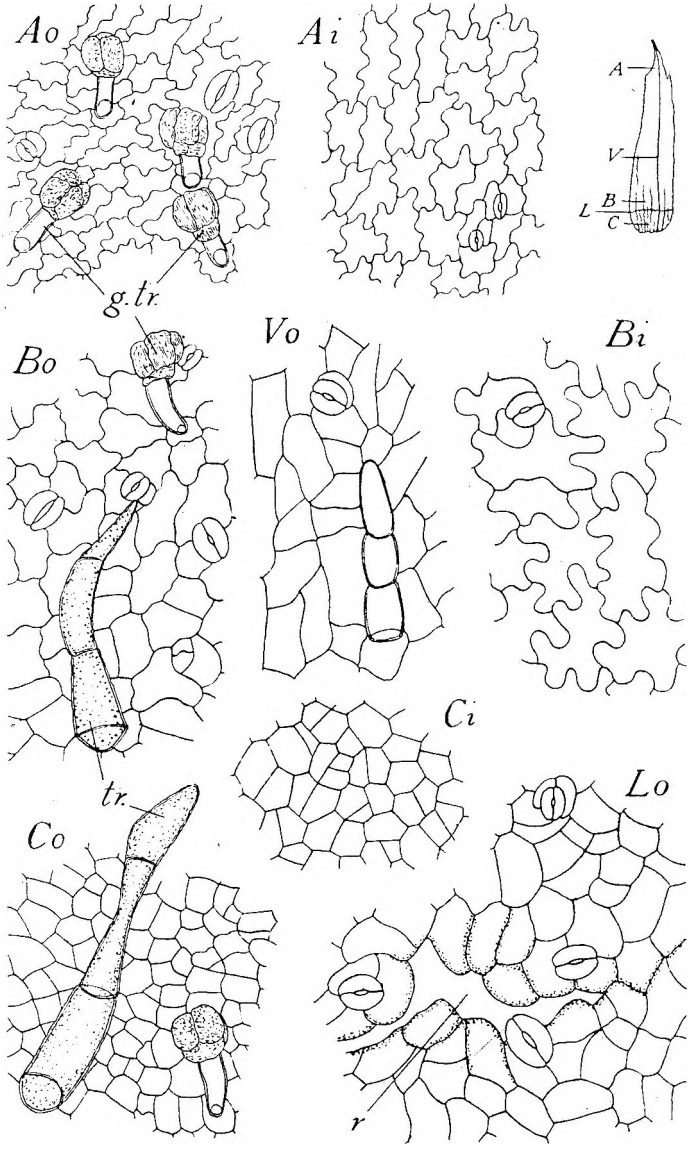


Fig. 1. *Datura stramonium* L., Calyx. Ao, outer epidermis at position A (as marked on diagram of one sepal, top right); Ai, inner epidermis at A; Bo, outer epidermis at B; Vo, outer epidermis over vein at V; Bi, inner epidermis at B; Co, outer epidermis at C; Ci, inner epidermis at C; Lo, outer epidermis at line of abscission at L; *g.tr.*, clavate glandular trichome; *r*, rupture at line of abscission; *tr*, warty covering trichome. All × 160.

forming a wide projecting rib on the inner surface of the lower part of the corolla tube. *Anthers*, about 2 to 5 mm. long, basifixed, dehiscence lateral, along two longitudinal splits.

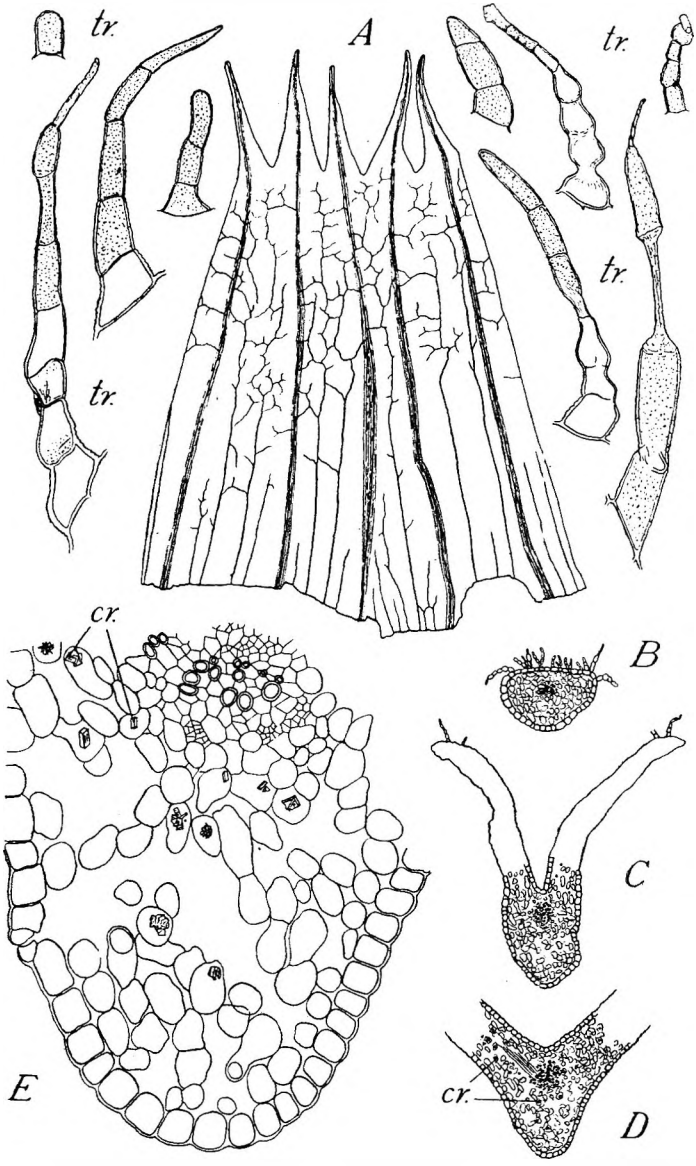


Fig. 2. *Datura stramonium* L., Calyx. A, calyx above the line of abscission, spread out to show its form and the venation, the shaded areas representing the five projecting ridges (× 2·5). B, C and D, transverse sections through the midrib at the tip of the lobe, the middle of the lobe and just above the line of abscission respectively (× 30). E, part of section D further magnified (× 160), to show the vascular strand and the cells of the mesophyll with inclusions, *tr*, peculiar trichomes on the edge of the lobes (× 160).

Gynœcium (Fig. 7A). *Ovary*, rounded-pyramidal, about 4 to 7 mm. long and 5 to 5 mm. in diameter at the widest part; covered by numerous erect spines in 4 patches along the sides; bilocular in the upper part and quadrilocular in the lower, due to the growth of false septa from the dorsal sutures of the carpels to the axile placentæ (Fig. 6, F, G, H and I); placentation axile, ovules numerous. *Style*, arising from the apex of the ovary, about 3 to 4 cm. in length increasing in diameter towards the apex, firm and almost erect, terminating in a bilateral, ovoid, capitate *stigma*.

Fruit. The ovoid fruit about 3 to 3·5 cm. long, surrounded below by the persistent base of the calyx which is strongly recurved; it is covered by numerous, unequal, sharp and rigid spines. The capsule opens by 4 valves extending from the apex to about half-way down. *Seeds*, numerous, brown or brownish-black, laterally flattened and subreniform, with shallow indefinite reticulate depressions on the surface, which is, in addition, finely pitted. The seeds measure about 3 to 3·7 mm. in length, 2·5 to 3 mm. in width and 1 to 1·6 mm. in thickness.

Histology of the Calyx

Outer (abaxial) epidermis. On the lobes, the epidermal cells are small and have very sinuate anticlinal walls (Fig. 1, Ao); they measure approximately L and T = 15 to **21** to **30** to 75 μ and R = 21 to 30 μ.* The cells on the upper part of the tube are similar to, but larger than those on the lobes; towards the base of the tube the cells have less sinuous anticlinal walls and at the very base, i.e., just above the line of abscission, the anticlinal walls are almost straight or slightly curved (Fig. 1, Bo). The cells on the persistent part of the calyx are smaller and polygonal in surface view (Fig. 1, Co). The cells at the middle of the tube measure approximately L and T = 30 to **45** to 90μ and R = 21 to 36 μ; in the region of the line of abscission, they measure L and T = 18 to **30** to **45** to 60 μ, R = 21 to **24** to 30 μ. The epidermal cells on the ridge over the midrib on the tube are rectangular to polygonal in surface view with straight or slightly curved anticlinal walls (Fig. 1, Vo). *Stomata* are frequent on the lobes and on the tube, but become less numerous or rare below the line of abscission; they are usually cruciferous (anisocytic). They are rare on the ridges over the midribs. *Trichomes*. Small *glandular* trichomes such as occur on the foliage leaves, having a short unicellular stalk and a comparatively large 5 to 6-celled pyriform head, occur frequently on the lobes and upon the tube, being less numerous towards the base. Conical warty *covering* trichomes occur on the lower half of the tube but rarely on the lobes; those on the ridge over the midribs are often blunt at the apex. The persistent part of the calyx bears small glandular trichomes as well as covering ones.

At the line of abscission, a granular substance appears along the walls of certain cells (Fig. 1, Lo); this appears to be the product of enzyme

* When recording measurements, the letters L, T and R are used to indicate measurements in a longitudinal, tangential and radial direction respectively, the directions having reference to the axis of the relevant plant member.

action, which dissolves the middle lamella and leads eventually to the detachment of the calyx. The cells, after abscission, retain their form and bear no evidence of tearing. The granular substance was found

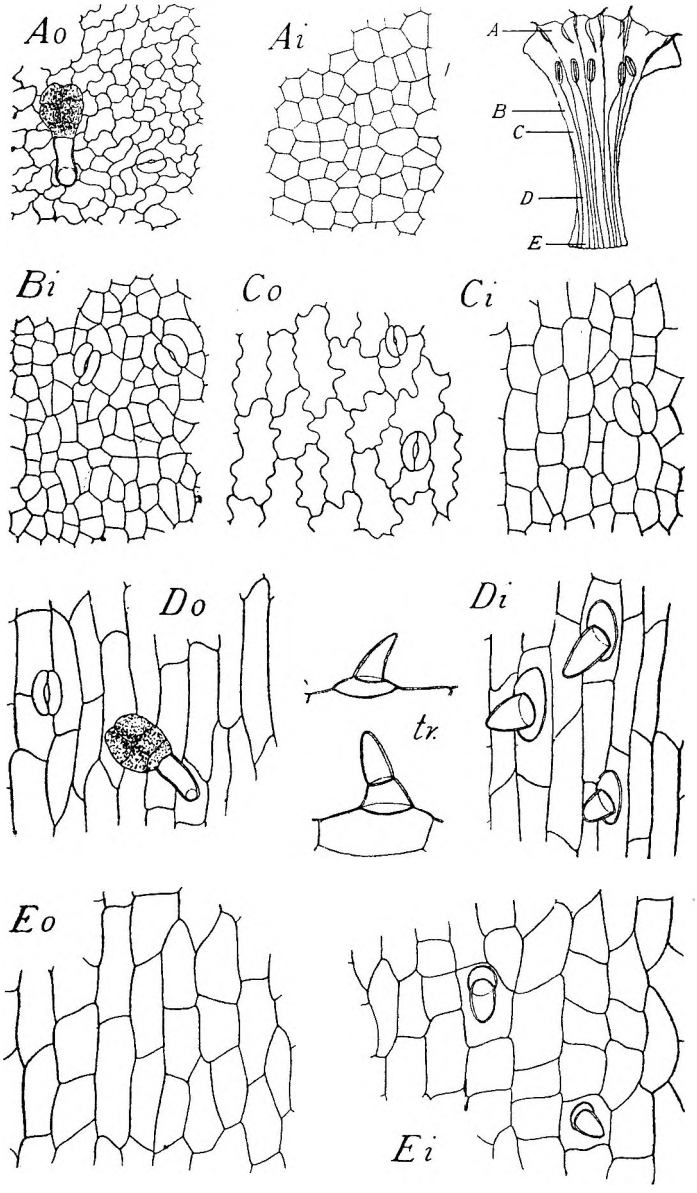


Fig. 3. *Datura stramonium* L., Corolla. Ao, outer epidermis at A (as indicated on diagram, top right); Ai, inner epidermis at A; Bi, inner epidermis at B; Co, outer epidermis at C; Ci, inner epidermis at C; Do, outer epidermis at D; Di, inner epidermis at D; Eo, outer epidermis at E; Ei, inner epidermis at E, *tr*, covering trichomes on the inner epidermis at the base. All × 160.

