BRITISH PHARMACEUTICAL CONFERENCE NOTTINGHAM, 1952

Chairman: H. B. MACKIE

CHAIRMAN'S ADDRESS

AN EDUCATION FOR A PHARMACIST

I HAVE entitled my address "An Education for a Pharmacist," not, you will observe, Pharmaceutical Education or even Education for Pharmacy. I have chosen this title to indicate that I desire rather more latitude than an exact interpretation of the other two would permit.

If what I have to say appears to be provocative, I hope it will be provocative of thought and discussion rather than disagreement. An Education, then, for a Pharmacist.

The person I have in mind is a professional man who understands thoroughly what he is doing, who comprehends the scientific basis of drugs and drug action, who is able to evaluate critically the products he handles, who is competent to advise physicians concerning drugs and their uses, who works at his profession creatively and advances its service.

Education concerns two spheres, one has to do with the human spirit, the other with material requirements. Any profession to be long-lived must insist on education and on education in this sense. Merely to train competent practitioners is only part of our purpose, and not, I think, the most important part.

EARLY EDUCATION

The early education of the future pharmacist should be as broadly based as possible, designed to discover aptitudes and latent potentialities, and should seldom degenerate into formalised and didactic instruction. Making the necessary allowances for all generalities, I hold it true that you can only teach a man what he knows, which is only another way of saying that the word education means exactly what it says. We all have our proper gift of God, and it should be the purpose of early education to discover that gift and to help and encourage the possessor to make the most of it.

The last years at school should be spent in the study of exact sciences, where the discipline of attention to detail is cardinal, but room must be found for the pursuit of purely cultural studies. I would like to see Latin restored to its original place, but failing this, the wide sweep of world history will give that sense of the past and feeling of continuity that the study of Latin manages so subtly to convey. Language is basic to communication and young people who lack Latin have a hard road to travel to be completely literate or even to express themselves clearly in an examination paper.

It should be possible to determine before the end of a pupil's schooldays

whether or not he is likely to succeed in Pharmacy. There is no lack of warning about this.

Burns:— What's a' your jargon o' your schools
Your Latin names for horns and stools
If honest nature made you fools
What sair's your grammars?
Ye'd better ta'en up spades and schols, or
knappen hammers.

Schiller's cry from the heart:—

Mit der Dummheit kämpfen Götter selbst vergebens;

and the devastating statement in the Old Testament:—

Though thou shouldest bray a fool in a mortar among wheat with a pestle, yet will not his foolishness depart from him.

The test of a successful early education is not the amount of knowledge that a pupil takes away from school, but his appetite to know and his capacity to learn. If the school sends out young people with a desire for knowledge and some idea of how to acquire and use it, it will have done its work.

Professional Studies

With young people who have had this type of early training and who have consciously selected pharmacy, we can now proceed. The whole field of training is too wide for my time and too long for your patience. I propose to deal in some detail with that aspect in which I am most interested, galenical pharmacy, but if we are to get this subject in proper perspective we must examine, however briefly, the basic sciences of pharmacology and pharmacognosy.

Pharmaceutical Chemistry

Concerning pharmaceutical chemistry, I have little to say, partly because the ground covered by this subject is fairly clearly delimited, partly because the matter has been discussed by others from this chair, chiefly because the able persons who practise this section of Pharmacy occupy dominating positions; so that there is little likelihood of this aspect of training being inadequate. The danger is of quite a different order: the tendency for pharmaceutical chemistry to acquire parts of the training that logically belong elsewhere. This has two bad effects; it overweights this part of the syllabus and robs other subjects of the key points that would give them real significance. Let us now turn to the other subjects.

Pharmacology and Pharmacognosy

Used in the broadest sense pharmacology and pharmacognosy mean the same thing, "Knowledge about Drugs," but each of these terms has gradually assumed an acquired meaning. What the pharmacist needs to know about drugs is their identity, their purity and their potency; especially their potency. This is what I understand by "Knowledge about

Drugs" and our scheme of education must be designed to inculcate the theoretical information and practical techniques on which such knowledge is based. The historical background, the botanical or zoological origin, the geographical source, methods of cultivation and preparation, is knowledge that a well informed pharmacist ought to possess, both for its cultural value and its commercial importance.

Pharmacology is important because it provides the link in the chain of knowledge between pharmacy and medicine. A modern education for a pharmacist must be designed to strengthen this link and by bringing him into closer contact with the physician ensure his professional status. Pharmacology is the youngest of the preclinical sciences and the most important factors in its early development were the rise of biochemistry and the pursuit of research in pharmaceutical laboratories.

The vast, ever enlarging, always changing field of therapeutic agents confronting the physician makes it imperative that he have assistance in finding his way through the labyrinth of complex and controversial materials. The need for an authentic and unprejudiced source of information increases daily and we must organise our training so that the pharmacist can be that source. Within recent years much publicity has been given to all scientific discoveries. This desire to keep the public informed of new developments is both commendable and dangerous. The danger lies in the fact that newspaper reports of scientific results are frequently garbled and never complete or adequate. This adds a further burden to the responsibilities of the professional man. Now it is his further duty to guide the public and keep people informed as to the merit of new therapeutic agents.

A knowledge of pharmacology is essential in the formulation of new medicaments. The preparation of solutions for the eye involving considerations such as the optimum *p*H for therapeutic action without loss of stability, the use of effective and non-irritant preservatives, and the adjustment of osmotic pressure; the preparation of injectable materials of every kind, the choice of vehicles for all types of preparations including ointments and creams—all these require a knowledge of pharmacology, if the work is to be intelligently performed.

The pharmaceutical curriculum includes many courses which contribute to a sound foundation for the study of Pharmacology and I share the opinion that true pharmacology may well find its fullest development in the field of pharmacy. An adequate background of the chemistry of natural products is essential in order to make possible the teaching of pharmacology on a high level and it is desirable that the pharmacognosy course be revised and modernised to contribute more effectively to this end.

We could probably all agree upon certain fundamental objectives. We have a right to demand of any course of applied science (1) that it will provide information that will be useful to the student in solving problems, not only of to-day but also of tomorrow, and (2) that the teaching of principles should be preferred to the communication of isolated facts. Facts are quickly forgotten and in any case can be found in hand-books;

understanding is more difficult to acquire but once acquired becomes an integral part of the student's mind.

The use of drugs has a very long history but pharmacognosy as a science dates from the beginning of the nineteenth century (the term "pharmacognosy" was introduced by Seydler in 1815). Although the chemical knowledge of natural products was constantly increasing during that century, these developments had very little influence on the teaching of Pharmacognosy. Plant descriptions, morphological and anatomical studies of crude drugs continued to be the essential features of the courses. There is however an approach to pharmacognosy that would permit the integration of this important subject with pharmacology to the immense benefit of both. The pharmacological actions of natural products derive from definite chemical entities elaborated by living cells. These active constituents also provide the centre of interest for the pharmacognosist, since the existence of these substances is the very reason that the drug is included in pharmacognosy. These constituents however are not only of interest because of their effect on the animal body; they have a function in the body of the producing organism too. An alkaloid, a glycoside, a vitamin, should be considered as part of a living organism, as functional parts of the cells.

Doubts have been expressed about the necessity of pharmacognosy for the practising pharmacist. A reduction of the taxonomical, morphological and anatomical emphasis in the syllabus and modernising the training by taking a more chemical, biochemical and physiological approach would change it into a subject whose significance and importance would be evident to all. Pharmacognosy is pharmacy's specific and peculiar contribution to the cause of Science and it behoves us to esteem it well.

PHARMACY

Galenical pharmacy enables us to present trains of thought and methods of scientific manipulation which as yet lie outside the domain of equations and formulae. Many remedial agents have crept into existence and become established through empirical experimentation, and the teacher who makes light of this, and who allows the wealth that came into his hands from days gone by to sink out of sight, wrongs himself and the community of which he is a part. Who knows the inter-structural relationships that exist in the simplest galenical preparations? Consider the terms:—tinctures, syrups, infusions, liquid and solid extracts. These and such as these apply to a complex association of a whole mass of materials derived from the vegetable structures that contain or yield them. Processes such as those indicated have been employed since the beginning of man's historical record. It would appear that galenical pharmacy is, and always has been, based on colloidal complications, and inasmuch as the normal structure and juices of most, if not all, plants are colloidal, it follows that the study of pharmacy as applied to natural substances is that of colloidal research and colloidal manipulation.

Within recent years such reasoning has been more generously received,

partly because of the clearer views that now prevail regarding the value of "structureless" compounds, and, partly, because advanced thought in pharmacy more fully comprehends its inherited opportunity. Pharmaceutical effort in this field of colloidal structures, long dormant in its original home, has elsewhere been very active. Accepting that non-crystalline substances compose the major part of plant tissues, it becomes the pharmacist's duty to disentangle these complicated structures and, in doing so, to provide a product which has not undergone fundamental rearrangements. We must cease to teach that a simple macerate or percolate is anything other than a crude beginning. There is, of course, no Alkahest, no Universal Solvent such as was dreamt of by Paracelsus. In the process of extraction of natural colloidal groups, the most promising neutral liquids are included in the list of fourteen laid down by Uri Lloyd. By means of these solvents successively applied most plants may be virtually exhausted of their contents. Each saturate is, however, not an ultimate, but in itself may constitute a group of associated constituents, which by further subdivision is capable of yielding yet more closely related substances. By such manipulative processes and without the use of energetic chemicals, colloidal plant structures may be dissected and individualised to a degree of pharmaceutical satisfaction, even if not to absolute chemical perfection. A tentative beginning was made when ergot, colchicum and strophanthus seed were freed from inert material by extraction with a neutral liquid before percolation with alcohol. The use of a neutral liquid as an excluder is exemplified in ox bile and the manipulation of different strengths of alcohol to include and exclude at will is carried further in the extraction of liver. Another facet of this idea is shown in the piecemeal separation of certain of the alkaloids of opium and their reassembly to give the well known injectable products.

I am satisfied that given the right training and outlook there is practically no limit to what may be done in producing medicaments which evoke the precise therapeutic response that is desired, plus the synergistic effect that frequently accompanies this, and which are free from inhibiting or damaging reactions. Some of us have been making a study of the solanaceous group of drugs. We find that by the use of quite simple successive solvents, we can produce, in the form of colloidal scales, a substance miscible with every type of solvent and which appears to possess all the properties of the original drug. This is but a beginning. By varying the solvent or slightly shifting the *p*H, medicaments may be provided to give immediate or prolonged action. This is shown in the cinchona and ipecacuanha group. At one time we were inclined to look askance on the use of sherry as a vehicle but recent experience has sent us back to examine this problem with fresh eyes. Even such an allegedly simple drug as liquorice presents problems little dreamed of a few years ago. The presence of potassium citrate, syrup or glycerin as part of the vehicle not only gives, in many cases, a better preparation, but one that tolerates the addition of iron salts.

I have said that much of this type of work lies outside the realm of formulae and equations, but a beginning has been made in the case of glycerin and sugar in the stabilising of tannin-containing galenicals. It

has been shown that the effect is proportional to the molecular concentration and can be expressed:—

$$T = KM$$

where T is the time in days, K a constant and M the molecular concentration. As time goes on, no doubt what is obscure or empirical will be reduced to laws expressible in this form.

The discovery of the alkaloidal affinities of hydrous aluminium silicate has opened a new field which at last is being energetically tilled by young pharmacists. Although it is not yet possible to be certain, there seems little doubt that this also will provide a method of obtaining plant substances in a purified and active form. The process of dialysis, once official in the pharmacopœia, must again take its place as an important pharmaceutical process.

Colloidal chemistry is based upon the fact that quantity is but one factor that determines chemical or therapeutic action. The condition of a substance is of first importance in its therapeutic application. Consider the case of mercury, an over-simplification perhaps, but it illustrates the point I am trying to make. Five ounces of mercury have been swallowed without appreciable result, while a few grains finely divided forms an active agent. The same effect of physical state is true of the purgative resins. The condition of the active substances will thus determine to a large extent the quality of the galenical, and this brings into prominence the relation between strength and quality. The strength of a vintage wine is based on its alcoholic content but its quality is determined by quite other considerations. There is no difficulty in doubling the alkaloidal strength of a nux vomica galenical by adding a few grains of strychnine, but the question arises, has the quality been improved?

STANDARDISATION

The attempt to standardise a preparation by a single dominating constituent is but a struggle towards a pharmaceutical standard of excellence in which the therapeutic quality should be the ideal. Until we have a far greater knowledge of the chemistry of plants, not only of the constituents but of the manner of their occurrence, and a far clearer picture of the exact therapeutic action of these constituents considered separately or in groups than we now possess, we must in our scheme of training stress the importance of quality. In doing so we shall be dealing with one of our most important pharmaceutical problems for, in addition to finding new remedial agents, we must aim at giving to the users of medicines, the wealth that comes from manipulative pharmacy and balanced research applied directly to the study of qualities. It is impossible to tell what will be the course of the development of our knowledge but it seems likely that in a few decades we shall have a much clearer picture of the whole field. The details are likely to become ever more complicated but that should not worry us if we can adopt the natural historian's approach. We can be sure that new physical, chemical and physiological principles of very general importance will emerge from such new knowledge.

Is it fanciful to believe that there is some analogy between the elaborately processed "foods" and the substitution of synthetic chemicals in medicine for the naturally occurring substances in the plant and animal kingdom. No one really believes that sodium *cyclo*hexylsulphamate does in fact replace cane sugar and honey in human metabolism or that glyceryl monostearate or polyoxyethylene compounds are not a very poor substitute for natural fats and oils. I am not here discussing the synthetic carcinogenetic substances that were added to foods, azo-dyes butter yellow or the nitrogen trichloride that "improves" our bread and isoloxic to dogs. To carry the point even further from our present discussion, I was once sufficiently close to agriculture to look with misgiving on the use of chemical substitutes for the natural fertilisers of the soil.

We are now upon the threshold of an era in pharmacy in which the crudeness of the past will rapidly disappear and in which our medicines will be known and valued in accordance with their actual conditions. In these circumstances it behoves us to look again at our pattern of research and the following quotation from a lecture recently delivered by Dr. R. L. M Synge at the Royal Institute of Chemistry, seems to be very pertinent to this matter.

"I will end by pleading for a change of emphasis in research in these fields. Glancing through Chemical Abstracts (which is a fair summary of *published* work), one gets the impression of an enormous misdirection of scientific effort. People are so keen to sell a new wonder drug or vitamin, or discover 'the cure' for cancer, that relatively few are studying naturally occurring substances in their natural environment. Detailed chemical analysis of biological material is exacting work. Most of these numberless dreary papers on 'The interaction of synthetic analogues of antibiotic W and vitamin X on ABC having a Y-induced requirement for Z' are getting us nowhere at great trouble and expense. The end observation is growth or failure to grow. More detailed and painstaking analysis of biological systems may not help anybody rapidly to create a market for W, X, Y, or Z. It may be three times more difficult and lead to writing only one-fifth as many papers, but in the long run the time spent will be vastly more productive both of real wonder drugs and real cancer cures. We shall also gain a new depth of understanding of the beauty, the simplicity and the complexity of living things."

The Future Pharmacist

It has been stated by knowledgeable persons that the future pharmacist will be chiefly occupied in counting tablets, mostly phenobarbitone, and dissolving a sterile substance in sterile water. I see a very different picture: I see the future Pharmacognosist and the future Pharmacologist working together to find and prove new remedial agents from biological sources that will be as different from our present galenicals as a spiders web is from a modern hemostatic. I see remedial agents that will require for their preparation all the knowledge and all the skill that a pharmacist can acquire. It may be true to-day as when it was written in mediæval times that Contra malum mortis non est medicamen in hortis." But we are

the prisoners of hope, we must take Voltaire's advice and continue to cultivate our gardens.

THE TEACHER

With permission, I would like to end by addressing directly the men and women engaged in my own section of pharmacy. I am well aware that what I have been saying will call for exertions and adaptations that may at first be resented. It is one of the compensations of a teacher's life that he is using his mind on valuable subjects. All over the world people are spending their lives at work where their minds must be kept numb all day, or else on highly rewarding activities (monetarily), which are tedious, or frivolous or worse.

Greatly daring we have elected to join a famous company; some of the most important men in history have been teachers. Many of the biggest advances in civilisation have been the chief work, not of politicians or inventors, not even of artists, but of teachers.

We must, therefore, have courage equal to the responsibilities we have undertaken. That we have difficulties to overcome goes without saying; lack of adequate accommodation is general in most institutions to-day. Storr Jordan's famous dictum "Have your university in sheds, have it in tents, but have the masters there" is not so helpful when you are relying on accurate laboratory work as your teaching method.

If however the early training follows the line I have suggested, and if, in particular, the young people who are ill equipped are diverted to other paths our work will become more rewarding. Not perhaps in a financial sense; that cannot, and in my opinion should not, be the inducement to become a teacher. We have Ruskin's assurance that pay alone never made a better soldier, a better artist or a better teacher.

We are the servants of reality, our responsibility is to the young and through them to the future, a future we can envisage but may not be able to share. The race that we are asked to run with patience is not a hundred yards individual sprint but a relay race that began long before us and will continue long after. The idea has been well expressed in the noble words of Havelock Ellis:—"For a brief space it is granted to us, if we will, to enlighten the darkness that surrounds our path. . . . We press forward, torch in hand along the path. Soon from behind comes the runner who will outpace us. All our skill lies in giving into his hand the living torch, bright and unflickering, as we ourselves disappear in the darkness."

SCIENCE PAPERS AND DISCUSSIONS

THE DETECTION AND DETERMINATION OF *ISO*NICOTINYL HYDRAZIDE

By P. G. W. Scott

From the Analytical Control Division of May and Baker, Ltd.

Received July 7, 1952

*iso*Nicotinyl hydrazide has recently been introduced as an anti-tuberculosis drug, but very little has been published concerning its qualitative reactions and quantitative determination. Although the tests and methods described in this preliminary communication have not yet received exhaustive study it is hoped they might provide a basis on which to gain further experience. Since one of the main routes for the administration of *iso*nicotinyl hydrazide is oral, its determination in the presence of tablet excipients is of particular importance and hence this aspect has been given special consideration.

1. Qualitative Tests

*iso*Nicotinyl hydrazide was expected to show reducing properties associated with the hydrazide side chain, and also to give reactions characteristic of the pyridine nucleus. Accordingly the qualitative tests proposed fall into 2 groups as shown in Table I.

TABLE I

Qualitative tests for *iso*nicotinyl hydrazide depending on (*a*) the hydrazide side chain, and (*b*) the pyridine nucleus

	Test*	Result
(*a*)	1. Add 1 ml. of solution of silver nitrate.	White precipitate, which is slightly soluble in water, and soluble in dilute nitric acid; on warming a silver mirror forms.
	2. Add 1 ml. of a 5 per cent. aqueous solution of selenium dioxide.	Red precipitate.
	3. Add 1 ml. of a saturated solution of 1:2-naphthaquinone-4-sulphonic acid in ethanol (50 per cent.), followed by a few drops of sodium hydroxide solution.	A bright red colour.
	4. Add 1 ml. of a 1 per cent. aqueous solution of sodium pentacyanoammine ferroate and stand for about 10 minutes.	A red colour.
	5. Reflux 0·1 g. of *iso*nicotinyl hydrazide with 20 ml. of 5N sodium hydroxide for 15 minutes. Dilute 1000 times with water. To 20 ml. add 5 ml. of a 2 per cent. solution of *p*-dimethylaminobenzaldehyde in a 10:1 mixture of ethanol and hydrochloric acid; stand for a few minutes.	A yellow colour.
(*b*)	6. Add 1 ml. of a 1 per cent. solution of mercuric chloride.	White precipitate.
	7. To 0·1 g. of borax in a test-tube, add 5 ml. of ethanol containing about 0·15 mg. of *iso*nicotinyl hydrazide and 5 ml. of 5 per cent. solution of 1-chloro-2:4-dinitrobenzene in ethanol. Heat to dryness on a boiling water bath and then for a further 10 minutes. Add 10 ml. of methanol and stir.	Reddish-purple solution.

* Unless otherwise stated all the reactions were carried out by adding the reagent to 5 ml. of a 5 per cent. aqueous solution of *iso*nicotinyl hydrazide.

Specificity of the Tests

Tests 1 and 2. These tests are of low specificity and serve simply to characterise the reducing properties of *iso*nicotinyl hydrazide.

Test 3. 1:2-Naphthaquinone-4-sulphonic acid reacts in alkaline solution with compounds which contain 2 removable hydrogen atoms attached to 1 nitrogen atom or 1 carbon atom.

Test 4. This reaction is given by aliphatic and aromatic hydrazines,[2] red to violet colours being formed by many hydrazine derivatives and hydrazides, e.g., phenylhydrazine, semicarbazide, benzoyl hydrazide.

Test 5. In this reaction *iso*nicotinyl hydrazide is hydrolysed to give hydrazine which then reacts with the *p*-dimethylaminobenzaldehyde to give an azine,[3] $(CH_3)_2N{\cdot}C_6H_4{\cdot}CH{:}N{\cdot}N{:}CH{\cdot}C_6H_4{\cdot}N(CH_3)$.

Test 6. Mercuric chloride forms a double salt with pyridine,[4] and pyridine carboxylic acids,[5] and, in a similar manner, with *iso*nicotinyl hydrazide.

Test 7. This reaction is fairly specific, being given by only γ-picoline and γ-ethylpyridine out of about 30 pyridine derivatives tried.[8]

2. Quantitative Determination

(a) Chlorodinitrobenzene Method

This method was evolved from the qualitative test and optimum conditions for it are incorporated in the procedure given below. This method is applicable to the determination of *iso*nicotinyl hydrazide and to its determination in the presence of the usual tablet excipients.

Reagent. 1-Chloro-2:4-dinitrobenzene solution, 5 per cent. w/v in dehydrated ethanol.

Apparatus. Water bath fitted with a 2½ inch wide brim to prevent ethanol vapour escaping over the side of the bath and igniting.

Procedure. Weigh accurately an amount of finely ground tablet containing about 0·06 g. of *iso*nicotinyl hydrazide into a 100-ml. flask, add 80 ml. of dehydrated ethanol, shake for 5 minutes and make up to 100 ml. with ethanol. Dilute 5·0 ml. to 100 ml. with ethanol and transfer 5·0 ml. of this dilution to a dry test-tube containing 0·1 g. of borax. Add 5 ml. of the chlorodinitrobenzene solution, mix and place the tube in a boiling water bath for 15 minutes. Cool in ice for 1 minute, add 25·0 ml. of methanol, shake and filter. Read the extinction of 1 cm. of the filtrate at about 530 mμ or, using a suitable filter, against a blank prepared as above omitting the sample, and obtain the amount of *iso*nicotinyl hydrazide present from a calibration curve (Fig. 1).

Notes on method. 1. The water bath is preferably heated over a flame which is adjusted so that the water is kept just at the boil initially, to prevent the ethanol from boiling out of the tubes. Under these conditions

the ethanol evaporates off in about 5 minutes, leaving a dry, purple coloured mass in the tube.

2. The colour of the final solution decreases by about 3 per cent. in 1 hour on standing in daylight.

3. The reproducibility is about ±3 per cent., and recoveries of 102·5 per cent. and 101·3 per cent. were obtained in 2 determinations on known mixtures of *iso*nicotinyl hydrazide with tablet excipients.

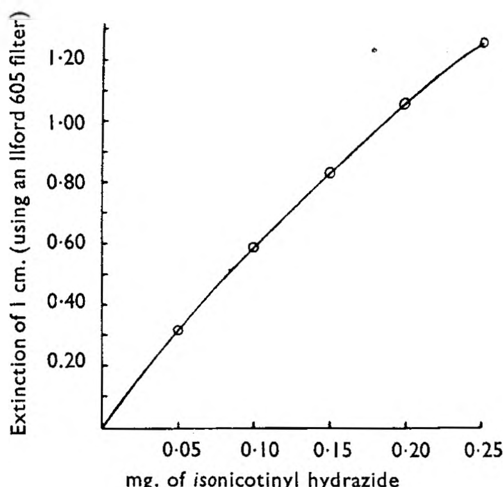


FIG. 1. Calibration curve for the determination of *iso*nicotinyl hydrazide by the chlorodinitrobenzene method. (Each point is the mean of three determinations.)

(*b*) *Azine Method*

Pesez and Petit[3] have described a method for the determination of hydrazine by coupling with *p*-dimethylaminobenzaldehyde to give an azine (*vide supra*) and since *iso*nicotinyl hydrazide can be quantitatively hydrolysed to give hydrazine with either acid or alkali, this method is applicable to the determination of *iso*nicotinyl hydrazide. If sugars or other aldehydes or ketones are present, reaction occurs during hydrolysis with the hydrazine, which precludes the use of this method for the determination of *iso*nicotinyl hydrazide in tablets containing sugars.

Reagents. 1. Sulphuric acid 20N.

2. Sodium hydroxide 5N.

3. *p*-Dimethylaminobenzaldehyde reagent, freshly prepared each day. Dissolve 0·4 g. of *p*-dimethylaminobenzaldehyde in a mixture of 20 ml. of absolute ethanol and 2 ml. of hydrochloric acid.

Procedure. Weigh accurately about 0·1 g. of *iso*nicotinyl hydrazide into a 100-ml. quickfit conical flask, add either 20 ml. of 5N sodium hydroxide or 10 ml. of 20N sulphuric acid, attach an air condenser and boil gently for 15 minutes. Cool, add 40 ml. of water down the condenser, mix well and transfer to a 100-ml. flask, washing in with water and making up to 100 ml. Dilute 10·0 ml. to 100 ml. with water and dilute 5·0 ml. of this to 100 ml. with water. Transfer 20·0-ml. to a 50-ml. flask,

add 5 ml. of *p*-dimethylaminobenzaldehyde reagent, stand for 10 minutes and dilute to 50 ml. Read the extinction of 1 cm. using a suitable filter against a blank prepared by diluting 5 ml. of the reagent to 50 ml. with water. Obtain the amount of *iso*nicotinyl hydrazide present from a calibration curve (Fig. 2).

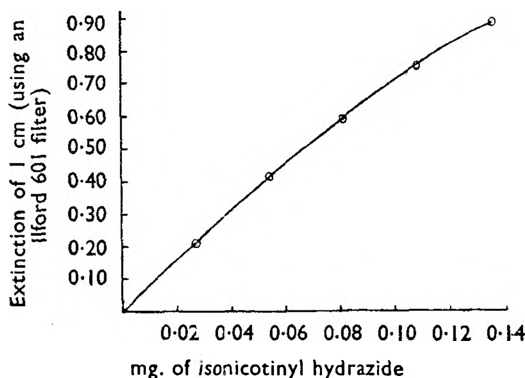


FIG. 2. Calibration curve for the determination of *iso*nicotinyl hydrazide by the azine method. (Each point is the mean of three determinations.)

Notes on method. 1. The colour of the final solution remains constant for at least 1 hour on standing.

2. The calibration curve can be prepared using hydrazine sulphate, since complete hydrolysis of the *iso*nicotinyl hydrazide to hydrazine occurs.

3. The reproducibility of the determination is about ± 1 per cent.

(c) *Spectrophotometric Determination*

The ultra-violet absorption spectrum of *iso*nicotinyl hydrazide in 0·01N hydrochloric acid has $\lambda_{max.} = 266$ mμ (Fig. 3) and since in neutral and alkaline solution a slightly lower intensity of absorption is found, acid solution using 0·01N hydrochloric acid was utilised for its determination. A plot of optical density against concentration of *iso*nicotinyl hydrazide is linear over the concentration range of 0·0007 per cent. to 0·0012 per cent. and the $E^{1 \text{ per cent.}}_{1 \text{ cm.}}$ at 266 mμ calculated from the optical density of an 0·001 per cent. solution of recrystallised *iso*nicotinyl hydrazide is 429·2. The method was applied successfully to the determination of *iso*nicotinyl hydrazide in known mixtures with tablet excipients and 3 determinations gave recoveries of 99·95, 99·9 and 99·1 per cent.

Procedure. Weigh accurately an amount of finely ground tablet containing about 0·1 g. of *iso*nicotinyl hydrazide into a 100-ml. flask. Add about 80 ml. of water, shake for 5 minutes, and dilute to 100 ml. Dilute 10·0 ml. to 100 ml. with water and transfer 10·0 ml. of this dilution to a 100 ml. flask. Add 10·0 ml. of 0·1N hydrochloric acid and dilute to 100 ml. with water. Prepare a blank from the tablet excipients in an exactly similar manner. Read the optical density of 1 cm. at 266 mμ against the blank.

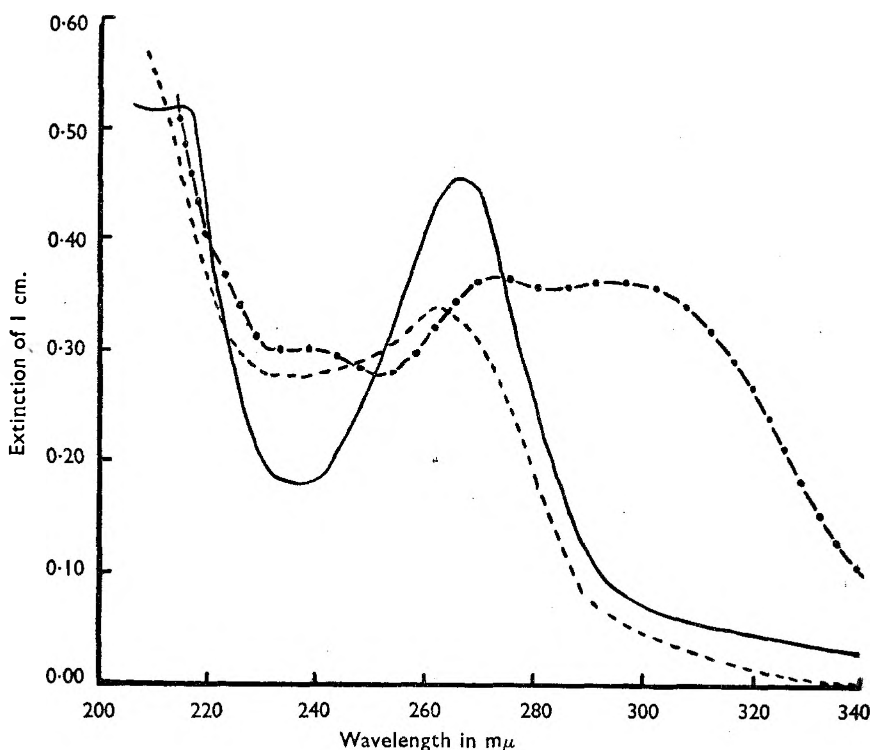


Fig. 3. Ultra-violet absorption curves of *iso*nicotinyl hydrazide.
——— 0·001 per cent. solution in 0·01N hydrochloric acid.
— — — 0·001 per cent. solution in water.
—●—●— 0·001 per cent. solution in 0·01N sodium hydroxide.

(d) Polarographic Determination

*iso*Nicotinyl hydrazide in solutions of *p*H from 1 to 8 is reducible at the dropping mercury electrode giving 2 waves about 0·2 volt apart; in solutions of *p*H greater than 8 the 2 waves tended to coalesce. With the cathode ray polarograph[9] the peak potentials for reduction in 0·01N hydrochloric acid/N potassium chloride using a silver chloride anode, were 0·55 volt and 0·75 volt. The second wave was chosen for quantitative purposes because of a small subsidiary wave on the first wave at low concentrations. In the supporting electrolyte mentioned the plot of current against concentration was linear over the concentration range 0·0001 per cent. to 0·02 per cent. The *iso*nicotinyl hydrazide content of tablet granules was determined with a precision of ± 1 per cent. the excipients having no interfering effect.

(e) Titration Methods

Cänback[6] has described a method of determination for *iso*nicotinyl-hydrazide by titration with iodine in slightly alkaline solution and his results indicate a reproducibility of about 0·5 per cent. He has applied this method to the determination of *iso*nicotinyl hydrazide in tablets.

A preliminary study has been made of two alternative methods which might be of value for the determination of *iso*nicotinyl hydrazide in the presence of substances which would interfere with the iodine titration. These are based on (*a*) acidimetric titration in a non-aqueous solvent with perchloric acid and (*b*) titration with nitrite. In the first method, excess of perchloric acid in glacial acetic acid is added to the sample dissolved in a mixture of equal volumes of monochlorobenzene and glacial acetic acid, followed by the potentiometric back titration with a standard solution of pyridine in monochlorobenzene. Glass and silver/silver chloride electrodes are used and the end-point is obtained graphically from a plot of potential against volume of pyridine solution added. 4 titrations of pure recrystallised *iso*nicotinyl hydrazide gave results with a maximum spread of 1·5 per cent. and provided an empirical factor of 1 ml. of 0·1N $\equiv$ 0·007114 g. of $C_6H_7ON_3$, compared with the theoretical value of 0·006857 calculated from an equivalent weight equal to half the molecular weight of *iso*nicotinyl hydrazide.