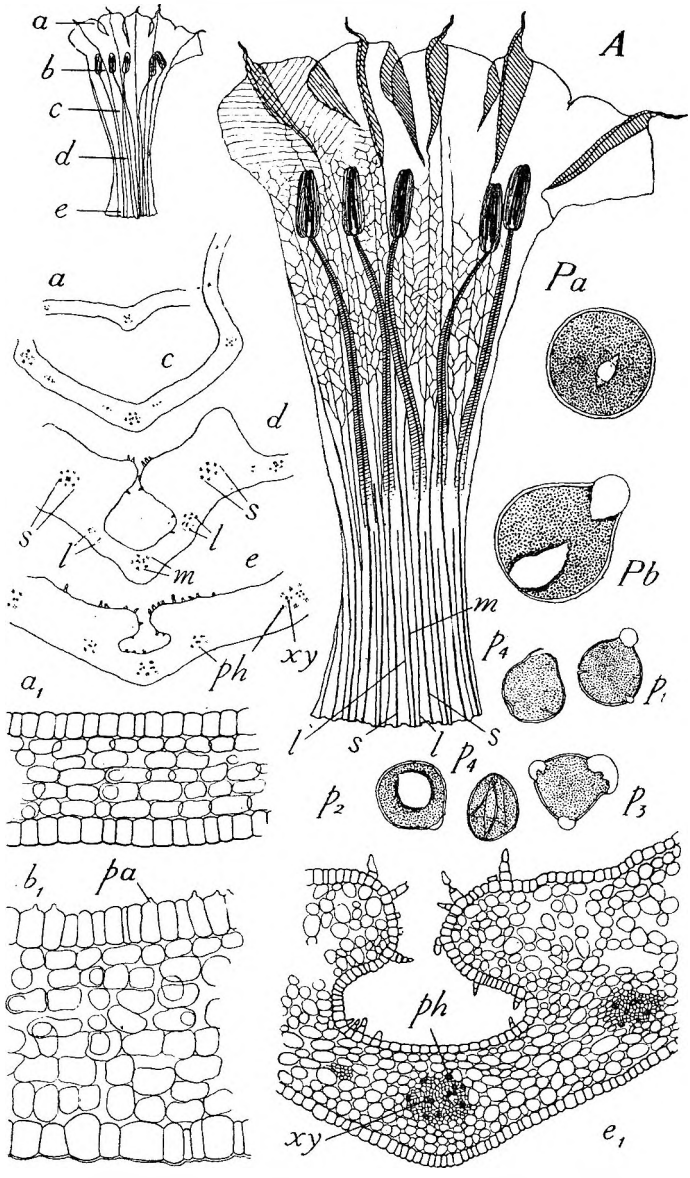


Fig. 4. *Datura stramonium* L. A, corolla above the line of abscission, spread out showing venation and epipetalous stamens, the venation of the lobes is shown on the extreme left lobe only (× 2). *a*, *c*, *d* and *e*, a series of diagrams of transverse sections through the corolla at various levels indicated in the diagram, top left (× 15). a_1, b_1 and e_1, sections at *a*, *b* and *e*, further magnified to show the details of the mesophyll, papillæ and vascular strands, a_1 and b_1 × 160, e_1 × 80. Pa, pollen grain showing indistinct furrow and pore (× 280); Pb, pollen showing intine extruding from the pore (× 280); p_1, p_2, p_3, pollen grains showing forms of pores and furrows (× 160); p_4, empty burst exteriors of pollen grains (× 160); *l*, lateral vein branching from the base of the midrib; *m*, midrib; *pa*, papilla; *ph*, phlœm; *s*, vein leading to a stamen; *xy*, xylem.

to dissolve when preparations were left in solution of chloral hydrate and glycerin for a few days.

Inner (adaxial) epidermis. The cells on the lobes and on the upper part of the tube are very sinuous; they become gradually larger towards the base of the tube (Fig. 1, Ai and Bi). The cells show elongation over the midribs in the tube, accompanied by a slight reduction in sinuosity. On the lower region of the persistent part of the calyx, the cells are polygonal with straight anticlinal walls (Fig. 1, Ci). The cells of the inner epidermis measure approximately L and T = 21 to **75** to **120** to 180 μ and R = 18 to **24** to 36 μ; on the lobes they approximate the lower limits. Cruciferous (anisocytic) *stomata* occur occasionally on the lobes and on the tube, but are absent below the line of abscission. *Trichomes* are very rare or absent. On the margin of the calyx lobes, there is an abundance of covering trichomes of various forms, which differ from those on other parts of the calyx (Fig. 2, tr.).

Trichomes. The dimensions of the clavate *glandular* trichomes are as follows: the stalk is about 30 to 45 μ in length and 15 μ in diameter, the head about 36 to 60 μ in diameter. The *covering* trichomes of the outer epidermis are 2 to 5-celled, uniseriate, conical and warty; those over the midrib have a rounded tip and sometimes a smooth cuticle. The covering trichomes along the edge of the lobes have a variety of forms; some are 1– to 3–celled with a blunt rounded tip; others are 3– to 8–celled, the cells being often collapsed and flattened alternately at right angles to one another while some cells have localised rounded enlargements; the majority of the cells of these trichomes are covered with a warty cuticle and the cuticle over many of the basal cells is transversely striate; very rarely a branched trichome occurs. The covering trichomes of the calyx measure about 60 to **165** to 630 μ in length and 24 to 36 μ in basal diameter.

Mesophyll (Fig. 2, B, C, D and E). The mesophyll consists of about 3 to 6 layers of parenchyma having large intercellular spaces. In the ridge over each midrib the spongy parenchyma is more loosely arranged than in the corresponding tissue in the interneural regions. The cells of the mesophyll have numerous *crystals* either as solitary prisms or other irregular shapes or aggregates of prisms (Fig. 2, D and E, cr). The crystals become more abundant towards the base and also along the midribs, where single prisms become more frequent. Below the line of abscission, idioblasts filled with micro-sphenoidal crystal sand are common.

Venation (Fig. 2, A). Each sepal has a well-marked midrib which runs almost directly into the tip of each lobe. Smaller veins arising at the base from various points between the midribs run parallel to the latter and terminate at various levels in the tube. All veins have fine branches which anastomose with each other. There are no marginal veins on the lobes. In transverse section, each midrib has a small vascular bundle consisting of an arc of slender spirally-thickened xylem vessels surrounded by groups of phloem tissue.

Histology of the Corolla

Outer (abaxial) epidermis. On the lobes and on the throat of the tube, the cells are small, sub-rectangular to polygonal with slightly sinuous anticlinal walls (Fig. 3, Ao); they measure approximately L and T = 9 to **12** to **21** to 45 μ and R = 15 to 24 μ. On the tube, half way down the corolla, the cells are larger and sub-rectangular with sinuous anticlinal walls (Fig. 3, Co); they measure approximately L and T = 15 to **30** to **45** to 60 μ and R = 24 to **30** to 48 μ. From the middle of the corolla tube to the base the cells are rectangular to polygonal, with almost straight anticlinal walls (Fig. 3, Do and Eo); the cells on the lower half of the tube measure approximately L = 60 to **90** to 225 μ and T = 15 to **24** to 39 μ while at the base of the tube, L and T = 9 to **24** to **36** to 45 μ, and R = 24 to **30** to 45 μ. Ranunculaceous (anomocytic) *stomata* are present on the lobes and upon the throat of the tube; cruciferous (anisocytic) stomata occur at the middle of the tube, but are less numerous; stomata are absent at the base. Small glandular *trichomes*, similar to those on the calyx lobes, are present in small numbers on the outer epidermis of the corolla lobes.

Inner (adaxial) epidermis. On the lobes, the cells are small and polygonal with straight anticlinal walls (Fig. 3, Ai); they measure approximately L and T = 9 to **15** to **18** to 30 μ and R = 15 to 24 μ. On the middle of the corolla tube, the cells are somewhat larger, sub-rectangular to polygonal, with straight or slightly curved anticlinal walls (Fig. 3, Bi and Ci); they measure approximately L and T = 18 to **21** to **30** to 60 μ and R = 18 to **27** to 36 μ. Many cells on the throat of the corolla and in certain specimens on the lobes as well, have small inconspicuous papillae (Fig. 4, b_1). At a point about two-thirds down the length of the tube, the cells are sub-rectangular and axially elongated; further down near the base, they are more isodiametric (Fig. 3, Di and Ei). On the lower part of the corolla, the cells measure approximately L = 30 to **75** to 120 μ, T = 15 to **21** to 24 μ and R = 15 to **24** to 30 μ. *Stomata* are absent on the lobes; a few ranunculaceous (anomocytic) *stomata* occur at the middle of the tube; they are absent towards the base. Covering *trichomes* are fairly numerous on the lowest third of the tube; they are 1 to 3-celled uniseriate and conical, with a short dome-shaped basal cell and a small conical cell at the apex; they measure about 15 to 30 μ in length and 24 to 30 μ in basal diameter (Fig. 3, tr).

Mesophyll (Fig. 4, a, b, c, d, a_1, b_1 and e_1). The mesophyll of the tube consists of 5 to 8 layers of thin-walled spongy parenchyma; that of the lobes consists of about 5 layers of smaller, more closely arranged cells, forming a thinner structure. The outgrowths caused by the adnated filaments consist of a spongy mesophyll having large intercellular spaces. *Calcium oxalate* occurs as small single crystals or as aggregates in all parts of the corolla and is more abundant towards the base. Large crystalline masses of unknown composition, such as were found by Timmerman[4] in the leaves of *D. metel*, occur sporadically in various parts of the mesophyll of the corolla tube.

Venation (Fig. 4, A). Each petal has a well-marked midrib running directly into the tip of the lobe. Two main branches arising on either side of each midrib near the base, terminate at the base of the lobe; in the upper half of the tube all the veins have lateral branches which anastomose freely. In the lobes, each midrib gives out, almost at right angles to itself, parallel branches which extend to the edge of the lobe. In transverse section, the midrib shows a xylem consisting of a few slender spiral vessels, surrounded by groups of phloem which are separated by thin-walled small-celled parenchyma (Fig. 4, d, e and e_1).

Histology of the Stamens

Filament. The epidermal cells on the free part of the filament are sub-rectangular to polygonal in surface view and are elongated along the direction of the axis (Fig. 5, B); they are covered by a thin cuticle which in certain specimens shows longitudinal striations; the cells measure approximately L = 120 to **180** to 300 μ T = 15 to 30 μ and R = 24 to **30** to 42 μ. The epidermal cells on the adnated part of the filament resemble the neighbouring cells on the corolla. *Stomata* and *trichomes* are absent on the free part of the filament; 1– to 3–celled conical covering trichomes are present on the adnated part of the filaments and resemble those of the corolla tube, associated with these are many larger 1– to 6–celled uniseriate covering trichomes; the trichomes measure approximately 28 to 700 μ in length and 14 to 100 μ in basal diameter (Fig. 5, tr). The *cortex* as seen in transverse section, consists of about 7 rows of thin-walled rounded paraenchyma loosely arranged; the cells are about 15 to **42** to 60 μ in diameter. The vascular strand resembles that of the corolla midribs (Fig. 5, A).

Anther. The *connective* has an epidermis consisting of sub-rectangular cells elongated in the direction of the axis; the cuticle is striated longitudinally. The vascular strand consists of slender spiral vessels appearing in transverse section (Fig. 5, C), in the form of an ellipse and surrounded by several small groups of phloem; at the centre a lacuna is usually present. The vascular cylinder is surrounded by a few layers of parenchymatous cells; the remainder of the tissue consists of spongy parenchyma. The outer epidermis of the *lobes* consists of sub-rectangular to polygonal cells usually elongated at right angles to the axis (Fig. 5, D and H); the cells measure approximately L = 15 to 30 μ, T = 45 to **60** to 120 μ and R = 15 to 30 μ; cruciferous (anisocytic) *stomata* occur occasionally. The lobes bear frequent slender, finely warty, 2– to 5–celled uniseriate covering trichomes on the outer epidermis near the line of dehiscence; the trichomes have micro-crystals in some of the cells; they measure approximately 140 to 420 μ in length and 6 to 15 μ in basal diameter. The *fibrous layer* (Fig. 5, E, F, G and H) is one–cell wide near the line of dehiscence, but gradually becomes 2– to 4–cells wide near the connective The fibrous layer often extends quite across the dorsal surface of the connective; the cells give only a slight reaction for lignin. The cells of the fibrous layer measure approximately L = 15 to 24 μ, T = 30 to **45** to

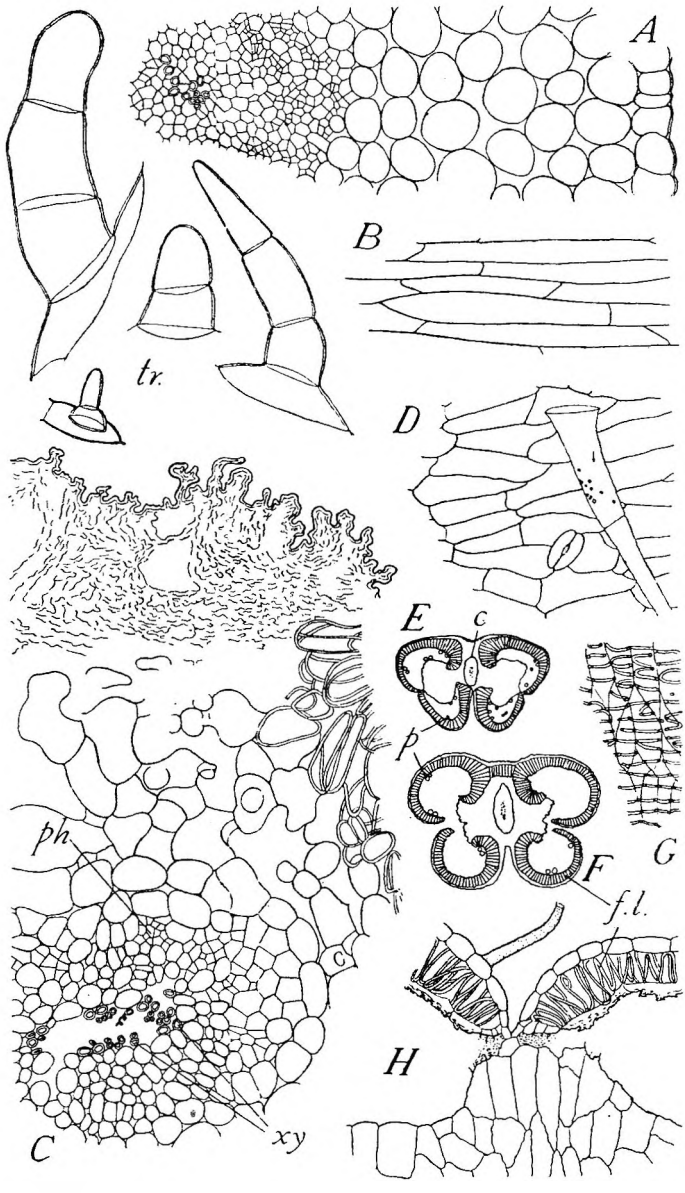


Fig. 5. *Datura stramonium* L. Stamen. A, transverse section through filament. B, epidermis from filament; C, transverse section through connective of the anther; D, epidermis of the anther lobe bearing trichome with crystals in the basal cell; E and F, diagrams of sections through the anther of a bud and of a flower respectively (× 15); G, fibrous layer of anther in surface view; H, transverse section of anther wall at line of dehiscence; *c*, connective; *f.l.*, fibrous layer; *p*, pollen grains; *ph*, phlœm; *tr.* trichomes on adnated part of filament; *xy*, xylem. All × 160, except E and F.

60 μ and R = 15 to **36** to 60 μ. Remains of the tapetum are often visible lining the inner surface of the pollen sac.

The *pollen grains* (Fig. 4, P and p) are spherical, their diameter being 48 to **60** to 78 μ when mounted in lactophenol. When boiled in solution of chloral hydrate the diameter is greater by about 7 μ and if the boiling is vigorous many of the grains are ruptured. There are three pores which vary in distinctness and in size, measuring from 12 to 28 μ in diameter; the margin of the pore is sometimes strongly marked (Fig. 4, P_b, P_2 and p_3) and in other pollen grains scarcely visible (Fig. 4, P_a and P_1). Germinal furrows are faintly outlined and are usually only slightly larger than the pores (Fig. 4, P_a and p_1); in many grains, where the intine has become extruded, the furrows appear as splits in the circumference of the pore outline (Fig. 4, P_b). Zander[5] was unable to see furrows, but careful investigation indicates that they are present, though small and often indistinct. The exine has a markedly irregularly warty surface. The pollen contains oil globules and minute starch grains, the latter measuring about 1 to 5 μ after treatment with a solution of chloral-iodine.

Histology of the Carpels

The Ovary. The cells of the *outer epidermis* (Fig. 6, A and B), of the ovary wall and of the numerous spines which cover the ovary, are polygonal in surface view with straight or slightly curved anticlinal walls; they measure L and T = 6 to **18** to 30 μ and R = 15 to 24 μ. *Stomata* are rare and ranunculaceous (anomocytic). Trichomes (Fig. 6, t_1, t_2 and t_3) vary in frequency of occurrence, they are mostly glandular with a 1– to 4–celled uniseriate stalk and a 1– to 5–celled multicellular rounded or clavate head. A few small 1– to 3–celled uniseriate trichomes with a rounded apex are present, as also are a few 2– to 3–celled warty conical covering trichomes. The *inner epidermis* consists of slightly smaller polygonal cells with almost straight walls (Fig. 6, C). *Stomata* occur occasionally; they are comparatively larger than the surrounding cells and ranunculaceous (anomocytic). The *mesophyll* consists of round–celled parenchyma which contains numerous minute starch grains; calcium oxalate occurs as prisms or irregularly shaped pieces and there are frequent idioblasts containing crystal sand (Fig. 6, G, id.). The *spines* (Fig. 6, F, sp., D and E) at a quite early stage of their growth show in transverse section about 15 groups of procambial tissue arranged in a circle near the periphery; these groups develop into vascular strands with spiral xylem elements in the very young fruit. The epidermal cells of the *septum* are similar to those of the ovary wall. Starch grains and crystals, similar to those in the ovary wall, are present in the mesophyll of the septum and of the placenta. The young *ovules* consist of undifferentiated parenchyma.

The Style (Fig. 7, A, B and G) is erect, firm and slightly thicker towards the apex; the epidermis resembles that of the filaments, but is covered with a longitudinally striated cuticle. The epidermal cells measure approximately L= 150 to **225** to 300 μ, T = 16 to **24** to 30 μ and R= 15 to 30 μ. *Stomata* (cruciferous) are rare and *trichomes* are absent from the style.

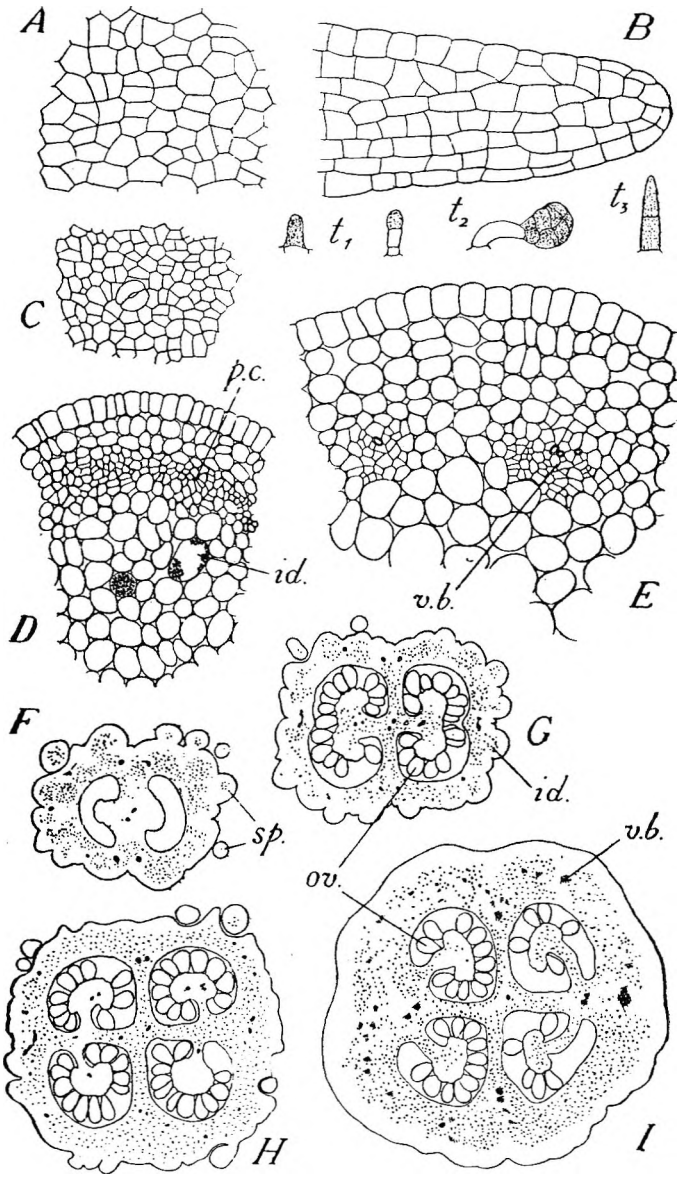


Fig. 6. *Datura stramonium* L., Ovary. A, outer epidermis of the ovary wall; B, epidermis of a developing spine; C, inner epidermis of the ovary wall; D, transverse section through a young spine of the ovary showing procambial strands; E, transverse section through an older spine from a very young fruit showing development of vascular strands; F, G, H and I, series of transverse sections through the ovary showing the variation in the number of loculi and the presence of a false septum in the lower part of the ovary (× 15); *id*, crystal-sand idioblast; *ov*, ovules; *p.c.*, procambial strand; *sp*, spine; t_1, t_2, t_3, covering, glandular and warty trichomes respectively from the outer epidermis of the ovary and of the spines; *v.b.*, vascular bundle. All × 160, except F, G, H and I.

The ground tissue consists of a round-celled parenchyma, many cells of which contain calcium oxalate in the form of small isolated crystals, either as prisms or of irregular shapes; small starch grains are present in many of the cells. Occasionally, large crystalline masses of unknown composition occur in different parts of the style (Fig. 7, B, cr.). In transverse section (Fig. 7, B and G), the style shows a central oval region of very thin-walled cells about 3 to 9 μ in diameter; frequently there is an irregular lacuna at the centre; on each side of the oval region is a vascular strand; the remaining tissue consists of a layer of about 12 rows of

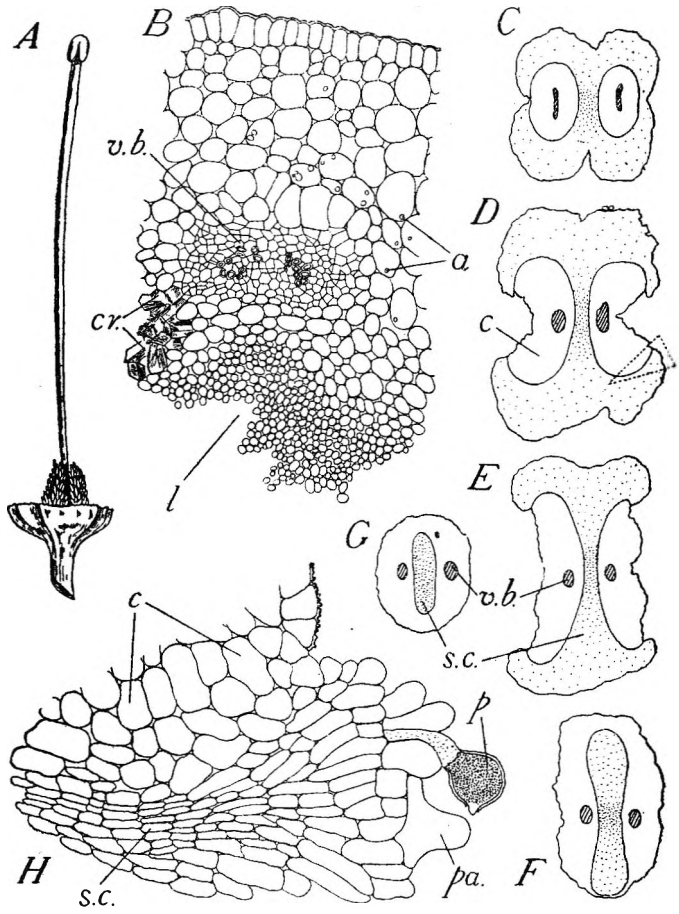


Fig. 7. *Datura stramonium* L. A, entire gynœcium with persistent part of the calyx (× 2); B, transverse section through the style (× 160); C, D, E, F and G, diagrams of series of sections from the apex of the stigma to the style, showing the proliferation of the small-celled tissue in the centre of the stigma resulting in the formation of the papillose surface (× 15); H, triangular area outlined by dotted lines in D, further enlarged (× 160); *a*, starch grains; *c*, ground tissue; *cr*, crystalline mass of unknown composition; *l*, lacuna; *p*, pollen with developing pollen-tube; *pa*, papillose cell; *s.c.*, small-celled tissue; *v.b.*, vascular bundle.

larger thin-walled parenchymatous cells, about 15 to 30 μ in diameter, with small intercellular spaces.

The Stigma (Fig. 7, A, C, D, E, F and H) is ovoid in shape and is covered by a papillose receptive surface which has on each side a small vertical groove which increases in width towards the base; the papillæ are short, cylindrical and rounded at the apex, the papillose cells measuring approximately 15 to 30 to 75 μ in length and 15 to 30 μ in diameter at the base. The stigma has a vascular strand on either side of a central region of small, thin-walled, loosely-arranged cells; this central region extends its growth at right angles to the plane containing the vascular strands until it reaches the epidermis which is then ruptured and the small-celled tissue emerges to form a papillose cap to the stigma. Pollen grains can often be observed attached to the papillose surface with the pollen tube growing into the style. The cuticle covering the epidermis in the grooves of the stigma is strongly striated.

Datura tatula

Linnaeus, in his original description of the plant *Datura tatula* in his *Species Plantarum*, second edition[6], 1762, states that it is similar to *D. stramonium* with the difference of purple colouration of the stem and the pale blue corolla. The description of the drug, Stramonium, in the British Pharmacopoeia for 1932 and 1948 includes the species *D. tatula*. Various workers have expressed the opinion that since *D. tatula* Linn. is identical in all respects with *D. stramonium* Linn., but for the purple colouration, it may be regarded as a variety of *D. stramonium*; Timmerman[4] found a slight difference in the size of the trichomes of the leaves of the two species. The following description of the flower of *D. tatula* is comparative with that of the flower of *D. stramonium* immediately preceding this description.

Material. The only available specimens used for this study were obtained from the Chelsea Physic Garden in July, 1949.

Histology

Calyx. The calyx shows no difference from that of *D. stramonium* except that in addition to the characteristic trichomes described under *D. stramonium* as occurring along the edge of the lobes, there are also in this position a few trichomes having multicellular glandular heads (Fig. 8, tr_1). The covering trichomes are about the same length as those of *D. stramonium* but are broader at the base, measuring up to 90 μ in basal diameter.

Corolla. No difference in structure from that of *D. stramonium* was observed.

Andrœcium. The only slight difference observed was in the trichomes on the adherent part of the filament, some cells of which are somewhat barrel-shaped instead of terete (Fig. 8, tr_2). The *pollen grains* are identical with those of *D. stramonium* in all respects except that the exine on a few grains appears to have a tendency towards streaky striations instead of the usual irregular markings.