In the second method *iso*nicotinyl hydrazide in dilute hydrochloric acid solution is titrated with 0·05M sodium nitrite solution, using starch-iodide as external indicator. 6 titrations gave a maximum spread of about 1 per cent. and 3 titrations using recrystallised *iso*nicotinyl hydrazide provided an empirical factor of 1 ml. of 0·05M $\equiv$ 0·006553 g. of $C_6H_7ON_3$, compared with a theoretical factor of 0·006857 calculated on the assumption that 1 mol. of sodium nitrite reacts with 1 mol. of *iso*nicotinyl hydrazide.

Summary and Conclusions

1. Qualitative reactions for *iso*nicotinyl hydrazide are given.
2. 2 colorimetric methods of determination, based on reaction with (*a*) 1-chloro-2:4-dinitrobenzene and (*b*) *p*-dimethylaminobenzaldehyde, and ultra-violet spectrophotometric and polarographic methods are proposed.
3. The spectrophotometric, polarographic and chlorodinitrobenzene methods have been applied to the determination of *iso*nicotinyl hydrazide in tablets.
4. 2 titration procedures for *iso*nicotinyl hydrazide, (1) with perchloric acid in a non-aqueous solvent, and (2) with nitrous acid, are given.

I am indebted to Mr. H. A. Glastonbury for providing the section on the polarographic determination of *iso*nicotinyl hydrazide, to Dr. E. F. Hersant and Mr. C. W. Ballard, for helpful criticisms and suggestions, and to the Directors of Messrs. May and Baker, Ltd., for permission to publish this work.

References

1. Feigl, *Qualitative Analysis by Spot Tests*, Elsevier Publishing Co. Inc., 1947, 389.
2. Feigl, *ibid.*, 1947, 385.
3. Pesez and Petit, *Bull. Soc. Chim. Fr.*, 1947, 122.
4. Bernthsen, *Organic Chemistry*, Blackie and Son, Ltd., 1941, 682.
5. Bernthsen, *ibid.*, 1941, 687.
6. Cänback, *J. Pharm. Pharmacol.*, 1952, **4**, 407.
7. Fritz, *Anal. Chem.*, 1950, **22**, 1028.
8. Ballard and Scott, *Chem. Ind.*, 1952, 715.
9. Glastonbury, *in the press.*

THE ESTIMATION OF *ISO*NICOTINYL HYDRAZIDE

By E. A. HAUGAS and B. W. MITCHELL

From the Research Department, Herts Pharmaceuticals, Ltd., Welwyn Garden City

Received July 7, 1952

THE introduction of the antitubercular substance *iso*nicotinyl hydrazide into pharmacy has necessitated an investigation of methods for its assay. The oxidation of the material by bromine and iodine has formed the basis of these studies and a method based on the oxidation by standard iodine solution has recently been described by Canbäck.[1] Having chosen for routine estimation a bromimetric method of assay and having obtained consistent figures for assaying the drug both as the pure substance and in the form of tablets, it is of interest to report a comparison of this method with that described by Canbäck.

In the case of the pure material, the bromimetric assay gave results which were significantly higher than those obtained by the iodimetric procedure. A more detailed study showed that whereas the iodimetric assay varied with the reaction time, the bromimetric assay remained virtually constant with reaction times greater than 10 minutes. These results are summarised in Table I.

TABLE I

EFFECT OF REACTION TIME ON THE IODIMETRIC AND BROMIMETRIC ASSAYS

	Iodimetric assay		Bromimetric assay	
Time minutes	Titre difference ml.	Assay per cent.	Titre difference ml.	Assay per cent.
5	25·12, 25·20	92·52	26·88, 26·89	99·49
10	25·83, 25·77	94·88	26·98, 26·99	99·93
15	25·90, 25·93	95·31	26·95, 26·90	99·71
20	26·47, 26·54	97·48	26·94, 26·97	99·84
40	26·86, 26·85	98·77	26·92, 26·94	99·74
	Blank titre = 46·90 ml. Factor of 0·05N $Na_2S_2O_3$ = 1·066 Weight of drug/titre = 49·71 mg.		Blank titre = 46·88 ml. Factor of 0·05N $Na_2S_2O_3$ = 1·067 Weight of drug/titre = 49·42 mg.	

TABLE II

THE ESTIMATION OF *iso*NICOTINYL HYDRAZIDE IN TABLETS (50 mg.)

Source of tablet	Iodimetric assay		Bromimetric assay	
		Average mg.		Average mg.
A (British	51·3, 51·1	51·2	50·1, 50·1	50·1
B (British)	50·2, 50·3	50·3	49·9, 49·9	49·9
	49·4, 50·1	49·7	49·4, 49·4	49·4
C (Swiss)	50·8, 50·6	50·7	50·5, 50·6	50·6

However, when the two methods were compared using various brands of 50 mg. tablets of the drug, it was found that the two assays agreed quite closely, the iodimetric assay tending to be slightly higher. Table II summarises the results obtained on tablets from three different sources.

It seemed most probable that the cause of the increased iodimetric assay of the drug in tablet form was due to the excipient used in tabletting the drug. For the 3 types of tablet examined, the excipient consisted mainly of lactose and starch and the effect of the reducing sugar lactose on the 2 assays was then investigated. Since the tablets contained 20 per cent. of drug, and in the case of two varieties of tablets the content of lactose was about 60 per cent., mixtures of *iso*nicotinyl hydrazide and lactose in the ratio 1:3 were assayed by the two methods. Table III shows the effect of lactose on the assay of *iso*nicotinyl hydrazide obtained from three different manufacturers.

TABLE III

THE EFFECT OF LACTOSE ON THE ESTIMATION OF *iso*NICOTINYL HYDRAZIDE

Source of drug	Iodimetric assay		Bromimetric assay	
	Normal	+ 150 mg. of lactose	Normal	+ 150 mg. of lactose
1 (German)	98·17 ± 0·1	101·5 ± 0·5	99·65 ± 0·1	99·80 ± 0·1
2 (British)	98·65 ± 0·07	100·35 ± 0·05	99·70 ± 0·02	99·75 ± 0·07
3 (Italian)	98·76 ± 0·18	101·8 ± 0·3	99·86 ± 0·02	99·88 ± 0·01

A comparison of the effect of the sugars glucose, lactose and sucrose on the assay procedures was then carried out in the absence of *iso*nicotinyl hydrazide. It appears that the alkaline medium used in the iodimetric procedure is necessary for the oxidation of glucose and lactose since iodination in the presence of excess acid (similar to the conditions used in the bromimetric assay) resulted in practically no oxidation of these sugars. The figures are only comparative, since in the presence of *iso*-nicotinyl hydrazide the quantity of excess halogen is rapidly reduced. The results are summarised in Table IV.

TABLE IV

THE OXIDATION OF SUGARS BY BROMINE AND IODINE

Sugar (150 mg.)	Iodine in bicarbonate solution		Bromine in acid solution		Iodine in acid solution	
	Observed titre difference ml.	Equivlaent as drug mg.	Observed titre difference ml.	Equivalent as drug mg.	Observed titre difference ml.	Equivalent as drug
Glucose	6·78 6·40	12·4 11·7	0·04 0·04	0·07 0·07	0·00 −0·03	nil
Lactose	4·41 5·08	8·1 9·3	0·01 0·01	0·02 0·02	−0·05 −0·08	nil
Sucrose	0·13 0·18	0·2 0·3	−0·01 0·00	nil	−0·07 −0·02	nil

During the course of these investigations, it appeared that the iodimetric assay was susceptible to temperature changes and a series of estimations were carried out on a sample of *iso*nicotinyl hydrazide using both assay methods at various temperatures. Whereas the iodimetric assay increased from 92·1 per cent. to 98·8 per cent. between 10° and 30° C., the bromimetric assay only showed an increase from 98·7 per cent. to

99·5 per cent. in the same temperature range. The results are given in Table V.

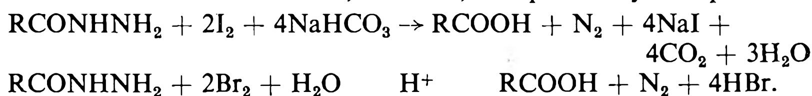
TABLE V

EFFECT OF TEMPERATURE ON THE ESTIMATION OF *iso*NICOTINYL HYDRAZIDE

Temperature °C.	Iodimetric assay per cent.		Bromimetric assay per cent.	
		Average mg.		Average mg.
10	91·64, 92·45	92·1	98·88, 98·47	98·7
20	97·52, 97·81	97·7	99·33, 99·56	99·4
30	98·79, 98·72	98·76	99·40, 99·61	99·5

The mechanism of the reaction between *iso*nicotinyl hydrazide and bromine appears to be complete oxidation of the hydrazide group and formation of *iso*nicotinic acid. We have found that under the conditions used in the bromimetric assay, one mole of nitrogen is evolved per mole of *iso*nicotinyl hydrazide oxidised. The presence of *iso*nicotinic acid in the oxidised mixture can be demonstrated by treating the hydrazide in acid solution with bromine water and evaporating to dryness. The hydrobromide obtained is dissolved in water, neutralised and then brought to *p*H 3 with hydrochloric acid when *iso*nicotinic acid is precipitated (melting point = 319° to 320° C. undepressed when mixed with authentic *iso*nicotinic acid).

It is interesting to note that in the determination of the drug in biological fluids Rubin *et al.*[2] first oxidise the hydrazide to *iso*nicotinic acid with potassium permanganate. The fact that in both assay procedures 4 equivalents of halogen are used, suggests that the mechanism is the same in both cases. The reactions can, therefore, be expressed by the equations:—



$$RCONHNH_2 + 2I_2 + 4NaHCO_3 \rightarrow RCOOH + N_2 + 4NaI + 4CO_2 + 3H_2O$$

$$RCONHNH_2 + 2Br_2 + H_2O \quad H^+ \quad RCOOH + N_2 + 4HBr.$$

We suggest that the bromimetric assay should be used for the estimation of *iso*nicotinyl hydrazide, particularly when in the form of tablets since the presence of lactose has been shown to interfere with the iodimetric assay.

EXPERIMENTAL

Iodimetric Assay (according to Canbäck[1])

About 0·5 g. of pure *iso*nicotinyl hydrazide, accurately weighed, was dissolved in water and made up to exactly 250 ml. 25 ml. of this solution was pipetted into a 250-ml. iodine flask, and 1 g. of sodium bicarbonate together with 25 ml. of water added. 25 ml. of 0·1N iodine solution were pipetted into the mixture and the flask stoppered and allowed to stand 15 minutes. 10 ml. of 5N hydrochloric acid were added slowly and the excess iodine titrated with 0·05 sodium thiosulphate. A blank titration was carried out at the same time.

Percentage of *iso*nicotinyl hydrazide =

$$\frac{\text{Titre Difference} \times \text{Factor of } 0{\cdot}05N\ Na_2S_2O_3 \times 1{\cdot}715}{\text{Weight of sample taken in g.}}$$

Bromimetric Assay

About 0·4 g. of the hydrazide, accurately weighed, was dissolved in water and made up to 250 ml. 25 ml. of this solution was pipetted into a 250-ml. iodine flask together with 25 ml. of 0·1N potassium bromide/potassium bromate solution. 5 ml. of concentrated hydrochloric acid was added, the flask stoppered immediately and allowed to stand for 10 to 15 minutes. 5 ml. of 20 per cent. potassium iodide solution was slowly admitted to the flask (care is needed since there is a slight positive pressure in the flask due to the formation of nitrogen). The liberated iodine was then titrated with 0·05N sodium thiosulphate, a blank titration being carried out at the same time.

Percentage of *iso*nicotinyl hydrazide =

$$\frac{\text{Titre Difference} \times \text{Factor of } 0{\cdot}05N\ Na_2S_2O_3 \times 1{\cdot}715}{\text{Weight of sample taken in g.}}$$

The Estimation of isoNicotinyl Hydrazide in Tablets

All samples examined were 250 mg. tablets containing 50 mg. of drug. 10 tablets were weighed and powdered as finely as possible. About 2·0 g. of the powder accurately weighed, was suspended in water and diluted to exactly 250 ml. After shaking thoroughly, the insoluble matter was allowed to settle for ½ hour and 25 ml. of the supernatant liquid pipetted into an iodine flask. The estimation was completed as described above, using 0·1N iodine or 0·1N potassium bromide/bromate solution.

Weight of drug in mg. per tablet =

$$\frac{\text{Titre Difference} \times \text{Factor of } 0{\cdot}05N\ Na_2S_2O_3 \times 17{\cdot}15 \times \text{Average weight of Tablet}}{\text{Weight of sample taken in g.}}$$

The Effect of Sugars on the Estimation

Aqueous solutions containing 0·6 per cent. of glucose, lactose and sucrose respectively were prepared (25 ml. ≡ 150 mg. of sugar). Estimations were carried out as follows:—

Iodine in bicarbonate solution. In the iodimetric assay procedure, 25 ml. of the hydrazide solution was replaced by 25 ml. of the requisite sugar solution.

Bromine in acid solution. In the bromimetric assay procedure, 25 ml. of the hydrazide solution was replaced by 25 ml. of the requisite sugar solution.

Iodine in acid solution. The procedure used was the same as for iodine in bicarbonate solution except that 1 g. of sodium bicarbonate was replaced by 5 ml. of concentrated hydrochloric acid and 5 ml. of 20 per cent. potassium iodide solution.

Iodimetric assay of the drug and lactose. In the iodimetric assay procedure, 25 ml. of water was replaced by 25 ml. of 0·6 per cent. lactose solution.

Bromimetric assay of the drug and lactose. 150 mg. of lactose powder was added to the flask before the addition of 5 ml. of concentrated hydrochloric acid.

The Effect of Temperature on the Estimation

3 water baths were maintained at the temperatures required (10° C., 20° C. and 30° C.) by addition of ice or slight warming. In the case of the iodimetric assay, the titration flask and contents were placed in the bath 10 minutes before the addition of the 0·1N iodine. The reaction was then allowed to proceed for 15 minutes in the bath. For the bromimetric assay, the flask and contents were placed in the bath 10 minutes before adding the concentrated hydrochloric acid, and then left a further 10 minutes for the reaction to proceed.

Estimation of Nitrogen formed in the Reaction

0·6857 g. of *iso*nicotinyl hydrazide was dissolved in water containing 1·4 g. of potassium bromate and 7·0 g. of potassium bromide and diluted to 100 ml. The reaction bulb of a micro Van Slyke apparatus was filled with 5N hydrochloric acid and the gas burette and connecting tubes filled with water. 2 ml. of the hydrazide/bromate/bromide solution were admitted to the reaction bulb and the contents shaken mechanically for ½ hour. The gas evolved was driven over into the burette and the volume measured at atmospheric pressure. 3 estimations were carried out giving the following results.

Weight of drug per estimation = 13·71 mg. = 0·1 millimole.
Pressure = 762 mm. Temperature = 25° C.

Estimation	1	2	3
Volume of nitrogen	2·43 ml.	2·40 ml.	2·49 ml.
Mg. of nitrogen	2·71	2·67	2·77
Moles of nitrogen/mole drug	0·97	0·95	0·99

The authors wish to thank Mr. D. E. Seymour for his advice and criticisms in preparing this paper and the Directors of Herts Pharmaceuticals Ltd. for permission to publish these results.

REFERENCES

1. Canbäck, *J. Pharm. Pharmacol.*, 1952, **4**, 407.
2. Rubin *et al.*, *Dis. Chest.*, 1952, **21**, 439.

DISCUSSION

The two papers on *iso*nicotinyl hydrazide were discussed together.

The first paper was presented by MR. P. G. W. SCOTT, and the second by MR. B. W. MITCHELL.

DR. C. H. HAMPSHIRE (London), in a written contribution, drew attention to the international non-proprietary name isoniazid, and pointed out that, if the drug fulfilled the high expectations entertained, pharmacopœial monographs would be required. He quoted a letter from Dr. T. Canbäck, who had published a suggested assay and had seen the proofs of both of the papers, in which he stated that a study of the time function of the reaction had shown that 10 minutes was enough for completion of the oxidation with iodine in slightly alkaline solution.

Dr. Canbäck had repeated the time function studies and quoted figures which suggested that a second reaction started after about 35 minutes. The figures led to the conclusion that the sample examined contained about 97 per cent. of isoniazid and a few per cent. of a second compound which reacted with the iodine after 30 to 40 minutes. The same sample, tested by the authors' bromimetric method, gave 95·7 per cent. after 10 minutes, rising to 98·5 per cent. after 18 minutes, results which again suggested that there was a time factor in the bromimetric procedure and that there was present a second compound which was more difficult to oxidise. The compound used was not absolutely pure. For practical purposes both methods were, he thought, acceptable, but he hoped, for scientific reasons, that both methods would be further investigated. For tablets containing excipients it was never safe to rely more on bromine in acid solution than on iodine in slightly alkaline solution.

DR. G. E. FOSTER (Dartford) said he believed that the bromimetric method was the better one. He gave details of the old and more recent methods of preparing standard bromine solutions, and asked Mr. Mitchell whether he had tried the bromimetric assay using both solutions and, if so, whether he had obtained similar results. In his experience with other substances the solutions could give different results.

DR. E. M. BAVIN (Welwyn) asked Mr. Scott whether he had used the chlorodinitrobenzene method for the estimation of *iso*nicotinyl hydrazide in biological fluids. He wondered whether that reagent could be used, as difficulties were encountered with the *p*-dimethylaminobenzaldehyde method and with the old method using cyanogen bromide.

MR. N. L. ALLPORT (London) said he thought that Mr. Scott's claim of an error of not greater than $\pm$ 3 per cent. was optimistic. He would have thought a figure of $\pm$ 5 per cent. more likely.

MR. P. G. W. SCOTT, in reply, said that he had not applied the chlorodinitrobenzene method to the determination of *iso*nicotinyl hydrazide in biological fluids, but hoped to do so. It was not likely that many substances would be found in biological fluids which would interfere with that colorimetric determination. He felt that $\pm$ 5 per cent. was unduly wide, and that by tightening up the conditions of determination it should be possible to obtain $\pm$ 2 per cent.

MR. W. MITCHELL, in reply, referred to the fact that Dr. Canbäck used rather impure material, and said that it was essential that an assay method should be capable of giving good results with impure as well as pure material. He had used commercial material from various sources and had obtained results of over 99 per cent. with the bromimetric method. He could not comment on the variations with time using the bromimetric method, but was pleased to learn that the subject was being investigated. In answer to Dr. Foster, he had not investigated bromine solution prepared with potassium hydroxide and bromine. In his method excess of bromine was present all the time and appeared to have no deleterious effect.

SOME OBSERVATIONS ON THE STRUCTURAL REQUIREMENTS FOR ANTIBIOTIC ACTIVITY IN THE CHLORAMPHENICOL SERIES. PART II*

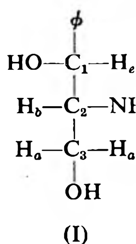
By R. J. Collins, B. Ellis, S. B. Hansen, H. S. Mackenzie, R. J. Moualim, V. Petrow, O. Stephenson and B. Sturgeon

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

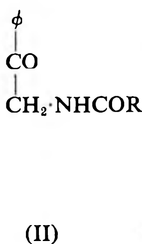
Received June 30, 1952

In Part I[1] an attempt was made to define broadly the structural features associated with antibiotic activity in the chloramphenicol series. Since then additional studies have been carried out in this and other laboratories which permit further elaboration of the concepts developed in the earlier publication. The present investigation is concerned largely with the preparation of some analogues of chloramphenicol (I) in which the hydrogen atoms marked *a*, *b* and *c* are replaced by methyl groups. Interpretation of the results obtained is included in the discussion.

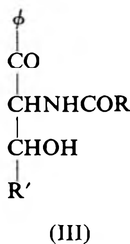
Experiments on the synthesis of 3-methylchloramphenicol (V; R = $-CHCl_2$; R′ = –Me) in which H*a* of (I) is replaced by Me, have been recorded by Rebstock,[2] who obtained a compound of unknown configuration, hereafter termed the "α"-isomer, by reduction of *iso*nitrosobenzoylacetone, followed by nitration and dichloroacetylation of the resulting amino-alcohol. The asymmetric configuration of the structural type (V), however, provides for the existence of 4 pairs of enantiomorphs, of which 2 pairs may be regarded as derived from *erythro-* and two from *threo*-2-acetamido-1-*p*-nitrophenylpropane-1 : 3-diol. We have, therefore, carried out experiments in order to prepare further compounds of this type.



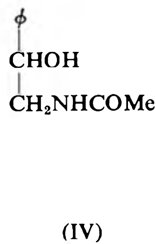
(I)



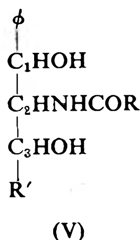
(II)



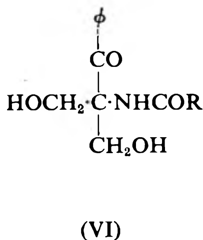
(III)



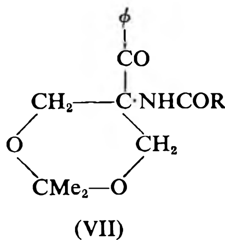
(IV)



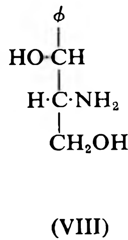
(V)



(VI)

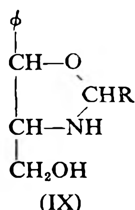


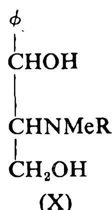
(VII)

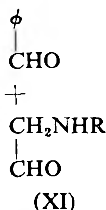


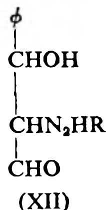
(VIII)

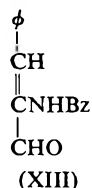
* *This Journal*, 1951, **3**, 149, is considered as Part I.

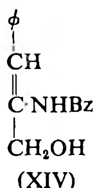

(IX)

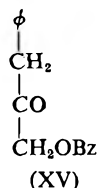

(X)

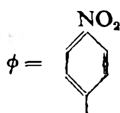

(XI)


(XII)


(XIII)


(XIV)


(XV)



Condensation of acetaldehyde with ω-acetamido-*p*-nitroacetophenone (II; R = Me) in aqueous ethanol in the presence of sodium bicarbonate led to the formation of 1-acetamido-2-hydroxy-*p*-nitrobutyrophenone (III; R = R′ = Me) in *ca.* 60 per cent. yield. The product thus formed proved less stable than the corresponding formaldehyde condensation product (III; R = Me; R′ = H) in that on Ponndorf reduction in boiling *iso*propanolic solution it underwent smooth fission and reduction to give 2-acetamido-1-*p*-nitrophenylethan-1-ol (IV)[4] in excellent yield. Reduction at room temperature was next examined, when it was hoped that fission of (III; R = R′ = Me) into its component parts might be avoided. The complex mixture obtained, however, still contained substantial quantities of (IV) admixed, in this instance, with *ca.* 10 per cent. of a new isomer of (V; R = R′ = Me) which we designate "β"-2-acetamido-1-*p*-nitrophenylbutane-1:3-diol. Hydrolysis of this compound with 6 N hydrochloric acid furnished "β"-2-amino-1-*p*-nitrophenylbutane-1:3-diol, from which "β"-2-dichloroacetamido-1-*p*-nitrophenylbutane-1:3-diol ("β"-3-methylchloramphenicol) (V; R = $-CHCl_2$; R′ = Me) was obtained by reaction with methyl dichloroacetate.

Attempts to simplify the preparation of "β"-3-methylchloramphenicol by employing ω-dichloroacetamido-*p*-nitroacetophenone[5,6] (II; R = $-CHCl_2$) as starting material proved unsuccessful. Reaction of (II; R = $-CHCl_2$) with acetaldehyde in alkaline solution offered no difficulty, 1-dichloroacetamido-2-hydroxy-*p*-nitrobutyrophenone (III; R = $-CHCl_2$; R′ = Me) being readily obtained in 50 per cent. yield. Ponndorf reduction of this product at room temperature, however, led to a complex mixture from which only (II; R = $-CHCl_2$) was isolated in *ca.* 25 per cent. yield.

It is, of course, not possible to decide the stereochemical configuration of "β"-3-methylchloramphenicol from its mode of synthesis alone. At the same time it seems likely that the compound has the *erythro*-configuration at C_1:C_2 on the basis of the following evidence. Whereas Ponndorf reduction of 1-(acet)-amido-2-hydroxy-*p*-nitropropiophenone (III; R = Me; R′ = H) gives DL-*threo*-2-(acet)-amido-1-*p*-nitrophenylpropane-1:3-diol[4] (≡ DL-chloramphenicol base) with only small quantities of the *erythro*-isomers, Ponndorf reduction of 1-acetamido-2-*acyloxy*-

p-nitropropiophenone gives products in which the *erythro*-forms predominate (Ellis and Sturgeon, *unpublished observations*). Increase in molar volume of the terminal $-CH_2OH$ group of (III; R = Me; R′ = H) is thus seen to favour production of *erythro*-forms. Extending this concept further, it seems reasonable to conclude that the terminal methyl group (R′) of (III; R = R′ = Me) may likewise exert a comparable steric effect on the Ponndorf reduction of this compound with formation of an *erythro*-isomer of (V; R = R′ = Me). Again, *erythro*-compounds of type (I) are known to undergo conversion into the corresponding *threo*-derivatives under the action of thionyl chloride.[7] As this reagent converts "β"-3-methylchloramphenicol into a new isomer, "γ"-3-methylchloramphenicol, it seems likely that the latter compound is a *threo*-form of (V; R = $-CHCl_2$; R′ = Me) (relative to C_1 and C_2), and that the "β"-isomer is its *erythro*-analogue.

The second *threo*-enantiomorph of (V; R = $-CHCl_2$; R′ = Me) is probably represented by the "α"-3-methylchloramphenicol of Rebstock.[2] This view derives from consideration of the von Auwers-Skita generalisation[8,9] that catalytic hydrogenation (of *iso*nitrosobenzoylacetone[2]) in neutral media may be expected to lead to the formation of *trans*-(*i.e.*, *threo*)-isomers.

In addition to the foregoing series of transformations, we have also studied the condensation of (II; R = Me) with chloral, benzaldehyde, and *p*-nitrobenzaldehyde, hoping thereby to obtain chloramphenicol analogues (V) in which R′ = $-CCl_3$, –Ph, and $-C_6H_4NO_2$(*p*). Reaction between ω-acetamido-*p*-nitroacetophenone (II; R = Me) and chloral readily occurred in the presence of sodium bicarbonate as condensing agent to give 1-acetamido-2-hydroxy-3:3:3-trichloro-*p*-nitrobutyrophenone (III; R = Me; R′ = $-CCl_3$). Ponndorf reduction of this compound gave 2-acetamido-1-*p*-nitrophenyl-4:4:4-trichlorobutane-1:3-diol (V; R = Me; R′ = $-CCl_3$), hydrolysed by dilute hydrochloric acid to the corresponding amine. Dichloroacetylation of the latter furnished 2-dichloroacetamido-1-*p*-nitrophenyl-4:4:4-trichlorobutane-1:3-diol (V; R = $-CHCl_2$; R′ = $-CCl_3$) of presumed *threo*-configuration, as it was recovered unchanged after treatment with thionyl chloride. Employment of benzaldehyde and *p*-nitrobenzaldehyde in the above reaction with (II; R = Me) led to the formation of 2-phenyl-(III; R = Me; R′ = –Ph) and 2-*p*-nitrophenyl-1-acetamido-2-hydroxy-*p*-nitropropiophenone (III; R = Me; R′ = $-C_6H_4NO_2$(*p*)). Ponndorf reduction of these compounds to the corresponding amino-diols (V; R = Me; R′ = Ph and $-C_6H_4NO_2$) could not be accomplished, however, as fission occurred in both cases with regeneration of the component parts, which severally underwent reduction in the normal way (see Experimental).

Attention was next directed to the preparation of 2-methyl-chloramphenicol (I; H_b replaced by –Me) from 1-acylamidopropiophenone by the general procedure of Long and Troutman.[10] Before this objective could be attained, however, a publication appeared by Huebner and Schultz[11] which not only covered essentially the same ground, but also revealed that the desired compound was inactive antibacterially. Our

own experiments were therefore discontinued. Interest in a 2-substituted chloramphenicol was nevertheless revived shortly afterwards when a publication (dated 1950) by Šorm, Gut, Suchý and Reichl[6] became available, in which it was claimed that "introduction of a second hydroxymethyl group on carbon atom 2 does not alter or even enhances the activity" (p. 508). As it seemed difficult to reconcile this result with conclusions reached from a study of 2-methylchloramphenicol, an attempt was made to repeat and hence confirm the startling and challenging claim put forward by the Czech workers.

2-Acetamido-2-*p*-nitrobenzoylpropane-1:3-diol (VI; R = Me) was prepared in *ca.* 40 per cent. yield by hydroxymethylation of ω-acetamido-*p*-nitroacetophenone (II; R = Me) by short warming with excess of formaldehyde in ethanolic solution in the presence of sodium bicarbonate as catalyst. Alternatively, the "monohydroxymethylation product" (III; R = Me; R′ = H) could be employed as starting material. The corresponding dichloroacetyl- (VI; R = $-CHCl_2$) (Šorm *et al*[6]) and propionyl- (VI; R = –Et) derivatives were readily prepared in the same way but in somewhat better yields, as the intermediate monohydroxymethylation products were more soluble in the reaction media employed than in the case of (II; R = Me), thereby facilitating introduction of the second hydroxymethyl-group. Ponndorf reduction of the acetyl-compound (VI; R = Me) employing the experimental conditions used by Šorm *et al.*[6] for the dichloroacetyl analogue (*vide infra*) led to the formation of a gummy product smelling strongly of formaldehyde from which no crystalline material could be obtained. Elimination of a hydroxymethyl group as formaldehyde was likewise observed during reduction of the propionyl-analogue (VI; R = Et) when only DL-*threo*-1-*p*-nitrophenyl-2-propionamidopropane-1:3-diol (V; R = Et; R′ = H) was isolated in low yield. The constitution assigned to this compound was confirmed by comparison with an authentic specimen prepared by propionylation of DL-*threo*-1-*p*-nitrophenylpropane-1:3-diol. Ponndorf reduction of 2-dichloroacetamido-2-*p*-nitrobenzoylpropane-1:3-diol (VI; R = $-CHCl_2$) (Šorm *et al.*[6]) followed an identical pattern with elimination of one hydroxymethyl group and formation of DL-chloramphenicol in 35 per cent. yield. No evidence for concomitant production of "2-hydroxymethylchloramphenicol" was obtained.

The facility with which compounds of type (VI) lose one molecule of formaldehyde during Ponndorf reduction leads us to doubt the validity of the Czech claims. Their identification of "2-hydroxymethylchloramphenicol" was based solely upon analyses for carbon, hydrogen, and nitrogen. The analytical figures for "2-hydroxymethylchloramphenicol," however, are not markedly different from those required by chloramphenicol and thus cannot serve alone to distinguish between the two compounds:—

	Per cent. C	Per cent. H	Per cent. N
Hydroxymethylchloramphenicol, $C_{12}H_{14}O_6N_2Cl_2$	40·8	4·0	7·9
Chloramphenicol, $C_{11}H_{12}O_5N_2Cl_2$	40·9	3·8	8·7
Product obtained by Šorm *et al.*	40·5	3·6	7·9

In addition, the melting point of their so-called "hydroxymethylchloramphenicol" was the same as that of chloramphenicol itself, with which their product is almost certainly identical. The term "hydroxymethylchloramphenicol" should not, therefore, be applied to the Ponndorf reduction product of (VI; R = $-CHCl_2$) unless evidence confirming this designation becomes available. Attempts to prevent elimination of formaldehyde from (VI) during Ponndorf reduction by stabilising the structure as the *iso*propylidene derivatives (VII) proved unsuccessful, (VII; R = –Me or –Pr) being recovered unchanged after treatment with aluminium *iso*propoxide in the usual way. No acetone was detectable in the distillates.

Experiments to prepare *N*-methylchloramphenicol (I; H_c replaced by –Me) by extension of the Long and Troutman procedure[10] to *N*-methylphenacylamine[12] and *p*-nitro-*N*-methylphenacylamine proved impracticable, as we were unable to obtain these alternative starting materials in more than minute yield by reaction of methylamine with the corresponding ω-bromoacetophenone. Attention was therefore directed to the *N*-methylation of DL-*threo*-2-amino-1-*p*-nitrophenylpropane-1:3-diol (VIII). Reaction of (VIII) with conventional methylating agents proved unsuccessful, intractable gums being obtained. Employment of methyl *p*-toluenesulphonate gave what appeared to be a dimethyl-product. In an attempt to control the methylation, (VIII) was condensed with anisaldehyde to give the oxazolidine [XI; R = $-C_6H_4OMe$ (*p*)]. The latter passed readily into the quaternary salt on treatment with methyl iodide, but attempts to decompose this product with water led only to liberation of anisaldehyde without concomitant production of the *N*-methylated base. Success was achieved by condensing (VIII) with formaldehyde, when the oxazolidine (IX; R = H) was presumably formed, followed by reduction with formic acid. The latter was then removed and the residue treated with one molar proportion of alkali to saponify the *O*-formyl esters present. DL-*threo*-2-Form-methylamido-1-*p*-nitrophenylpropane-1:3-diol (X; R = –CHO) thus obtained was hydrolysed with hydrochloric acid to give (X; R = H), which was characterised as the picrate and hydrochloride.