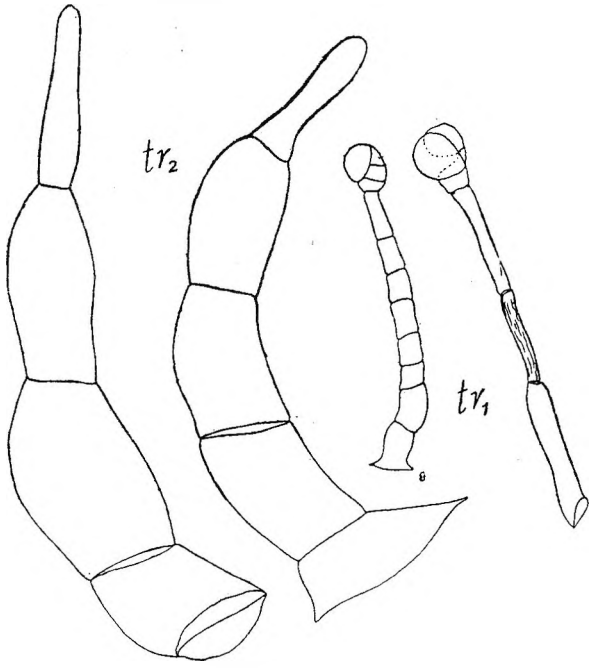


Fig. 8. *Datura tatula* L. tr_1, glandular trichomes from the edge of the calyx lobes; tr_2, covering trichomes from the adnated part of a filament (× 100).

Gynœcium. No features of anatomy could be observed which distinguish the gynœcium of *D tatula* from that of *D. stramonium.*

Summary and Powder

The diagnostic characters of the powdered flowers may be regarded as supplying also a summary of the important histological features of the unground flowers.

Powdered Flowers. Dried flowers of *D. stramonium*, reduced to No. 90 powder, yield a greyish-green powder with an irritant and typically leafy odour. The usual reagents were used for the examination of the powder and the characters helpful in identification are listed in order of importance, chloral hydrate being the mountant.

1. Pollen grains, spherical, about 55 to 63 to 80 μ in diameter, having 3 pores, with the intine often extruding from the pores; the exine has an irregularly warty surface.

2. Broken stalks of covering trichomes with a warty surface, rarely smooth, some fragments with callapsed cells; occasionally a detached glandular head of a clavate trichome.

3. Numerous fragments of the calyx intersected by slender spiral vessels, having wavy-walled epidermal cells with cruciferous (anisocytic) stomata and containing calcium oxalate crystals in the mesophyll in the form of isolated prisms or typical aggregates of prisms or irregular-shaped

crystals. The arrangement of the crystals in the vein-islets is similar to that in the leaf.

4. The only feature which distinguishes the powder of the dried flowers of *D. tatula* from that of *D. stramonium* is that fragments of corolla and anther lobes yield a transient pink colouration when mounted in cold solution of chloral hydrate.

References

1. Linnæus, 1753, *Species Plantarum*, ed. I, pp. 179-180.
2. Bentley and Trimen, 1880, *Medicinal Plants*, 3, 192. (*D. stramonium*).
3. Fischer, W., 1937, *Beiträge zur Morphologie von Datura stramonium L.* (Inaugural Dissertation, Universität Basel).
4. Timmerman, *Pharm. J.*, 1927, (a) pp. 741 and 742 (crystalline masses); (b) p. 738 (*D. tatula*).
5. Zander, 1935, *Beiträge zur Herkunftsbestimmung bei Honig, Pollengestaltung und Herkunftsbestimmung bei Blütenhonig*, Berlin, 279.
6. Linnæus, 1762, *Species Plantarum*, ed. II, p. 256.

THE COLORIMETRIC DETERMINATION OF METHYLTHIOURACIL AND PROPYLTHIOURACIL IN TABLETS USING 2 : 6-DICHLOROQUINONE-CHLOROIMIDE

By RONALD A. MCALLISTER and KENNETH W. HOWELLS

From the Biochemical Laboratory, Royal Samaritan Hospital, Glasgow

Received November 26, 1951

THE recent paper by Bergren and Kirsten[1] on the errors involved in the titrimetric determination of propylthiouracil in tablet form, due to the presence of magnesium stearate, has prompted us to report on the colorimetric determination of methylthiouracil and propylthiouracil using the chloroimide reaction. One of us (McAllister[2,3,4,5]) has already described the chloroimide reaction for antithyroid compounds of both the thiouracil and mercaptoimidazole series, and methods have been elaborated for the quantitative determination of these. During the course of this work much information has been accumulated on the reaction involved, and we have been able to isolate the coloured product of the reaction with propylthiouracil in crystalline form. The present paper describes methods for the colorimetric determination of methylthiouracil and propylthiouracil in tablet form together with other work not previously reported.

PROCEDURES

Reagents. 1. 0·4 *per cent. solution of* 2:6-*dichloroquinone chloroimide in aldehyde-free absolute ethanol.* The colour reagent will keep for about 4 weeks if stored in a brown bottle.

2. *Buffer-chloride solution, p*H 8·0. To 50 ml. of 0·2 M boric acid in 0·2 M potassium chloride add 4 ml. of 0·2 N sodium hydroxide. Then add 100 ml. of a 20 per cent. solution of sodium chloride in water. Make the volume of the solution to 200 ml. with distilled water. Check the *p*H of the solution and adjust it to 8·0 by the addition of 0·1 N sodium hydroxide.

3. *Aldehyde-free absolute ethanol.* Prepared according to the method of Callow, Callow and Emmens.[6] To 500 ml. of absolute ethanol add 2 g. of *m*-phenylenediamine and allow the mixture to stand for 1 week in a dark cupboard. The prolonged digestion time may be replaced by boiling for 1 hour under a reflux condenser.[7] Distil the mixture in an all-glass still. If stored in a dark bottle, the solution will keep indefinitely.

4. *Chloroform B.P.*

5. *Standard solutions.* (*a*) *Propylthiouracil.* Dissolve 25 mg. of the compound in 5 ml. of aldehyde-free absolute ethanol. It is advisable to use a small beaker for the purpose to minimise deposition of the substance on the sides of the beaker due to evaporation. Add 100 ml. of distilled water, and allow to stand for 10 minutes. Make the volume to 250 ml. with distilled water. 1 ml. of this solution contains 100 μg. of propylthiouracil. The solution will keep for about 2 days. (*b*) *Methylthiouracil.* Weigh out 100 mg. of 4-methyl-2-thiouracil. Add 80 ml. of water and 0·8 ml. of concentrated ammonia solution and mix until

dissolved. Make the final volume to 1 l. with distilled water. 1 ml. of this is equivalent to 100 μg. of methylthiouracil. This solution will not keep.

Standard graphs. Dilute both standards suitably and take amounts up to 100 μg. of each. Adjust the volume of each to 5 ml. with water. Apply the same procedure to these as is given in the section dealing with the analysis of tablets.

Methods of Assay

(*a*) *Methylthiouracil.* One tablet of methylthiouracil containing 100 mg. of the active compound is crushed in 80 ml. of water and 0·8 ml. of concentrated ammonia solution (sp.gr. 0·880) added. The solution is stirred for 20 minutes, and the volume then made up to 1 l. with distilled water. The solution is then filtered to remove undissolved tablet base. For the assay this solution is diluted 1 in 4 with distilled water and 1, 2, 3, and 4 ml. taken. The volume of each of these containing 25, 50, 75 and 100 μg. of methylthiouracil respectively, is adjusted to 5 ml. with water. 5 ml. of the buffer-chloride solution, *p*H 8·0, and 0·1 ml. of the 0·4 per cent. chloroimide reagent are added to each. The solutions are well mixed and the colour reaction in each allowed to proceed for 45 minutes at room temperature. Then 10 ml. of chloroform is added to each and the tubes well shaken. Once all of the yellow colour in each has been extracted the chloroform extracts are allowed to settle, and the aqueous supernatant liquid in each removed by suction. The chloroform extracts are then filtered through small Whatman No. 42 filter-papers. The optical density in each is read in a Spekker absorptiometer, using a Spekker filter (violet, O.B.1). The concentration of methylthiouracil in each is determined by reference to a standard graph.

(*b*) *Propylthiouracil.* A tablet containing 25 mg. of the active compound is crushed in a beaker with a glass rod and 5 ml. of aldehyde-free absolute ethanol added. The solution is stirred well and allowed to stand for 20 minutes. Stirring is maintained during this period. The volume of the solution is then made up to 250 ml. with distilled water and filtered. Dilute this solution 1 in 4 with water and take 1, 2, 3 and 4 ml. of it. This gives a range of 25, 50, 75 and 100 μg. of propylthiouracil. Adjust the volume of each of these to 5 ml. with water and proceed as described under methylthiouracil. The colour is the same therefore the same absorptiometer filter is used as for methylthiouracil.

Results. The recovery values obtained in the analysis of tablets are as follows:—methylthiouracil, 100 mg.; recovered 86, 98, 96·2, 94·3 per cent.; propylthiouracil, 25 mg.; recovered 96, 100, 97·2, 96 per cent.

The colour reaction. In a suitably buffered solution, 2 : 6-dichloroquinone-chloroimide condenses with methylthiouracil[2] and propylthiouracil[3] with the formation of a yellow-coloured complex. The latter is removable from the reaction mixture by means of chloroform. The solvent extraction renders the colour reaction specific for thiouracils and mercaptoimidazoles.[5]

Preparation of the Thiouracil Complexes

(*a*) *Propylthiouracil.* 50 mg. of 6-*n*-propylthiouracil was dissolved in 10 ml. of aldehyde-free absolute ethanol and 100 ml. of the buffer-chloride mixture, *p*H 8·0, added. The mixture was cooled in ice and 20 ml. of a 0·4 per cent. solution of 2 : 6-dichloroquinone chloroimide in aldehyde-free absolute ethanol added slowly, and with constant stirring. The orange-red solution was then extracted with chloroform. The latter was then taken down to near dryness *in vacuo*. From this mixture the propylthiouracil–chloroimide complex was isolated in orange-red needles; m.pt. 172° C., with decomposition. These dissolved readily in chloroform to give intensely coloured yellow solutions.

(*b*) *Methylthiouracil.* A similar procedure applied to 4-methyl-2-thiouracil gave very concentrated solutions of the pigment, but in our hands, the pigment could not be crystallised from the reaction mixture.

As far as we are aware the products of the reactions above have not been previously isolated. Fearon[8] working on the 2 : 6-dichloroquinone chloroimide reaction for uric acid was unsuccessful in an attempt to isolate the coloured product, but his work was hindered by the insolubility of both the reactants.

Specificity of the colour reaction. This has already been reported in some detail by one of us (McAllister[2,3,5]). Here we have investigated the effect of magnesium stearate and lactose on the colour development with methylthiouracil and propylthiouracil and have found no interference. As regards the presence of magnesium stearate in tablets, it may be noted that in the initial dissolving of the thiouracils any stearate present is removed during the removal of insoluble tablet base by filtration.

Summary

1. A colorimetric method for the determination of methylthiouracil and propylthiouracil in tablet form is presented.

2. Recovery values obtained with the method are given.

3. The isolation of the coloured complex formed in the reaction between propylthiouracil and 2 : 6-dichloroquinone chloroimide is described.

References

1. Bergren and Kirsten, *Farm. Revy*, 1951, **50**, 245.
2. McAllister, *Nature, Lond.* 1950, **166**, 789.
3. McAllister, *J. clin. Path.*, 1951, December issue.
4. McAllister, *Nature, Lond.* 1951, **167**, 863.
5. McAllister, *J. Pharm. Pharmacol.*, 1951, **3**, 506.
6. Callow, Callow and Emmens, *Biochem. J.*, 1938, **32**, 1312.
7. Nicholls, *Aids to the Analysis of Food and Drugs*, 6th Ed., Baillière, Tindall and Cox, London, 1942.
8. Fearon, *Biochem. J.*, 1944, **38**, 399.

THE BACTERIOSTATIC ACTION OF BASIC DI- AND TRIPHENYLMETHANE DERIVATIVES

PART I. THE INHIBITION OF BACTERIOSTATIC ACTION BY INACTIVE COMPOUNDS OF SIMILAR STRUCTURE

BY E. FISCHER, M. GENSELOVICH and P. RONA

From The Research Department of Szabó Hnos., Kessler & Cia., Buenos Aires, Argentina

Received November 26, 1951

DURING our earlier experimental work on the bacteriostatic activity of certain di- and triphenylmethane dyes[1,2,3,4] we have observed some facts suggesting that, among methane carbon atom derivatives of these dyes, only those with a "potential quinoid structure" were able to inhibit the growth of *Staphylococci* and *Streptococci*. We have noted[1,2] that the leucobases (R_3CH), the leucoamines ($R_3C{\cdot}NH_2$) and the leucocyanides ($R_3C{\cdot}CN$) of basic triphenylmethane dyes were practically inactive, while the corresponding dye cation ($R_2C = R$), carbinol bases ($R_3C{\cdot}OH$), leucosulphonic (bisulphite) and leucosulphinic (hydrosulphite) derivatives ($R_3C{\cdot}SO_3Na$, $R_3C{\cdot}SO_2Na$) exerted a strong bacteriostatic effect of equal degree. It is a well known fact that carbinol bases possess a tautomeric quinoid ionised form whose cation appears in their dye salts. Bisulphite and hydrosulphite derivatives, for their part, are unstable compounds, transformed more or less rapidly, in contact with air, into coloured dye salts (vat dyes).

Still more conclusive have been the results of experiments with diphenylmethane derivatives.[3,4] The bacteriostatic activity of these is far weaker than that of the triphenylmethane homologues, but the stability of their methane carbon atom derivatives allows a more clear cut separation between "essentially non-quinoid" and "potentially quinoid" structures. The non-quinoid compounds R_2CH_2 (bis[dimethylaminophenyl]methane) and $R_2CH{\cdot}NH_2$ (leuco-auramine) were practically negative, whereas both the compounds R_2CHOH (the "potentially quinoid" Michler's hydrol) and $R{\cdot}C(NH_2){=}R$ (the quinoid auramine dye) showed bacteriostatic effects of the same degree. The sulphonic derivative ($R_2CH{\cdot}SO_3H$), which is, contrasting with its unstable triphenylmethane homologues, a stable non-quinoid compound, did not exert, correspondingly, any noticeable bacteriostatic activity. The non-quinoid Michler's ketone ($R_2C = O$) was also inactive.

The most important common feature of active di- and triphenylmethane derivatives, which distinguish them from inactive compounds of similar structure, is constituted in our opinion by their "potentially quinoid" character. An actually quinoid structure, however, cannot be looked upon as a requisite for the bacteriostatic action, since quinoid dye cations and actually non-quinoid carbinol bases have a quantitatively equal effect. Especially noteworthy is, from this point of view, the similarity of the bacteriostatic action of Michler's hydrol, non-quinoid at the *p*H of the bacteriological medium (7·0) and that of auramine, quinoid cation at the

same *p*H. Marini-Bettòlo,[7] confirming the comparable antimicrobic efficacy of triphenylmethane dyes and the corresponding carbinol bases, considers their higher state of oxidation responsible for this phenomenon. We should emphasise that the circumstance that both dyes, cation and carbinol basis, are oxidised compounds, does not account for their similar bacteriostatic efficiency, as Michler's ketone, a still more highly oxidised derivative, is inactive. It would be necessary to admit that a degree of oxidation represented by both the quinoid dye cation and the carbinol basis is needed for the efficiency. We mention in this connection, that Ingraham[5] tried to explain the antimicrobic activity of triphenylmethane dyes by postulating a balancing effect on the oxidation-reduction potential (*cf.* our discussion of Ingraham's work[6]).

Our experimental results, on the inhibition of the bacteriostatic effect of triphenylmethane dyes by their leucobases,[3,4] are also connected with this problem. The demonstration of such an inhibition acquires special importance in view of the well known theory of Fildes and Woods (competition with essential metabolites), applied with great success to explain the antimicrobic effects of sulphanilamides and other substances. Northey,[8] referring to our work, remarks that "there are cases of antagonism between similarly constituted chemotherapeutic drugs where it is difficult to picture the antagonist as being essential to the metabolism of the parasite."

It seemed advisable, therefore, for us to reinvestigate the antagonism between active and inactive derivaties in a more quantitative manner. As shown in a previous paper,[2] experiments with triphenylmethane leucobases are technically difficult because their very low solubility in water does not allow quantitative measurements.

In the hope of encountering more favourable conditions among diphenylmethane dyes, we have investigated the leucobases of both the bis-4:4′-(dimethylaminophenyl)methane and bis-4:4′-(aminophenyl)-methane series. The very slightly water-soluble leucobases of the former series, i.e., tetramethyl-*pp*′-diaminodiphenylmethane and leucoauramine, did not offer any advantage over leucomalachite green and leucomethyl violet. We had more success with the corresponding member of the second series, *pp*′-diaminodiphenylmethane, a fairly water-soluble compound, which had no bacteriostatic effect on *Staphylococcus aureus* in concentrations as high as 1 : 5000 (0·02 per cent.), while a 0·0025 to 0·005 per cent. concentration prevented the growth inhibition of a 0·005 per cent. concentration of Michler's hydrol. To counteract the effect of a 0·0025 per cent. concentration of the latter, a 0·0025 to 0·00125 per cent. concentration of diaminodiphenylmethane was required (see Table I). We deduce from these results, that one molecule of the reduced substance is sufficient to suppress the action of one or two molecules of the oxidised compound. The antagonistic action of diaminodiphenylmethane is not limited only to diphenylmethane derivatives, for 3000 molecules of it inhibited also the action of one molecule of methyl violet, a triphenylmethane dye of high bacteriostatic efficiency (1 : 8,000,000) (see Table II).

The antagonism between the reduced and oxidised forms may, perhaps,

TABLE I

GROWTH OF *Staphylococcus aureus* IN BROTH

Michler's hydrol alone		Michler's hydrol and					
		DIAMINODIPHENYLMETHANE			DIAMINOBENZOPHENONE		
Dilution of Michler's hydrol		1:20,000	1:40,000	1:80,000	1:20,000	1:40,000	1:80,000
1:20,000	−	+	±	−	+	−	−
1:40,000	−	+	+	±	+	+	±
1:80,000	+	+	+	+	+	+	+
0	+	+	+	+	+	+	+

\+ Normal growth in 24 hours
− No growth in 24 hours
± Growth in some experiments and no growth in others

TABLE II

GROWTH OF *Staphylococcus aureus* IN BROTH

Methyl violet alone		Methyl violet and	
		DIAMINODIPHENYLMETHANE	DIAMINOBENZOPHENONE
Dilution of methyl violet		1:5,000	1:5,000
1:2,000,000	−	−	−
1:4,000,000	−	−	−
1:8,000,000	−	+	+
1:16,000,000	+	+	+
0	+	+	+

suggest that these exert opposite influences on the oxidation-reduction potential. Nevertheless, such a possibility has been excluded by subsequent experiments realised with *pp'*-diaminobenzophenone, a non-methylated homologue of Michler's ketone. This substance is a highly oxidised non-quinoid derivative of the diaminodiphenylmethane series that has been reported as slightly active by Auhagen[9] and by Kuhn, Möller and Beinert[10] against *Streptobacterium plantarum*. This activity corresponded to the sulphanylamide type, having been opposed by a 1:2000 concentration of *p*-aminobenzoic acid. Jensen and Schmith[11] did not observe, however, any bacteriostatic effect of this substance on *Pneumococcus*. In our own experiments with *Staphylococcus aureus*, diaminobenzophenone did not exert a growth-inhibiting action in concentrations as high as 1:5000 (0·02 per cent.), behaving, in this respect, exactly like diaminodiphenylmethane. Applied simultaneously with Michler's hydrol and with methyl violet, diaminobenzophenone showed, furthermore, an antagonistic effect of the same magnitude as diaminodiphenylmethane, one molecule inhibiting the effect of 1 or 2 molecules of the hydrol, and 3000 molecules inhibiting the effect of one molecule of methyl violet (Tables I-II).

These results confirm our working hypothesis, according to which the bacteriostatic effect of basic di- and triphenylmethane derivatives is linked in some way to their "potentially quinoid structure," being the essentially non-quinoid substances of the same series—independently of their lower or higher state of oxidation—not only non-bacteriostatic, but even inhibitory for the bacteriostatic effects of active "potentially quinoid"

derivatives. It is difficult to consider this kind of antagonism as an example of competition with essential metabolites and it does not seem to have anything to do with opposing effects of reduced and oxidised substances on oxidation-reduction potential. We think it more likely that the non-toxic derivatives block the way for the penetration of the chemically related toxic agents to the susceptible parts of the cell. The toxicity of di- and triphenylmethane derivatives for bacterial cells is probably connected with their specific chemical configuration and not with the degree of their basicity or state of oxidation.

References

1. Fischer, Hoffmann and Prado, *Science*, 1944, **100**, 576.
2. Fischer, Hoffmann, Prado and Boné, *J. Bact.*, 1944, **48**, 439.
3. Fischer, Garcés and López, *Science*, 1945, **102**, 507.
4. Fischer, Garcés and López, *J. Bact.*, 1946, **51**, 1.
5. Ingraham, *J. Bact.*, 1933, **26**, 573.
6. Fischer and Muñòz, *J. Bact.*, 1947, **53**, 381.
7. Marini-Bettòlo, *Ist. botan. univ. lab. crittogam.* (*Pavia*), 1947, **3**, 265.
8. Northey, *The Sulfonamides and Allied Compounds*, Reinhold, 1948, 1st ed., 492.
9. Auhagen, *Z. physiol. Chem.*, 1942, **274**, 48.
10. Kuhn, Möller and Beinert, *Ber. dtsch. chem. Ges.*, 1942, **75B**, 711.
11. Jensen and Schmith, *Z. Immunitätsf.*, 1942, **102**, 261.

A NOTE ON BENZYLPENICILLIN DIETHYLAMINOETHYL ESTER

By Aa. Thiel Nielsen and K. Roholt

From the Biological Department, Leo Pharmaceutical Products, Copenhagen

Received December 3, 1951

The diethylaminoethyl ester of benzylpenicillin (PDE) differs from other benzylpenicillin derivatives, in that it has a pronounced affinity for lung tissue.[1] It is used clinically in the form of the hydriodide.* As a carboxylic acid ester of an amino-alcohol it might be expected to have some pharmacological activity.

The physico-chemical properties, in particular the solubility and rate of hydrolysis, will be described in detail elsewhere (Juhl Nielsen *et al.*[2]). In 0·9 per cent. sodium chloride solution at *p*H 7·3 and 37° C. it undergoes 50 per cent. hydrolysis within 23 minutes to form penicillin and diethylaminoethanol. Control experiments have shown that the pharmacological activity of the hydrolysis products in equimolar amounts is not detectable at the levels used in this work. The effects depend greatly on the mode of administration and the salt used, the hydriodide is sparingly soluble while the hydrochloride is highly soluble in aqueous solution.

Oral administration. The hydrochloride and hydriodide are slowly absorbed and partly hydrolysed in the gastrointestinal tract. Consequently after oral administration, blood levels are low and toxic effects are only seen with extremely high doses.

Parenteral administration. The slightly soluble hydriodide is less toxic than the highly soluble hydrochloride. In mice, rats and rabbits the LD50 (subcutaneous administration) is the same for these species: about 2000 mg. per kg. and about 1500 mg. per kg. for the hydriodide and hydrochloride respectively. The effects of intravenous administration were investigated with the more soluble hydrochloride. In the rat, mouse and rabbit the LD50 was found to be approximately 20 to 40 mg. of the hydrochloride per kg. (injection time, 5 seconds). Death occurs within a few seconds after intravenous injection with clonic and tonic convulsions. A pharmacological investigation has been undertaken to determine the cause of death.

Circulatory and respiratory effects. Intravenous injection of 10 mg. of the hydrochloride per kg. (rabbit, urethane) produces a sudden drop in arterial blood pressure and changes in the electrocardiogram. Depressive effects were shown on the conducting system, first the sino-auricular node and thereafter the auriculo-ventricular node. The effect is temporary and circulatory function is rapidly restored. Higher doses may lead to complete cardiac arrest, respiratory failure and death. Intravenous administration could not be used in studies on respiration because of the speed with which the animals recovered (or died) after injection. The effects on respiration were investigated after intramuscular injection

* "Leocillin" (Denmark), "Estopen" (England).

(rabbit, urethane). Large doses, about 1 g. of the hydrochloride per kg., gave rise to symptoms similar to those observed after intravenous injection but the course of events was slowed enabling more detailed studies to be made. The first effect is a drop in arterial blood pressure, at this time respiration is unaffected or slightly stimulated. Failure of respiration does not occur until immediately before death when the blood pressure has fallen to zero. The main toxic effect is therefore on the circulation. The anoxia consequently produced may explain the respiratory depression seen in anæsthetised animals and the convulsions noted in unanæsthetised animals.

The action on the circulation could be caused by either a central or a peripheral effect. A primary central effect is probably excluded by the observations that injection into the carotid artery does not cause a depression of blood pressure while intravenous injection into the spinal rabbit is immediately followed by depression of blood pressure.

Isolated heart. A Ringer-perfused rabbit heart was employed. Benzylpenicillin diethylaminoethyl ester at a concentration of 10 μg./ml. caused distinct inhibition of contraction while 100 μg./ml. completely arrested contraction.

Isolated rabbit ear. Addition to the Ringer perfusion fluid caused no changes in the vascular tone. Yet, a possible inhibition of the normal tone (controlled by the autonomic system) will not be observed in experiments with an isolated vascular bed. Therefore, the experiments are not conclusive. However, the effects on the heart appear to be sufficient to explain all the observed effects on the circulation without postulation of any action on the peripheral vascular system.

Adrenergic blocking. Using the method of Hunt[3] no anti-adrenaline effect could be demonstrated.

Cholinergic blocking. In vitro experiments with intestine of rat, rabbit and monkey showed some atropine-like action in depressing methacholine-induced contractions. In these experiments it was shown to have about 1/500 to 1/1000 of the activity of atropine.

In vivo experiments in rats demonstrated that it could not inhibit the production of chromodacryorrhea induced by methacholine. Likewise no mydriatic effects could be demonstrated (mice).

Summary

1. The diethylaminoethyl ester of benzylpenicillin is a substance of low toxicity when administered orally, subcutaneously or intramuscularly.

2. Intravenous administration may—according to the dose—cause either a transient drop in arterial blood pressure or complete circulatory failure due to cardiac arrest.

References

1. Jensen *et al.*, *Acta Path. Microbiol. Scand.*, 1951, **28**, 407.
2. Juhl Nielsen *et al.*, to be published.
3. Hunt, *J. Pharmacol.*, 1949, **95**, 117.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Acetaldehyde and Acetone, Determination of. S. D. Nogare, T. O. Norris and J. Mitchell, Jr. (*Anal. Chem.*, 1951, **23**, 1473.) The method is based on the reaction of acetaldehyde and acetone with hypoiodite solution to give iodoform which is measured spectrophotometrically at 347 mμ. Iodoform absorption is characterised by three well defined maxima occurring at 347, 307 and 274 mμ, the absorption peak at 347 mμ being the most sensitive to changes in iodoform concentration and obeying Beer's law for amounts of iodoform from 0 to 3 mg. Under controlled conditions acetaldehyde and acetone gave reproducible iodoform yields of 58 and 108 per cent. respectively, on a molecular basis. Interference was encountered from compounds which gave the iodoform reaction; a list of compounds which interfered, together with the concentration at which interference was appreciable, is given. The procedure could be used to determine 1:2-propylene glycol alone or in the presence of ethylene glycol; the glycols were first oxidised with periodate in order to convert them quantitatively to acetaldehyde and formaldehyde, acetaldehyde being then determined by the iodoform method. R. E. S.