Dichloroacetylation of (X; R = H) presented initial difficulty, as boiling with ethyl dichloroacetate in ethanolic solution for 30 hours led to the formation of DL-*threo*-2-dichloroacetmethylamido-1-*p*-nitrophenylpropane-1:3-diol (X; R = $-COCHCl_2$) in only 10 per cent. yield. Pentachloroacetone, previously employed by Fritsch[13] to dichloracetylate aniline, proved more satisfactory, reacting rapidly with (X; R = H) to give DL-*N*-methylchloramphenicol (X; R = $-COCHCl_2$) in very high yield.

Inter alia we examined the possibility of preparing chloramphenicol by aldol condensation between *p*-nitrobenzaldehyde and derivatives of aminoacetaldehyde (XI) to give (XII), followed by Ponndorf reduction to (I). Unfortunately, it proved impossible to stop the initial condensation at the aldol stage (XII), dehydration to (XIII) invariably taking place. 2-Benzamido-*p*-nitrocinnamaldehyde (XIII), obtained in this way,

was reduced by the Ponndorf method to the corresponding cinnamyl alcohol (XIV). Hydrolysis of the latter compound with hydrochloric acid led to the formation of 2-keto-3-*p*-nitrophenylpropyl benzoate(XV), together with a second compound, $C_{17}H_{16}O_4N_2$, which was not fully characterised.

Biological study of the above compounds by Dr. S. W. F. Underhill and his staff failed to reveal marked antibiotic activity.

Discussion

Antibiotic action is now generally thought to be due to interference by the antibiotic in an enzyme system essential to the cellular organism. Enzymes themselves are specific, catalytically-active proteins, which often require association with relatively simple molecules, known as prosthetic groups or coenzymes, for their activity. The terms "prosthetic groups" and "coenzyme" are not, strictly speaking, synonymous. The former designation is usually applied to those groups which are sufficiently firmly bound to the protein to be considered as part of the enzyme molecule. The term "coenzyme," in contrast, is reserved for those cases in which the association is so loose that in solution the enzyme exists largely in the free state. The spatial relationship between the enzyme and its related coenzyme or prosthetic group has been discussed by Pauling, who assumes that the protein molecule forms a polypeptide chain, rolled up and packed and held in a specific shape by hydrogen bonding and other forces between the polypeptide groups and other polar functions. Embedded in this protein matrix lie the coenzyme molecules at such points where a transfer of hydrogen ions to and from the peptide chain can be effected, and closely associated with these lie strategically placed "cavities" which represent the active centres through which the enzymes perform their catalytic functions.

The mechanism of enzyme action may then be pictured as taking place in the following sequence: (i) approach of the metabolite molecule or substrate to the active centre or "cavity," (ii) combination between the metabolite molecule and the active centre, which is preceded by close juxtaposition between certain polar groups present in the substrate with complementary groups present in the enzyme, (iii) occurrence of the specific enzyme reaction, and (iv) release of the modified metabolite. In order to interfere with such an enzyme system, an antimetabolite must fulfil certain conditions. Firstly, it must show close spatial corre-dence to at least that part of the metabolite molecule which is in close juxtaposition to complementary polar groups present in the "cavity" of the enzyme. Secondly, it must be able to form a complex with the enzyme which is either irreversible, or else cannot be converted into the normal metabolite transformation product. Compounds which function in this way by blocking the approach of the metabolite to its particular "cavity" in the enzyme molecule are known as "structural analogues antagonistic to metabolites," or more simply, as "antimetabolites." Thus chloramphenicol is thought to function as an antimetabolite by interfering with enzymic transformations undergone by an essential metabolite probably

related to phenylalanine.[14,15] The exact structure of the metabolite has yet to be determined. It is, nevertheless, possible from the present evidence to draw certain conclusions regarding the nature of the spatial relationship between the antibiotic and the active enzymic centre or "cavity" to which its unique structure is so delicately adjusted. Combination between chloramphenicol and enzyme involves all the polar groups of (I) (Part I[1]) which must be in close juxtaposition to complementary groups within the enzyme matrix. Such a situation obtains if the molecule assumes the planar form shown in Figures 1 and 1*a*, the face represented in Figure 1 lying in contact with the enzyme. In this position all the polar groups of (I) are available for complex formation with the enzyme, whilst H_a, H_c, H_d, and H_e are all directed away from the enzyme surface (cf. Fig. 1*a*).

Pronounced regularities in biological activity are often evident in closely related series of compounds. If, therefore, Figure 1 and 1*a* represent the spatial relationship between the antimetabolite and the enzyme matrix, it follows that replacement of H_a, H_c, or H_d, respectively, by methyl should lead to homologues with biological activity. Work outlined in the preceding section, however, shows clearly that replacement of H_a, H_b, H_c, or H_d by methyl leads, in all cases, to loss of antibiotic action. These facts point to the conclusion that combination between antimetabolite and enzyme is such that H_a, H_b, H_c, and H_d are *embedded within* the protein matrix. If this is indeed the case, it follows that their replacement by methyl must hinder close juxtaposition between antimetabolite and enzyme, and thus destroy the basis upon which antibiotic action depends.

It is, of course, possible to picture the situation described in the foregoing paragraph in terms of the spatial configuration represented in Figures 1 and 1*a*. At the same time the facts are better accommodated by means of the "buckled" structure represented in Figures 2 and 2*a*, in which the propane side chains of the antibiotic, and hence H_a, H_b, H_c, and H_d are assumed to lie within the body of the enzyme. The conclusions thus reached on biological grounds gain added significance from studies described by Dunitz[16] on the crystal structure of chloramphenicol. His results show that (I) exists in the crystal state in a spatial form almost identical with that shown in Figures 2 and 2*a* and that this configuration is especially suitable for interaction with the polar groups of a protein chain.

Somewhat different considerations apply to the *p*-nitrophenyl portion of the molecule. In contrast to the propanediol side chain, important but limited changes in structure may be effected in this part of the molecule with retention of biological activity. Thus replacement by *p*-chlorophenyl-,[17] *p*-dichloroacetylamido-,[18] 4′-nitro-4-biphenyl-,[19] 4-nitro-1-naphthyl-,[20] etc., leads to compounds with some antibiotic action. Whilst, therefore, the propanediol side chain represents a *specific* pharmacodynamical portion of the molecule, the nitro(phenyl)-group represents a relatively *non-specific* pharmacodynamical structure. The relative non-specificity of the *p*-nitro(phenyl)-portion of the molecule is best

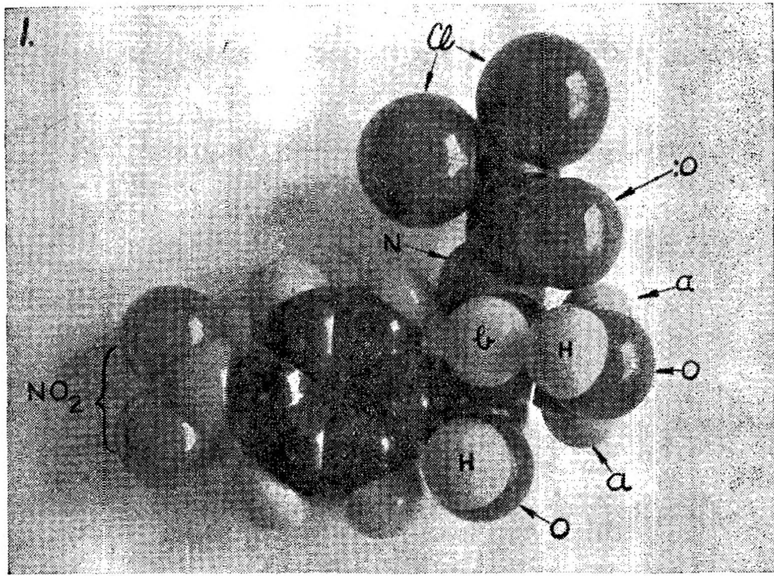


FIG. 1.

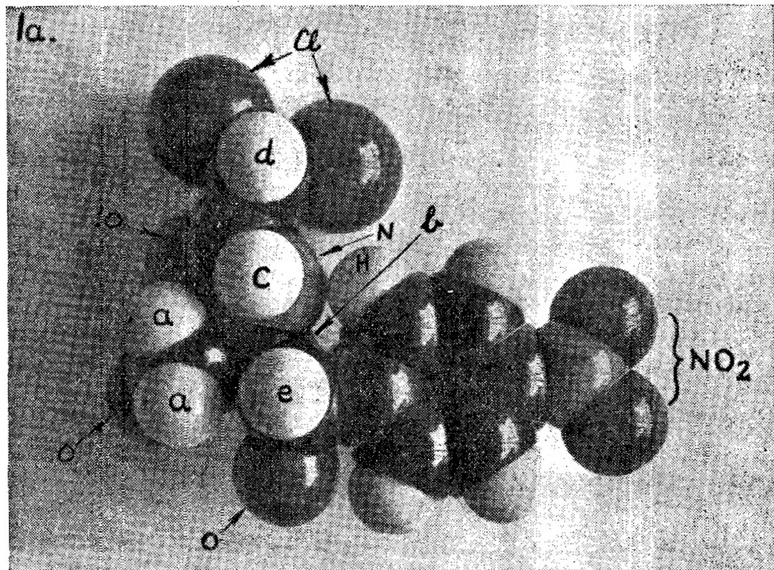


FIG. 1*a*.

interpreted by the assumption that this part of the antimetabolite is not embedded within the protein matrix but held upon its surface. The *p*-nitro(phenyl) group may therefore be likened to a grappling hook

which holds the antibiotic to the surface of the enzyme. Its structural requirements will therefore be less rigid than those required by the propanediol side-chain which lies embedded in the protein matrix. The

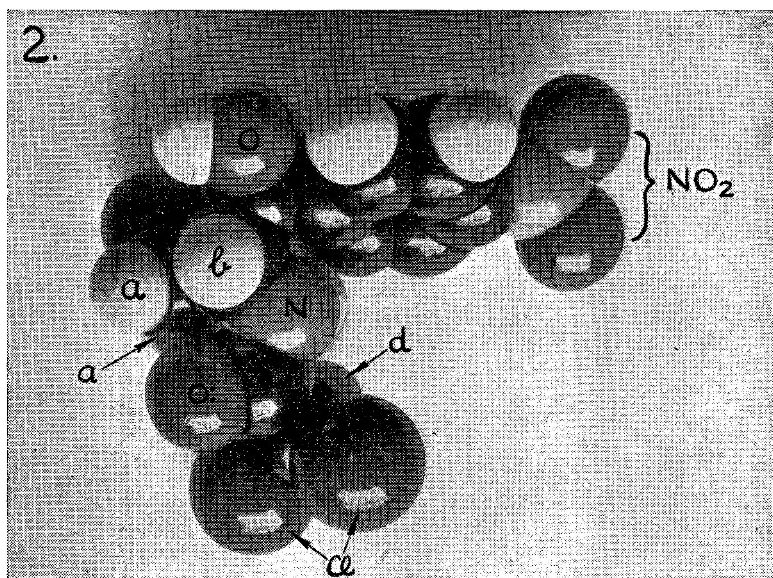


FIG 2.

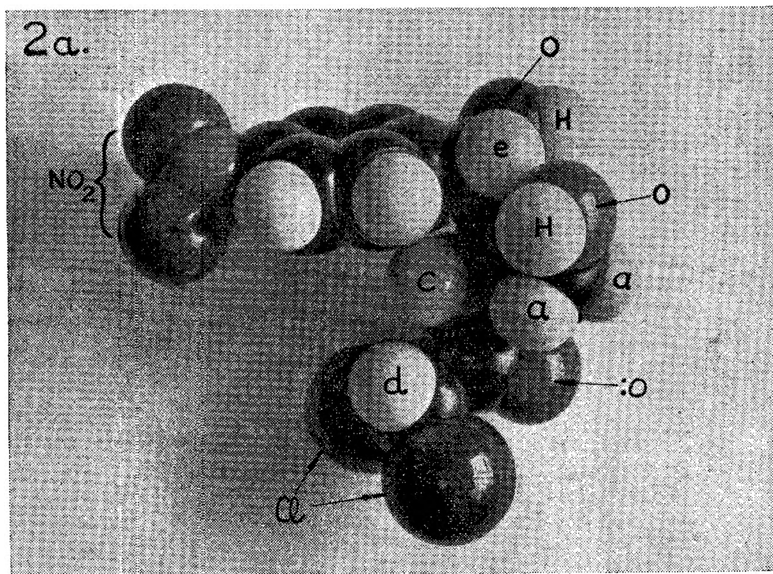


FIG. 2*a*.

concepts formulated above thus imply a fundamental difference in the functions associated with specific and non-specific pharmacodynamical groups. It is therefore suggested that these contrasting types be differentiated by use of the prefixes σ (specific) and υ (non-specific).

EXPERIMENTAL

M.pts. are uncorrected. Microanalyses are by Drs. Weiler and Strauss, Oxford.

1-*Acetamido-2-hydroxy*-p-*nitrobutyrophenone* (III; R = R′ = Me).—A mixture of ω-acetamido-*p*-nitroacetophenone (20·0 g.), ethanol (95 per cent.) (80 ml.), acetaldehyde (8·0 g.) and water (16 ml.) was stirred mechanically at 35° C. when sodium bicarbonate (1·3 g.) was added. Stirring at 35° C. was continued for a further 2 hours, when the mixture was cooled to 5° C. The separated solids were collected and crystallised from ethanol to give 1-*acetamido-2-hydroxy*-p-*nitrobutyrophenone*, needles, m.pt. 192° to 194° C. Found: C, 54·1; H, 5·2; N, 10·5. $C_{12}H_{14}O_5N_2$ requires C, 54·1; H, 5·3; N, 10·5 per cent.

Ponndorf reduction of 1-*acetamido-2-hydroxy*-p-*nitrobutyrophenone*.—A suspension of the foregoing compound (98 g.) in *iso*propanol (1900 ml.) containing redistilled aluminium *iso*propoxide (120 g.) was stirred for 5 days at room temperature. The red mixture was treated with water (400 ml.), stirred for 24 hours, filtered through a pad of filter aid, and the filtrate concentrated *in vacuo*. Separation of yellow granular solids occurred when the volume had been reduced to *ca*. 150 ml. The material (*Fraction A*; 19 g., m.pt. 145° C.) was removed, the filtrate taken to dryness, and the residue treated with ethyl acetate (100 ml.). The insoluble portion (*Fraction B*; 8·7 g., m.pt. 180° C.) was collected and the mother liquors concentrated to half bulk to give more solids (*Fraction C*; 4·5 g., m.pt. 150° C.).

Purification of *Fraction A* from ethyl acetate gave needles of 2-acetamido-1-*p*-nitrophenylethan-1-ol, m.pt. 160° C. (not depressed in admixture with an authentic specimen[4]). Found: C, 53·8; H, 4·7; N, 12·9. Calculated for $C_{10}H_{12}O_4N_2$: C, 53·6; H, 5·4; N, 12·5 per cent.

Fraction B was treated with hot ethyl acetate (60 ml.) and the insoluble portion crystallised from methanol-ethyl acetate-light petroleum. "β"-2-*Acetamido*-1-p-*nitrophenylbutane*-1:3-*diol* (7·7 g.) was obtained in plates, m.pt. 195° C. Found: C, 53·2; H, 6·1; N, 10·4. $C_{12}H_{16}O_5N_2$ requires C, 53·7; H, 6·0; N, 10·4 per cent.

Fraction C was extracted with hot ethyl acetate (40 ml.). Purification of the insoluble fraction gave a further quantity (2·5 g.) of the compound, m.pt. 195° C. The ethyl acetate extract, on cooling, deposited the compound m.pt. 160° C.

"β"-2-*Amino*-1-p-*nitrophenylbutane*-1:3-*diol*.—The acetyl-derivative (1·5 g.) in 6N hydrochloric acid (10 ml.) was heated under reflux for 1 hour and the solution (charcoal) taken to dryness *in vacuo*. The residue was dissolved in water (5 ml.) and treated with 2N sodium hydroxide to *p*H 12. Crystallisation of the precipitate from water gave

"β"-2-*amino*-1-p-*nitrophenylbutane*-1:3-*diol* as needles, m.pt. 155° to 156° C. Found: C, 53·0; H, 6·0; N, 12·4. $C_{10}H_{14}O_4N_2$ requires C, 53·1; H, 6·2; N, 12·4 per cent.

"β"-2-*Dichloroacetamido*-1-p-*nitrophenylbutane*-1:3-*diol* crystallised from ethyl acetate-light petroleum in plates, m.pt. 109° to 110°C. Found: C, 42·6; H, 4·5; N, 8·0; Cl, 19·1. $C_{12}H_{14}O_5N_2Cl_2$ requires C, 42·7; H, 4·2; N, 8·3; Cl, 21·0 per cent.

1-*Dichloroacetamido*-2-*hydroxy*-p-*nitrophenylbutyrophenone* (III; R = –$CHCl_2$; R′ = Me), formed prismatic needles, m.pt. 170° to 172° C. after crystallisation from methanol. Found: C, 42·9; H, 3·6; N, 8·0; Cl, 19·9. $C_{12}H_{12}O_5N_2Cl_2$ requires C, 43·0; H, 3·6; N, 8·4; Cl, 21·2 per cent.

Ponndorf reduction at room temperature yielded 1-dichloroacetamido-*p*-nitroacetophenone (II; R = –$CHCl_2$), needles, m.pt. 146° to 147° C., after crystallisation from ethyl acetate-hexane. Found: C, 41·5; H, 3·0; N, 9·3; Cl, 23·8. Calculated for $C_{10}H_8O_4N_2Cl_2$: C, 41·2; H, 2·7; N, 9·6; Cl, 24·4 per cent. The m.pt. was not depressed in admixture with an authentic specimen.[21]

"γ"-2-*Amino*-1-p-*nitrophenylbutane*-1:3-*diol*.—A solution of "β"-2-acetamido-1-*p*-nitrophenylbutane-1:3-diol (V; R = R′ = –Me) (5 g.) in redistilled thionyl chloride (15 ml.) was kept for 1 hour at room temperature, when it was treated with water (15 ml.) added drop by drop. The mixture was heated for 1 hour on the steam bath and the solution (charcoal) made alkaline with 20 per cent. aqueous sodium hydroxide. The crystalline solids (2·0 g.) were purified from hot water giving "γ"-2-*amino*-1-p-*nitrophenylbutane*-1:3-*diol* in needles, m.pt. 109° to 110° C. Found: C, 53·4; H, 6·3; N, 12·9. $C_{10}H_{14}O_4N_2$ requires C, 53·1; H, 6·2; N, 12·4 per cent.

"γ"-2-*Dichloroacetamido*-1-p-*nitrophenylbutane*-1:3-*diol* formed rhombs, m.pt. 128° C. after crystallisation from ethanol-light petroleum. Found: C, 42·6; H, 4·4; N, 8·8; Cl, 21·2. $C_{12}H_{14}O_5N_2Cl_2$ requires C, 42·7; H, 4·2; N, 8·3; Cl, 21·0 per cent.

1-*Acetamido*-2-*hydroxy*-3:3:3-*trichloro*-p-*nitrobutyrophenone* (III; R = –Me; R′ = –CCl_3), after crystallisation from methanol, was obtained (60 per cent.) in glistening needles, m.pt. 180° to 181° C. (decomp.). Found: C, 39·0; H, 3·2; N, 7·5; Cl, 28·3. $C_{12}H_{11}O_5N_2Cl_3$ requires C, 39·0; H, 3·0; N, 7·6; Cl, 28·8 per cent.

2-*Acetamido*-1-p-*nitrophenyl*-4:4:4-*trichlorobutane*-1:3-*diol* (V; R = –Me; R′ = –CCl_3) was obtained (25 per cent.) by Ponndorf reduction of the foregoing compound. After crystallisation from ethyl acetate it formed plates, m.pt. 188° to 189° C. Found: C, 39·1; H, 3·5; N, 7·6; Cl, 28·4. $C_{12}H_{13}O_5N_2Cl_3$ requires C, 38·8; H, 3·5; N, 7·5; Cl, 28·7 per cent.

2-*Amino*-1-p-*nitrophenyl*-4:4:4-*trichlorobutane*-1:3-*diol* was obtained by hydrolysis of the foregoing compound with 7·5 per cent. hydrochloric acid for 3 hours on the steam bath. After crystallisation from ethyl acetate-hexane it formed needles, m.pt. 149° to 150° C. Found: C, 36·8; H, 3·6; N, 8·5; Cl, 31·7. $C_{10}H_{11}O_4N_2Cl_3$ requires C, 36·4; H, 3·3; N, 8·5; Cl, 32·3 per cent.

2-*Dichloroacetamido*-1-p-*nitrophenyl*-4:4:4-*trichlorobutane*-1:3-*diol* (V; R = $-CHCl_2$; R′ = $-CCl_3$), prismatic needles, m.pt. 174° to 175° C., after crystallisation from ethyl acetate. Found: C, 32·9; H, 2·5; N, 5·6; Cl, 40·3. $C_{12}H_{11}O_5N_2Cl_5$ requires C, 32·7; H, 2·5; N, 6·3; Cl, 40·3 per cent.

2-*Phenyl*-1-*acetamido*-2-*hydroxy*-p-*nitropropiophenone* (III; R = Me; R′ = Ph) was purified from methanol to give (25 per cent.) prismatic needles, m.pt. 172° to 173° C. Found: C, 62·8; H, 4·9; N, 9·0. $C_{17}H_{16}O_5N_2$ requires C, 62·2; H, 4·9; N, 8·5 per cent.

2-p-*Nitrophenyl*-1-*acetamido*-2-*hydroxy*-p-*nitropropiophenone* (III; R = Me; R′ = $-C_6H_4NO_2$) separated from ethanol in needles, m.pt. 175° C. Found: C, 54·0; H, 4·0; N, 11·4. $C_{17}H_{15}O_7N_3$ requires C, 54·7; H, 4·0; N, 11·3.

2-p-*Nitrophenyl*-1-*dichloroacetamido*-2-*hydroxy*-p-*nitropropiophenone* (III; R = $-CHCl_2$; R′ = $-C_6H_4NO_2$), after crystallisation from a large volume of ethanol, formed pale yellow leaflets, m.pt. 211° C. Found: C, 47·0; H, 2·7; N, 9·3. $C_{17}H_{13}O_7N_3Cl_2$ requires C, 46·2; H, 2·9; N, 9·5 per cent.

ω-Dichloroacetamidoacetophenone was prepared from the tin salt complex of ω-aminoacetophenone by the procedure of Long and Troutman.[10] Recrystallisation from light petroleum afforded needles, m.pt. 122° to 123° C. Found: C, 49·1; H, 3·5; N, 5·6; Cl, 28·7. $C_{10}H_9O_2NCl_2$ requires C, 48·8; H, 3·7; N, 5·7; Cl, 28·9 per cent.

1-*Dichloroacetamido*-2-*hydroxy*-2-p-*nitrophenylpropiophenone*, prepared by treating ω-dichloroacetamidoacetophenone (600 mg.) and *p*-nitrobenzaldehyde (370 mg.) in ethanolic solution with 1 drop of piperidine, was purified from methanol to form crystals (80 per cent.), m.pt. 174° to 175° C. Found: C, 51·5; H, 3·8; N, 6·4; Cl, 16·6. $C_{17}H_{14}O_5N_2Cl_2$ requires C, 51·4; H, 3·5; N, 7·0; Cl, 17·9 per cent.

1-*Acetamido*-2-*hydroxy*-2-p-*nitrophenylpropiophenone* formed yellow crystals, m.pt. 171° to 172° C. Found: C, 61·9; H, 5·0; N, 8·7. $C_{17}H_{16}O_5N_2$ requires C, 62·2; H, 4·9; N, 8·5 per cent.

2-*Acetamido*-2-p-*nitrobenzoylpropane*-1:3-*diol* (VI; R = Me).—ω-Acetamido-*p*-nitroacetophenone (22·2 g.) in hot ethanol (100 ml.), was treated with 36 per cent. solution of formaldehyde (100 ml.; *ca.* 12 mole), the solution cooled to 45° C., and sodium bicarbonate (1·0 g.) added. After standing for 2 days at room temperature, the dark-coloured mixture was saturated with salt and the product extracted with ethyl acetate. Crystallisation from ethanol-light petroleum (b.pt. 60° to 80° C.) furnished 2-*acetamido*-2-p-*nitrobenzoylpropane*-1:3-*diol* as needles, m.pt. 151° to 154° C. Found: C, 50·7; H, 5·3; N, 9·9. $C_{12}H_{14}O_6N_2$ requires C, 51·1; H, 5·0; N, 9·3 per cent. The m.pt. varies somewhat with the rate of heating.

The iso*propylidene* derivative (VII; R = Me) was prepared by shaking the foregoing compound (2·2 g.) in dry acetone with phosphorus pentoxide (2·0 g.) for 30 minutes at room temperature. After decanting from the gum and shaking with solid sodium carbonate until neutral, the mixture was concentrated and diluted with water. The product,

after crystallisation from methanol, formed needles, m.pt. 199° to 200° C. Found: C, 56·1; H, 5·7. $C_{15}H_{18}O_6N_2$ requires C, 55·9; H, 5·6 per cent.

*ω-Propionamido-*p-*nitroacetophenone* (II; R = Et). ω-Amino-*p*-nitroacetophenone hydrochloride (from 200 g. of *p*-nitroacetophenone) was thoroughly stirred with water (2 l.) for 10 minutes when propionic anhydride (260 g.) followed by sodium acetate trihydrate (240 g.) in water was added. The *product*, collected after stirring for 2 hours, was recrystallised from ethyl acetate forming cream needles (132 g.), m.pt. 128° C. Found: C, 55·5; H, 5·1; N, 12·1. $C_{11}H_{12}O_4N_2$ requires C, 55·9; H, 5·1; N, 11·9 per cent.

2-*Hydroxy*-1-*propionamido*-p-*nitropropiophenone*, prepared from the foregoing compound, was purified from chloroform-light petroleum, forming prismatic needles, m.pt. 116° C. Found: C, 54·0; H, 5·3; N, 10·1. $C_{12}H_{14}O_5N_2$ requires C, 54·1; H, 5·3; N, 10·5 per cent.

2-p-*Nitrobenzoyl*-2-*propionamidopropane*-1:3-*diol* (VI; R = Et), prepared (48 per cent.) by heating ω-propionamido-*p*-nitroacetophenone (5 g.), ethanol (20 ml.), solution of formaldehyde (7 ml. of 37 per cent.; *ca.* 4 moles) and sodium bicarbonate (200 mg.) for 2 hours at 40° C., crystallised from benzene-methanol in prismatic needles, m.pt. 172° to 174° C. Found: C, 53·3; H, 5·2; N, 9·5. $C_{13}H_{16}O_6N_2$ requires C, 52·7; H, 5·4; N, 9·5 per cent.

The iso*propylidene* derivative, after crystallisation from ethanol-light petroleum, formed rosettes of felted needles, m.pt. 169° to 170° C. Found: C, 57·2; H, 5·6; N, 8·3. $C_{16}H_{20}O_6N_2$ requires C, 57·2; H, 5·8; N, 8·3 per cent.

Ponndorf reduction of (VI; R = Et) gave DL-*threo*-1-p-*nitrophenyl*-2-*propionamidopropane*-1:3-*diol*, fibrous needles, m.pt. 131° to 132° C., after crystallisation from ethyl acetate. Found: C, 53·3; H, 5·8. $C_{12}H_{16}O_5N_2$ requires C, 53·7; H, 6·0. The compound thus obtained gave no depression of m.pt. in admixture with a sample prepared in the following way. DL-*threo*-2-Amino-1-*p*-nitrophenylpropane-1:3-diol (3·8 g.), suspended in a mixture of ethyl acetate (12 ml.) and ethanol (3·8 ml.), was treated with propionic anhydride (9·6 ml.) and warmed to boiling point. After allowing to cool, the product was collected and crystallised from ethyl acetate, to give needles, m.pt. 132° C. Found: C, 53·3; H, 5·8; N, 10·3. $C_{12}H_{16}O_5N_2$ requires C, 53·7; H, 6·0; N, 10·5 per cent.

2-*Dichloroacetamido*-2-p-*nitrobenzoylpropane*-1:3-*diol* (VI; R = –$CHCl_2$) was recrystallised from benzene-methanol to give feathery needles, m.pt. 136° to 137° C. Found: C, 41·2; H, 3·6; N, 7·8; Cl, 20·2. Calc. for $C_{12}H_{12}O_6N_2Cl_2$: C, 41·0; H, 3·4; N, 8·0; Cl, 20·2 per cent. Šorm *et al.*[6] give m.pt. 134° C.

Ponndorf reduction of (VI; R = –$CHCl_2$) furnished DL-chloramphenicol, identified by m.pt. and mixed m.pt. with an authentic specimen.

DL-threo-1-p-*Nitrophenyl*-2-p-*toluenesulphonamidopropane*-1:3-*diol*, prepared by heating the DL-*threo*-amine (6·3 g.), tosyl chloride (6·0 g.), sodium acetate (3·0 g.) and ethanol (40 ml.) under reflux for 1 hour, was crystallised from ethyl acetate-methanol to give silvery platelets,

m.pt. 210° C. Found: C, 51·9; H, 4·9; N, 7·8; S, 8·6. $C_{16}H_{18}O_6N_2S$ requires C, 52·5; H, 4·9; N, 7·7; S, 8·7 per cent.

O-*Formyl*-DL-*chloramphenicol*, prepared by heating DL-chloramphenicol (1 g.) with formic acid (10 ml.) under reflux for 1 hour, crystallised from aqueous ethanol in flat needles, m.pt. 146° C. Found: C, 41·0; H, 3·4; N, 8·1; Cl, 20·0. $C_{12}H_{12}O_6N_2Cl_2$ requires C, 41·0; H, 3·4; N, 7·9; Cl, 20·2 per cent.

4-p-*Nitrophenyl*-5-*dichloroacetamido*-1:3-*dioxane* was prepared by heating DL-chloramphenicol (1 g.) with solution of formaldehyde (2 ml. of 36 per cent.) and formic acid (10 ml.) for 1 hour on the steam bath. After crystallisation from aqueous ethanol it formed long flat needles, m.pt. 147° to 148° C. Found: C, 42·9; H, 3·5; N, 8·5; Cl, 21·1. $C_{12}H_{12}O_5N_2Cl_2$ requires C, 43·0; H, 3·6; N, 8·4; Cl, 21·2 per cent.

DL-threo-2-*Form-methylamido*-1-p-*nitrophenylpropane*-1:3-*diol* (X; R = –CHO).—The DL-*threo*-amine (10 g.) was covered with ethanol and then treated with solution of formaldehyde (5 ml. of 36 per cent., 1 mol.), when immediate reaction occurred with dissolution. After heating for 10 minutes at 100° C., formic acid (50 ml.) was added and heating continued for a further 90 minutes. After evaporation of most of the formic acid the residue was dissolved in water (50 ml.) and neutralised with potassium carbonate. Potassium hydroxide (2·5 g.) was then added and the mixture heated to saponify any *O*-formates present. After allowing to cool the product was collected and crystallised from ethyl acetate-methanol. DL-threo-2-*Form-methylamido*-1-p-*nitrophenylpropane*-1:3-*diol* (3·2 g.) formed cream prisms, m.pt. 196° C. Found: C, 51·4; H, 5·4; N, 11·1. $C_{11}H_{14}O_5N_2$ requires C, 52·0; H, 5·5; N, 11·0 per cent.

DL-threo-2-*Methylamino*-1-p-*nitrophenylpropane*-1:3-*diol hydrochloride*, prepared by hydrolysis of the foregoing compound (2·8 g.) with 4 N hydrochloric acid (30 ml.) for 1 hour at 100° C., formed needles (2·2 g.), m.pt. 176° to 177° C., after crystallisation from ethyl acetate-methanol. Found: C, 45·3; H, 5·7; N, 10·5; Cl, 13·6. $C_{10}H_{14}O_4N_2{\cdot}HCl$ requires C, 45·7; H, 5·7; N, 10·7; Cl, 13·5 per cent.

The corresponding *base* (X; R = H) was crystallised from ethyl acetate forming prisms, m.pt. 107° C. Found: C, 53·1; H, 6·2; N, 12·4. $C_{10}H_{14}O_4N_2$ requires C, 53·1; H, 6·3; N, 12·4 per cent. Its *picrate* separated from water in flat yellow needles, m.pt. 186° to 187° C. Found: C, 42·1; H, 4·0; N, 15·1. $C_{10}H_{14}O_4N_2$ requires C, 42·2; H, 3·7; N, 15·4 per cent.

DL-threo-2-*Dichloroacetmethylamido*-1-p-*nitrophenylpropane*-1:3-*diol* (X; R = $-COCHCl_3$) was prepared by treating the corresponding amine (1·0 g.), suspended in cold ethanol (10 ml.), with pentachloroacetone/tetrachloroacetone (3·5 g. of 33 per cent. pentachloroacetone; 1 mole) when a vigorous reaction took place. After 15 minutes, light petroleum (20 ml.) was added and the product collected after 24 hours at 0° C. Purification from ethanol-light petroleum gave prisms, m.pt. 141° to 142° C. Found: C, 42.6; H, 4·0; N, 8·0; Cl, 20·6. $C_{12}H_{14}O_5N_2Cl_2$ requires C, 42·7; H, 4·2; N, 8·3; Cl, 21·1 per cent.