Arsenic in Bismuth Compounds, Detection of. J. A. C. Van Pinxteren and G. Schallenberg-Heertjes. (*Pharm. Weekbl.*, 1951, **86**, 701.) The following method is recommended: 0·2 g. of the inorganic compounds (nitrate, carbonate, hydrate) is ignited and to the residue 3 ml. of stannous chloride containing hydrochloric acid, 3 ml. of water and granulated zinc is added. If 1 μg. of arsenic is present a yellow stain is produced on mercuric chloride paper. Of the organic preparations (salicylate, tannate, gallate, oxyiodetogallate) 0·2 g. is ignited with 0·2 g. of magnesium peroxide and the residue treated as described above. In all cases it was possible to detect 1 μg. of As in 0·200 g. of the bismuth compound. J. R. F.

Coumarin in the Presence of Vanillin, Test for. M. Nivoli. (*Annali Chim.*, 1951, **40**, 642.) To 1 ml. of the ethanolic solution to be tested add 1 ml. of a 1 per cent. aqueous solution of sodium hydroxide and 4 or 5 drops of a 1 in 1000 solution of colourless *p*-aminophenol in 95 per cent. ethanol. In the presence of coumarin a blue colour is obtained which reaches its maximum intensity in a few minutes. The reaction is given by 0·020 mg. of coumarin, and vanillin, even in very large excess, does not interfere. H. D.

Fluorine in Organic Compounds, Determination of. R. Belcher and J. C. Tatlow. (*Analyst*, 1951, **76**, 593.) Decomposition of the compound is effected by heating with metallic sodium in a specially designed nickel bomb, details of which are given, at a high temperature for 1 hour, and cooling. The fluoride ion is then determined gravimetrically as lead chlorofluoride. The method is suitable for the assay of the most strongly bonded organic compounds of fluorine. J. R. F.

Monosaccharides, a Colorimetric Method for the Determination of, in Organic Solvents for use in Partition Chromatography. S. Gardell. (*Acta chem. scand.*, 1951, **5**, 1011.) The determination of certain monosaccharides in solution in organic solvents, as collected during flowing chromatogram techniques, is described. The method, which is suitable for the estimation of aldopentoses, methylpentoses and aldohexoses in concentrations of 10 to 300 μg./ml. depends upon the colour reaction obtained with aniline trichloroacetate in a strong solution of trichloroacetic acid. The colour is not given by fructose and in the case of both pentoses and hexoses it shows the strongest light absorption at 370 mμ. The various factors affecting the formation of the colour are discussed in some detail. J. B. S.

Nitrates and Nitrites, Colour Reaction for. H. Barnes. (*Analyst*, 1951, **76**, 666.) It was found that a brilliant purple colour was produced by nitrates with a sulphuric acid solution of the nitrite reagent, *N*(1-naphthyl)ethylenediamine hydrochloride. For the test, 2·25 ml. of an aqueous solution was used and 2·75 ml. of nitrogen-free sulphuric acid were added, followed by 1·0 ml. of a 0·02 per cent. solution of the reagent in sulphuric acid. With 2·5 μg. of nitrate nitrogen per ml. in the test solution the liquid turns to a distinct purple in 90 seconds and an intense purple in 3 minutes. The colour is stable for some time and the limit of sensitivity is about 0·25 μg. of nitrate nitrogen per ml., although at these low concentrations the colour takes longer to develop. Nitrites gave the same reaction but potassium iodate was the only other oxidising agent tested that produced a purple colour. A table is given showing the behaviour of a number of oxidising agents under the conditions of the test. R. E. S.

Nitrites, Determination of. H. Barnes and A. R. Folkard. (*Analyst*, 1951, **76**, 599.) A number of modifications of the Griess-Ilsovay reaction for the estimation of nitrites have been examined. Of the four techniques compared, the method of Rider and Mellon showed the most sensitive results, giving an optimum absorption with an Ilford green filter (604) and a maximum colour development in a coupling time of about 25 minutes at 18° C. to 25° C. A modification of the Shinn method was also applied. The rate of colour development is extremely rapid, the maximum intensity being obtained in 10 minutes at 25° C., and the test is slightly more sensitive than the Rider-Mellon method, but the coupling reagent discolours on keeping, giving a large blank reading. J. R. F.

Particle Size Analysis, Approximate. E. I. Johnson and J. King. (*Analyst*, 1951, **76**, 661.) A simple method is proposed for this determination, attempting to separate those particles that have a greater Stokes' diameter than a predetermined limit. It is believed, in conjunction with the apparatus described, to be particularly well suited for routine control use, the simplicity of the apparatus and its ease of operation compensating for the loss of some of the information obtainable by more elaborate methods. A weighed sample of powder is dispersed in water or other suitable suspending medium and placed in a fat extraction tube; two marks 12 cm. apart are made and the time t in seconds required for a particle of the size limit chosen to travel down the tube from the top mark to the lower mark is calculated from a formula derived from Stokes' law. The layer above the lower mark is blown off by the usual method, and the tube is refilled to the top mark with water, the procedure

being repeated until all fine particles are removed and the only ones left are those falling below the lower mark in time t; four such extractions are usually sufficient and the weight of coarse particles remaining in the tube is then estimated by a convenient method. Results are given for comparative determinations on two α-naphthylthiourea powders using the present method and that due to Andreason (*Ber. dtsch. keram. Ges.*, 1930, **11**, 249). R. E. S.

Phosphorus, Estimation of, by Ceric Sulphate. G. S. Deshmukh. (*Analyst*, 1951, **76**, 604.) Ceric sulphate oxidises phosphorus quantitatively to phosphoric acid. An accurately weighed sample of red phosphorus was placed in a Kjeldahl type flask fitted to a Leibig's condenser. A known volume of ceric sulphate, sufficient to give excess, was added, and the solution heated under a reflux condenser until the red phosphorus dissolved. The solution was cooled and the unchanged ceric sulphate estimated by titration against standard ferrous ammonium sulphate with *o*-phenanthroline ferrous complex as indicator. It has not been possible to apply this method to the determination of white or yellow phosphorus owing to the difficulty of weighing in the dry form. The adaptation of the procedure to determine the solubility of phosphorus in various solvents has been suggested. J. R. F.

Sodium in Serum, Rapid Determination of. P. Trinder. (*Analyst*, 1951, **76**, 596.) A magnesium uranyl acetate reagent, containing 80 per cent. v/v of ethanol is used to precipitate the sodium and protein simultaneously. The precipitate is separated by centrifuging at moderate speed. The uranium is determined in the reagent and in the supernatant liquid by a photoelectric colorimetric method and the sodium content of the serum is calculated from the loss in concentration, by the use of a calibration graph prepared with solutions of known sodium content. A single determination can be completed within 15 minutes. J. R. F.

Testosterone Propionate in Vegetable Oil Solution, Determination of. J. J. Madigan, E. E. Zenno and R. Pheasant. (*Anal. Chem.*, 1951, **23**, 1691). An attempt was made to find a method for the quantitative determination of testosterone propionate by the isolation and identification of the 2:4-dinitrophenylhydrazone, from oil solutions; experiments failed to produce quantitative recoveries and attention was given to the formation of the semicarbazone. A detailed method is given whereby the testosterone propionate is quantitatively separated as the semicarbazone, which could be weighed and identified by its m.pt. (with characteristic colour change), or by ultra-violet absorption. The ultra-violet absorption spectrum was determined in a 0·001 per cent. solution in methanol, in which maximum absorption occurred at 268 to 269 mμ (specific absorption, $E_{1\text{ cm.}}^{1\text{ per cent.}} = 725 \pm 5$ per cent.) and negligible absorption from about 320 mμ upward. The m.pt. of the recovered semicarbazone should be between 207° and 217° C. corrected, when determined in a bath heated at 3° per minute, with the capillary inserted at 200° C. R. E. S.

ESSENTIAL OILS

Orange Oil, Fermentation-inhibiting Properties of. D. A. A. Mossel (*Nature, Lond.*, 1951, **168**, 999.) As the result of an observation that non-preserved fruit drinks prepared from emulsified orange oil inhibited the fermentation of an inoculum of *Saccharomyces cerevisiæ* the author made some quantitative experiments on similar lines. It was found that 0·01 per cent. or

more of Florida orange oil (aldehydes 1·6 per cent.) increased the time required for complete fermentation of an inoculum in a semi-synthetic medium from 48 hours to at least 96 hours. The terpenes and the fraction (3·6 per cent.) containing the oxygen derivatives extracted from New Guinea orange oil were tested separately and it was found that the terpenes possessed the same order of activity as the original oil. The oxygen-containing fraction was inactive in concentrations equivalent to 0·1 per cent. of original oil, thus confirming the statement made previously by Guenther (*The Essential Oils*, 1948, **1**, 81).

G. R. A. S.

ORGANIC CHEMISTRY

Camphor Substitutes, Water-soluble Synthetic. M. Fioretti, (*Boll. chim.-farm.*, 1951, **90**, 424.) Injections of camphor are painful and slow in action, so a water-soluble compound with the same pharmacological action is desirable. Camphoric acid is prepared by oxidising camphor with nitric acid. The m. pt. of that obtained from natural camphor is 187° C. while that from synthetic camphor is 202° C. and it is less soluble in water. This acid and its sodium salt have no action on the heart and are diuretic. Camphorsulphonic acid can be prepared by acting on camphor with concentrated sulphuric acid and acetic anhydride. The salts are soluble in water but are rapidly excreted in the urine and only a very slight camphor-like activity is shown. Another derivative is camphor-carbonic acid, prepared by the action of carbon dioxide on sodium camphor in an organic solvent. This compound, unlike the others, is not stable to heat and the solution begins to decompose at 80° C., but solutions can be heated for half-an-hour in sealed vessels in a current of steam. It is, however, without physiological action and is passed unaltered in the urine. Thus none of these bodies can be considered as a water-soluble substitute for camphor. H. D.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Dechlorogriseofulvin—A Metabolic Product of *Penicillium griseofulvum* Dierckx and *Penicillium janczewskii* Zal. J. MacMillan. (*Chem. Ind.*, 1951, 34, 719.) The isolation of a second "curling factor" from culture filtrates of both these organisms is recorded. Chloroform extracts of culture filtrates have been submitted to chromatography on alumina and have been shown to contain griseofulvin and also a new mould metabolite, dechlorogriseofulvin. Chromatography of similar extracts from the mycelium yielded only pure griseofulvin. Dechlorogriseofulvin, $C_{17}H_{18}O_6$, m.pt. 179° to 181° C. $[\alpha]_D^{19° C.}$ + 390 (*c.* 1·0 in acetone), is a neutral crystalline compound, containing three methoxyl groups. Hydrogenation with platinic oxide yielded tetrahydrodechlorogriseofulvin, identical with a compound obtained by reductive dechlorination of griseofulvin. A structure is suggested for dechlorogriseofulvin. It produces a typical griseofulvin-like response in *Botrytis allii*, although it is less active than griseofulvin itself. J. B. S.

Hydroxytyramine (β-3 : 4-Dihydroxyphenylethylamine) in Human Urine. U. S. Von Euler, U. Hamberg and S. Hellner. (*Biochem. J.*, 1951, **49**, 655.) By partition chromatography of human urine extracts on starch columns, hydroxytyramine, adrenaline and noradrenaline have been separated and identified. The excretion of hydroxytyramine in normal urine has been confirmed and has been found quantitatively to be the most important catechol

amine present. It occurs in urine largely in the free state. The daily output of hydroxytyramine is estimated at 0·1 to 0·2 mg. and exceeds considerably that of noradrenaline and adrenaline. The fraction with *R* values corresponding with those of hydroxytyramine gives a characteristic spot in paper chromatograms and shows the same behaviour as a hydroxytyramine on colorimetric estimation and biological assay. J. R. F.

Lactose, Biological Synthesis of, from Carbon-14 Glucose. T. H. French, G. Popjak and F. H. Malpress. (*Nature, Lond.*, 1952, **169**, 71.) Carbon-14 starch, prepared by photosynthesis, was administered to lactating rabbits by stomach tube, and milk collected after 6 hours. The analytical data confirm earlier conclusions that the lactose is derived solely from the glucose intake, since the specific activities of the carbon of the lactose samples, and of the glucose and galactose fractions prepared from them by acid hydrolysis, were all the same. It is also concluded that both moieties of the lactose molecule are found in equal measure from the same source. J. B. S.

Lyxoflavin, Vitamin Activity of. G. A. Emerson and K. Folkers. (*J. Amer. chem. Soc.*, 1951, **73**, 5383.) It was considered possible that lyxoflavin might be a vitamin because of the close similarity of structure of vitamin B_2 and lyxoflavin, because vitamin B_2, vitamin B_{12} and lyxoflavin contain a 1 : 2-diamino-4 : 5-dimethylbenzene moiety linked through a nitrogen atom to a pentose, and because all three are concerned with the human body. Synthetic lyxoflavin was found to be devoid of riboflavine activity when tested in conventional assays using rats and *L. casei*. It was shown to possess growth promoting or vitamin activity in a rat assay method, the details of which are given. A. H. B.

BIOCHEMICAL ANALYSIS

Antibiotics from Streptomyces. J. Berger, A. I. Rachlin, W. E. Scott, L. H. Sternbach and M. W. Goldberg. (*J. Amer. chem. Soc.*, 1951, **73**, 5295.) Three new crystalline antibiotics were isolated from cultures of three unidentified streptomyces. The three streptomyces were isolated from soil samples and grown on a variety of media in aerated submerged culture. Although the new antibiotics were chemically different, their biological activity and certain chemical properties are so similar that they are reported here as a group. They are colourless, optically active, organic acids, and their most likely empirical formulæ are $C_{46-47}H_{80-82}O_{13}$, $C_{25}H_{40}O_7$ and $C_{34}H_{52}O_8$. Only one has a characteristic ultra-violet absorption spectrum. They are active *in vitro* against certain Gram-positive bacteria and mycobacteria but inactive against Gram-negative bacteria and fungi, rather toxic, and inactive *in vivo* against a variety of bacterial and protozoan infections. A. H. B.

Blood in Urine, Chemical Tests for. H. Caplan and G. Discombe. (*Brit. med. J.*, 1951, **2**, 774.) Two tests are recommended. (1) Acidify 3 ml. of urine with a few drops of acetic acid and overlay with 1 to 2 ml. of 5 per cent. amidopyrin solution in 95 per cent. ethanol: allow 5 to 6 drops of 10 volume hydrogen peroxide to fall through the ethanolic layer. Allow to stand for a few minutes, a blue- or lilac-coloured ring is a positive reaction. This test is simple but comparatively insensitive. (2) Wash a white porcelain tile with cavities, rinse with 95 per cent. ethanol and allow to dry by evaporation. Place about 3 mg.

of *o*-tolidene hydrochloride in a cavity: add 1 drop of urine, stir with a glass rod and add 1 drop of a mixture of glacial acetic acid and 10 volume hydrogen peroxide in equal parts; a positive reaction appears as a blue colouration or spreading blue-green streaks which fade, after 5 to 30 minutes, to brown. A negative reaction is pale brown. J. R. F.

Calcium and Magnesium in Plasma, Simplified Titrimetric Techniques for the Assay of. E. S. Buckley, J. G. Gibson and T. R. Bortolotti. (*J. Lab. clin. Med.*, 1951, **38**, 751.) The techniques depend upon the fact that aqueous solutions of certain dyes at a critical *p*H have a characteristic colour which changes in the presence of a minute concentration of metal ions, and addition of sufficient ethylenediamine tetra-acetate results in a reversion to the original colour if the *p*H of the system is not altered. A description of the technique and reagents is given for the direct assay in plasma of calcium concentrations using murexide, and of the sum of calcium and magnesium concentrations using Eriochromschwarz-T. The methods were found to be reliable with concentrations in plasma of the two cations ranging from 0·10 millimolar for calcium, and 0·18 millimolar for the sum of calcium and magnesium to the clinically significant levels of 3·0 millimolar and 4·0 millimolar. Thus an indirect method for the estimation of plasma magnesium is available. A. H. B.

Indole, Colorimetric Estimation of, by the Xanthydrol Reaction. W. R. Fearon and J. A. Drum. (*Sci. Proc. R. Dublin Soc.*, 1951, **25**, 295) A sensitive method for the determination of indole, based on the production of a stable violet colour when indole and xanthydrol react in acid solution, is described. The details of the preparation of the reagents, and the method of obtaining indole from biological fluids, are given. A. H. B.

Indoxyl, Estimation of. J. A. Drum. (*Sci. Proc. R. Dublin Soc.*, 1951, **25**, 299.) The investigation of indole metabolism necessitated the estimation of urinary indican, and, therefore, the hydrolysis of indican and the condensation of isatin and indoxyl to form indirubin was examined. The replacement of hydrochloric acid, used by previous workers, by sulphuric acid was found to produce more consistent results. A colorimetric method for the estimation of urinary indoxyl, based on the above condensation, is described. The minimum quantity of indican which can be detected and estimated is of the order of 4 μg. A. H. B.

Procaine Penicillin, Determination of Total Penicillin in. A. M. Wild. (*J. appl. Chem.*, 1951, **1**, 329.) The method depends on the conversion of procaine penicillin to sodium penicillin by precipitation with sodium silicotungstate, the sodium penicillin being assayed iodimetrically. The sample of procaine penicillin is ground, weighed and stirred with water before adding the special silicotungstate reagent; the solution is filtered under specified conditions before making up to a definite volume. The assay is completed by the published Analysts Sub-Committee method (*Analyst*, 1949, **74**, 550; *J. Pharm. Pharmacol.*, 1950, **2**, 260). Using this method the total penicillin content of a sample of sodium penicillin was found to be 97·8 per cent. standard deviation 0·35; with added procaine penicillin a mean result of 97·3, standard deviation 0·32 was obtained. R. E. S.

Vitamin B_{12}, Determination of, with a Mutant Strain of *Escherichia coli*. P. R. Burkholder. (*Science*, 1951, **114**, 459.) The microbiological assay

technique used employed bacteria produced by selection from ultra-violet irradiated cultures of *E. coli* W. (ATCC 9637), one strain, 113–3, showing marked responses to both methionine and vitamin B_{12}. The strain 113–3 was employed in establishing dosage-response curves for different levels of vitamin B_{12} and for methionine. It was found that the great sensitivity of the organism to relatively low concentrations of vitamin B_{12} as compared with the requirement of much higher amounts of methionine permitted the development of specific vitamin B_{12} assays of many natural materials by appropriate dilution of samples. Proof of the response of strain 113–3 to vitamin B_{12} in complex materials was obtained by making parallel determinations at different levels for vitamin B_{12} with strain 113–3 and for methionine with another strain 26–18 which was stimulated by methionine or homocystine but not by vitamin B_{12}. Details are given of the media and of the method used for the assay (of the tube type) which employed turbidity measurement for the final vitamin B_{12} estimation. It was found that adequate growth could be obtained after 15 to 18 hours of incubation on a shaking machine with the temperature at 30° C. The half-maximum level of growth occurred at about 0·12 mμ of B_{12}/ml. Under the specified conditions of assay, the organism also responded to vitamins B_{12} and B_{12a}. In duplicated assays with *Euglena* and with *E. coli*, blood serum proteins, animal tissue extracts, and various preparations of bacteria and algæ yielded satisfactory results. Whole normal blood appeared to have amounts of vitamin B_{12} too low for direct assay in the presence of free methionine. Vitamin B_{12} bound in tissues and blood serum was released by heat or enzyme treatment before assay. R. E. S.

CHEMOTHERAPY

Dimethylaminoethyl-substituted Compounds, Antispasmodic and Local Anaesthetic Activity of. J. F. Reinhard, E. T. Kimura and J. V. Scudi. (*J. Pharmacol.*, 1951, **103**, 288.) The antispasmodic and local anæsthetic activity of compounds of the type $ROCH_2CH_2N(CH_3)_2$, where R = methyl, ethyl, decyl, phenyl, β-pyridyl, etc., and of the type $\begin{matrix}R_1\\R_2\end{matrix}\!\!>\!N\text{-}CH_2CH_2N(CH_3)_2$ when R_1 and R_2 = various aliphatic groups and cyclic structures, was measured. Guinea pig ileum was used in the tests for antispasmodic activity and guinea pig cornea for the tests for local anæsthetic activity. Of the compounds tested, 17 were active against histamine, 9 against acetylcholine and 5 against barium chloride. 8 members of the series had more than 20 times the local anæsthetic potency of cocaine. The relationship between structure and activity is discussed. A. H. B.

3:3-Diphenyl-propanolamines, -allylamines and -propylamines; Pharmacological Properties of. A. C. White, A. F. Green and A. Hudson. (*Brit. J. Pharmacol.*, 1951, **6**, 560.) Atropine-like activity, shown by antagonism of carbachol *in vitro* and by mydriasis, is greater in the propanolamines than in the allylamines and propylamines. 3:3-Diphenylpropan-3-oldiethylamine is as active as atropine sulphate on a molecular basis. Antihistamine activity is greater in the allylamines and propylamines than in the propanolamines, the pyrrolidine analogues, which are both a tenth to a fifth as active as mepyramine, being the most potent antihistamines in these series. The quaternary ammonium salts are, in general, less potent antihistamines than the tertiary amines. All series showed corneal anæsthetic properties, the greatest degree of activity being in the allylamines; the most active members are a twentieth as active

as cinchocaine and cause considerable conjunctival irritation; the quaternary ammonium salts are less active than the tertiary amines. No analgesic activity was found in these series. Pharmacological action varies greatly with the nature of the basic groups in these and other related series, and in any one property in a given species the optimal groups, though commonly allied, may not necessarily be the same even in closely related series. S. L. W.

Hexamethonium and Homologues, Actions of. R. Wien and D. F. J. Mason. (*Brit. J. Pharmacol.*, 1951, **6**, 611.) A study has been made of certain actions of hexamethonium and of homologues with ethyl substituents, in which one or more methyl groups on each nitrogen atom were replaced by ethyl groups. The bis-ethyldimethylammonium homologue (hexane-1:6-bis-ethyldimethylammonium dibromide dihydrate) was one and a half times to twice as potent as hexamethonium in paralysing autonomic ganglionic transmission in both sympathetic and parasympathetic ganglia, and had a similar type of action. The bis-triethylammonium homologue was much less potent and possessed neuromuscular blocking properties absent in the other compounds. The bis-diethylmethylammonium compound was as active as hexamethonium on parasympathetic ganglia but slightly less potent on sympathetic ganglia. It is shown that the nature of the terminal groupings as well as the distance between the two quaternary nitrogen atoms is a factor determining optimal activity. Some actions of hexamethonium, such as inhibition of salivary excretion and vasodilatation are very slight and play little part in the main action of the drug, but it has an appreciable mydriatic effect. It has little effect on the heart. Gastric motility may be excited or inhibited by hexamethonium dependent on the functional innervation of the stomach in different animals. In the cat, stomach movements were increased. Gastric secretion in the dog was inhibited by both hexamethonium and its bis-ethyldimethylammonium homologue; there were reductions in volume, free and total acidity and peptic power. No inhibitory effect was exerted on gastric secretion induced with histamine. Considerably less of the compounds was excreted in the urine after oral than after intravenous administration. S. L. W.

PHARMACY

NOTES AND FORMULÆ

Methimazole (Tapazole). (*New and Nonofficial Remedies*; *J. Amer. med. Ass.*, 1951, **147**, 1668.) Methimazole is 1-methyl-2-mercaptoimidazole, $C_4H_6N_2S$, and occurs as an almost odourless and tasteless, white to buff, crystalline powder; m.pt. 145° to 148° C., soluble in water (1 in 4·5), ethanol (1 in 5), chloroform (1 in 4·4) and ether (1 in 125); a 2 per cent. solution has *p*H 6·7 to 6·85. An aqueous solution at first decolorises solution of iodine and then yields a brown precipitate which becomes brownish black. A 0·0003 per cent. solution in water exhibits an ultra-violet absorption maximum at about 2520 Å ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ about 1357) and a minimum at 2200 Å. When dried *in vacuo* over phosphorus pentoxide for 24 hours, methimazole loses not more than 0·5 per cent. of its weight; it yields not more than 0·1 per cent. of sulphated ash. The amount of nitrogen present, determined by the Kjeldahl method, is 24·0 to 24·8 per cent., equivalent to 98·0 to 101·0 per cent. of methimazole. When assayed by dividing the absorption of a 0·0003 per cent. solution at 2520 Å by 135·7, it contains 96·5 to 102·5 per cent. of methimazole. Methimazole has the action and uses of propylthiouracil but is perhaps 20 times as potent. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

Antimonial Compounds, Action of, on the Liver Fluke *in vitro*. T. E. Mansour. (*Brit. J. Pharmacol.*, 1951, **6**, 583.) Using both Ringer's solution and a mixture of equal volumes of bovine serum and Ringer's solution as media, *in vitro* tests of the effects of certain antimonials were carried out on preparations of the liver fluke (*Fasciola hepatica*). The tests were conducted for a maximum period of 90 minutes and kymographic records were obtained of the movements of the fluke. At least 4 flukes were used in each test. In a saline medium, in a concentration of 1:1000, tartar emetic, stibophen and neostibosan all failed to cause paralysis of rhythmical movement. On the other hand, a definite lethal action was demonstrated to tartar emetic in the presence of serum, though not to stibophen or neostibosan. The fraction of serum responsible for this action was found to be dialysable through cellophane membrane against distilled water, but not against saline solution. S. L. W.

Atropine, Action of, on the Cardiovascular System. L. A. Nalefski and C. F. G. Brown. (*Arch. intern. Med.*, 1950, **36**, 898.) The effects of atropine sulphate given by intravenous or subcutaneous injection were studied in 133 normal subjects of both sexes and various age groups, with a view to assessing its role in the treatment of coronary thrombosis. Each subject was given 0·02 mg. of atropine sulphate per kg. of body weight—this dose was considered sufficient to produce a pronounced effect on the vagi. All subjects showed an initial drop in heart rate and a subsequent rise following the administration of atropine. The effects on the heart rate were more rapid and transient with intravenous than with subcutaneous injection, and response to atropine most pronounced in persons under twenty. Blood-pressure changes varied with mode of administration but were more brisk in ages ranging from 20 to 50 years. Systolic pressures either increased or decreased but diastolic pressures invariably increased. Salivary secretion of subjects up to the age of 20 was less affected by atropine than that of subjects in older age groups. The authors conclude that if the degree of vagal tone can be measured by the changes in heart rate noted after atropine administration, then these results indicate that vagal tone is greatest in childhood and gradually decreases through the succeeding decades. Their experiments lead them to believe that atropine sulphate should play a prominent part in the treatment of coronary thrombosis. G. R. B.