Phthalimido-acetal.—Potassium phthalimide (50 g.), bromoacetal (50 g.)

and potassium iodide (50 g.) were heated together in ethylene glycol (150 ml.) for 4 hours at 145° C. The mixture was poured into water and extracted with ether. The residue left after evaporation partially crystallised. The crystalline material was separated with the aid of light petroleum and the filtrate distilled. After a small forerun of bromo-acetal, *phthalimido-acetal* was collected at 180° C./0·5 mm. It was combined with the crystalline material and purified from light petroleum to give plates (30·0 g.), m.pt. 69° C. Found: C, 63·9; H, 6·5. $C_{14}H_{17}O_4N$ requires C, 64·0; H, 6·5 per cent.

Phthalimidoacetaldehyde.—(i) The foregoing compound (10 g.) and N hydrochloric acid (60 ml.) were heated at 100° C. for 20 minutes with stirring. The product crystallised on cooling. It was collected (7 g.) and purified from chloroform-light petroleum, m.pt. 112° C.

(ii) Phthalylglycyl chloride (100 g.), dry xylene (500 ml.), 5 per cent. palladium-barium sulphate catalyst and quinoline-sulphur catalyst poison[22] (1 ml.) were heated under reflux with stirring in a stream of hydrogen for 10 hours. The solution was filtered hot and cooled to 0° C. *Phthalmidoacetaldehyde* (45 g.) separated, a second crop (19 g.) being obtained by evaporation, m.pt. 111° C. Total yield 75 per cent. (cf. Radde[23]).

Phthalimidoacetaldehyde diacetate, prepared by treating the aldehyde (500 mg.) in cold acetic anhydride (5 ml.) with concentrated sulphuric acid (2 drops) for 12 hours at room temperature, was crystallised from chloroform-light petroleum to give prisms, m.pt. 128° to 129° C. Found: N, 4·7. $C_{14}H_{13}O_6N$ requires N, 4·8 per cent.

Acetamidoacetaldehyde.—A cooled solution of aminoacetal (6·6 g.) in ether (30 ml.) was treated with acetic anhydride (6·5 g.) in ether (10 ml.) with stirring. After 1 hour the solution was evaporated to dryness under reduced pressure and the residue distilled at 10 mm. Acetamidoacetal (7·3 g.) was obtained as a colourless oil, b.pt. 136° to 138° C./10 mm. Found: C, 54·6; H, 9·6; N, 8·2. $C_8H_{17}O_3N$ requires C, 54·9; H, 9·7; N, 8·0 per cent.

Addition of a small quantity of acetamidoacetal to 2:4-dinitrophenylhydrazine in 5 N hydrochloric acid led to separation of *acetamidoacetaldehyde 2:4-dinitrophenylhydrazone*, soft golden needles, m.pt. 182° C., after crystallisation from ethanol. Found: C, 42·9; H, 3·9; N, 25·0. $C_{10}H_{11}O_5N_5$ requires C, 42·7; H, 3·9; N, 24·9 per cent.

Attempts to liberate acetamidoacetaldehyde from its acetal proved unsuccessful owing to decomposition. Attempts to condense the crude material with *p*-nitrobenzaldehyde likewise proved unsuccessful.

Toluenesulphonamidoacetal was prepared by treating aminoacetal (2·6 g.) in dry pyridine with *p*-toluenesulphonylchloride (3·8 g.) added in portions with ice-cooling, followed by reaction at room temperature overnight. After crystallisation from ether-light petroleum, it formed flat needles, m.pt. 68° to 69° C. Found: C, 53·4; H, 6·8; N, 5·0; S, 11·5. $C_{13}H_{21}O_4NS$ requires C, 54·0; H, 7·3; N, 4·9; S, 11·1 per cent.

Toluenesulphonamidoacetaldehyde 2:4-dinitrophenylhydrazone, after

crystallisation from ethanol, formed yellow leaflets, m.pt. 184° to 185° C. Found: N, 17·3. $C_{15}H_{15}O_6N_5S$ requires N, 17·8 per cent.

Pivalyl-aminodiethylacetal.—Aminodiethylacetal (5·4 g.) in ether (35 ml.) was treated with 3 ml. of a solution of potassium hydroxide (6·5 g.) in water (15 ml.) with stirring and cooling at 0°. Pivalyl chloride (5 g.) and the remainder of the potassium hydroxide solution were added drop by drop simultaneously over 10 minutes with cooling and stirring. The mixture was stirred at room temperature for 4 hours. The ethereal layer was separated, washed twice with saturated sodium chloride solution, dried and the ether removed. The residual solids (8·04 g.) were crystallised from light petroleum (b.pt. 40° to 60° C.) at −70° C. to give pivalyl-aminodiethylacetal, needles, m.pt. 43° to 44° C. Found: C, 60·6; H, 10·6; N, 6·5. Calc. for $C_{11}H_{23}O_3N$: C, 60·8; H, 10·6; N, 6·5 per cent.

Attempts to hydrolyse the acetal to the corresponding aldehyde proved unsuccessful.

p-*Nitrobenzylidene-phthalimidoacetaldehyde* (cf. XIII).—Phthalimidoacetaldehyde (950 mg.) and *p*-nitrobenzaldehyde (750 mg.), dissolved in ethanol (8 ml.), were treated with 2 drops of piperidine. After allowing to evaporate spontaneously at room temperature for 7 days, the product (90 mg.) was collected and purified from ethanol. *p-Nitrobenzylidene-phthalimidoacetaldehyde* formed cream needles, m.pt. 176° to 177° C. Found: C, 63·3; H, 3·2; N, 8·7. $C_{17}H_{10}O_5N_2$ requires C, 63·3; H, 3·1; N, 8·4 per cent.

Replacement of piperidine by other basic catalysts failed to give products which could be identified.

2-*Benzamido*-p-*nitrocinnamaldehyde* (XIII).—Benzamidoacetaldehyde[24] (820 mg.) and *p*-nitrobenzaldehyde (750 mg.) in ethanol (5 ml.) containing collidine (4 drops) were heated under reflux for 7½ hours. After allowing to cool the separated solids (56 per cent.) were collected and purified from chloroform. 2-*Benzamido*-p-*nitrocinnamaldehyde* formed yellow microcrystals, m.pt. 193° C. Found: C, 64·8; H, 4·2; N, 9·6. $C_{16}H_{12}O_4N_2$ requires C, 64·9; H, 4·1; N, 9·5 per cent.

The *semicarbazone*, after crystallisation from methanol, formed felted needles, m.pt. 210° to 225° C. (decomp.). Found: N, 19·6. $C_{17}H_{15}O_4N_5$ requires 19·8 per cent.

2-*Benzamido*-p-*nitrocinnamyl alcohol* (XIII) was prepared by Ponndorf reduction of the foregoing compound. After crystallisation from benzene it formed microneedles, m.pt. 175° C. Found: C, 64·3; H, 4·8. $C_{16}H_{14}O_4N_2$ requires C, 64·4; H, 4·7 per cent.

The *acetate* was crystallised from aqueous ethanol to form silky needles, m.pt. 145° C. Found: C, 63·6; H, 4·5; N, 8·1. $C_{18}H_{16}O_5N_2$ requires C, 63·5; H, 4·7; N, 8·2 per cent.

Hydrolysis of (XIII).—the foregoing compound (1·0 g.) in ethanol was heated under reflux with concentrated hydrochloric acid (0·5 ml.) for 30 minutes. After allowing to cool the separated solids were removed (*fraction A*) and the filtrate neutralised with sodium bicarbonate when a precipitate (*fraction B*) was obtained.

Purification of *fraction A* from aqueous acetone gave *2-keto-3-p-nitrophenylpropyl benzoate*, plates, m.pt. 162° to 163° C. Found: C, 64·7; H, 4·8; N, 4·3. $C_{16}H_{13}O_5N$ requires C, 64·2; H, 4·3; N, 4·7 per cent. The compound formed an oxime, m.pt. 121° to 123° C., after crystallisation from aqueous ethanol. Alkaline hydrolysis led to the formation of benzoic acid.

Purification of *fraction B* from aqueous methanol gave brown prisms, m.pt. 121° C. Found: C, 65·3; H, 4·6; N, 8·9. $C_{17}H_{16}O_4N_2$ requires C, 65·4; H, 5·1; N, 9·0 per cent.

Summary and Conclusions

1. The preparation and biological activity of some methyl-homologues of chloramphenicol are described.

2. It is concluded that the pharmacodynamical groups present in the antibiotic fall into two categories (i) specific, and (ii) relatively non-specific, designated herein as σ- and ν-groups respectively.

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

References

1. Feitelson, Gunner, Moualim, Petrow, Stephenson and Underhill, *J. Pharm. Pharmacol.*, 1951, **3**, 149.
2. Rebstock, *J. Amer. chem. Soc.*, 1951, **73**, 3671.
3. Controulis, Rebstock and Crooks, Jr., *ibid.*, 1949, **71**, 2463.
4. Long and Troutman, *ibid.*, 1949, **71**, 2473.
5. Long and Troutman, *ibid.*, 1951, **73**, 481.
6. Šorm, Gut, Suchý and Reichl, *Collection Czech. Chem. Communs.*, 1950, **15**, 501.
7. Parke, Davis and Co., B.P. 673,864/1952.
8. von Auwers, *Liebig's Ann.*, 1920, **420**, 91.
9. Skita, *Ber. dtsch. chem. Ges.*, 1920, **53**, 1792.
10. Long and Troutman, *J. Amer. chem. Soc.*, 1949, **71**, 2469.
11. Huebner and Schultz, *ibid.*, 1951, **73**, 2089.
12. Rumpel, *Arch. Pharm.*, 1899, **237**, 222.
13. Fritsch, *Liebig's Ann.*, 1897, **297**, 312.
14. Wooley, *J. biol. Chem.*, 1950, **185**, 293; see also *Antimetabolites*, Wiley and Sons, Inc., 1952, p. 140.
15. Molho and Molho-Lacroix, *Bull. Soc. Chim. biol., Paris*, 1950, **32**, 680.
16. Dunitz, *J. Amer. chem. Soc.*, 1952, **74**, 995.
17. Buu-Hoi, Hoan, Jacquignon and Khoi, *C.R. Acad. Sci., Paris*, 1950, **230**, 662.
18. Parke, Davis and Co., U.S.P. 2,568,511/1951.
19. Parke, Davis and Co., U.S.P. 2,543,267/1951.
20. Long and Troutman, *J. Amer. chem. Soc.*, 1951, **73**, 542.
21. Long and Troutman, *ibid.*, 1951, **73**, 481.
22. *Organic Reactions*, Vol. **4**, 368.
23. Radde, *Ber. dtsch. chem. Ges.*, 1922, **55**, 3174.
24. *The Chemistry of Penicillin*, Princeton University Press, 1949, p. 483.

DISCUSSION

The paper was presented by Dr. F. Hartley.

Mr. D. E. Seymour (Welwyn) asked for more details of the biological screening tests which had been carried out to determine the activity of the various compounds. He thought it would also be interesting to know whether the reduction of the nitro-group of chloramphenicol would alter its activity, particularly to *M. tuberculosis*.

DR. A. H. BECKETT (London) welcomed the authors' emphasis on the stereochemical configuration, and asked whether the physicochemical properties of the substances had been dealt with before the stereochemical mechanism was postulated.

DR. F. HARTLEY, in reply, said that in Part I of this series of papers the general procedure for the biological examination of the compounds had been indicated. The majority had been tested against *M. tuberculosis* as well as *Entamœba histolytica.* The range of organisms in the later work had been extended to include *B. coli*, *B. dysenterica*, and *B. typhosus.* In each case the activity fell from about 1 in 125,000 to 1 in 4,000 (bacteriostatic) and from about 1 in 20,000 to about 1 in 1000 (bactericidal). With regard to the nitrogroup, many would like to see some means of removing this radical. In acylated reduced nitro-compounds some activity was retained and also in the iodo-compound, but not in the corresponding chloro- and bromo-compounds. The stereochemical problem was intriguing. The puzzling feature was that 3-methylchlorramphenicol had 4 possible DL forms, but only 3 had so far revealed themselves. The form isolated in America had, in the present authors' view, a "*threo*" configuration on the basis of the von Auwers-Skita generalisation regarding catalytic hydrogenation. The physico-chemical properties of the β-isomer now isolated were in line with those expected of an "*erythro*" form, in particular the solubility was greater than that of the γ-stereoisomer resulting on treatment with thionyl chloride. Although the "*erythro*" compounds in this series were in general more soluble than the "*threo*" compounds it was not easy to see why that should be so.

CHANGES IN THE HYDROCYANIC ACID CONTENT OF CHLORODYNE ON STORAGE

By H. A. GLASTONBURY

From the Analytical Control Division of May and Baker, Ltd.

Received July 9, 1952

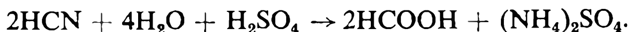
DURING an investigation into the possibility of the loss from Tinct. Chlorof. et Morph. B.P.C. (chlorodyne) of the volatile constituents after manufacture, a considerable decrease in the hydrogen cyanide content was observed in specially sealed control samples. This decrease was larger than could have occurred by volatilisation during the short periods for which samples were necessarily open during the experiments. 2 check samples drawn from stock manufactured at the beginning of this year and 3 samples of unknown age purchased from 3 other companies were found to have apparently lost between 80 per cent. and 93 per cent. of the theoretical hydrogen cyanide content. The more important reactions which could take place in chlorodyne resulting in a loss of free hydrogen cyanide have therefore been investigated.

THEORETICAL DISCUSSION

The 3 more important reactions which could lead to an apparent loss of hydrogen cyanide are (*a*) polymerisation, (*b*) hydrolysis and (*c*) addition to reducing sugars.

(*a*) Lewcock[1] showed that the apparent decomposition of aqueous solutions of hydrocyanic acid was due to the presence of traces of alkali cyanide or of alkali dissolved from the glass container and could be retarded by the addition of mineral acid; the addition of 1 per cent. of sulphuric acid (calculated on the hydrogen cyanide content) considerably retarded the decomposition and he suggested that "if as much as 10 per cent. of sulphuric acid (calculated on hydrogen cyanide content) could be added, such solutions for practical purposes would keep indefinitely." Walker and Eldred[2] have shown that the decomposition is due to polymerisation to a dark brown amorphous substance containing C, O, N and H in variable proportions. The polymerisation is initiated by alkaline impurities, and the polymer itself is a catalyst for the decomposition. Adams and Green[3] have shown that even traces of ammonia and ammonium salts catalyse the polymerisation "with hydrogen cyanide concentrations ranging from very dilute. . . ." Dilute hydrocyanic acid usually contains added sulphuric acid to inhibit polymerisation, and of the other constituents of chlorodyne chloroform is known to have the same effect.[4]

(*b*) Hydrogen cyanide can be lost by acid hydrolysis to formic acid and ammonia.



$$2HCN + 4H_2O + H_2SO_4 \rightarrow 2HCOOH + (NH_4)_2SO_4.$$

Krieble and McNally[5] and Krieble and Peiker[6] studied the kinetics of this reaction and found it to be catalysed principally by the undissociated

mineral acid molecule but that sulphuric acid is a poor catalyst compared with other mineral acids. Loss of hydrogen cyanide is possible by this reaction since chlorodyne may contain approximately 0·003 per cent. of sulphuric acid; up to 0·06 per cent. of sulphuric acid may be added to dilute hydrocyanic acid as a stabiliser.

(*c*) Killiani[7,8] showed that hydrogen cyanide reacts additively with dextrose to form a cyanhydrin, the reaction being accelerated by traces of ammonia. The function of the basic catalyst has been treated by Lapworth.[9] Cyanhydrins are hydrolysed to 2-hydroxy acids with liberation of ammonia,[7] and therefore hydrogen cyanide removed from solution by reaction with dextrose cannot be recovered by hydrolysis.

Experimental

The formula for chlorodyne requires 0·125 per cent. of hydrogen cyanide. Aqueous solutions of hydrogen cyanide of this concentration and freshly prepared chorodyne solutions were treated as shown in Tables I and II and stored in flasks having ground-glass stoppers which were sealed with soft paraffin after each withdrawal of samples for periodic determination of hydrogen cyanide. The reducing sugar content of the treacle and liquorice used and the ammonia content of the liquorice and of a chlorodyne sample before and after storage were also determined.

According to Childs and Ball[10] hydrogen cyanide is readily volatile in steam, and recovery by distillation is 99 per cent. quantitative. 30 ml. of mixtures of 50 ml. of chlorodyne diluted with 50 ml. of water were distilled into 20 ml. of water containing 5 ml. of dilute ammonia solution and 5 drops of potassium iodide solution and the distillate was titrated with 0·05N silver nitrate. Recovery experiments confirmed Childs and Ball's findings with a reproducibility of better than 1 per cent.

Appropriate dilutions of treacle and of liquorice were clarified with zinc acetate and potassium ferrocyanide,[11] the reducing sugar was determined by titrating Fehling's solution by Lane and Eynon's method[12] and calculated as dextrose.

Ammonia was determined by distillation from an alkaline solution of treacle and of liquorice into 0·1N sulphuric acid followed by back titration with 0·1N sodium hydroxide to screened methyl red indicator. The addition of a few drops of amyl alcohol to the contents of the distillation flask prevented excessive frothing.

Discussion of Results

From Table I it is seen that there is no loss of hydrogen cyanide by volatilisation under the experimental conditions (1), neither acid hydrolysis nor ammonia catalysed polymerisation affecting the hydrogen cyanide content, at least in the early stages (2) (3). The rate of the addition reaction with reducing sugar is small in the presence of mineral acid or ammonium salt (4), (5), but the presence of ammonia increases the rate of reaction probably by its catalytic effect on the mutarotation of glucose and on the addition reaction (6).

Table II shows that the loss of hydrogen cyanide in chlorodyne is

TABLE I

THE EFFECT OF ADDED REAGENTS ON THE HYDROGEN CYANIDE CONTENT OF 0·125 PER CENT. AQUEOUS SOLUTIONS

Added reagent	Time in days	Loss per cent.
1. Sulphuric acid, 0·0006 per cent. ..	15	Nil
2. Sulphuric acid, 0·06 per cent.	15	Nil
3. Ammonia, 0·02 per cent.	15	Nil
4. Dextrose, 1 per cent.	7	1
5. Dextrose, 1 per cent. ammonium chloride, 0·1 per cent. ..	7	1
6. Dextrose, 1 per cent.	0·1	38
ammonia, 0·1 per cent.	2	95

TABLE II

THE LOSS OF HYDROGEN CYANIDE FROM CHLORODYNE ON STORAGE AND THE EFFECT OF CONTAINER, CONSTITUENTS AND ADDED REAGENTS

	Time in days	Loss per cent.
1. Glass container	6	14
	14	39
	37	63
2. Glass container	5	16
	13	39
	21	51
3. Polythene container	4	13
4. Ammonium chloride, 0·1 per cent. added	4	12
	17	40
5. Ammonia, 0·1 per cent. added	0·05	30
	0·10	60
	0·15	80
	1	90
	3	95
6. Chlorodyne solution prepared without treacle	6	10
	14	26
	23	33
	28	42

progressive and the rate gradually declines with decreasing concentration (1), (2). Storage in an inert (polythene) container has no retarding effect (3). The addition of ammonium salt does not accelerate the reaction (4) but the effect of free ammonia is striking (5). The catalytic agent is present in the liquorice (6) which itself contains sufficient reducing sugar to react with the hydrogen cyanide.

The ammonia recovered by distillation from alkaline solutions of a sample of chlorodyne increased from 0·024 per cent. when freshly prepared to 0·076 per cent. after storage for 1 month, an increase of 0·052 per cent. The hydrogen cyanide content of the sample fell from 0·125 per cent. to 0·034 per cent., a decrease of 0·091 per cent. Had this quantity of hydrogen cyanide reacted with reducing sugar, alkaline hydrolysis of the cyanhydrin formed would have liberated an additional 0·057 per cent. of ammonia. The agreement between the calculated and the experimental figures is consistent with the postulated addition reaction.

The reducing sugar contents, calculated as dextrose, of the samples of treacle and liquorice used were 21 per cent. and 8 per cent. respectively. The ammonia content of the liquorice was 0·14 per cent. There is therefore a sufficiently large excess of each component to permit an ammonia catalysed addition reaction with the hydrogen cyanide.

Consideration has been given to the stabilisation of chlorodyne and a method of maintaining the theoretical hydrogen cyanide content has been examined; the results of this work will be published later.

SUMMARY

1. Samples of chlorodyne from various manufacturers have been found to have lost as much as 90 per cent. of the theoretical hydrogen cyanide content.

2. The possible reasons for this have been examined. The experimental evidence suggests that hydrogen cyanide is removed by reaction with the carbonyl group of dextrose catalysed by ammonia derived from the liquorice extract.

I am indebted to Dr. Hersant and Mr. Ballard for their interest in this work, to Miss J. Charlton and Miss S. Spice for technical assistance and to the Directors of Messrs. May and Baker for permission to publish.

REFERENCES

1. Lewcock, *Pharm. J.*, 1918, **101**, 50.
2. Walker and Eldred, *Industr. Engng. Chem.*, 1925, **17**, 1074.
3. Adams and Green, U.S.Pat. 2,069,543.
4. Bebie, *Manual of Explosives, Military Pyrotechnics and Chemical Warfare Agents*, Macmillan, N.Y., 1943, p. 160.
5. Krieble and McNally, *J. Amer. chem. Soc.*, 1929, **51**, 3368.
6. Krieble and Peiker, *ibid.*, 1933, **55**, 2326.
7. Killiani, *Ber. dtsch. chem. Ges.*, 1886, **19**, 767, 1128.
8. Killiani, *ibid.*, 1888, **21**, 956.
9. Lapworth, *J. chem. Soc.*, 1903, **83**, 995.
10. Childs and Ball, *Analyst*, 1935, **60**, 294.
11. Nicholls, *Aids to the Analysis of Food and Drugs*, Baillière, Tindall and Cox, 1942, 82.
12. Lane and Eynon, *Analyst*, 1923, **48**, 221.

DISCUSSION

The paper was presented by MR. H. A. GLASTONBURY.

DR. W. MITCHELL (London) said that he had confirmed the loss of hydrocyanic acid from chlorodyne and enquired whether the bases in liquid extract of liquorice had been determined by distillation with alkali, or by straight distillation without alkali, and whether ammonia had been identified in the distillate. In his own experiments with liquorice extract to which no ammonia had been added he had found 0·01 per cent. of bases calculated as ammonia by straight distillation and 0·14 per cent. by distilling with strong alkali. He could understand that free ammonia might catalyse the reaction, but doubted whether the bases naturally present in the liquid extract would do so. Chlorodyne made with this liquorice extract lost 5 per cent. of hydrocyanic acid in 24 hours. When chlorodyne was made with liquid extract of liquorice previously adjusted to *p*H 5·3 with either ammonia or caustic soda, a loss of 30 per cent. of hydrocyanic acid resulted in 24 hours, suggesting that the loss of stability was a *p*H effect.

DR. D. C. GARRATT (Nottingham) stated that if liquid extract of

liquorice were distilled in the presence of mild alkali, e.g., magnesium oxide, no ammonia was evolved. Ammonia was only obtained if a strong alkali were used. His associates had observed over many years that chlorodyne made with extract of liquorice to which no ammonia had been added lost its hydrocyanic acid content almost immediately. Within 2 days the hydrocyanic acid present had dropped from 1·25 per cent. to 0·04 to 0·05 per cent. He questioned the conclusion of the author that the loss of hydrocyanic acid was due to a reaction catalysed by ammonia.

MR. N. L. ALLPORT (London) asked whether the authors had made any chlorodyne with liquid extract of liquorice made strictly in accordance with B.P. instructions, because according to the B.P. specification ammonia should not be present.

DR. F. HARTLEY (London) asked whether, in view of the analytical findings of the author, there was any justification for including hydrocyanic acid in chlorodyne.

MR. J. H. OAKLEY (London) said that hydrocyanic acid was a nuisance from the forensic angle and the loss due to volatilisation must not be forgotten. He was not convinced that it was worth adding. If the loss be made up, was there a danger that the hydrocyanic acid added in the first instance might have entered into some combination which still had toxic properties? The analytical specification should be in accordance with the keeping properties of the preparation.

MR. V. REID (London) asked whether, in view of the fact that hydrocyanic acid showed such loss on storage it would be possible to use cyanate. Would this have the same therapeutic effect as well as preventing loss?

MR. A. F. CALDWELL (Singapore) emphasised that the keeping properties of preparations was an all-important question in tropical countries, and said that he could see no value from the pharmacological point of view in the inclusion of hydrocyanic acid in chlorodyne.

DR. F. WOKES (King's Langley) stated that there was evidence that cyanides might be important biologically, and before specifying a given quantity of free or combined cyanide it was necessary to consider the biological effect. Cyanates were comparatively inert in the human body, but there was still a lot to be learnt of the metabolism of both cyanides and cyanates. He drew attention to a discrepancy between the method of expressing the experimental figure for the loss of hydrocyanic acid and the statement in the summary that as much as 90 per cent. had been lost.

MR. H. A. GLASTONBURY, in reply, said he was not concerned with the pharmacological question as to whether hydrocyanic acid should be present or not. Determinations had been carried out of the ammonia content of liquid extract of liquorice supplied by different manufacturers, and in all cases 0·2 per cent. of ammonia was found to be present, as determined by distillation in the presence of caustic soda. Attempts had been made to determine ammonia by straight distillation from an aqueous solution, and only approximately 0·05 per cent. had been found but

because the liquorice caused excessive frothing, it had been necessary to discontinue the determination after half an hour. He had found that chlorodyne to which sulphuric acid had been added to bring the acidity to 0·1 N still showed similar loss of hydrocyanic acid and he did not consider the loss to be a *p*H effect. He still considered it probable that cyanide ions reacted with reducing sugars and that the reaction was catalysed by a basic substance, possibly ammonia.

THE APPLICATION OF EMISSION SPECTROGRAPHY TO PHARMACEUTICAL ANALYSIS

By L. Brealey, D. C. Garratt and K. A. Proctor

From the Physical Assay Division, Standards Department, Boots Pure Drug Co., Ltd.

Received July 2, 1952

Although spectrographic methods of analysis are initially expensive when compared with the more classical methods because of the high cost of much of the equipment used, nevertheless such methods, particularly those involving emission spectrography are becoming increasingly popular. This is because in some cases the work cannot be done by purely chemical means, while at other times emission spectrography may prove more accurate, and certainly in the majority of instances there is a considerable saving of time. The last point is of paramount importance, particularly in large laboratories concerned with the control of manufacturing processes. Another reason for favouring such methods is the small amount of sample needed and the fact that even on this small amount the presence of a considerable number of elements can be determined qualitatively or quantitatively.

Emission spectrography has been in use for some time for the determination of traces but its application to pharmaceutical analysis, which demands also the estimation of major constituents, has not been widely reported, probably because it was not considered sufficiently accurate for this purpose. The introduction of new techniques, particularly those involving solution methods has enabled these difficulties to be overcome. One of the most tedious and certainly the most difficult feature of methods involving the spectrographic analysis of powders is the preparation of homogeneous standards and samples, but the use of solution methods obviates this difficulty. In this way solution methods have a great advantage over the older powder methods and the increased accuracy associated with them makes them ideally suitable for application to pharmaceutical analysis. In our laboratory two such methods are finding increasing application and our experience has shown that in both cases an accuracy within 2 per cent. can be obtained in routine assays. These two methods are those involving flame photometry and the porous-cup technique.

Flame Photometry

The use of flame photometers of varying design for the simple determination of the alkalis has become fairly widespread. In planning and building the flame photometer in use in this laboratory a wider application was envisaged and already the range of elements examined by this method has been considerably extended. The instrument is one described by Brealey and Ross[1] and can be used with either an air–propane or an air–acetylene flame. The use of such an instrument for the estimation of sodium and potassium is well known and indeed it has proved so

successful in this laboratory that these two elements are not normally determined by any other method. The routine control of fertilisers is carried out by this method and Table I, taken from the paper by Brealey,[2] will serve to show the agreement between results obtained by the official[3] chemical method and by flame photometry.

TABLE I

Sample	Potassium oxide, K_2O	
	Flame photometry per cent.	Chemical per cent.
G1	8·07	8·12
G2	7·66	7·73
G3	8·77	8·61
G4	10·00	10·27
G5	7·95	7·96
G6	8·20	8·20
T1	11·65	11·58
T2	10·40	10·29
T3	12·25	11·93
T4	10·40	10·53
T5	9·05	8·83
T6	9·88	9·74

The starting material for the manufacture of all potassium salts is either potassium carbonate or potassium hydroxide, which always contain some of the corresponding sodium compound. This contamination subsequently appears to a greater or less extent in the finished product, depending on the particular salt being manufactured. A great deal of interest has been shown recently in this problem, and flame photometry offers the ideal method of determining sodium in potassium salts. The examination of a number of potassium salts (Table II) has revealed the fact that this contamination is often considerable and there is no doubt that the future will see ever-increasing use of this method for such determinations because of the small amount of time and labour needed in carrying them out once the method has been established. In the past such estimations have been somewhat neglected, for want of a quick, reliable method.

TABLE II

Potassium salt	*Corresponding sodium* salt per cent.
Citrate	2·4, 5·1, 7·6, 8·8
Acetate	5·4, 5·7
Iodide	0·31, 0·22, 0·42, 0·34
Chloride	0·72
Bromide	0·14, 1·2, 0·54, 1·0
Sulphate	7·1, 5·6, 3·1, 3·5
Chlorate	0·02
Bicarbonate	0·15
Acid tartrate	0·49
Carbonate	3·0, 4·5, 1·0, 1·9
Hydroxide	1·0, 0·91

The preparation of the samples for examination could not be more simple. In many cases all that is needed is the solution of the sample in water and it is a routine matter for one person to prepare and examine 20 such samples in an hour. When the sample is an organic salt, preliminary preparation is sometimes required and this entails ashing the sample or submitting it to a wet oxidation. It is usually an easy problem to find means of extracting any required element from an insoluble material.

Before the estimation of a particular element can become a routine process a certain amount of investigational work has to be carried out on the photometric side of the method in order to determine the sensitivity range of the element and also the extent of interference from extraneous elements. The following determinations of lithium may be cited as examples of how such problems are approached.

The first was a determination of lithium and was required for its estimation in "kidney pills" which contain 6 per cent. of lithium benzoate in a vegetable extract base. As no simple specific chemical method was

available the problem was submitted for investigation by flame photometry.

Since the vegetable extract contains sodium and potassium salts it was decided to use the air–propane flame because it is known that this flame exhibits less interference from cations than the hotter air–acetylene flame. Standard solutions of 1000, 100 and 10 p.p.m. of lithium in water were prepared and examined. It was found that a full scale deflection of the galvanometer could be obtained with the 10 p.p.m. standard using the 6707 Å line. Further standards of 2, 4, 6, 8 p.p.m. of lithium were then prepared and the readings obtained showed that lithium gives a linear response over the range 0 to 10 p.p.m.

The effect of the sodium, potassium and benzoate ions was then studied and it was shown that the benzoate ion in large excess of the amount actually combined with the lithium in the pills has no effect on the lithium standards. Similarly potassium has no effect but sodium causes a strong enhancement as shown by Table III.

TABLE III

Li (p.p.m.)	Interfering ion	Concentration of interfering ion (p.p.m.)	Error per cent.
2	Na^+	10	+40
2	,,	100	+70
2	,,	1000	+125
5	,,	10	+6
5	,,	100	+22
5	,,	1000	+58
8	,,	10	+1
8	,,	100	+14
8	,,	1000	>25

Having noted the serious errors caused by the presence of sodium, the actual amount present in the final solution to be used was determined and found to be only 2 p.p.m. It can be seen from Table III that the effect of this amount of sodium on 6 to 7 p.p.m. of lithium is negligible. The following method for the determination of the lithium content of the pills was then adopted: 20 pills were ground and 1 g. of the mixture heated with 100 ml. of water on a steam bath for 20 minutes. The solution was cooled, diluted to 500 ml. and filtered. The filtrate was examined on the flame photometer.

In the above example it can be seen that although interfering elements were present, the amounts were not sufficient to cause inaccuracies in the lithium estimation. These suitable conditions are not however always present and other methods have to be adopted to overcome interference effects.

An example of this is the estimation of lithium in effervescent granules of lithium citrate. These contain about 0·3 per cent. of lithium together with about 40 times as much sodium, and the possibility of determining the lithium content by flame photometry was investigated. It was already known from the work carried out on the "kidney pills" that over the range 0 to 10 p.p.m. lithium gives a linear response and that sodium has a considerable enhancement effect, which we think is due to the background

caused by the close proximity of the sodium doublet at 5890/6 Å to lithium at 6707 Å. Since the sodium could not be removed chemically the possibility of correcting in some way for its effect was examined.