Aureomycin, Topical Use of, in Skin Diseases. B. Solomons. (*Brit. med. J.*, 1951, **2**, 525.) A 3 per cent. aureomycin hydrochloride ointment in a petrolatum and wool fat base was employed in the treatment of 144 dermatological cases (sycosis barbæ, 22; folliculitis of other areas, 12; impetigo, 57; miscellaneous, 53). The ointment was applied thinly and gently to the affected areas twice a day. The most striking results were obtained in the treatment of sycosis barbæ; within a week improvement was noted in all cases, and in all except 3 cases the pustules had disappeared by the end of a fortnight. All the cases except one of folliculitis of other areas also responded to the treatment, and all except 2 of the cases of impetigo were cured within 4 to 7 days. Local sensitivity occurred in only 2 cases of the series. Aureomycin would appear to be the best available antibiotic for use in ointment form for pyogenic infections. S. L. W.

2-Carbethoxythio-1-methylglyoxaline, (C.G.1) Antithyroid Activity of. A. Lawson, C. Rimington and C. E. Searle. (*Lancet*, 1951, **261**, 619.) The antithyroid effects of this substance have been investigated in mice, rats and man.

The acute toxicity in mice is of the same order as that of thiouracil. The chronic toxicity has been examined in rats and considerable hypertrophy of the thyroid occurred; the gland concentrated only 11·5 per cent. as much radio-iodine as those of control animals. It has been compared with other anti-thyroid compounds by a new technique developed by the authors. Its activity in rats is about equal to that of 2-mercapto-1-methylglyoxaline, both being much more active than thiouracil. Its effect on the radio-iodine uptake in man was studied in a small number of subjects and the neck/thigh ratio count examined. Results showed a somewhat greater inhibition of radio-iodine take up than after 2-mercapto-1-methylglyoxaline. J. R. F.

Chorionic Gonadotrophin, Prolongation of Action. K. Didcock, J. M. Robson and A. A. Sharaf. (*Brit. J. Pharmacol.*, 1951, **6**, 445.) The period of action of chorionic gonadotrophin is considerably extended when the hormone is administered as a compressed implant made with magnesium monostearate. The duration of such implants was investigated by determining their effect in immature mice, implants weighing 1 mg. and containing one part of active material with 3 parts of excipient being inserted subcutaneously in the upper dorsal region. The active material was absorbed from the implant and produced an effect lasting for about 20 days. Other experiments suggest that the period of absorption depends on the relative proportion of active material and excipient, and that periods of action suitable for the clinical use of the material could be obtained by varying the relative proportions of the gonadotrophin and the magnesium monostearate. Macroscopic examination revealed no reaction round the implanation sites. S. L. W.

Cinchona Alkaloids, Oxytocic Action of. D. K. de Jongh, E. G. van Proosdij-Hartzema and A. Th. Knoppers. (*Arch. int. Pharmacodyn.*, 1951, **88**, 84.) The oxytocic action of quinine, hydroquinine, quinidine, cinchonine and cinchonidine, either alone or in combination with pituitary or ergometrine, was investigated *in vitro* (using uteri of rats and guinea pigs) and *in vivo* (using rabbits, cats, guinea pigs and rats). Oxytocic effects *in vitro* were regularly observed with quinine in concentrations of $1:5 \times 10^4$ and upwards; for the other alkaloids the minimum active concentration was $1:10^6$. The paralysing concentration amounted to $1:10^5$ for all the alkaloids. No potentiation by quinine of the pituitary or ergometrine effect was observed *in vitro*. Oxytocic effects were usually obtained *in vivo* with all the alkaloids in doses of 0·5 to 10 mg./kg. intravenously. Higher doses (20 mg./kg.) sometimes paralysed the uterus. There were no important differences between the 5 alkaloids. Quinine *in vivo* did not enhance the pituitary effect, though in rare instances it enhanced the ergometrine effect. S. L. W.

Cortisone, Antitoxic Action of. F. Boyer and L. Chedid. (*C.R. Acad. Sci., Paris*, 1951, **233**, 1232.) Cortisone protects mice against infection with *Salmonella typhi*, but is not as effective as chloramphenicol. Injections of 2 to 5 mg. of cortisone allowed 40 per cent. of mice infected with a culture to survive 8 days, while 10 mg. of chloramphenicol allowed 100 per cent. to survive from the same dose of culture, which killed 100 per cent. of the controls. With *Salmonella* toxin however 77 per cent. of those treated with 1 to 5 mg. of cortisone survived 48 hours, while only 37 per cent. of those receiving 10 mg. of chloramphenicol and 20 per cent. of the controls which received no treatment survived. With antigen O (Ty II) (1 mg. given by injection), 2 and 4 mg. of

cortisone gave 100 per cent. survival for 48 hours and 90 per cent. for 6 days; chloramphenicol 10 mg. *per os* gave 80 per cent. and 60 per cent. survival respectively, only 20 per cent. of the controls surviving. With antigen Vi, 2 mg. of cortisone caused 90 per cent. to survive 48 hours and 70 per cent. 6 days, with 0·25 mg. 1 hour before and 0·25 mg. 16 hours after there were no survivors, with chloramphenicol (10 mg. *per os*) 50 per cent. survived 48 hours and 40 per cent. 6 days. Against diphtheria toxin cortisone had no effect. H. D.

β-Naphthyl-di-2-chloroethylamine (R48); Oral Treatment of Polycythæmia Vera. K. Iversen and E. Meulengracht. (*Brit. med. J.*, 1951, **2**, 510.) 6 patients with polycythæmia vera of several years standing, most of whom had already received other treatment such as X-rays, intravenous nitrogen mustards, or repeated venesection, were treated orally with β-naphthyl-di-2-chloroethylamine in a dosage of 300 to 600 mg. daily. Treatments were given in series, alternating with free intervals, the course of the disease being followed with weekly blood counts; in only 2 patients was it possible to give the drug continuously (200 mg. daily). In 5 out of the 6 cases a marked fall in the percentage of hæmoglobin and red-cell counts occurred, and, parallel with this, a decrease in the redness and a subjective improvement. In one of the patients the remission has lasted for 15 months. The substance is a potential bone-marrow poison and a fall of leucocytes was observed in most cases, and it is essential that patients should be carefully and constantly controlled by blood counts during treatment. Gastro-intestinal symptoms were not observed during these trials. S. L. W.

Noradrenaline and the Suprarenal Medulla. D. M. Shepherd and G. B. West. (*Brit. J. Pharmacol.*, 1951, **6**, 665.) The sympathomimetic amine present in embryonic adrenal glands of cat, rabbit, guinea pig, dog and man is noradrenaline; very small amounts of adrenaline may also be present. Although no evidence has been obtained of the presence of hydroxytyramine and dihydroxyphenylalanine, large amounts of noradrenaline show that this amine must be a precursor of adrenaline in these mammals. Indeed, noradrenaline may itself be the hormone of the gland in the early days of life. It is suggested that in the adult glands the degree of methylation of noradrenaline is related to the relative cortical size. In animals where the cortex is large relative to the medulla, methylation of noradrenaline is almost complete and often only adrenaline is found in gland extracts. When there is little change with age in the ratio of cortical size to medullary size (as in the fowl) there is also little change in the relative amount of noradrenaline in the gland. Animal experiments show that exhausted adrenal glands have only about one-fourth the activity of healthy glands. As the total activity in exhausted adrenal glands of man is about 0·24 mg./g. (31 estimations) this would indicate that a total activity of about 1 mg./g. might be found in healthy individuals. S. L. W.

Procaine Amide for Cardiac Arrhythmias. J. M. Kinsman, W. R. Hansen and R. L. McClendon. (*Amer. J. med. Sci.*, 1951, **222**, 365.) Procaine has a digitalis-like action on the heart but the hydrochloride is rapidly hydrolysed in the blood and is acetylated in the liver so that its effects are of relatively short duration. Procaine amide (pronestyl) is hydrolysed only slowly in the body; it is excreted in the urine, and is not acetylated in the liver. It is readily absorbed from the gastro-intestinal tract and is therefore active when given orally but to secure rapid action intravenous administration is desirable and the authors studied its action in 41 patients with various cardiac arrhythmias when

given by this route. The compound was administered as a 10 per cent. solution at a rate of not more than 200 mg. per minute and usually at half this rate. Doses ranged from 0·5 to 2 g. Arrhythmias of supraventricular origin except auricular ectopic contractions and paroxysmal auricular fibrillation were not affected. Ventricular ectopic contractions were abolished in 12 out of 14 patients and ventricular tachycardia was abruptly stopped in 2 out of 4. Subjective toxic effects were rare but objective toxic effects were frequent; they included a fall in peripheral blood pressure and in pulmonary arterial pressure, a decrease in cardiac output and an increase in circulation time and in intraventricular conduction time. The authors conclude that the intravenous route should be reserved chiefly for patients with paroxysmal rapid heart action who are in immediate danger of death, and for patients under anæsthesia.

H. T. B.

Quinidine, Effects of Parenteral Administration of. H. Blinder, J. Burstein, W. Horowitz, E. Gersh, and R. Smelin. (*Arch. intern. Med.*, 1950, **36**, 917.) The authors studied the effects of a stable injection solution of quinidine lactate administered parenterally to 59 subjects divided into three groups. 22 had normal hearts, 15 abnormal (with regular sinus rhythm) and 22 with cardiac arrhythmias. The effects of the quinidine lactate were assessed by electrocardiogram. Special note was made of toxic effects. It was first given intravenously in a dosage of 0·65 g. The toxic effects encountered when given by this route make its therapeutic use dangerous, and after a limited trial its use was discontinued. Toxicity is directly related to speed of injection when quinidine is given intravenously. Intramuscularly, quinidine lactate (0·65 g.) is relatively painless and no more toxic than quinidine orally in similar dosage. This route of administration has the added advantages of providing more rapid and uniform absorption and of obviating possible gastric intolerance to the drug. In subjects with a normal sinus rhythm maximal cardiac effect is attained in about 30 minutes. The duration of peak effect is between 2 and 4 hours in subjects with normal hearts and at least 6 hours in those with abnormal hearts. A small but significant quinidine effect persists for at least 24 hours. The following dosage schedule is recommended for intramuscular injection: when rapid therapeutic effect is important, hourly administration; when a speedy response is not required, 3 to 4 hourly injections. G. R. B.

Streptomycin, Interference of Aureomycin, Chloramphenicol and Terramycin with the Action of. E. Jawetz, J. B. Gunnison and R. S. Speck. (*Amer. J. med. Sci.*, 1951, **222**, 404.) The authors find that each of the antibiotics aureomycin, chloramphenicol and terramycin reduces the bactericidal action of streptomycin *in vitro*, the effect being most marked during the first 12 hours of incubation. The antagonism is most pronounced with bacteriostatic concentrations; with concentrations which are themselves bactericidal the effect is much less. Chloramphenicol exhibits greater antagonism than an equal weight of either of the other two antibiotics. Chloramphenicol did not affect the activity of streptomycin on a suspension of *Klebsiella pneumoniæ* in Ringer's solution, in which no multiplication of the organism was taking place. The same effects were demonstrable in experimental infections in mice. The interference was observed only with organisms sensitive to streptomycin; with a strain of bacterium resistant to streptomycin, terramycin may have a synergistic action. With aureomycin and terramycin the antagonism is relatively slight and therefore may not be significant clinically but this may not be true of chloramphenicol. H. T. B.

BOOK REVIEWS

PHARMACOLOGY, by Michael G. Mulinos. Second edition. Pp. viii + 466 and Index. Oxford University Press, London. 1951. 40s.

This book is a synopsis of pharmacological facts assembled in a tabulated form. Its main purpose is to afford to the medical student and practioner a short manual of pharmacology. As such, it suffers from undue brevity and is difficult to understand without supplementary reading. The first edition has been revised by the simple expedient of interpolating extra pages, while retaining the original pages and page numbers. This has resulted in an unsatisfactory presentation. Too often sentences are guillotined in the middle, to be completed two or three pages further on, and a high percentage of blank pages appears. The book has not been brought up to date, which should have been possible with such a simple method of revision. For instance, the methods of biological assay for pituitary posterior lobe and digitalis are quoted from the United States Pharmacopœia XIII, although these have been superseded in the U.S.P. XIV published in 1950. No mention is made of the new neuromuscular blocking agents, or cortisone, yet these are described in books published in 1950. The terminology is open to criticism, for instance, the terms "adrenolytic," "sympatholytic," "cholinogenic" and "adrenogenic," while the inclusion of the ergot alkaloids and some of the vitamins under "hormones" excites surprise! In spite of its faults the book should be valuable for revision and reference purposes.

G. F. SOMERS.

HISTORY OF PHARMACY, by Edward Kremers and George Urdang. Second edition, revised and enlarged. Pp. xiv + 622 (including 30 illustrations). J. B. Lippincott Company, London. 1951. 60s.

In this edition, which is wholly the work of Dr. Urdang, owing to the death of Dr. Kremers shortly after the publication of the first edition in 1941, the original material has been revised and expanded by about one-third. Unfortunately even this enlargement has been insufficient to balance the disproportionate amount of space allotted in the first edition to American pharmacy as against European. Nevertheless it is reassuring to note the addition of a chapter on the development of pharmacy in Spain, an important subject most regrettably neglected hitherto by writers in the English language.

The book is divided into four parts, the first being concerned with the early pharmacy of the ancient civilisations. The second part (130 pages) deals with the rise of professional pharmacy in Europe, with special reference to Italy, France, Germany and Britain. In the last-named section the opportunity has been taken to correct one or two slips in the previous edition. Pharmacy in the United States occupies the whole of the third part and overlaps into the fourth, making a total of 277 pages. The last hundred pages are occupied by an extensive bibliography comprising over 800 references, a chronological table and a valuable biographical appendix. The revision of a book of this scope must have entailed an immense amount of careful work, and Dr. Urdang is to be congratulated on the highly successful result.

A. LOTHIAN.