The first method investigated, although abortive, is instructive as an illustration of the approach that is made to the solution of such problems. It was thought that a reading taken at the lithium wavelength (6707 Å) would represent the lithium plus sodium and that a reading taken at some nearby wavelength would represent the sodium alone, so that the difference between these two readings would give a measure of the lithium.

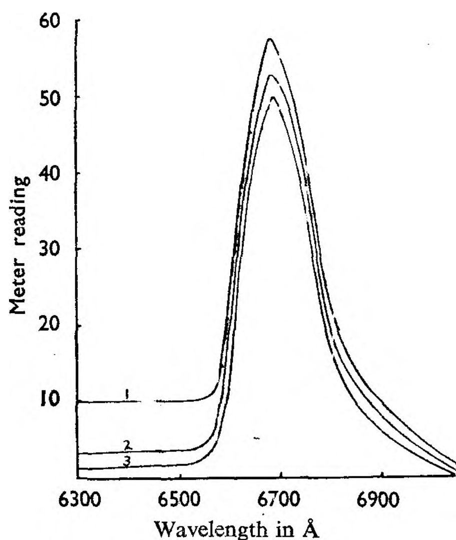


FIG. 1.

1. 5 p.p.m. of lithium + 300 p.p.m. of sodium.
2. 5 p.p.m. of lithium + 50 p.p.m. of sodium.
3. 5 p.p.m. of lithium.

The spectrum of combined lithium and sodium standards was therefore examined over the region of 6000 Å to 7000 Å with both the propane and acetylene flames and a typical result is shown in Figure 1. It seemed reasonable from these results that the difference between the readings at 6707 Å and 6500 Å could be taken as a measure of the lithium present. Standards of 5 p.p.m. of lithium plus 100, 500, 1000 p.p.m. of sodium were prepared and examined at 6707 Å and 6500 Å. The result using the propane flame is shown on Figure 2 together with the results obtained by subtracting the readings at the two wavelengths. It can be seen that in both cases this method gives an overcorrection as the difference in all cases should be equivalent to 5 p.p.m. of lithium (i.e., a galvanometer reading of 50). This overcorrection is probably due to the fact that there is another lithium line at 6104 Å which causes a small reading at 6500 Å so that the reading at this latter wavelength is not due to the sodium alone. This method was considered unsatisfactory.

TABLE IV

Li p.p.m.	+ Na p.p.m.	(A) Reading	Na p.p.m.	(B) Reading	(A)-(B)	Theoretical reading
3	100	41·0	100	10·5	30·5	30
3	500	52·0	500	22·0	30·0	30
3	1000	64·0	1000	34·0	30·0	30
5	100	60·5	100	10·5	50·0	50
5	500	72·0	500	22·0	50·0	50
5	1000	85·0	1000	34·0	51·0	50
7	100	81·0	100	10·5	70·5	70
7	500	91·0	500	22·0	69·0	70
7	1000	96·0	1000	34·0	62·0	70

In the next method investigated a reading was obtained at 6707 Å, (due to lithium plus sodium), and at the same wavelength a reading was obtained for the sodium alone. Since the reading for the sodium was comparatively low at this wavelength the difference was taken as a measure of the lithium present. The results obtained are shown in Table IV and it can be seen that except for one bad result at 7 p.p.m. of lithium plus 1000 p.p.m. of sodium the results obtained are very close to those expected on theoretical grounds.

This method was then applied to a batch of granules.

Experiment 1

10 g. was dissolved in water and diluted to 1 l. and 10 ml. of this was diluted to 100 ml. (solution A). A further dilution of 10 to 100 ml. of solution A was made to give solution B. The sodium content of solution B was estimated in the normal way on the flame photometer and found to be 14·6 p.p.m. Solution C, containing 146 p.p.m. of sodium, was then prepared. Solutions A and C were then read at 6707 Å and gave the following results: solution A, 50; solution C, 13; difference, 37 ≡ 3·7 p.p.m. of lithium. This is equivalent to a lithium citrate content of 5·0 per cent. for the granules. This result is rather higher than expected (theoretical content is 4·5 per cent.) but the accuracy of the method was eventually proved as follows.

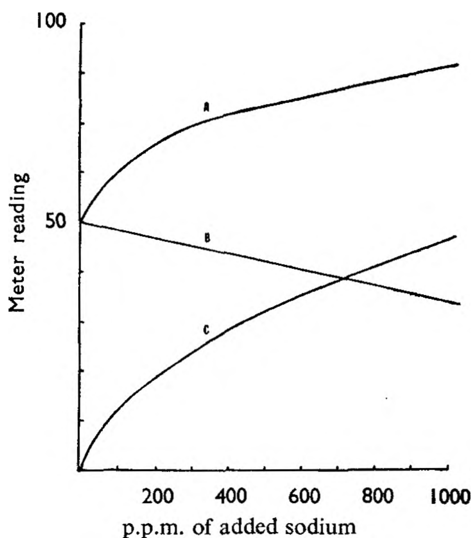


FIG. 2. A. 5 p.p.m. of lithium 6707Å.
B. Difference.
C. 5 p.p.m. of lithium 6500Å.

Experiment 2

Solution A (as in experiment 1) was made stronger by taking a dilution of 15 to 100 ml. of the original granule solution and solution C was 220 p.p.m. of sodium. These were read as in experiment 1 and gave the following results: solution A, 69; solution C, 14; difference 55 ≡ 5·5 p.p.m. of lithium. This is again equivalent to 5·0 per cent. of lithium citrate in the granules.

Experiment 3

This involved a recovery experiment and was carried out by using solution A as in experiment 1 to which the equivalent of 2·0 p.p.m. of lithium was added. Solution C was the same as in experiment 1 and the following results were obtained: solution A + 2 p.p.m. of lithium, 70;

solution C, 13; difference 57 ≡ 5·7 p.p.m. of lithium. The difference between this and solution A (3·7 p.p.m. of lithium) is 2·0 p.p.m. of lithium which is equivalent to a 100 per cent. recovery. From the results obtained there seems no doubt that this method of compensating for the interference effect of sodium on lithium provides a reliable means of estimating lithium in such granules.

TABLE V

Concentration of Ca (p.p.m.)	Normality of PO_4	Error per cent.
50	0·1	−61
50	0·01	−56
50	0·001	−13
5	0·1	−7
5	0·01	−5
5	0·001	−3

The estimation of calcium in the presence of phosphate provides a further means of overcoming the effect of an interfering ion. In this instance the interfering ion depresses the readings due to calcium as shown in Table V. Experiment showed that if increasing amounts of phosphate in the form of ammonium phosphate were added to a fixed amount of calcium a concentration of phosphate was eventually reached beyond which there was no further alteration of the calcium reading. This is illustrated in Table VI.

TABLE VI

Concentration of Ca (p.p.m.)	Concentration of PO_4 (mg./ml.)	Ca found (p.p.m.)
5	0·005	4·85
5	0·01	4·45
5	0·05	4·25
5	0·1	4·3
5	0·5	4·4
5	1·0	4·4
5	1·5	4·4
5	2·0	4·4

Similar results are obtained with solutions containing 50 p.p.m. of calcium and concentrations of phosphate in excess of 0·5 mg./ml. Since no sample examined contains such a high proportion of phosphate in relation to the calcium content, this concentration of phosphate is always added to the solutions of standards and samples prepared for flame photometry. This added phosphate being of very high concentration compared with that present in the sample, eliminates errors due to variations in the amount contained in the sample.

The estimation of the potash content of fertilisers and of the contamination of sodium and potassium salts has been referred to above. Besides these routine applications, flame photometry is used in this laboratory for the estimation of potassium in such preparations as compound syrup of hypophosphites, compound syrup of glycerophosphates and various non-official galenicals. The work has also been extended to the determination of iron and manganese in various preparations. Iron is one of the less sensitive elements when excited in the flame and thus the range of standards used for its determination is 0 to 200 p.p.m. at wavelength 3736 Å with the acetylene flame. Sodium, potassium and calcium all interfere strongly with iron in quantities greater than 100 p.p.m. in the solution being examined and thus the application of flame photometry in this respect is, at the moment, somewhat limited until more work can be carried out with a view to eliminating these effects. Manganese gives a linear response over the range 0 to 100 p.p.m. with

an acetylene flame at 4031 Å and has been estimated in ferrous sulphate after removal of the iron with results which agreed well with those obtained by chemical methods.

Many of the above estimations require nothing more than simple dilutions of the sample before submitting it to flame photometry. Other determinations require more complex manipulation, e.g., for copper in veterinary emulsions it was necessary to extract the sulphated ash 3 times with aqua regia, evaporate the extract to dryness and leach out the soluble copper salt with hot water. The range of standards used was 0 to 200 p.p.m. of copper at 3274 Å with the acetylene flame. The copper content of various bronzing powders has also been determined.

The great advantage of flame photometry lies in its application to routine analyses. However, from time to time, various problems arise which are not a routine matter but which can be most easily resolved by the use of flame photometry. In this laboratory, for instance, the calcium content of chicken bones has been examined as part of a large programme of investigational work and the sodium and potassium contents of various agars have been compared. Contamination problems of widely diverse natures have been investigated.

There can be no doubt of the superiority in many respects of flame photometry over other methods of analysis. The time-saving factor alone makes it worthy of serious consideration. Once solutions have been prepared the results on half-a-dozen samples can be reported within 15 minutes, which compares very favourably with the time taken, for instance, to estimate sodium, potassium and lithium in the presence of each other. In our opinion the latter estimations need the attention of skilled analysts, whereas flame photometric assays can be carried out by junior staff with comparatively little experience or skill. As far as accuracy is concerned flame photometry is at least as reliable as many other methods.

The Porous-cup Technique

Many pharmaceutical powder preparations contain magnesium and silicon, often in the form of magnesium trisilicate and the estimation of these elements by chemical methods is a laborious process. In this laboratory, which is dealing with the routine checking of a large number of process samples, delay was frequently encountered, so it was necessary to look for other means of analysis. The porous-cup technique due to Feldman[4] was investigated and it proved to be a reliable alternative to the chemical method. A modification of this method is now employed in this laboratory for the routine determination of magnesium and silicon in all powders containing magnesium trisilicate. If any technique is to be of use in such applications its accuracy must be high and in our opinion a standard deviation of 2 per cent. is the maximum permissible. It has been shown that the method is capable of this accuracy.

In essence the method consists of pipetting a small quantity of solution into a cup electrode in the form of a narrow graphite cylinder open at the top end and with a base of a standard uniform thickness. Before

use the base is made porous by heating, so that during the passage of a spark the solution in the electrode passes continuously through it into the discharge. The lower electrode consists of solid graphite with the end turned to an 80° cone with a slightly flattened tip. The cup electrodes are easily drilled on a lathe from graphite rods, 10 such electrodes being obtained from 1 rod. The thickness of the base is adjusted by means of a special gauge and checked with the aid of a micrometer. The actual base thickness is not critical and in practice limits of 40/1000 inch ± 3/1000 inch are used, but the base of each electrode must be of consistent thickness otherwise erroneous results are obtained. The lower electrodes are made from 3-inch lengths of graphite rod turned to the required angle with a simple cutter similar in principle to a pencil sharpener, the tip being flattened with a small file. These lower electrodes can be re-sharpened continually until they become too short for further use, but the cup electrodes can only be used once. The cup electrodes in use in this laboratory are somewhat larger than those used by Feldman and the dimensions are shown in Figure 3.

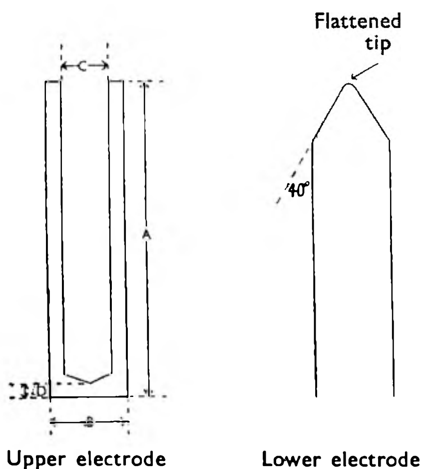


FIG. 3. A = 1 inch.
B = 1/4 inch.
C = 5/32 inch.
D = 40/1000 inch.

During sparking, the solution from the upper cup electrode is fed continuously into the discharge. The estimation may be divided into three stages.

(a) *The preparation of solutions.*—Various methods were tried, including carbonate fusions, but the following technique was eventually evolved and proved to be the most satisfactory in the majority of cases where insoluble materials containing silica were involved.

A quantity of powder estimated to contain between 0·1 g. and 0·15 g. of SiO_2 is accurately weighed into a platinum dish. 2 ml. of hydrochloric acid is added slowly and the mixture evaporated to dryness on a steam bath. 2·5 g. of potash pellets is added and warmed gently over a bunsen flame until liquid. After heating gently for a short time a clear solution results. The mass is allowed to cool and is then dissolved in 20 ml. of water. The resulting solution is acidified with 5 ml. of nitric acid, and transferred to a 50 ml. graduated flask containing 5 ml. of 0·5 per cent. sodium borate solution and 10 ml. of 1 per cent. potassium dichromate solution and diluted to volume. The boron acts as an internal standard for the estimation of the silicon and the chromium for the magnesium.

Standard solutions containing: (1) 0·015 per cent. of SiO_2 + 0·025 per cent. of MgO; (2) 0·025 per cent. of SiO_2 + 0·035 per cent. of MgO; (3) 0·035 per cent. of SiO_2 + 0·045 per cent. of MgO; and equivalent

amounts of potassium hydroxide, nitric acid, sodium borate and potassium dichromate are prepared. If difficulty is encountered in obtaining clear solutions it has been found advantageous to use a 1 : 1 mixture of 60 per cent. perchloric acid and concentrated hydrochloric acid for the initial breakdown of the powder before the treatment with potash is carried out.

(b) *Recording of the Spectrograms.*—Before use, the base of each cup electrode is heated to a dull red heat in a blow lamp for 5 seconds in order to make it porous. These electrodes are mounted in the upper of 2 special stainless steel electrode holders attached to a de Gramont stand placed in such a position that the electrodes are 20·7 cm. from the entrance slit of a Hilger medium quartz spectrograph. An F1028 lens, focal length 16·5 cm., is positioned 2 cm. from the slit. The lower conical electrode is then positioned in the lower holder so that there is a gap of 2 mm. between the top of this electrode and the base of the cup electrode. 0·15 ml. of solution is pipetted into the cup electrode, care being taken to prevent the formation of air pockets which would cause the solution to bubble over when the spark is applied. In order to facilitate the filling of these electrodes the end of the pipette is drawn out into an elongated jet so that it reaches the bottom of the electrode cavity. 30 seconds are allowed to elapse for the solution to seep through the base of the electrode before a spark is applied for 40 seconds. The source unit used is the Hilger 15,000 v. condensed spark with 0·03 mH inductance. Ilford thin film half-tone plates are used to record the spectrograms and it is possible, using a slit height of 1 mm., to obtain 36 such spectra usually consisting of 3 standards and 9 samples each in triplicate on one plate. The plates are developed for 5 minutes in ID2 developer at 18·5° C., fixed for 10 minutes, washed for 20 minutes and allowed to dry.

(c) *Evaluation of the spectrograms.*—From the spectrograms the density differences of the line pairs Si 2516·1Å/B 2497·7Å and Mg 2790·9Å/Cr 2830·4Å are calculated from densitometer readings. Curves are plotted of these density differences against percentages of SiO_2 and MgO and from these the strengths of the unknown solutions are computed.

Silicon and magnesium were the first elements for which this technique was worked out in this laboratory. This involved finding the means of getting the powders into solution, investigating the sensitivity ranges of all the elements concerned, finding suitable internal standards and finally determining the accuracy of which the method was capable. It is of interest to note here that until boron was adopted as the internal standard for the silicon the method proved quite unsatisfactory. No difficulty has been encountered from the presence of boron in the electrodes. It was also proved that there is no interference between the magnesium and silicon; in other words the amount of either element present does not affect the accuracy of the determination of the other.

Table VII shows the results obtained on a sample of powder containing magnesium trisilicate, magnesium carbonate and sodium bicarbonate

which was examined for the purpose of obtaining an estimate of the accuracy of the method:

TABLE VII

SiO_2 per cent.				
18·7	19·5	19·3	Plate 1	
18·6	17·8	17·9	„ 2	
17·7	17·6	18·5	„ 3	Average 18·5 per cent.
18·1	18·6	18·7	„ 4	
18·5	18·4	19·0	„ 5	

These results show a standard deviation of 3·3 per cent. for individual determinations which is reduced to 1·9 per cent. when the sample is examined in triplicate.

MgO per cent.				
24·7	23·8	24·7	Plate 1	
23·1	23·6	23·8	„ 2	
22·9	22·9	22·8	„ 3	Average 23·2 per cent.
23·8	22·0	23·1	„ 4	
22·1	22·8	21·5	„ 5	

The results show a standard deviation of 3·5 per cent. for individual determinations, 2·0 per cent. for triplicate determinations.

For comparison purposes Table VIII gives the results obtained spectrographically and chemically on 5 samples of compound powder of magnesium trisilicate.

TABLE VIII

Batch number	SiO_2 Chemically per cent.	SiO_2 Spectrographically per cent.
5006 H	13·3	13·7
5007 H	12·8	13·3
5008 H	13·6	14·2
5009 H	13·3	13·2
5011 H	13·8	13·9

It can be seen that this method provides a reliable alternative to chemical methods of assay. In practice 10 samples per day can be examined in triplicate for both the silicon and magnesium content by one person, showing a great saving in time over other methods. Once the technique had been thoroughly investigated and methods worked out, it became an easy matter to adapt it to the determination of other elements. For instance, the method has been used to determine the amount of aluminium and magnesium present in samples containing 1 per cent. of MgO and 3·5 per cent. of Al_2O_3. The solution of the samples presented no difficulty when the method already outlined for magnesium trisilicate powders was adopted. Aluminium was found to be extremely sensitive and the following composite standards were employed: (1) 0·015 per cent. of MgO + 0·004 per cent. of Al_2O_3; (2) 0·020 per cent. of MgO + 0·008 per cent. of Al_2O_3; (3) 0·025 per cent. of MgO + 0·012 per cent. of Al_2O_3; each with the addition of 0·1 per cent. of potassium dichromate.

Chromium was used in this case as internal standard for both the magnesium and aluminium.

This method also gives a considerable saving in time over the chemical assay and it is possible for one person to carry out the examination in about 3½ hours. It would be possible for about a dozen samples to be examined in 6 hours. One particular sample was examined both chemically and spectrographically and gave the following results: MgO

per cent.—chemically, 1·06, spectrographically, 1·11; Al_2O_3 per cent.—chemically, 3·44, spectrographically, 3·36.

The uses of the porous-cup technique already described prove it to be a speedy and accurate method for the determination of major constituents. The method can also be used for the determination of traces. An example of this is the estimation of zinc in iron salts. These estimations were formerly carried out polarographically but the great advantage of a spectrographic determination is that the presence of zinc can be positively identified by the presence of a trio of lines at 3282·3Å, 3302·0Å and 3345·0Å even in the presence of a small amount of iron whereas the polarographic method is not necessarily specific in the possible presence of other impurities. Zinc standards in the range 100 to 1000 p.p.m. are employed and as the method permits the concentration or dilution of the sample a considerable range of zinc content can be determined. This range has been found the most suitable for the majority of samples, but by sparking 0·25 ml. of solution for 2 minutes the range 20 to 200 p.p.m. has also been used. Once again the method has proved to be sufficiently speedy to permit the investigation by one person of up to 10 samples in triplicate in one day.

Because of the complex iron spectrum the main problem in the application of the method is the removal of the greater part of the iron and this is accomplished as follows. A suitable amount of the sample (usually 5 g. for solids and 10 ml. for solutions) is dissolved in 10 ml. of hydrochloric acid and warmed on the steam bath. Sufficient nitric acid is then added to ensure complete oxidation of the solution which is then cooled, transferred to a separator and extracted with 50 ml. of ether. After separation of the aqueous phase a further 10 ml. of hydrochloric acid is added and the solution again extracted with 50 ml. of ether. Further extractions are carried out, if necessary, in the same way until the aqueous phase is colourless, after which it is evaporated to dryness. The residue is dissolved in a suitable quantity of 0·25 per cent. cadmium sulphate solution (usually 5 ml.) and centrifuged if necessary to obtain a clear solution. 0·15 ml. quantities of this solution are sparked for 40 seconds, the cadmium serving as internal standard.

Summary

1. The introduction of new techniques has improved the accuracy of certain spectrographic methods. Two such techniques of recent development are described, namely flame photometry and the "porous-cup" method, and these are considered sufficiently accurate for the determination of major as well as minor constituents in pharmaceutical materials.

2. The problems encountered in the application of these methods to the determination of certain elements in a number of pharmaceutical materials are described.

3. The given methods show a considerable saving of time over the normal chemical methods hitherto employed.

REFERENCES

1. Brealey and Ross, *Analyst*, 1951, **76**, 334.
2. Brealey, *ibid.*, 1951, **76**, 340.
3. The Fertilisers and Feeding Stuffs Regulations, 1932.
4. Feldman, *Anal. Chem.*, 1949, **21**, 1041.

DISCUSSION

The paper was presented by DR. D. C. GARRATT.

DR. F. HARTLEY (London) said that Dr. Garratt in his own way made the method sound simple, but in his propaganda for the spectrographic method he tended to minimise two aspects. One was the initial investigations of the interference by extraneous elements. It would be helpful if Dr. Garratt could say something about the influence of different anions on the determination of a particular cation. Secondly, it was to be hoped that the author would give some assistance in the future in assessing the proportion of impurities in cations by analytical methods. When revealed by emission spectrophotometric methods some shock might be sustained with regard to metallic impurities in preparations hitherto thought to contain a few parts per million.

DR. G. E. FOSTER (Dartford) pointed out that there were spectrophotometers available in which the lamp housing could be replaced by flame photometer accessories, and he asked the author whether such an arrangement would be suitable in a routine laboratory for use with both emission and absorption spectrophotometric problems. He was interested in the porous cup technique and enquired whether it was now possible to obtain silicon-free graphite electrodes.

MR. N. L. ALLPORT (London) asked whether the instruments manufactured for flame photometry, one in particular, were capable of doing the work which was described in the paper. The instrument described by Mr. Brealey some time ago appeared to be very elaborate and outside the possibilities of any but the largest firms. With regard to the question of precision, the actual method of flame photometry had such a sensitive reaction it meant that in calculating the results it would be necessary to multiply by a correction factor. He would like to hear whether precision could be maintained when using such very delicate reactions as sodium and potassium flame colours.

MR. M. DOMBROW (London) asked whether the authors had determined traces of impurities, particularly of lead, copper and zinc in magnesium trisilicate or other materials which were powerful absorbents. A problem which would have to be investigated was whether the large quantities of apparently harmful metallic impurities present in powerful absorbents were sufficiently released for them to be as dangerous as spectrophotographic figures might suggest.

DR. D. C. GARRATT, in reply, said that the techniques introduced were intended for routine work. With regard to the question of the cost of the instrument, when one considered the amount of time spent in chemical analysis, it was negligible. In answer to Dr. Hartley, the problem of

anionic interference was overcome by various methods, and that subject had been dealt with by Mr. Brealey in a recent publication. Dealing with Dr. Foster's points, he had not used the photoelectric instrument for that work and, in fact, did not consider that it could be adapted for use in emission work. Pure graphite electrodes for the porous cup technique were now easily obtainable. No attempt had been made to work out trace elements in other absorbents than magnesium trisilicate.

Mr. L. Brealey, in reply, said that units were available for converting spectrophotometers into flame photometers. It was true that the ideal flame photometer was produced by adding a flame unit to the spectrophotometer in place of the lamp house. It would appear that Mr. Allport had the filter instrument in mind, but in his (Mr. Brealey's) opinion the only element which could be determined on a filter instrument with any precision was potassium. A monochrometer should always be used for elements other than potassium. With regard to accuracy, highly dilute solutions were used with the flame photometer which gave the required precision. The fact that very dilute solutions were being used was a desirable feature of flame photometry.

ETHYL ESTERS OF HYDNOCARPUS OIL STABILISED WITH CREOSOTE

BY G. E. FOSTER, E. L. KENDRICK, E. WALTON AND W. D. WILLIAMS

From the Wellcome Chemical Works, Dartford

Received June 16, 1952

THE use of chaulmoogra and hydnocarpus oils in the treatment of leprosy has been long established and the oils are still extensively used, in spite of the introduction of modern drugs of the sulphone type. These oils, which consist largely of the glyceryl esters of chaulmoogric and hydnocarpic acids,[1] containing characteristic unsaturated 5-membered rings in their molecules, undergo oxidation and become acid on storage. On this account the oils usually cause irritation on injection and much work has been done in attempting to remedy this defect.

Hofmann,[2] after attempting to isolate the active principle of chaulmoogra oil, approached the problem by isolating the fatty acids obtained by saponification of the oil and esterifying them with ethanol. The resulting mixture of ethyl esters consisted of a low-boiling oil, easily purified by distillation under reduced pressure, and it quickly found a place under the name "Antileprol" in the treatment of leprosy. Subsequently, it was found that the ethyl esters prepared similarly from hydnocarpus oil were equally effective and they have since been included in the British Pharmacopœia.

The ethyl esters, during the past 25 years, have not entirely fulfilled their early promise, for on storage in the presence of air they develop peroxides[3] and become acid. Such esters cause much pain and irritation when injected. Purification by redistillation or by treatment with reducing agents, such as sodium bisulphite solution, is of little value unless the product is used immediately. Iodination of the esters[4] was successful in reducing the incidence of irritation but the iodised esters gave rise to discolouration at the site of injection and never enjoyed great popularity. A more fruitful approach consisted in the addition of anti-oxidants to inhibit oxidation and, in our experience, the best stabiliser of this type is creosote. The use of creosote in Brazil, where a combination of esters of chaulmoogra oil with 4 per cent. of creosote was being used, came to our knowledge in 1941. During the past decade the ethyl esters of hydnocarpus oil stabilised with creosote have enjoyed wide use by leprologists and it is the purpose of this communication to give some account of the stability and standardisation of the preparation.

STABILITY TESTS

For our study of the stability of the ethyl esters of hydnocarpus oil, with and without the addition of creosote, it was decided to use three grades of esters which had been prepared by (1) using no special precautions, (2) refining by chemical treatment and (3) purifying by redistillation. Deterioration was assessed by observing the increase in acid value of the preparations and the development of peroxide. For

the latter determination a test based upon the B.P. method for determining the ascaridole content of chenopodium oil was found convenient and was first brought to our notice by Mr. Y. T. Kruithof, of the Medical Service, Netherlands Guiana, to whom we are indebted. Details of the test are as follows. Into a glass stoppered tube, of about 25 ml. capacity, place 10 ml. of 30 per cent. w/v solution of potassium iodide and 3 ml. of 4N hydrochloric acid. Add 10 ml. of ethyl esters of hydnocarpus oil, shake for 10 minutes and titrate the liberated iodine with 0·1N sodium thiosulphate, using mucilage of starch as indicator.

The preparations, stored in completely filled stoppered bottles and in open half-filled bottles, were kept at 40° C. for 3 months after which they were re-examined. The results are summarised in Table I.

TABLE I

KEEPING TESTS ON SAMPLES OF ETHYL ESTERS OF HYDNOCARPUS OIL

Sample	Initial examination		Examination after 3 months at 40° C.					
			Filled and stoppered			Open and half filled		
	Peroxide Test 0·1N sodium thiosulphate ml.	Acid Value	Reference Letter	Peroxide Test 0·1N sodium thiosulphate ml.	Acid value	Reference Letter	Peroxide Test 0·1N sodium thiosulphate ml.	Acid value
Esters	13·0	0·59	A.	12·2	0·89	B.	25·7	2·46
Esters + 4 per cent. v/v of Creosote ..	12·6	1·02	C.	7·7	1·64	D.	8·8	1·64
Refined esters ..	1·1	0·58	E.	0·6	1·42	F.	22·9	3·18
Refined esters + 4 per cent. v/v of creosote ..	1·0	1·04	G.	0·4	3·12	H.	0·7	3·16
Redistilled esters	0·35	0·61	I.	0·1	0·61	J.	19·2	1·47
Redistilled esters + 4 per cent. v/v of creosote	0·43	1·14	K.	0·4	2·01	L.	1·5	2·62

From these experiments it is evident that samples kept in completely filled stoppered bottles exhibit good preservation, even when no creosote is added. Refined esters became coloured during the test and were less satisfactory than the redistilled product. When air is present, the stabilising effect of added creosote is marked.

At the completion of the keeping test the samples were sent to Dr. A. C. White, of the Wellcome Research Laboratories, Beckenham, who carried out irritation tests by intracutaneous injection of 0·1 ml. of a 25 per cent. dilution of each preparation in liquid paraffin into guinea-pigs. His results are summarised in Table II, in which the numbers beside the letters in the two columns are based upon the intensity of raction in 2 series of experiments involving 12 guinea-pigs.

TABLE II

IRRITATION TESTS

(Letters refer to samples in Table I)

B (26)	>	A (10)
D (10)	>	C (7·5)
F (27)	>	E (10·5)
G (7·8)	>	H (6·5)
J (31·5)	>	I (6·5)
K (7·5)	>	L (6·2)

The preparations fell roughly into two groups and B, F and J were clearly the most irritant samples tested.

It seemed of interest to complete our stability studies by examination of samples of esters which had been kept at room temperature for periods up to 5 years. As was to be expected from our experiments at 40° C., it was found that the esters, with and without creosote, kept satisfactorily in completely filled and well closed containers, very little increase in peroxide and only slight rises in acid values occurring. As an example of what can happen, however, a sample of esters, stored for 5 years at room temperature, was found to give a reading in the peroxide test of 140 ml. of 0·1N sodium thiosulphate. A sample of the corresponding product, containing 4 per cent. v/v of creosote, prepared from the same esters and stored under identical conditions for the same period gave a reading of only 0·15 ml. of 0·1N sodium thiosulphate when tested.

Standardisation

The analytical control of ethyl esters of hydnocarpus oil stabilised with creosote is surrounded by difficulties. Both constituents are mixtures of indefinite composition and no specific methods for their estimation are available. Application of the usual analytical methods for oils and fats, however, has provided a useful control for purposes of manufacture. Table III summarises data on 10 freshly prepared batches of esters, selected at random from many, and Table IV gives data for the corresponding preparations containing creosote. The reference numbers of the samples are correlated; for example, sample No. 1 in Table IV was prepared with sample No. 1 of esters in Table III.

TABLE III

Analytical data on samples of ethyl esters of hydnocarpus oil

Sample	Specific Gravity at 15·5° C.	Saponification value	Acid value	Iodine value	Optical rotation	Refractive Index at 20° C.	Peroxide test 0·1 N sodium thiosulphate ml.
1	0·9105	193·3	0·42	92·2	+45·64°	1·4580	1·6
2	0·9099	193·2	0·22	92·0	+46·37°	1·4600	0·6
3	0·9100	193·0	0·20	91·1	+46·33°	1·4610	0·2
4	0·9100	193·6	0·29	90·5	+46·41°	1·4605	1·0
5	0·9100	192·6	0·2[illegible]	92·9	+46·51°	1·4605	0·75
6	0·9101	192·1	0·22	92·6	+46·45°	1·4605	0·9
7	0·9102	192·0	0·20	93·0	+46·25°	1·4595	1·6
8	0·9106	190·6	0·1[illegible]	91·0	+46·8°	1·4580	0·5
9	0·9099	193·3	0·2[illegible]	90·1	+46·96°	1·4605	0·5
10	0·9111	192·3	0·23	91·3	+47·4°	1·4601	0·4

Estimation of Creosote

We have spent much time in search of a reliable method for the estimation of creosote in the stabilised esters but we have discovered no entirely satisfactory assay process. It is possible to remove creosote from a solution of the esters in petroleum ether by shaking with N sodium hydroxide solution, the creosote being recovered from the alkaline extract by acidifying and extracting with ether. It is difficult, however, to remove the solvent from the final extract without loss of creosote. In some experiments, recoveries of creosote amounting to 100 per cent.

TABLE IV

ANALYTICAL DATA ON SAMPLES OF ETHYL ESTERS OF HYDNOCARPUS OIL WITH 4 PER CENT V/V OF CREOSOTE

Sample	Specific gravity at 15·5° C.	Acid value	Optical rotation at 20° C.	Peroxide test 0·1N sodium thiosulphate ml.
1	0·9177	1·2	+43·89°	1·0
2	0·9173	0·72	+44·53°	0·65
3	0·9173	0·73	+44·45°	0·25
4	0·9170	0·85	+44·55°	0·65
5	0·9171	0·79	+44·6°	0·9
6	0·9173	0·69	+44·1°	0·2
7	0·9170	0·75	+44·44°	0·15
8	0·9177	0·34	+44·62°	0·45
9	0·9168	0·6	+45·04°	0·4
10	0·9180	0·74	+45·4°	0·3

were obtained, but in others the results were less satisfactory. The separated esters may also be recovered and weighed by removal of the solvent from the initial petroleum ether extract; the residue being dried at 100° C. for 15 minutes. Results correct to within 1 per cent. of theory were obtained for the ester content so determined.

More promising results were obtained by use of the reaction between nitrous acid and phenols employed by the B.P. for the estimation of morphine in some opium preparations. Details of this process are as follows.

Reagents.

1 per cent. w/v Solution of Sodium Nitrite
Dilute Phosphoric Acid B.P.
Dilute Solution of Ammonia B.P.

Calibration Curve. Prepare a 0·1 per cent. w/v solution of creosote B.P. in water containing a few drops of sodium hydroxide solution. Place 0, 1, 2, 3, 4 and 5 ml. in 50-ml. stoppered cylinders and dilute each to 20 ml. Add 8 ml. of sodium nitrite solution and 6 ml. of dilute phosphoric acid to each cylinder, mix and allow to stand for 15 minutes. Render alkaline by the addition of 12 ml. of dilute ammonia solution, adjust the volume to 50 ml., mix well and allow to stand for 15 to 20 minutes. Measure the light absorption of the reaction mixtures with a Spekker Absorptiometer using 1 cm. cells and a combination of Ilford 603 and heat resisting H503 filters. Construct a calibration curve from the readings obtained.

Assay of Ethyl Esters of Hydnocarpus Oil with Creosote. Transfer a quantity of esters containing 3 to 4 mg. of creosote to a separating funnel and dilute with 20 ml. of ether. Add 15 ml. of water, 8 ml. of sodium nitrite solution and 6 ml. of dilute phosphoric acid. Shake for 15 minutes, add 12 ml. of dilute ammonia solution, mix, allow to separate and run the lower layer into a stoppered cylinder. Wash the ether extract with two quantities, each of 2 ml., of water, adding the washings to the cylinder. Adjust the volume to 50 ml., mix well and allow to

stand for 15 to 20 minutes. Measure the light absorption of the reaction mixture, as above, and estimate the creosote content of the esters from the calibration curve. Use the appropriate specific gravities to calculate the creosote content as per cent. v/v.

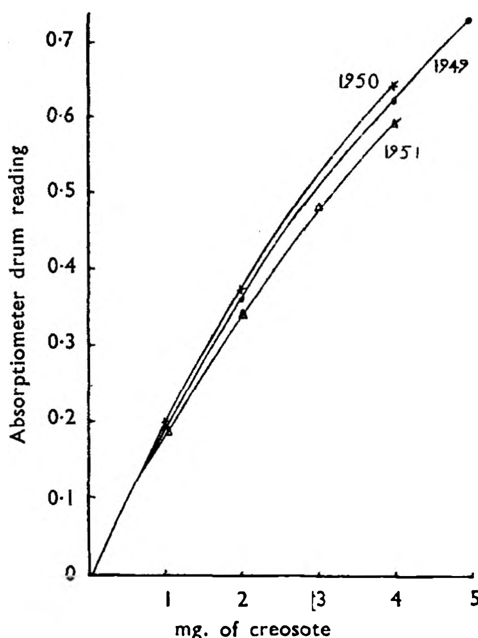


FIG. 1. Calibration curves for batches of creosote; with Ilford 603 and heat resisting H503 filters.

This colorimetric assay process yields good results providing the calibration curve is constructed using a sample of the same creosote as is used for stabilising the esters examined. Unfortunately, the curves for batches of creosote vary, as will be seen by reference to Figure 1. Using an average curve we have obtained figures of 4 to 4·5 per cent. for preparations containing 4·0 per cent. of creosote.

DISCUSSION

The work described in the present paper emerged from the irritation repeatedly observed when hydnocarpus oil and its derivatives were injected during clinical practice. There is little doubt[5] that the principal cause of excessive irritation is the presence of oxidation products of the unsaturated acids of which the oils are esters. The stability experiments performed have afforded conclusive evidence that the ethyl esters of hydnocarpus oil keep well at room temperature when stored in completely filled and well closed containers but, on exposure to air, oxidation readily takes place with the initial formation of organic peroxides. This deterioration can be very largely eliminated by the addition of 4 per cent. of creosote to the esters; at the same time the development of irritant properties is retarded.

In view of the extent to which the stabilised esters have been used in leprosy it is desirable that suitable analytical methods should be available to control manufacture. This raises difficulties owing to the constituents of the preparation being mixtures of varying composition. From the analytical data presented in Tables III and IV it is evident that application of the usual methods for the analysis of oils and fats can afford useful information. In particular these data show that both the specific gravity and the optical rotation of the esters are significantly affected by the addition of creosote. An average difference of 1·95° between the optical rotations of the esters, before and after stabilisation, is observed

and this corresponds to approximately 4 per cent. of added creosote. Similarly, taking the specific gravity at 15·5° C. of creosote as 1·086, the average increase in specific gravity roughly agrees with the amount of added creosote. The presence of organic peroxides should be controlled and it would be reasonable to impose a limit of 2 ml of 0·1N sodium thiosulphate for the titration obtained by the peroxide test described. Addition of creosote tends to increase the acid value but there is no reason for the acid value of the stabilised product to be greater than that allowed (1·0) for ethyl esters of hydnocarpus oil B.P. Application of the nitrous acid reaction, employed for morphine estimations, has afforded a useful colorimetric method for the determination of creosote in the stabilised esters but its application is limited owing to the need to prepare a calibration curve using a sample of the actual creosote present in the esters if accurate results are to be obtained.

The success which attended the addition of creosote to the ethyl esters directed attention to stabilising hydnocarpus oil by similar means. This problem is complicated by the fact that the oil, unlike the esters, cannot be commercially purified by re-distillation. The only practical way of overcoming the difficulty is to add creosote to the oil immediately after expression from the seeds of *Hydnocarpus Wightiana* and before oxidation has commenced. This, in fact, has been done and many tons of hydnocarpus oil containing creosote have been used. The problems of analytical control of this preparation are similar to those of the stabilised ethyl esters and have been largely solved by application of the methods described for the esters, with appropriate modification.

Summary

1. The stability of the ethyl esters of hydnocarpus oil B.P. with and without added creosote, has been investigated by keeping samples at 40° C. for 3 months and at room temperature for 5 years.

2. The esters when kept in completely filled well-closed containers undergo little deterioration on storage.

3. When exposed to air the esters rapidly develop peroxides and become acid in reaction. Such esters cause excessive irritation on injection.

4. Addition of 4 per cent. v/v of creosote to the esters very largely inhibits the oxidation which occurs when the esters are kept in partly filled containers.

5. Analytical data are presented for the standardisation of the esters stabilised with 4 per cent. v/v of creosote.

6. A colorimetric method for the determination of creosote in the esters is described.

We wish to thank the Directors of The Wellcome Foundation Ltd. for permission to publish this paper.

REFERENCES

1. Barrowcliff and Power, *J. chem. Soc.*, 1907, **91**, 557.
2. Hofmann, *Z. Angew. Chem.*, 1920, 33, 276.
3. Baranger, *Internat. J. Lep.*, 1949, **17**, 49.
4. Cole, *Philippine J. Sci.*, 1931, **46**, 377.
5. Paget, Trevan and Attwood, *Internat. J. Lep.*, 1934, **2**, 149.

DISCUSSION

The paper was presented by DR. G. E. FOSTER.

The CHAIRMAN asked whether guaiacol had been considered as a stabiliser, as it had a local anæsthetic effect and was therefore less painful on injection, and whether other phenolic substances had been investigated in that respect.

MR. A. F. CALDWELL (Singapore) stated that, in Malaya, creosote had been added to hydnocarpus oil. Work had not been done with the official oil because supplies from India were not free from peroxide, but oil from *Hydnocarpus anthelmintica* was found to be satisfactory and of a better quality than the hydnocarpus oil from India. In the treatment of leprosy most patients had some nerve involvements and the clinical results might therefore be different from tests on experimental animals. He referred to the difficulty of trying to assess clinical pain and described his own experience of issuing on one occasion a large number of bottles of hydnocarpus oil known to be free from peroxides. The oil had come back with the complaint that it was very painful. It had then been divided into two portions and reissued. They were subsequently informed that both batches were perfectly satisfactory. It had been found from experience that hydnocarpus oil, sterilised and packed in 1 oz. bottles completely filled, remained free from peroxide for a period of up to 10 years.

DR. F. HARTLEY (London) suggested that it was unexpected to find that creosote was the chosen antioxidant, and asked what criteria the authors had in mind when they referred to it as the "best stabiliser." He was surprised by the degree of precision indicated in the results of the irritation tests.

DR. R. E. STUCKEY (London) asked for information about the method used in selecting creosote as the antioxidant. Recent work had shown that there was a synergistic effect between one class of antioxidants, phenolic bodies, and some acidic substances such as phosphoric or citric acid. It had been shown that citric acid removed the metallic ions which catalysed the oxidation so increasing the efficiency of the phenolic antioxidant. Had the authors tried any such combinations in their experiments? Was there any evidence of the synergistic effect of an acid, such as citric acid, and creosote in stabilising the ester?

DR. G. E. FOSTER, in reply, said that guaiacol had not been tried instead of creosote. The anæsthetic effect mentioned by the Chairman was interesting, because in 1927 a great deal of pain was reported when the ethyl esters were being used. The addition of creosote was tried as

something which would minimise pain owing to its very weak anæsthetic properties, but it was given up at that time because it seemed to be of no particular value. It was not until 1940 that creosote was used in Brazil with beneficial results in reducing the incidence of irritation and with antioxidant effect. He had established that 1 per cent. of creosote was no less irritant than 4 per cent. when added to the preparation, and clinical trials showed the same.

It was gratifying to learn that some of the results obtained by Mr. Caldwell coincided with their own.

Dr. Hartley and Dr. Stuckey had mentioned the use of other antioxidants and he would give them fullest information after he had had an opportunity of consulting his colleagues at Dartford.

THE DETERMINATION OF ASCARIDOLE IN OIL OF CHENOPODIUM AND IN SOLUTION OF OIL OF CHENOPODIUM IN CASTOR OIL

By A. H. BECKETT and M. DOMBROW

From the Pharmaceutical Chemical Laboratories, School of Pharmacy, Chelsea Polytechnic, Manresa Road, London, S.W.3

Received July 10, 1952

A SOLUTION of oil of chenopodium in castor oil is used in veterinary practice as an anthelmintic and an assay of the ascaridole content was required for the British Veterinary Codex. A reliable assay is especially important because the anthelmintic effective dose is stated to be near to the toxic dose for certain animals.

The B.P. 1948 and U.S.P. (XII) method for the determination of ascaridole in oil of chenopodium, depending upon the liberation of iodine from potassium iodide solution, is obviously inapplicable in the presence of castor oil. Although the oil of chenopodium could probably be separated from the castor oil by distillation, the application of any prolonged heat treatment in an assay process would be unwise because of the ease of rearrangement or decomposition of the ascaridole molecule.

A number of methods have been used previously for the assay of ascaridole, of which the method of Cocking and Hymas,[1] upon which the B.P. method is based, has found widest acceptance, despite the fact that an empirical rather than a stoichiometric factor has to be used. Furthermore, the conditions must be controlled accurately if reproducible results are to be obtained. Halpern[2] has studied the iodination of terpenes and has shown that the non-stoichiometric behaviour of ascaridole towards the iodide ion cannot be explained by the simple iodination of olefines present in the oil of chenopodium. Do Vale[3] has modified the above method slightly by determining the liberated iodine colorimetrically. Nelson's method[4] is based on the solubility of ascaridole in 60 per cent. acetic acid, but other substances present in oil of chenopodium are also soluble in this solvent. Paget[5] used the reduction with titanous chloride as a method of assay, but here too, an empirical factor had to be applied. A colorimetric method based on the brown colour arising from the action of concentrated hydrochloric acid on a 1 per cent. ethanolic solution of ascaridole has been described.[6] Reports have appeared[7,8,9,10,11] dealing with the above methods of assay of ascaridole, and these reports indicate the limitations of these methods. The method for the assay of chenopodium involving the use of bisulphate solution and the measurement of the undissolved oil[12] is obviously not suitable for precise work. A number of colorimetric assays have been described based on the colour produced with ascaridole and strong sulphuric acid,[13] and 2:6-dichlorophenolindophenol.[14] Recently a gravimetric assay[15] has been used which involves the addition of mercuric chloride in ethanol (90 per cent.) and filtration after 48 hours in the dark.

It is apparent from a detailed consideration of the above briefly mentioned methods that a precise, reliable and relatively straightforward assay process for ascaridole has not yet been devised. Consequently we have investigated the application of polarography to the determination of ascaridole in oil of chenopodium because this approach would probably be applicable in the presence of castor oil. Since this work was commenced, the paper by Bitter[16] dealing with the polarography of some essential oils, including oil of chenopodium, came to our attention. He showed that ascaridole gave a reduction step in absolute ethanol and that polarography was a suitable method for its assay, but few details were given and the effect of varying conditions upon the results was not investigated. We now report the results of our investigations and suggest a routine method suitable for the analysis of ascaridole in oil of chenopodium and in solutions of oil of chenopodium in castor oil.

EXPERIMENTAL

Materials

Reagent chemical grade—lithium chloride dihydrate, lithium hydroxide monohydrate.

Analar grade—mercury, calomel, and potassium chloride.

B.P. grade—acetic acid, agar, castor oil and oil of chenopodium.

Ethanol, redistilled from commercial absolute ethanol.

Ascaridole. A pure sample was obtained in 2 ways from ascaridole kindly supplied by Dr. Foster of the Wellcome Chemical Works.

(*a*) Fractional distillation under reduced pressure. The fraction b.pt. 84° C./3 mm., $n_D^{22° C}$ 1·4725 gave the highest polarographic wave.

(*b*) Chromatography using alumina and benzene/light petroleum.

Polarography indicated that the sample obtained by chromatography had a purity about 1 per cent. greater than the sample obtained by fractional distillation. This work will be reported in detail in a subsequent paper dealing with the chemistry and stability of ascaridole.

Apparatus

Current-voltage curves were recorded using a Tinsley Polarograph, Mark 12. Capillary constant, $m = 1·94$ mg. per second, $t = 3·54$ seconds, on open circuit, in ethanol solution of the electrolyte. The temperature was controlled at 20° ± 0·5° C. The solutions were deoxygenated with oxygen-free nitrogen which had previously been passed through 97 per cent. ethanol. The recorder pen was set at zero with the electrodes disconnected.

RESULTS AND DISCUSSION

Choice of solvent. Owing to the low water solubility of castor oil and oil of chenopodium, a solvent of low water content was required. This limited the choice of electrolyte and lithium chloride was used. Of the various solvents tried, well formed waves were given by oil of chenopodium only in methanol, industrial methylated spirit and ethanol, the latter being chosen owing to minor waves occurring at 1·7 volts in the first 2 solvents.

Buffering. The most satisfactory buffer was found to be a mixture of lithium acetate and acetic acid, each 0·01N. Neither the half wave potential nor the diffusion current of ascaridole was affected by changes of acidity or alkalinity within the range of this buffer or an ammonium chloride-ammonium hydroxide buffer of the same strength but, with the latter, maxima occurred at 1·65 volts. A second wave, due to reduction of the electrolyte, began at 2·1 volts (approx.) in these buffered solutions but was displaced to 1·8 volts in 0·15N acetic acid, thereby seriously curtailing the limiting current plateau of the first wave. Hydrochloric acid 0·01N, unbuffered, gave an interfering hydrogen wave.

Rancidity of the castor oil may give rise to a small quantity of weak acid. A sample just within the B.P. acid value limit of 4 would give a solution 0·004N in acid at the castor oil strength used in the suggested method (see below). Acetic acid 0·03N and ricinoleic acid 0·1N were found to be without effect on the diffusion current. The lithium acetate buffer was therefore used primarily as a precaution against contamination with strong acids.

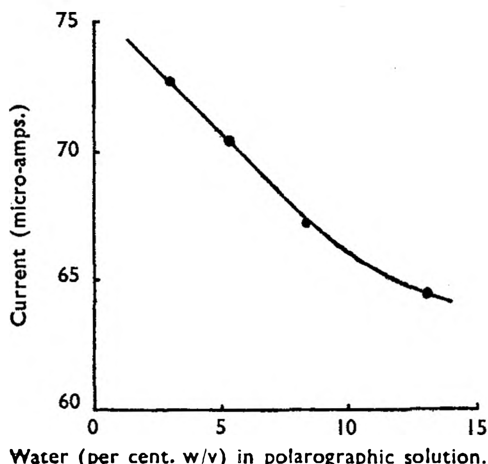


FIG. 1. Effect of water on diffusion current of ascaridole.

Water content of solvent. The effect of water on the diffusion current of ascaridole in ethanol containing the above buffer and 0·2N lithium chloride is shown in Figure 1.

The diffusion current fell by about 1·5 per cent. for a 1 per cent. increase in water content. Castor oil had little effect upon the slope. The water content was most conveniently controlled by the use of constant boiling ethanol (see section on reagents).

Electrolyte concentration and cell resistance. The half wave potential, corrected for *iR* drop, occurred at about 1·04 volts (against S.C.E.) in 0·2N lithium chloride and fell by about 50 millivolts as the electrolyte concentration was reduced to 0·05N. The main objection to low concentrations of electrolyte is the high cell resistance occurring with ethanol solutions. With the particular cell used in this work, 0·1N electrolyte was found satisfactory, but with large cells, a stronger solution would be desirable.

Temperature effect. By measurement of the diffusion current over a range from 12° to 28° C., the temperature coefficient was found to be about 1 per cent. per degree C.

Use of pool anode. Some difficulties were experienced with the agar solidified aqueous salt bridge as a result of precipitation effects at the

junction, particularly if the bridge was left in the solution during bubbling. This resulted in a fall of current due to displacement of the cathode potential to less negative values by the greatly increased iR drop. The trouble was eliminated by the use of a pool anode, which had the further advantage of reducing the cell resistance to 3200 ohms at an interval of about 2 cm. between the electrodes. The potential of the pool, measured against the saturated calomel electrode, varied slightly with applied voltage and current but did not exceed ± 30 millivolts (against S.C.E.) with currents up to $80 \mu A$ and was generally much less.

Effect of castor oil. A method of assay was required by the British Veterinary Codex for a castor oil solution of oil of chenopodium containing 3·25 per cent. of ascaridole. Polarograms obtained with a fixed concentration of ascaridole and varying quantities of castor oil showed that an increase of 1 per cent. in the castor oil concentration depressed the diffusion current by 2 per cent. (Fig. 2).

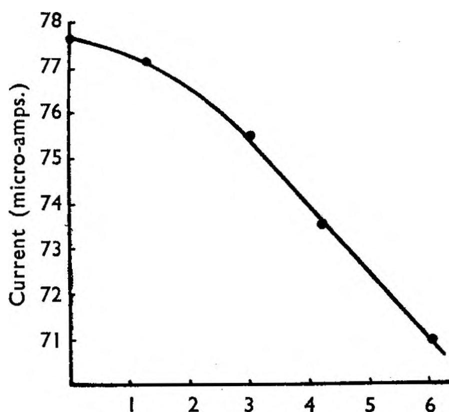


FIG. 2. Effect of castor oil on diffusion current of ascaridole.

A precision of $\pm 0·5$ per cent. will hence be achieved, in respect of the effect of castor oil concentration, in the proposed method (below) by controlling the sample weight to within ± 5 per cent.

Calibration curves. Full polarograms were recorded for ascaridole concentrations from zero to 0·2 per cent. (*a*) in the absence, (*b*) in the presence of 6 per cent. of castor oil and the diffusion currents at various voltages plotted against the concentration (Fig. 3) without correction for the residual current. On first short-circuiting the cell at zero applied voltage, a small negative current flowed, which fell to zero at 0·05 volts, after which a normal residual current curve was obtained, rising to $0·5 \mu A$ at 0·5 volts and $1·5 \mu A$ at 1·65 volts in a solvent blank. Incomplete deoxygenation could therefore be detected by the presence of an oxygen wave below 0·5 volts. The current commenced to rise rapidly at 1·7 volts as decomposition of the solvent electrolyte began. This solvent wave occurred at the same voltage with low ascaridole concentrations, and was displaced progressively to above 2 volts at medium and high concentrations. The uncorrected half wave potential of ascaridole itself rose from 1·03 to 1·13 volts over the same concentration range, but on correction for the iR drop, a value of $1·01 \pm 0·02$ volts (against S.C.E.) was obtained.

The plots were very nearly linear whether the current was read at potentials of 1·35 and 1·50 volts (corrected for iR drop) or applied voltages

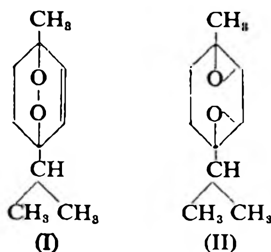
of 1·60 and 1·70. The latter plots showed slight curvature at low concentrations (under 0·1 per cent. ascaridole) with better precision on the linear portion. The former plots were more precise at the lower concentrations but showed less overall precision than the uncorrected curves. There was little to choose between results obtained at 1·6 and 1·7 volts. The values of the average current, obtained with damping, were of slightly lower precision than the instantaneous maximum currents, recorded with no damping and corresponding to maximum drop size. They were 80 per cent. of the maximum currents, slightly less than the values predicted by the Ilkovic equation.

It is clear that a calibration curve, plotted at voltages of 1·6 or 1·7, uncorrected for *iR* drop, using a cell of resistance 3200 ohms, may be used as the basis of a polarographic method for the determination of ascaridole. There is a danger with polarographic cells of high resistance, and at high values of current, that the readings may be taken slightly below the plateau of the wave, which will reduce precision, although not necessarily accuracy, in a relative method. For this reason, the higher voltage was chosen, the electrolyte concentration increased, and the residual current read on a solvent blank at 1·5 volts to allow for the slight *iR* drop in the stronger solutions.

Diffusion current constants (i_dC) at 1·7 volts were calculated from the data used in plotting the calibration curves (Fig. 3) after subtracting the residual current of 1·5μA. The values for ascaridole showed an average deviation from the mean of $\pm$0·8 per cent., with no damping. With damping, the average deviation was $\pm$1 per cent. upon excluding the last 3 points obtained at very low ascaridole concentrations. Further work is being carried out to see whether the Ilkovic equation may be applied, and a comparison solution eliminated, by the use of a diffusion current coefficient for ascaridole.

Linear calibration curves of excellent precision were also obtained in the presence of a constant amount (6 per cent.) of castor oil (Fig. 3) without correction for the *iR* drop.

Ascaridole in oil of chenopodium. Oil of chenopodium contains, in addition to ascaridole (I), other constituents including *p*-cymene, *l*-limonene, *d*-camphor and cineole, as well as ascaridole glycol anhydride (II) and ascaridole glycol arising from the rearrangement of ascaridole during distillation or steam distillation.



It appears unlikely that any of these constituents will give a reduction step in the polarographic method of assay. However, this point was investigated further by heating a sample of oil of chenopodium containing 60 per cent. of ascaridole (determined polarographically) at 150° C. under reflux. Heating is known to cause rearrangement of ascaridole to ascaridole glycol anhydride (II).[17] After 2½ hours the oil was found to possess a constituent (presumed to be ascaridole)

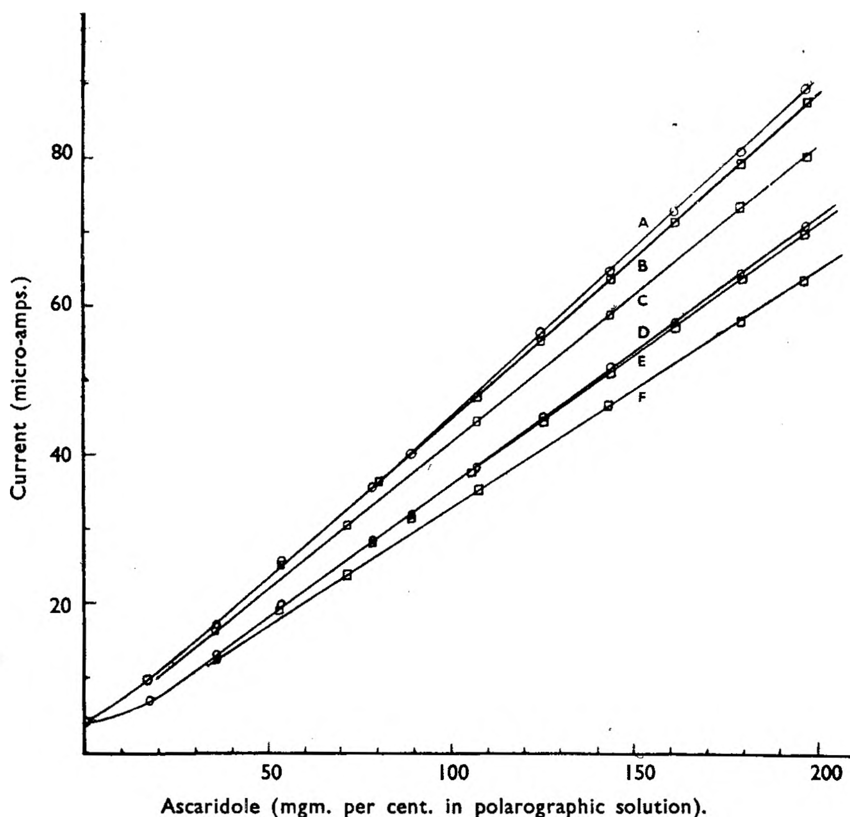


FIG. 3. Calibration curves for ascaridole in ethanol (97 per cent.) with lithium chloride 0·1N, lithium acetate 0·01N, acetic acid 0·01N.

Current at maximum drop size, undamped. A. 1·7 volts. No castor oil. B. 1·6 volts. No castor oil. C. 1·7 volts. 6 per cent. of castor oil.

Arithmetical mean current, damped. D. 1·7 volts. No castor oil. E. 1·6 volts. No castor oil. F. 1·7 volts. 6 per cent. of castor oil.

(All voltages uncorrected for iR drop and pool anode potential. m = 1·94 mgm. per second. t = 3·0 seconds at 1·6 volts, 2·9 seconds at 1·7 volts. Temperature = 20° C. ± 0·5.)

which gave a polarographic wave identical with ascaridole and the results indicated a content of 10 per cent., which fell to 4 per cent. after 4 hours heating and to about 1 per cent. after 8 hours heating. Although it is possible that this heat treatment may have caused changes in other constituents as well as in ascaridole, it is presumed that this polarographic method does in fact measure only the ascaridole content of oil of chenopodium. In connection with the possible presence of substances in oil of chenopodium or castor oil which might interfere with the polarographic determination of ascaridole, the recent publication by Willits *et al.*[18] is of interest. These authors established that a number of substances related chemically to rancidity products of fats and oils and including cumene, unsaturated esters, and epoxy and hydroxy

derivatives of fatty acids and alcohols are not reduced polarographically in a lithium chloride-methanol-benzene solvent.

Stability of ascaridole solution. Experiments are under way to test the stability of ascaridole under varying conditions. In view of the suggested use of a standard ascaridole solution, it is of interest that a 1·0 per cent. solution of ascaridole in ethanol (97 per cent.), which was prepared 2 months ago and has been exposed to sunlight and to the atmosphere frequently, still gives the same diffusion current as when it was first prepared.

Proposed Method for the Determination of Ascaridole

Solutions

Lithium acetate buffer solution (lithium chloride 0·5N, lithium acetate 0·025N, acetic acid 0·025N)

Lithium chloride dihydrate, 39·5 g.
Glacial acetic acid, B.P., 3·0 g.
Lithium hydroxide monohydrate, 1·1 g.
Ethanol (97 per cent. v/v) to 1 l.

Standard ascaridole solution

Ascaridole (freshly fractionated), 0·5 per cent. w/v in ethanol (97 per cent. v/v).

Castor oil solution

Castor oil, B.P., 30 per cent. w/v in ethanol (97 per cent. v/v).

Ethanol (97 per cent. v/v)

Prepared by dilution from absolute ethanol and subsequent distillation at atmospheric pressure, rejecting first and last portions.

Determination of ascaridole in oil of chenopodium

Weigh accurately 0·13 to 0·15 g. of oil of chenopodium into a 50 ml. graduated flask, add 20 ml. of *Lithium Acetate Buffer Solution* and dilute to 50 ml. with *Ethanol* (97 per cent. v/v). Transfer a portion to a polarographic cell, deoxygenate by bubbling with oxygen-free nitrogen which has previously been passed through a wash bottle containing *Ethanol* (97 per cent. v/v). Measure the diffusion current at 20° C. (±0·5°) and an applied voltage of 1·7 using a dropping mercury cathode and large pool anode, about 2 cm. apart or less. If the diffusion current is less than 30μA, repeat the determination using a larger weight of sample. Deduct the residual current given at 1·5 applied volts by a solvent blank, prepared and treated exactly as the above solution except that the sample is omitted. From the net diffusion current, calculate the weight of ascaridole present, either by using a calibration curve or a standard comparison solution.

Standard comparison solution. Determine the net diffusion current exactly as described above using, in place of the sample, 20 ml. of *Standard Ascaridole Solution* or an equivalent quantity of pure ascaridole. The net diffusion currents given by the sample and standard will be in the same ratio as the weights of ascaridole present in the respective solutions.

Calibration curve method. A calibration curve showing the relationship between "net current" and "weight of ascaridole in 50 ml. of prepared solution" may be prepared by carrying out the determination exactly as described above using suitable quantities of *Standard Ascaridole Solution* in place of the sample. Volumes between 12 and 20 ml. should cover normal variations in sample strength. The curve will be valid only for the particular electrode capillary used and the polarographic cell resistance should not vary appreciably.

Determination of ascaridole in castor oil solution

Carry out the determination exactly as described above using 2·6 to 2·8 g. of sample (castor oil containing 3·25 per cent. of ascaridole) in place of the oil of chenopodium.

Standard comparison solution and calibration curve. In preparing the standard comparison solution, or the solutions used in plotting a calibration curve, add 9 ml. of *Castor Oil Solution* or an equivalent weight of castor oil in addition to the other reagents, before adjusting to 50 ml.

Solutions of ascaridole in castor oil stronger than 4 per cent. may be determined by using smaller weights of sample and making a corresponding reduction in the quantity of castor oil used in any standard comparison solutions. (A correction based on the data plotted in Figure 2 may otherwise be applied.)

SUMMARY

1. The methods which are available for the determination of ascaridole are briefly reviewed.

2. The possible application of a polarographic assay of ascaridole in oil of chenopodium and in solutions of oil of chenopodium in castor oil is investigated and the effects of various conditions upon this assay are examined.

3. A method suitable for the routine determination of ascaridole in the above solutions is proposed.

REFERENCES

1. Cocking and Hymas, *Analyst*, 1930, **55**, 180.
2. Halpern, *J. Amer. pharm. Ass.* (Sci. Ed.), 1948, **37**, 465.
3. Do Vale, *Noticias farm.* (Portugal), 1948, **14**, 397; *Chem. Abst.*, 1949, **43**, 8421 g.
4. Nelson, *J. Amer. pharm. Ass.*, 1921, **10**, 836.
5. Paget, *Analyst*, 1926, **51**, 170.
6. Knaffl-Lenz and Hoffmann, *Arch. Pharm.*, 1929, **267**, 117.
7. Broughton and Weiland, *J. Ass. off. agr. Chem.*, 1930, **13**, 334.
8. Broughton, *ibid.*, 1931, **14**, 341.
9. Bodendorf, *Apoth. Ztg.*, 1930, **45**, 1636.
10. Simmons *et al.*, *Analyst*, 1936, **61**, 179.
11. Reindollar, *J. Amer. pharm. Ass.*, 1939, **28**, 589.
12. Dodge, *Drug Cosmetic Ind.*, 1940, 46, 414.
13. Tartsitano, *Boll. soc. ital. biol. sper.*, 1945, **20**, 101; *Chem. Abst.*, 1946, **40**, 7078.
14. Bohme and van Emster, *Arch. pharm. Berl.*, 1951, **284**, 171.
15. Sanna and Marchi, *Boll. chim.-farm.*, 1950, **89**, 447; *Chem. Abst.*, 1951, **45**, 2146.
16. Bitter, *Coll. Czech. chem. Communs.*, 1950, **15**, 677.
17. Henry and Paget, *J. chem. Soc.*, 1921, **119**, 1722.
18. Willits, Ricciuti, Knight and Swern, *Anal. chem.*, 1952, **24**, 785.

DISCUSSION

The paper was presented by DR. A. H. BECKETT.

DR. D. C. GARRATT (Nottingham) said that, apart from the desirability of standardising ascaridole analytically, the work which Dr. Beckett had undertaken arose from a specific request from the appropriate committee of the Veterinary Codex for a close assessment of the ascaridole content of oil of chenopodium in castor oil as distinct from the chenopodium oil content. That was because of the assertion that deaths had occurred as a result of the varying ascaridole content of the chenopodium oil used. In fact the ascaridole content of B.P. oils varied only within a few units per cent. It appeared that the therapeutic dose of ascaridole must be so close to the toxic dose that the conclusion might be reached that it was undesirable to use it in veterinary practice at all. Polarographic methods were not specific, and he felt that the point had been reached at which it was desirable to pause in their use. Although in the routine determination a standard ascaridole solution was desirable, who was to keep the standard, and would it deteriorate over a long period? Further, what proof had the authors that the ascaridole which they purified was in fact pure ascaridole and how did they prove that the ascaridole was still as such and was not an isomer or polymer giving the same deflection? Lastly, he asked how the results of their method compared with those obtained by chemical methods over a range of B.P. oils.

DR. G. E. FOSTER (Dartford) said that he had tried Dr. Beckett's method of assay and confirmed that a straight line calibration curve was obtained, but when the estimations were repeated it was found that the calibration curve was not altogether reproducible. After completing a number of determinations on one series of solutions one day, the next day the actual calibration was slightly different, and far from being able to estimate the ascaridole content to $\pm 0{\cdot}5$ per cent., he was of the opinion that it was not possible to get results nearer than ± 10 per cent.

DR. J. M. ROWSON (London) asked the authors whether they had any comparative figures either of the oils or anthelmintic preparations from them analysed by the polarographic method and by the pharmacopœial method? Had the pharmacologist or veterinary worker attempted to evaluate with reasonable probability the anthelmintic action of the oil and compared it with the iodimetric and polarographic methods?

MR. W. H. STEPHENSON (Nottingham) asked the authors whether they had done any work on old samples of oil and different grades of castor oil, with a view to ascertaining the interference of any peroxides which might be present.

DR. A. H. BECKETT, in reply, said that when using the B.P. method of determination of ascaridole it was possible to obtain any result, depending upon the weight of the sample taken. For a comparison between the polarographic and B.P. methods much closer standardisation of the conditions of the B.P. assay was required. It was considered

that the B.P. method gave results about 15 per cent. too high for oils containing 60 to 70 per cent. of ascaridole and 12 per cent. too high for oils containing 80 per cent. of ascaridole. Furthermore, when ascaridole was dissolved in 90 per cent. acetic acid, it decomposed very rapidly. Therefore, any comparison between the polarographic and B.P. methods was difficult. He was not certain that ascaridole had ever been obtained 100 per cent. pure, but he was continuing his work on the purification. He was investigating old samples of oils.

MR. M. DOMBROW (London), in reply, disagreed with Dr. Garratt's statement that polarographic methods were not specific. The development of polarography was more recent than that of spectrophotometry, but a great deal of information was being accumulated to suggest that polarography was quite specific under certain conditions and for certain groupings. Polarography of an oil in anhydrous ethanol was less straightforward than in aqueous solvents.

THE POLAROGRAPHIC DETERMINATION OF RIBOFLAVINE IN COMPRESSED TABLETS AND AMPOULE SOLUTIONS

By Sylvia Fowler and R. C. Kaye

From the Department of Pharmacy, Bradford Technical College

Received June 30, 1952

Several methods are at present available for the estimation of riboflavine, including microbiological, fluorimetric, and spectrophotometric assay. These rather complex techniques have been used primarily for the determination of riboflavine in foods, where, along with many other substances, it is present in relatively small amounts. The U.S.P. XIV prescribes the fluorimetric and microbiological methods for the assay of riboflavine in compressed tablets. In view of the increasing medicinal importance of riboflavine, and the proposal to include compressed tablets containing it in the British Pharmacopœia, it seems desirable to have a simpler and more rapid method for its determination in such products. The U.S.P. XII included a colorimetric method of estimating riboflavine in compressed tablets which has the merit of simplicity, but which, in our experience, does not give good results.

Lingane and Davies[1] have drawn attention to the possibility of determining riboflavine polarographically. Their work was not extended to the estimation of riboflavine in natural products, or pharmaceutical preparations. The present investigation was prompted by the desire to find the most suitable conditions for the polarographic determination of riboflavine in compressed tablets. The determination of riboflavine in ampoule solutions was also investigated on a more limited scale. Some time was also devoted to the photo-decomposition of riboflavine. This latter portion of the work is not complete, but because of its pharmaceutical interest, some results obtained have been incorporated into this paper.

Experimental

A manually operated polarograph was employed. The cell was similar to one described by Kolthoff and Lingane,[2] and was constructed from a B.40 Pyrex glass joint. The capillary constants determined on open circuit conditions in 0·1 N potassium chloride solution were as follows: m. = 0·969 mg. sec.$^{-1}$, t. = 3·37 sec., where m. = the rate of mercury flow, and t. = the drop-time. Current was measured by means of a calibrated damped Cambridge "spot" galvanometer. All experiments were carried out at 25° C. Air was removed from the cell solution by passing cylinder nitrogen, purified by passage through a series of gas-washing bottles containing alkaline hydrosulphite solution. The potential of the dropping mercury electrode (d.m.e.) was adjusted by means of a tapped resistance network, forming a potential divider. In obtaining current-potential curves, potential increases of about 20 mV. were usually employed. All dropping mercury electrode potentials were measured against an immersion type saturated calomel electrode.

For the examination of the current-time relationship during the life of a single mercury drop, an Ultrascope Mark I cathode-ray oscilloscope was employed, in conjunction with an external time base of about 5 seconds traversing time. Though non-linear, this time base was found adequate. The cell current was amplified by means of a simple D.C. amplifier employing two EF 50 valves, constructed in the laboratory. The output of the amplifier was fed to the Y plates of the cathode-ray tube.

Sörensen's buffer solutions served as supporting electrolyte. For many of the determinations, urea was also present as a co-solvent for the riboflavine. In preparing the cell solutions, the usual technique was to dissolve the riboflavine, with the aid of gentle heat, in a 10 per cent. solution of urea in buffer solution. This solution was then made up to final volume with plain buffer solution, giving a solution of known urea content. For the removal of tablet base debris a porosity 4 sintered glass filter was chosen in preference to filter paper which adsorbs riboflavine.

Two batches of compressed tablets, and two batches of ampoules were examined. For both tablets and ampoules the different batches will be designated A and B. Tablets and ampoules of batch A were of one manufacture, and the batch B products of another. The tablets of batch A were uncoated; those of batch B had a deep orange coating. All the tablets were stated to contain 3 mg. of riboflavine. The ampoules contained 2 ml. of sterile solution of riboflavine, strength 5 mg./ml.

Results and Discussion

Riboflavine is only sparingly soluble in water at about *p*H 7. It is very much more soluble in acid and alkaline solvents, in which it may exist as a cation or an anion. It was shown by Brdička and Knobloch[3] that in acid solution, the riboflavine step is complicated by the presence of an anomalous fore-wave, later shown by Brdička[4] to be due to the adsorption of the reduction product on the surface of the mercury drop. This anomalous fore-wave was not present in the case of solutions of *p*H higher than about 6. In order to avoid possible complications due to these adsorption effects, it seemed desirable to employ solutions of *p*H greater than 6. In alkaline solution, however, riboflavine undergoes rapid photolysis. For these reasons, it was decided to work with approximately neutral solutions, and to employ urea to increase the solubility of riboflavine.

The Effect of Urea and Pyridine on the Polarographic Behaviour of Riboflavine.—In order to determine the effect of urea on the polarographic reduction of riboflavine, a series of solutions containing varying amounts of urea with the same concentration of riboflavine was prepared and examined polarographically. No variation of wave height was observed, nor was there any shift of the half wave potential (− 465 mV.). It may be inferred from these results that close compound formation between riboflavine and urea does not occur; such compound formation would almost certainly be accompanied by a shift of half-wave potential, and a lowering of the diffusion coefficient of the reducible substance, leading

to a diminution of wave height. No reduction of urea was observed over the range of potential covered, i.e., up to — 1·8 V.

Another point of interest was to examine the effect of urea on the adsorption of riboflavine on the mercury drop. It has been shown by Kaye and Stonehill[5] that the addition of ethanol to solutions of acridine, by increasing the solubility of the latter, and its reduction products, prevented the adsorption of electro-active material on the mercury drop surface, and enabled a normal polarographic wave to be obtained. These workers also found that a normal polarogram was obtainable when a limited amount of adsorption of electro-active material occurred, and that the best indication of adsorption on the dropping mercury electrode was the form of the current–time relationship during the life of a single mercury drop. It might therefore be supposed that urea, by increasing the solubility of riboflavine, would have a similar effect in preventing

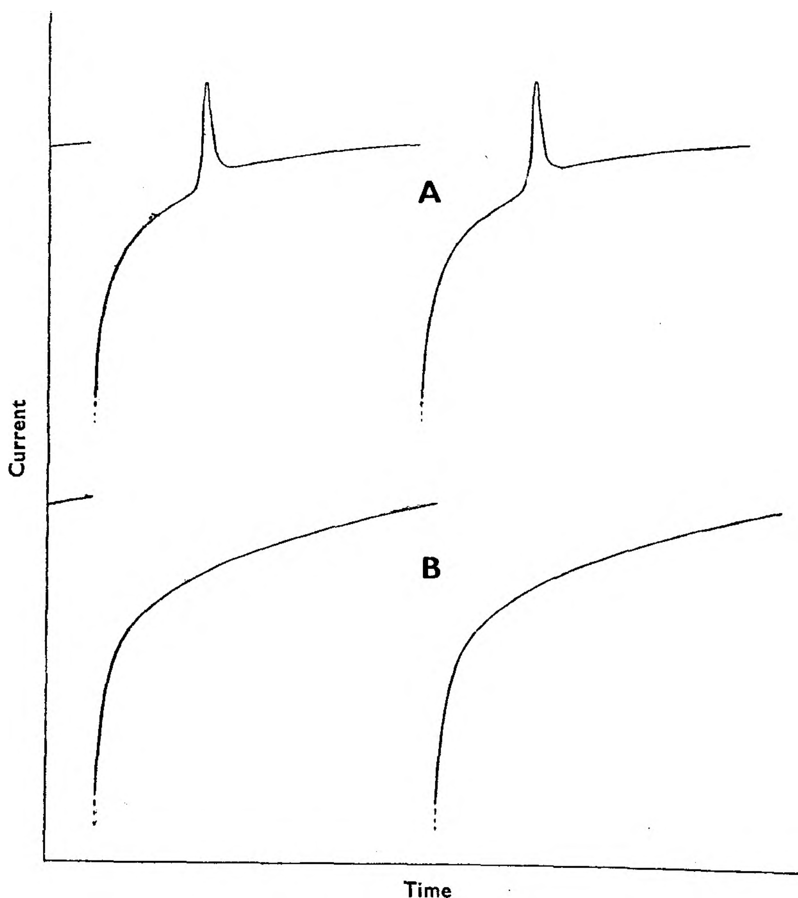


FIG. 1. Time-current relationships during the reduction of riboflavine.
A. With or without addition of urea.
B. With addition of pyridine.

adsorption of the latter, and possibly its reduction product on the mercury drop. To test this supposition, the current–time relationships during the reduction of riboflavine (4×10^{-4} M) in *p*H 7·38 buffer solution in the presence and absence of urea were examined on the oscilloscope, and the tracings photographed. The current–time relationship during the life-time of a single drop was of the form shown in Figure 1A. The sudden surge of current, which occurs early in the life of the mercury drop and is probably due to the adsorption of oxidant at the mercury surface, may clearly be seen. The form of this current–time relationship was not altered by the presence of up to 25 per cent. of urea in the solution, showing that urea does not affect the adsorption of electro-active material on the drop surface. Another experiment with a solution of riboflavine in a buffer-pyridine mixture showed that the inclusion of 20 per cent. of pyridine, which is a good solvent of riboflavine, eliminated entirely the adsorption of electro-active material on the mercury drop, and enabled a normal current–time curve to be obtained (Fig. 1B). Measurements showed that the inclusion of the somewhat basic pyridine in the solution did not significantly alter the *p*H. The elimination of adsorption was not therefore due to the existence of riboflavine in a different ionic state in the pyridine-buffer mixture. These results show that the reactions of urea and pyridine as co-solvents with riboflavine are quite different. Since the limited amount of adsorption occurring at *p*H 7·38 was found not to cause any distortion of the polarogram, and because of the objectionable nature of pyridine, it was decided to employ urea as an aid in the preparation of tablet solutions for polarographic assay.

The diffusion current constant ($\frac{I_d}{c}$) for riboflavine was found by Lingane and Davies (*loc. cit.*) to vary slightly with *p*H, and it was therefore important to know how the addition of small amounts of urea affected the *p*H of the buffer solution used. That this effect was little if any, was already apparent from the independence of the half-wave potential on the presence of urea; any alteration of *p*H would be accompanied by a corresponding shift of the $E\frac{1}{2}$ value, since hydrogen ions are involved in the electro-reduction. Measurements with a glass electrode *p*H meter confirmed this.

Having found the inclusion of urea in the solution to be free from objection, it was decided to employ a standard base solution consisting of Sörensen's phosphate buffer solution, *p*H 7·38, with the addition of 2 per cent. of urea. A satisfactory method was to dissolve the riboflavine in 10 ml. of buffer solution containing 10 per cent. of urea, followed by adjustment to volume with plain buffer solution. Using this technique, a series of solutions containing 1×10^{-4} M. to 6×10^{-4} M. of a B.P. sample of riboflavine, dried over sulphuric acid, was prepared and polarograms obtained. The residual current was obtained for the buffer-urea mixture, and subtracted from the measured wave height to obtain corrected values of the latter for different concentrations of riboflavine. The relationship between $\bar{i}_d$ (the limiting value of the diffusion

current), and concentration was found to be linear. In order to assess the accuracy with which the diffusion current could be measured, several determinations were made using the same solution. For riboflavine concentrations of 10^{-4} M to 10^{-3} M the accuracy of measurements was $\pm$ 2 per cent.

The effect of soluble tablet excipients on diffusion current.—A variety of materials is likely to be encountered in compressed tablets, and it is important to know if any of the ingredients present in commercially produced tablets would affect the polarographic behaviour of riboflavine. It might be expected, for example, that acacia and similar substances, by increasing the viscosity of the solution slightly, would lower the value of the riboflavine diffusion coefficient and therefore the diffusion current, which is governed by the diffusion coefficient in accordance with the Ilkovič equation $\bar{i}_d = 605\, n\, D^{\frac{1}{2}}\, C\, m^{\frac{2}{3}}\, t^{\frac{1}{6}}$; where $\bar{i}_d$ = diffusion current in microamperes; C = concentration of reducible substance in millimols per litre; n = number of electrons involved in the electro-reduction; D = diffusion coefficient; t = drop time; and m = rate of mercury flow in mg./sec.$^{-1}$ In the case of coloured sugar-coated tablets there is the additional possibility of electro-reducible dyes being present in the coating. To decide these points, a tablet from batch A was disintegrated in 10 per cent. urea solution in *p*H 7·38 buffer, and the riboflavine dissolved. The solution was clarified as described and adjusted to 50 ml. The amount of riboflavine was determined polarographically. 3 mg. of riboflavine, accurately weighed, was then dissolved in the solution, and the total riboflavine content obtained. The difference between the two determinations gave the added amount of riboflavine as 3·07 mg., showing any soluble component of the tablet base to be without effect. A second experiment with orange-coloured sugar-coated tablets from batch B gave a similar result. To determine if the orange dye in the coating was reducible, the dye coat was dissolved in 10 per cent. urea in buffer solution (the tablet removed before any riboflavine dissolved) adjusted to volume with plain buffer and assayed polarographically. The dye was found to be non-reducible over the potential range covered.

Comparison of the Polarographic and U.S.P. colorimetric methods.—The U.S.P. XII colorimetric method consists of comparing the colour of a filtered tablet solution with that of two standard solutions containing 20 per cent. less and 20 per cent. more than the amount of riboflavine expected to be present in the tablets. The comparisons are made in matched tubes. Using this method the riboflavine content of a batch A tablet was found to be about 2·4 mg. Polarographic analysis of the same tablet solution gave the result as 2·01 mg. In the colorimetric method it was found difficult to distinguish colour difference between the solutions used when the comparison was carried out in tubes, and different workers failed to agree about the colour matching. In order to reduce the subjective errors of this method a Duboscq colorimeter was employed to determine the riboflavine content of a number of tablets. The same tablet solutions were then estimated polarographically, and the two sets of results compared (Table I).

Some difficulty was experienced when using the Duboscq colorimeter, the tablet solutions appearing slightly opalescent. This imparted a slightly grey appearance to the solution in the colorimeter. It was found impossible to clarify the solutions perfectly using a porosity 4 sintered glass filter. The rate of filtration, moreover, was extremely slow. All the colorimetric results were considerably lower than those obtained polarographically. To determine to what extent this was due to imperfect clarification of the tablet solutions, a further series of comparisons of the two methods was made using carefully weighed samples of pure riboflavine. The results are given in Table II.

TABLE I

UNCOATED RIBOFLAVINE TABLETS 3 MG., BATCH A

Colorimetric method mg.	Polarographic method mg.
2·01	2·46
2·08	2·40
1·75	2·37
1·84	2·07
1·38	1·73

TABLE II

Weight of sample used mg.	Result of colorimetric determination mg.	Result of polarographic determination mg.
3·8	4·43	3·76
4·03	4·50	4·13
3·67	3·89	3·76

In the case of the weighed samples of pure riboflavine, the colorimetric results are higher than the polarographic. It is evident that the presence of minute amounts of suspended material from the tablet base exerts a marked influence on the results obtained colorimetrically. In addition to this difficulty, subjective errors were apparent in using the Duboscq colorimeter. The results obtained by different observers differed considerably.

The effect of suspended tablet debris on polarographic analysis.—As stated above, the effect of minute amounts of suspended tablet debris on the colorimetric determination was appreciable. Since clarification through a No. 4 sintered glass filter was always tedious, it was decided to examine the effect of the presence of tablet debris during the polarographic determination of riboflavine, with a view to eliminating altogether the clarification process. Accordingly, an uncoated 3-mg. tablet from batch A was disintegrated in 10 per cent. urea solution in *p*H 7·38 buffer, care being taken to dissolve all the riboflavine. The solution was adjusted to 50 ml. and placed in the cell without clarification. After bubbling nitrogen through the liquid, rapid settling of the larger particles occurred, leaving a translucent supernatant liquid. Current-potential readings were obtained. 3 mg. of riboflavine was then dissolved in the liquid, and a second determination made. The difference between the two diffusion currents enabled the amount of added riboflavine, as determined in the presence of tablet debris, to be calculated. The experiment was repeated with a sugar-coated tablet from batch B. The uncoated tablet

batch A showed 3·08 mg. of added riboflavine, estimated in the presence of tablet debris and the coated tablet batch B showed 2·95 mg. These results show that the presence of suspended tablet debris has little influence on the polarographic determination of riboflavine. A possible objection to the technique is that the tablet debris, by displacing a small volume of liquid, will affect the concentration of dissolved riboflavine. Experiments showed that the average volume occupied by the debris from both batch A and batch B tablets was approximately 0·05 ml. The volume of the solution plus tablet debris was 50 ml. so that the error introduced by leaving the tablet debris in the solution is less than 0·2 per cent.—considerably less than the errors involved in measuring the diffusion current. Using this technique the remaider of the tablets in batch B (sugar-coated) were assayed. The results are given in Table III.

TABLE III

Corrected wave height (galvanometer scale readings)	Riboflavine content mg.
2·0	2·60
2·26	2·95
1·94	2·52
2·35	3·09
1·95	2·54
2·08	2·70
2·13	2·77
2·26	2·95
2·20	2·88
2·32	3·03

Estimation of riboflavine in ampoule solutions.—2 batches of ampoules of different manufacture were examined. The ampoules were stated to contain 10 mg. of riboflavine in 2 ml. of solution. Using a method similar to that employed for compressed tablets, it was found that the solutions in both batches of ampoules contained nothing which affected the riboflavine diffusion current, or significantly affected the pH of the cell solution. The solutions for examination were prepared by taking 1 ml. of the ampoule solution and making up to 50 ml. with pH 7·38 buffer solution. The amount of riboflavine present was estimated both polarographically and colorimetrically, using a Duboscq colorimeter. The results for ampoules of batch A are given in Table IV.

TABLE IV

Polarographic result mg./ml.	Colorimetric result mg./ml.
2·26	2·75
2·45	2·63
2·26	2·58
2·58	2·68
2·69	2·75

As was found previously for samples of pure riboflavine, the colorimetric results are considerably higher than the polarographic. Several of the ampoules of batch A showed a black deposit, which dispersed on shaking; this might account for the serious shortage of riboflavine in the ampoules. There was also a slight difference in the colour quality of the diluted ampoule solution, and the standard solution employed for the colorimetric determination. The contents of the ampoules of batch B were also examined by the two techniques. The colour of the solution diluted with pH 7·38 buffer, was orange yellow, compared with the greenish-yellow colour of the standard solution prepared for the colorimetric assay. It was found quite impossible to obtain any reliable results by colorimetry. The polarographic results for 4 of the ampoules of batch B were:—4·97, 4·98, 5·12, 5·02 mg./ml.

The riboflavine content of the ampoules was thus in accordance with

the statement of strength on the label. No black deposit or other signs of deterioration were observed.

The effect of light on the polarographic behaviour of riboflavine.—Solutions of riboflavine are usually regarded as being unstable to light, especially if alkaline. Since the water bath used for maintaining the contents of the cell at 25° C. was heated by two carbon filament lamps, it was important to know if decomposition was likely to occur during the determination. To determine this, a 4×10^{-4} M solution of riboflavine was prepared in *p*H 7·38 buffer using urea as co-solvent. The urea concentration in the final solution was 2 per cent. Current-potential curves were obtained both before and at intervals during exposure to a 60-watt lamp, the maximum time of exposure being 44 hours. The resulting polarograms showed a slight but quite definite modification after exposure of the solution to light, this consisting of a more rapid decrease in the slope of the curve towards the crest of the wave, suggesting the formation of a second and ill-defined wave. There was also a very small reduction of the total wave height. Further experiments showed that exposure to the light of the heating lamps, in the water bath, for a moderate period of time produced no polarographically detectable change in the solution.

In view of the possibility of using the polarographic method to detect and to estimate the photolytic products from riboflavine, a series of experiments with an alkaline solution of riboflavine was undertaken, photolysis being much more rapid at high *p*H. Riboflavine was dissolved in *p*H 11 buffer solution and determined polarographically, the solution being protected from light. The solution was then divided into two portions, one being exposed to the light of a 60-watt lamp under standard conditions, and the other stored in the dark. Current-potential curves for both solutions were obtained periodically. A few hours exposure to light resulted in the appearance of a second wave with a half-wave potential more negative than that of the main riboflavine wave. Continued exposure to light caused an increase in the height of the second wave, and a corresponding decrease of the main riboflavine wave. It seemed likely that the new substance was lumiflavine. To confirm this a small sample of the solution was taken, neutralised with hydrochloric acid and extracted with chloroform, in which lumiflavine is soluble. The separated chloroform layer was yellow. The chloroform layer was distilled off, and the residue taken up in *p*H 11 buffer solution. This yielded a single polarographic wave, of half-wave potential identical with that of the second wave described above. Continued exposure of the solution to light resulted in the appearance of a third wave, at still more negative potential, and a corresponding decrease in the height of the lumiflavine wave, indicating a further breakdown of the lumiflavine, and the formation of a third reducible substance. These changes are shown in Figure 2. The points of inflection at the top of the third wave in polarograms 5 to 9 are probably due to incipient maxima. Figure 3 shows how the diffusion currents, and hence the concentrations of riboflavine, lumiflavine, and its breakdown product alter with time.

FIG. 2. Current potential curves for a solution of riboflavine exposed to light. A. Zero. B. 18 hours. C. 68 hours. D. 92 hours. E. 108 hours. F. 144 hours. G. 255 hours. H. 473 hours. I. 947 hours.

The potential scale is marked off at 100 mV. intervals. Each curve commences at — 400 mV.

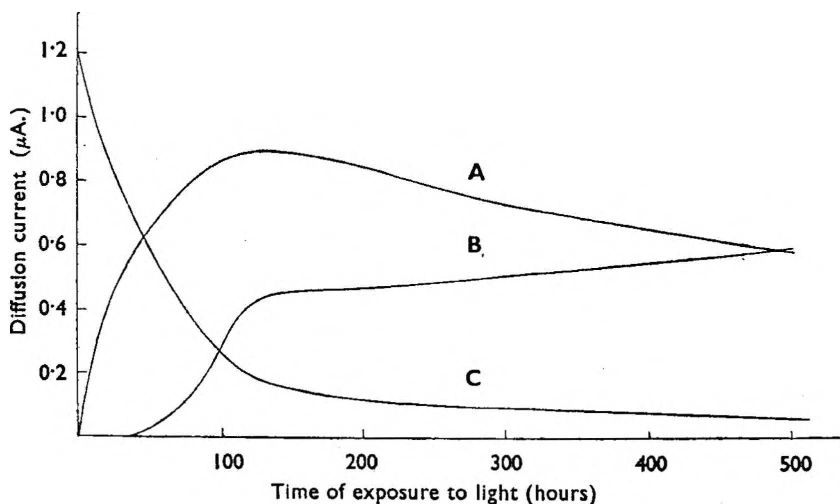
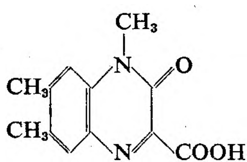


FIG. 3. Changes of diffusion current with time of exposure of solutions to light.
A. Lumiflavine.
B. Breakdown product of lumiflavine.
C. Riboflavine.

Although the production of lumiflavine from riboflavine in alkaline solution is primarily a photo-chemical process, we have no evidence suggesting that the further break-down of lumiflavine depends on exposure to light. The solution stored in the dark was examined periodically. Polarograms showed a gradual loss of riboflavine, but at a much slower rate than in the case of the solution exposed to light. Disappearance of riboflavine was accompanied by the formation of another reducible substance which, from its half-wave potential, appeared to be identical with the break-down product of lumiflavine present in the solution exposed to light. No lumiflavine could be detected in the solution stored in the dark. It seems probable therefore that this break-down product has the structure:—



which is known to be formed by the action of alkali on both riboflavine and lumiflavine (Kuhn and Rudy[6]). It is interesting to note that the polarographic evidence of the existence of this further break-down product supports the observations of Daglish, Baxter, and Wokes,[7] who, from evidence obtained spectroscopically, suggested the existence in alkaline riboflavine solutions of some decomposition product other than lumiflavine.

The small, ill-defined wave, noted on polarograms obtained after exposure of an approximately neutral solution to light, is probably not due to the presence of lumiflavine, since its $E\frac{1}{2}$ value was only about 60 m. more negative than that of the main riboflavine wave. It may be attributed to the presence of a small amount of lumichrome, which is formed from riboflavine by irradiation in neutral or acid solutions.

The polarograms of Figure 2 show that the *total* wave height at first increases with the progressive decomposition of riboflavine. This is to be expected, since the decomposition products, of smaller molecular weight than the parent substance, would have larger diffusion coefficients. After 144 hours exposure to light, however, the total wave height decreases. The most likely explanation of this is that further decomposition occurs, yielding products which are not detectable polarographically.

This study by the polarographic method of the decomposition of riboflavine is incomplete, and further investigation is in progress. The results obtained so far show that polarography is suitable for the detection of some of the break-down products of riboflavine. None of these products was found in the compressed tablets and ampoule solutions examined.

SUMMARY

1. Conditions suitable for the polarographic estimation of riboflavine in compressed tablets have been determined.

2. Polarography has been found to be more reliable than the simple colorimetric procedure of the U.S.P. XII. It is more rapid than the fluorimetric, microbiological, and spectrophotometric methods of riboflavine estimation.

3. Two batches of commercially produced tablets, and two of ampoules have been examined. One batch of tablets and one of ampoules were found to be seriously deficient in riboflavine. There exists a need for more stringent control of such products.

4. Some decomposition products of riboflavine may readily be detected and estimated polarographically. The decomposition of riboflavine in alkaline solution, when exposed to light and also when stored in the dark, has been followed polarographically. The results are in agreement with those of spectroscopy.

5. The inclusion of pyridine in a solution of riboflavine at *p*H 7·38 has been found to prevent adsorption of electro-active material at the surface of the mercury drop.

References

1. Lingane and Davis, *J. biol. Chem.*, 1941, **167**, 567.
2. Kolthoff and Lingane, *Polarography*, Interscience Publishers N.Y., 1946.
3. Brdička and Knobloch, *Ztschr. Electrochem.*, 1941, **47**, 721.
4. Brdička, *Ztschr. Electrochem.*, 1942, **48**, 686.
5. Kaye and Stonehill, *J. chem. Soc.*, 1951, 27.
6. Kuhn and Rudy, *Ber., dtsch. chem. Ges.*, 1934, **67**, 892.
7. Daglish, Baxter and Wokes, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 344.

DISCUSSION

The paper was presented by Miss S. Fowler.

Dr. F. Wokes (King's Langley) suggested that the authors might in their future work obtain more information from spectrophotometrical results. He pointed out that they had given results in respect of two samples of tablets and ampoules only, and on the basis of those results had found one deficiency. The samples might be deficient, but as soon as possible more information should be obtained on a much wider range of samples. What were the actual wavelengths at which the authors radiated their riboflavine?

Mr. T. D. Whittet (London) asked whether the authors had tried the possible effect of nicotinamide in their determinations as he had used nicotinamide and urea together to solubilise riboflavine.

Mr. M. Dombrow (London) said that the authors mentioned that an anomalous fore-wave which occurred early in the life of the drop was probably due to the absorption of oxygen on the mercury surface. Early in the paper there was a reference to the work of Brdička in which it was suggested that the anomalous fore-wave occurring was due to absorption of the reduced material, in particular the leuco or semi-quinone form. There appeared to be some discrepancy in that connection and it would be interesting to have some information concerning evidence that the absorption was due to the oxidant and not to the reductant. With regard to the statement that the reactions of urea and pyridine as co-solvents for riboflavine were different due to the fact that in the presence of urea the anomalous fore-wave occurred, while in the presence of pyridine it was dissipated, had they different actions as co-solvents?

MISS S. FOWLER, in reply, said that the polarographic method was adopted because it had the merit of simplicity in the preparation of solutions and in the actual reading of the final results. With regard to the comparison with the spectrophotometric method, facilities were now available for comparison with that method and work would be carried out. Nicotinamide had not been used as a solvent for riboflavine, but it would be borne in mind. With regard to the standard conditions for radiation, light was provided from a 60 watt lamp in which there would not be a great deal of ultra-violet light. Their aim had been to produce such substances as lumiflavine by some artificial means as quickly as possible. She was not in a position to say whether it was the oxidant or reductant product which was absorbed. The assertion that the actions of urea and pyridine as solvents were different was based on spectroscopic evidence.

THE DETERMINATION OF THYROXINE WITH SPECIAL REFERENCE TO TABLETS

By D. C. M. Adamson, A. P. Domleo, J. P. Jefferies and W. H. C. Shaw

From Glaxo Laboratories, Ltd., Greenford, Middlesex

Received July 3, 1952

Chemical methods for the determination of thyroxine are generally based on measuring the organically combined iodine. As a rule organic matter is destroyed by ignition with one or more of the alkali carbonates[1] (cf. the methods specified for thyroid in the British Pharmacopœia, 1948, and in the United States Pharmacopœia XIV). The iodide resulting from the ignition is determined directly[2,3] or, more frequently, by subsequent oxidation to iodate with potassium permanganate,[4,5] hypochlorite[1,6] or bromine[7]; after acidification and removal of excess of oxidising agent, iodide is added, the liberated iodine being determined volumetrically or colorimetrically. Various other methods have been suggested[8,9,10] to avoid the preliminary ignition.

Although capable of high accuracy, determination of thyroxine by ignition with alkali suffers from interference by inorganic iodides and other organic iodine compounds, so that with impure materials preliminary separation of the thyroxine is usually necessary.[11,12,13,14] Moreover, the method is tedious for large numbers of determinations.

The recent commercial availability of synthetic L-thyroxine[15] led us to investigate other possible methods of determination, particularly those of potential value for the analysis of tablets. Since the activity of L-thyroxine is extremely high, the amounts available for analysis are often small (e.g., 50 to 100 μg. of L-thyroxine per tablet) and a satisfactory method must be of high sensitivity. Three methods have been studied: ultra-violet absorption, polarography and a colorimetric procedure based upon the orange colour developed by treating thyroxine with nitrous acid and then with ammonia.[16]

Preparation of purified sodium L-*thyroxine.*—Specially purified sodium L-thyroxine, prepared by the method of Chalmers *et al.*,[15] was dried at 40° C. Found: Na, 2·34; I, 57·2; H_2O, 10·2 per cent. $C_{15}H_{10}O_4NI_4Na, 5H_2O$ requires Na, 2·58; I, 57·2; H_2O, 10·1 per cent.

Ultra-violet Absorption

The ultra-violet absorption of thyroxine has been studied by a number of workers,[17,18,19,20] and curve A in Figure 1 is in close agreement with the results recently published by Reinecke and Turner[21] for wavelengths above 260 mμ. Besides the maximum at 325 mμ a second much larger maximum occurs at approximately 227 mμ ($E_{1\,cm.}^{1\,per\,cent.} = 620$), but this is of limited value, since interference by irrelevant absorption is likely at such wavelengths and similar maxima are exhibited by closely related substances (B and C, Fig. 1).

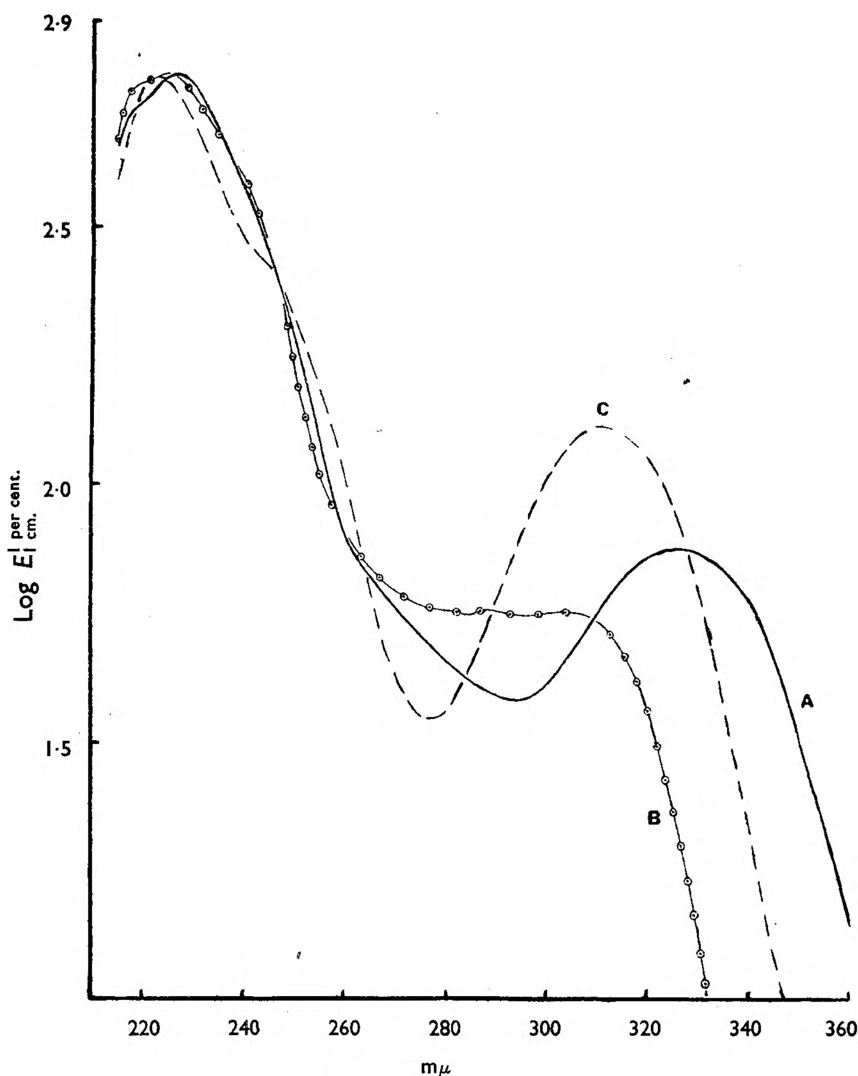


FIG. 1. Ultra-violet absorption spectra in 0·1 N sodium hydroxide.
A, sodium L-thyroxine. B, 3;5-diiodo-L-thyronine. C, 3;5-diiodo-L-tyrosine.

In practice, ultra-violet absorption has not been found of great value for the analysis of thyroxine, though the ratio of the extinction at 325 mμ (maximum) to that at the minimum near 295 mμ provides useful information. Any marked deviation from a value of 1·98 for this ratio suggests the presence of an impurity. The extinction of sodium L-thyroxine at 325 mμ is relatively low ($E_{1\,cm.}^{1\,per\,cent.} = 76$); nevertheless, this property may be made a basis for determination in suitable preparations, though interference by tablet excipients limits the value of the method.

POLAROGRAPHY

The polarographic reduction of thyroxine was first reported by Simpson and Traill,[22] who used a base solution containing 1 per cent. of tetramethylammonium bromide in a mixture of ethanol (2 vols.) and 0·5 N sodium carbonate (3 vols.). Borrows, Hems and Page[23] retained the tetramethylammonium bromide, but used a final concentration of 0·5 N sodium carbonate in 20 per cent. v/v *iso*propanol. They reported three steps having half-wave potentials at − 1·12 V, − 1·30 V and − 1·51 V measured against a saturated calomel electrode, the second step being surmounted by a prominent maximum. The height of either the first or the total step could be used for quantitative purposes, though the first was preferred, because it was not affected by the presence of 3:5-diiodotyrosine. 3:5-Diiodothyronine, however, interfered, giving steps at − 1·18 V and − 1·37 V.

Preliminary trials with the method using commercial samples of sodium L-thyroxine disclosed considerable discrepancies in the ratios of the first to the total step height. This was apparently due to the presence of maxima of varying heights on the first step. Specially purified samples gave polarograms with a pronounced maximum on the first and a large one on the second step (A, Fig. 2). Further investigation into the effect of differences in composition of the base solution appeared desirable.

A Cambridge pen-recording polarograph was used throughout. The capillary constants in 0·1 N potassium chloride at 25° C. on open circuit were $m = 1{\cdot}83$ mg./sec., $t = 3{\cdot}15$ sec., and all polarograms were recorded at 25 (± 0·1) °C. against a saturated calomel electrode. Oxygen was removed from all solutions by thorough bubbling with nitrogen. Purified sodium L-thyroxine prepared as described above was used as standard.

(*a*) iso*Propanol concentration.*—*iso*Propanol was purified by distillation over sodium hydroxide and zinc dust. Differences in concentration in the base solution affected the height of both the first and total steps to the same extent, as shown in Table I, where 20 per cent. v/v is regarded as standard.

(*b*) *Tetramethylammonium bromide concentration.*—Omission of this ingredient caused much distorted polarograms (E, Fig. 2): but between 0·75 and 1·25 per cent. w/v the concentration is not critical.

(*c*) *Sodium carbonate concentration.*—Variations in strength from 0·4 to 0·6 N had no effect on the shape of the polarogram nor on the step height.

(*d*) *Suppression of the maxima.*—By addition of graded amounts of gelatin, the maxima on both first and second steps may be suppressed (B, C and D, Fig. 2). However, the amount required must be very carefully adjusted, since too little gives a fictitiously high first step and too much causes distortion. The variations in the maxima observed on the first steps given by commercial preparations of sodium L-thyroxine may well be due to traces of impurities acting in a similar manner. Although the shape of the unsuppressed first step may be of possible value as a guide to quality, it has been found of little value for quantitative

purposes. Figure 2 and Table I show the effects of varying the concentration of gelatin. Methylcellulose, carboxymethylcellulose, α-naphthol alizarin, methyl red and bromophenol blue were tried as suppressors but all were inferior to gelatin.

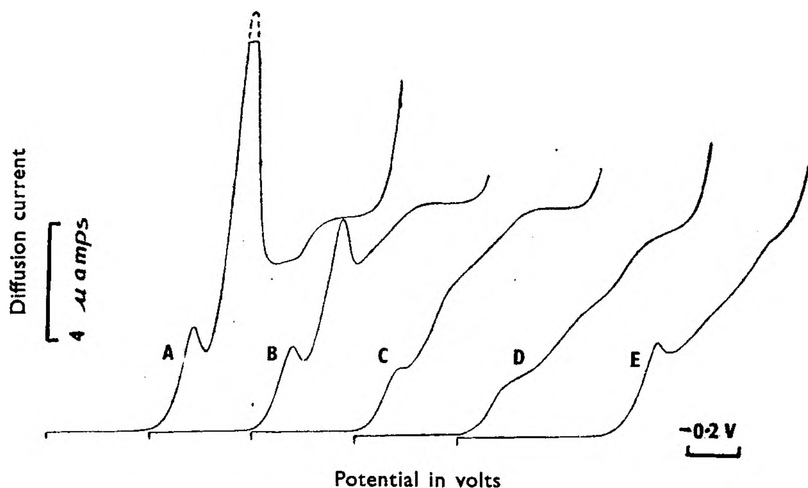


Fig. 2. Polarograms for sodium L-thyroxine, 0·06 per cent. w/v at *p*H 11·6.
A, without gelatin. B, 0·0025 per cent. C, 0·0065 per cent. D, 0·030 per cent. of gelatin. E, without tetramethylammonium bromide.
All polarograms start at −0·6V.

(*e*) *Variation in* p*H*.—The sodium carbonate base solution recommended by Borrows *et al.*[23] has *p*H 11·6. Since the height of the first step at that *p*H is affected by the presence of a maximum, and suppression is extremely critical, the total step height is preferably used for quantitative purposes. The effect of varying the *p*H (Fig. 3) shows that at *p*H 10

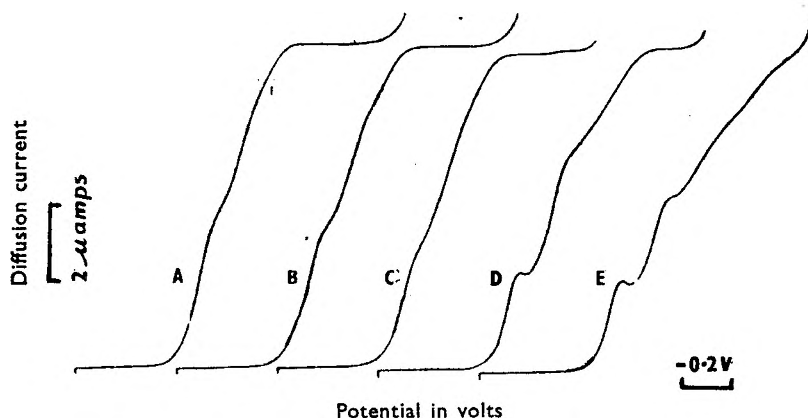


Fig. 3. Effect of variation of *p*H on polarograms for sodium L-thyroxine (0·06 per cent. w/v).
A, *p*H 9·4. B, *p*H 10·0. C, *p*H 10·3. D, *p*H 11·6. E, *p*H 13·2.
All polarograms start at −0·6V.

thyroxine gives only two steps with half-wave potentials at − 1·11 V and − 1·32 V. This first step is also unsuitable, but the total step is more satisfactory than the one occurring at *p*H 11·6.

The following base solution has been found satisfactory:—

		ml.
2·5 N Sodium carbonate		5·0
1·0 N Sodium bicarbonate		12·5
*iso*Propanol		10·0
Tetramethylammonium bromide solution (10 per cent. w/v)		5·0
Gelatin solution (0·1 per cent. w/v)		2·0
Water	to	50·0

Note.—The sample (about 30 mg. of sodium L-thyroxine pentahydrate) should be dissolved in the ingredients (other than the sodium bicarbonate) diluted with about 10 ml. of water. The sodium bicarbonate is added and the solution is then diluted to volume.

Variation of any constituent of the above solution gives results similar to those in Table I for a base solution of *p*H 11·6, but the concentration of gelatin may be varied between 0·002 per cent. and 0·010 per cent. w/v without significantly affecting the step height.

TABLE I

EFFECT OF VARIATION IN CONCENTRATION OF *iso*PROPANOL AND GELATIN

*iso*Propanol concentration per cent. v/v	Gelatin concentration per cent. w/v	Percentage of standard step height*	
		First step	Total step
10	0·0065*	123	124
15	0·0065	108	111
20	0·0065	100*	100*
25	0·0065	92	92
30	0·0065	85	86
20	nil	125	97
20	0·0015	115	103
20	0·0025	110	104
20	0·0045	105	101
20	0·0065	100*	100*
20	0·010	99	99
20	0·030	(not measurable)	approx. 89

* 20 per cent. v/v of *iso*propanol and 0·0065 per cent. w/v of gelatin yield the most satisfactory polarograms at *p*H 11·6 and the step heights at these concentrations are here regarded as 100.

Application to tablets.—The polarographic method with the base solution given above has been successfully adapted to the analyses of thyroxine tablets, and good agreement with other methods has been obtained (Table II).

Preliminary separation from the excipients is usually necessary, since common ingredients such as magnesium stearate or buffering compounds interfere. Although satisfactory separations can be devised for tablets of known composition, the scope of the method is limited since it appears that each formulation would require separate consideration.

TABLE II

ANALYSES OF SODIUM L-THYROXINE TABLETS

Sample	Labelled strength (per tablet)	Thyroxine content (mg. anhydrous sodium L-thyroxine per tablet)		
		By iodine determination	Polarographic method	Colorimetric method
A	0·1 mg.	0·097 0·097	0·098 0·103	0·098 0·097
B	0·1 mg.	0·099 0·100	0·100 0·102	0·101 0·100
C	0·1 mg.	0·094 0·095	0·096 0·098	0·095 0·093
D	0·05 mg.	0·048 0·049	0·049 0·048	0·049 0·048

COLORIMETRIC DETERMINATION

Like many other 2:6-diiodophenols, thyroxine gives a red colour when treated first with nitrous acid and then with ammonia. This reaction, originally noted by Kendall and Osterberg,[24] was used for the photometric determination of thyroxine and diiodotyrosine by Morton and Chaikoff.[25] It has recently been the subject of a detailed study by Roche and Michel[10] for use in the analyses of thyroid gland and iodinated proteins. Other colorimetric methods of determination are based upon the red colour developed by treatment with freshly-prepared diazobenzenesulphonic acid in alkaline solution,[26] and a similar method makes use of the diazo derivative of *N*′-diethylsulphanilamide.[27] Thyroxine gives no colour with Millon's reagent, which may therefore be used for determining other reactive phenols in the presence of thyroxine.[16] However, thyroxine is converted to a reactive phenol by prolonged treatment with alkaline stannite and a colorimetric method based on this procedure has been suggested.[28]

Of these methods, the one based on the nitrite-ammonia reaction appeared to be the most suitable for the determination of synthetic sodium L-thyroxine and of particular value for application to tablets. Although not as selective for thyroxine as that described by Winikoff and Trikojus,[27] the method is simple, rapid and sufficiently sensitive. The procedure detailed by Roche and Michel[16] was found essentially satisfactory for maximum colour development, as shown by the results in Table III.

We prefer, however, to replace the prescribed sodium hydroxide-hydrochloric acid reagent by an equivalent amount of sodium chloride and to increase the proportions of hydrochloric acid and of ammonia: any buffering effect of tablet excipients can then be ignored. In addition, 20 minutes contact with nitrous acid is more satisfactory. The colour produced by thyroxine is photolabile, particularly after addition of ammonia, and all operations should be carried out in diffuse light. Although unlikely to be encountered in synthetic sodium L-thyroxine, 3:5-diiodotyrosine gives a more intense colour than thyroxine,[10] while we have found that 3:5-diiodothyronine gives about 65 per cent. of the colour of thyroxine.

TABLE III

THE EFFECT OF VARIATION IN CONDITIONS FOR DEVELOPMENT OF NITRITE-AMMONIA COLOUR

Condition varied		Percentage of maximum colour
1. Concentration of hydrochloric acid at nitrosation stage	approximately 0·035N	73
	" 0·07N	100*
	" 0·10N	100
	" 0·50N	100
	" 1·00N	98
2. Concentration of sodium chloride at nitrosation stage	approximately 0·75N	100
	" 1·5N	100*
	" 2·25N	102
3. Concentration of ethanol (95 per cent.) in final solution	15·0 per cent. v/v	93
	20·0 " " "	97
	25·0 " " "	99*
	27·5 " " "	100
	30·0 " " "	100
	32·5 " " "	98
	35·0 " " "	97
	37·5 " " "	93
4. Volume of sodium nitrite solution (1 per cent. w/v) used	1·0 ml.	99
	2·0 ml.	100*
	3·0 ml.	100
5. Time of contact with sodium nitrite solution (2 ml. 1 per cent. w/v)	2·5 minutes	69
	5 "	96
	10 "	98*
	15 "	100
	20 "	100
	25 "	100
6. Stability of colour from time of preparation	2 minutes	100
	8 "	100
	20 "	99

* Conditions prescribed by Roche and Michel.[16]

Extraction of the thyroxine from tablets in a form suitable for colorimetric estimation usually presents little difficulty. Most of the commonly used excipients are either soluble in the reagents without interfering or can be removed by some simple extraction and filtration technique. The colorimetric method is thus more rapid and selective for thyroxine than determination of the organically combined iodine and is more accurate and less likely to suffer from interference by tablet excipients than the polarographic method. It is also more sensitive than either procedure and gives a linear calibration over the range 0 to 0·8 mg. of sodium L-thyroxine.

The following method has been found satisfactory for commercial sodium L-thyroxine tablets:—

Reagents

(1) *Sodium chloride reagent.*—Dissolve 170 g. of sodium chloride (A.R.) in sufficient N hydrochloric acid to produce 1000 ml.

(2) *Sodium nitrite solution.*—1 per cent. w/v, freshly prepared.

Weigh and powder a sufficient number of tablets. Transfer an accurately weighed quantity of the powder, equivalent to 1·5 to 2·0 mg. of anhydrous

sodium L-thyroxine, to a 50-ml. graduated flask. Add 17·5 ml. of ethanol (95 per cent.) and 25 ml. of sodium chloride reagent. Heat the flask in a boiling water bath until the solution boils, cool and dilute to volume with sodium chloride reagent. Filter and collect 35 ml. of filtrate. Transfer 15·0 ml. of clear filtrate to a 20-ml. graduated flask, add 2·0 ml. of sodium nitrite solution, mix and allow to stand in the dark for 20 minutes. Dilute to volume with strong ammonia solution (32·5 per cent. w/w of NH_3), mix and determine the optical density of the solution in a 2-cm. cell using spectrum blue (602) filters. Carry out a blank determination with 2·0 ml. of water in place of the 2·0 ml. of sodium nitrite solution and apply the necessary correction to the optical density.

Calculate the sodium L-thyroxine content of the tablets by reference to a calibration graph prepared with known amounts of pure sodium L-thyroxine (0·5 mg. normally gives an optical density of about 0·33).

Summary

1. Methods for the determination of sodium L-thyroxine have been examined, particularly those of potential value for the analysis of tablets prepared from synthetic material.

2. Ultra-violet absorption, polarography and a colorimetric method based upon nitrosation of thyroxine have been applied. Of these, the colorimetric method has been found the most satisfactory.

References

1. Hunter, *J. biol. Chem.*, 1910, 7, 321.
2. Hilty and Wilson, *Industr. Engng. Chem., Anal. Ed.*, 1939, **11**, 637.
3. Sappington, Halperin and Salter, *J. Pharmacol.*, 1944, **81**, 331.
4. Fabre and Pénau, *C.R. Soc. Biol.*, 1922, **87**, 1026.
5. Pickworth, *Biochem. J.*, 1925, **19**, 768.
6. Beal and Skalkowski, *J. Amer. pharm. Ass.*, 1935, **24**, 742.
7. Schulek and Stasiak, *Pharm. Zentralh.*, 1928, **69**, 513.
8. Shahrokh, *J. biol. Chem.*, 1943, **147**, 109.
9. Paal and Motz, *Biochem. Z.*, 1935, **279**, 106.
10. Nicklaus and Tippett, *J. Amer. pharm. Ass., Sci. Ed.*, 1940, **29**, 124.
11. Harington and Randall, *Quart. J. Pharm. Pharmacol.*, 1929, **2**, 501.
12. Leland and Foster, *J. biol. Chem.*, 1932, **95**, 165.
13. Blau, *ibid.*, 1933, **102**, 269.
14. Blau, *ibid.*, 1935, **110**, 351.
15. Chalmers, Dickson, Elks and Hems, *J. chem. Soc.*, 1949, 3424.
16. Roche and Michel, *Biochim. Biophys. Acta*, 1947, **1**, 335.
17. Hicks, *J. chem. Soc.*, 1925, **127**, 771.
18. Abderhalden and Haas, *Z. physiol. Chem.*, 1927, **166**, 78.
19. Heidt, *J. biol. Chem.*, 1936, **115**, 223.
20. Ungande, *J. Amer. chem. Soc.*, 1941, **63**, 2091.
21. Reinecke and Turner, *Res. Bull. Agric. Exp. Station Coll. Agric. Univ. Missouri*, No. 355, 1942.
22. Simpson and Traill, *Biochem. J.*, 1946, **40**, 116.
23. Borrows, Hems and Page, *J. chem. Soc.*, 1949, S204.
24. Kendall and Osterberg, *J. biol. Chem.*, 1919, **40**, 265.
25. Morton and Chaikoff, *ibid.*, 1943, **147**, 1.
26. Moser, *Experentia*, 1947, **3**, 119.
27. Winikoff and Trikojus, *Biochem. J.*, 1948, **42**, 475.
28. Brand and Kassell, *J. biol. Chem.*, 1939, **131**, 489.

DISCUSSION

The paper was presented by MR. D. C. M. ADAMSON.

The CHAIRMAN remarked that it was interesting to see how close were the results given by the three methods of determination, namely, ultra-violet absorption, polarography and colorimetry.

DR. F. WOKES (King's Langley) said that in Figure 1 the absorption band due to thyroxine was overlapped to a considerable extent by the band for 3:5-diiodo-L-tyrosine. The spectrographic method might possibly still be employed if, instead of using the minimum at 295 mμ (as compared with the maximum at 325 mμ), the authors used the top part of the curve and applied a correction. They could then work with a much narrower band.

MR. T. D. WHITTET (London) asked whether the authors had tried tests on the new substance triiodothyronine which had been found to be more active than thyroxine. It had been detected polarographically in thyroid extracts.

MR. M. DOMBROW (London) said it was difficult to reconcile the half-wave potentials recorded with the actual figures in the diagrams. He asked how the *p*H was determined. Was it measured with a *p*H meter or was it calculated from a mixture of known buffers?

DR. G. E. FOSTER (Dartford) enquired whether thyroid tablets had been assayed by the method described.

DR. N. EVERS (Hertford) said that if Mr. Adamson and his colleagues had found a method which would replace the present B.P. method they deserved thanks, because there was no method which took up so much time and gave such unsatisfactory results in the hands of different analysts.

MR. D. C. M. ADAMSON, in reply, said there had been a plea for spectrophotometric methods with the Morton correction. Ultra-violet spectrophotometry had found its place in industrial and other laboratories in recent years, but those with experience of the difficulty of determining vitamin A would not wish to become involved with a Morton's correction for another substance. The method had not been applied to triiodo-thyronine. For routine use he preferred to increase the height of the wave at higher potentials—more than half-wave. It was not claimed that the method worked with thyroid tablets; it might do so, but probably it would be necessary to extract the thyroxine.

THE PARTITION CHROMATOGRAPHY OF ALKALOIDS

PART IV. THE ASSAY OF SOLANACEOUS DRUGS

BY W. C. EVANS and M. W. PARTRIDGE
From the University, Nottingham

Received June 25, 1952

CHEMICAL methods for the standardisation of solanaceous drugs have hitherto usually involved determination of the total alkaloids by a suitable adaptation[1] of the classical Stas-Otto process. Useful improvements have been effected by chromatographic adsorption of the total alkaloids on an alumina column,[2] by precipitation of their silicotungstates[3] and, for tropates, by application of the Vitali-Morin colour test[4] or direct determination of the tropic acid liberated by hydrolysis.[5] For the separate determination of hyoscine and hyoscyamine, differences in their dissociation constants and solubilities[6] and in the solubilities of their picrates in chloroform[7] have been exploited. Chromatographic separation of the bases has been achieved by means of a silica column[8] and of the hydrochlorides by means of partition columns.[9]

It is known that hyoscine and hyoscyamine exhibit qualitative differences in their pharmacological actions, particularly on the central nervous system, and that important variations in the relative proportions of these alkaloids within a given species are frequently encountered.[10,11,12] Accordingly it was considered worth while to adapt the partition chromatographic procedure described in Parts I and II[13,14] to the routine determination of hyoscine and hyoscyamine in solanaceous drugs. Factors influencing the quantitative separation of the alkaloids and the extraction of the drug have been examined and an assay process based on these observations has been applied to a range of samples of solanaceous drugs.

SEPARATION OF HYOSCINE AND HYOSCYAMINE

As an extension of the experiments recorded in Part I,[13] a systematic study was made of the variables which appeared most likely to operate in the separation of hyoscine and hyoscyamine by elution development of a partition column. The arbitrary, standard partition chromatographic system adopted for comparison consisted of a column of 10 g. of kieselguhr ("Hyflo Super-cel"), mixed with 3·2 ml. of 0·25 M phosphate buffer of pH 7·0, packed[15] in a tube 1·7 cm. in internal diameter; a mixture of 14·6 mg. of hyoscyamine and 5·8 mg. of hyoscine dissolved in 2 ml. of ether was chosen as the standard load and the ether eluate was collected in fractions of 2·5 ml. The experimental technique employed in following the course of fractionation and the method of presentation of the results were the same as those described in Part I.

The results of experiments concerned with systematic variations in the rate of flow of the eluting solvent, in the quantity of buffer distributed on the kieselguhr, in the load of alkaloids, in the concentration of the buffer and in its pH value and in the dimensions of the column are

summarised in Tables I, II, III, IV, V and VI. A buffer containing disodium hydrogen phosphate and citric acid was employed for pH values below 5·8 (Table V). Results obtained with the arbitrarily chosen standard system are printed in italics.

TABLE I

VARIATION IN THE RATE OF FLOW OF THE ELUANT

Rate of flow of eluant ml./minute	Hyoscine 1·8 mg./ml. of buffer		Hyoscyamine 4·6 mg./ml. of buffer	
	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
2·5	*3*	*17·5*	*29*	*210*
6·6	3	17·5	26	210
13·0	3	17·5	21	210

Column, 10 g. of kieselguhr, 3·2 ml. of 0·25 M phosphate buffer, pH 7·0; eluant, ether; eluate fractions, 2·5 ml.

TABLE II

VARIATION IN THE QUANTITY OF BUFFER

0·25 M phosphate buffer pH 7·0 ml.	Hyoscine			Hyoscyamine		
	Hyoscine per ml. of buffer mg.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Hyoscyamine per ml. of buffer mg.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
0·0	0	—	—	‡	3	37·5
1·0	5·7	3	70*	14·4	—	—
2·0	2·9	3	175†	7·3	11	—
2·5	2·5	3	17·5	6·2	16	180
3·0	1·9	3	17·5	5·0	18	195
3·2	*1·8*	*3*	*17·5*	*4·6*	*29*	*210*
3·5	1·6	3	17·5	4·2	37	215
4·0	1·5	3	17·5	3·8	42	275
5·0	1·1	3	17·5	2·9	50	285

* Volume required to elute both alkaloids without separation.
† Volume required to elute both alkaloids with partial separation.
‡ 15 mg. on plain kieselguhr.
Column, 10 g. of kieselguhr; eluant, ether; eluate fractions, 2·5 ml.

TABLE III

VARIATION IN THE LOAD OF ALKALOIDS

Weight of kieselguhr g.	Volume of buffer ml.	Hyoscine			Hyoscyamine		
		Hyoscine per ml. of buffer mg.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Hyoscyamine per ml. of buffer mg.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
15·6	5·0	1·2	4	20·0	3·2	66	275
12·5	4·0	1·6	3	20·0	3·9	41	250
10·0	*3·2*	*1·8*	*3*	*17·5*	*4·6*	*29*	*210*
10·0	3·2	0·9	3	17·5	2·6	55	195
10·0	3·2	2·5	3	17·5	6·6	25	230
10·0	3·2	3·3	3	15·0	9·0	12	240
10·0	3·2	4·2	3	250*	11·6	—	—
9·4	3·0	2·1	3	17·5	5·2	26	205
7·8	2·5	2·6	3	17·5	6·4	17	180
6·3	2·0	3·1	3	15·0	7·5	9	140
3·1	1·0	6·2	3	30·0*	15·6	—	—

* Volume required to elute both alkaloids without separation.
Column, 0·25 M phosphate buffer, pH 7·0; eluant, ether; eluate fractions, 2·5 ml.

TABLE IV

VARIATION IN THE CONCENTRATION OF THE BUFFER

Molarity of buffer solution *p*H 7·0	Hyoscine 1·8 mg./ml. of buffer		Hyoscyamine 4·6 mg./ml. of buffer	
	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
0·5	3	17·5	90	240
0·25	*3*	*17·5*	*28*	*210*
0·125	3	17·5	17	215
0·063	3	255†	15	—
0·032	3	50*	—	—

* Volume required to elute both alkaloids without separation.
† Volume required to elute both alkaloids with partial separation.
Column, 10 g. of kieselguhr, 3·2 ml. of phosphate buffer; eluant, ether; eluate fractions, 2·5 ml.

TABLE V

VARIATION IN THE *p*H VALUE OF THE BUFFER

*p*H value of buffer	Hyoscine 1·8 mg./ml. of buffer		Hyoscyamine 4·6 mg./ml. of buffer	
	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
7·7	3	75*	—	—
7·2	3	200†	13	—
7·0	*3*	*17·5*	*28*	*210*
6·8	3	17·5	59	200
6·3	8	22·5	103	30§
5·8	21	47·5	105	75§
5·8‡	23	50	106	55§
5·5‡	56	80	117	100§
5·0‡	104	27·5§	163	210§

* Volume required to elute both alkaloids without separation.
† Volume required to elute both alkaloids with partial separation.
‡ Citrate buffer.
§ Volume of chloroform required for elution after 100 eluate fractions with ether.
Column, 10 g. of kieselguhr, 3·2 ml. of phosphate or citrate buffer; eluant, ether; eluate fractions, 2·5 ml.

TABLE VI

VARIATION IN THE DIMENSIONS OF THE COLUMN

Length of column cm.	Diameter of column cm.	Hyoscine 1·8 mg./ml. of buffer		Hyoscyamine 4·6 mg./ml. of buffer	
		Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.	Position of maximum on elution curve. Eluate fraction number	Volume of eluant for complete elution ml.
33·2	1·0	3	12·5	22	165
15·5	1·4	3	17·5	26	200
11·7	*1·7*	*3*	*17·5*	*28*	*210*
9·0	2·0	3	17·5	32	210
4·1	2·9	3	22·5	39	270

Column, 10 g. of kieselguhr, 3·2 ml. of phosphate buffer, *p*H 7·0; eluant, ether; eluate fractions, 2·5 ml.

The quality of kieselguhr employed as carrier for the buffer has been found to be of great importance. Of 6 commercial samples examined, only 2 grades proved satisfactory, namely "Hyflo Super-cel" and "Celite

No. 545."* With columns prepared from other varieties, the alkaloids were strongly adsorbed and their elution with ether or chloroform was either very slow or impossible. Attempts to improve such varieties by washing with acid, elutriation or calcination were unsuccessful. The following simple, empirical test, based on the adsorptive capacity of kieselguhr for hyoscyamine, served to distinguish between satisfactory and unsatisfactory grades:—6 ml. of 0·004 per cent. solution of hyoscyamine in water is shaken with 1 g. of the kieselguhr; 5 ml. of solvent ether is added and the mixture is again shaken for 1 minute. The ether layer is allowed to separate and 2 ml. is transferred to 7 ml. of water containing 2 drops of solution of bromocresol green adjusted to the transition colour (*p*H 4·4). On shaking, the aqueous layer turns blue with satisfactory grades of kieselguhr; with unsatisfactory grades there is no colour change.

Extraction of Alkaloids from the Crude Drug

The choice of a base for the liberation of the alkaloids in the drug and of a solvent for their extraction was examined with samples of *Datura stramonium*, *Atropa belladonna* and *Hyoscyamus niger* which had been assayed for total alkaloids by the Pharmacopœial method. The alkaloids extracted under a given set of conditions were determined after chromatographic separation on a column consisting of 10 g. of kieselguhr on which was distributed 3·2 ml. of 0·25 M phosphate buffer of *p*H 6·0. In a lengthy series of experiments, which do not merit detailed description, it was found that a number of features could interfere with the convenient operation of a chromatographic assay of hyoscine and hyoscyamine.

Liberation of the alkaloids with ammonia was unsuitable, since its removal, prior to chromatographic separation of the alkaloids, necessitated heating the residue obtained by evaporation of the percolate. Calcium hydroxide was found to be entirely satisfactory for this purpose; the volume of solvent required for complete extraction was less than when ammonia was used, and the amount of colouring matter extracted was reduced.

The large amounts of non-alkaloidal extractive matter obtained when mixtures of ether and ethanol were employed for extraction rendered such solvents useless. Ether alone was found to comply with all requirements except for the volume needed for complete extraction. By appropriate modification of the extraction technique, details of which are given below, this volume could be reduced. The turbid percolate obtained with certain samples of *A. belladonna* and *H. niger* could be clarified by filtration through No. 42 Whatman filter-paper. Failure to remove the material responsible for this turbidity resulted in a greatly reduced rate of flow of the mobile phase through the chromatographic column.

Chromatography of the Total Alkaloids of Solanaceous Drugs

Two problems were involved in the chromatography of the alkaloids extracted from solanaceous drugs, namely, separation of the hyoscine

* Messrs. Johns-Manville Co. Ltd., Artillery House, Artillery Road, London, S.W.1.

and hyoscyamine from colouring matter and, in some samples of these drugs, from other weakly basic material. In systematic experiments on these problems, the residue left after evaporation of the percolate was dissolved in the solvent selected as eluant, the solution was transferred to the column and the eluate from the column was collected in fractions and titrated in the usual way. Sufficiently sharp separation of the alkaloids from the colouring matter could not be achieved with ether, light petroleum or benzene as eluting solvents. Elution first with carbon tetrachloride, then with ether and finally with chloroform afforded satisfactory results with a buffer of *p*H 5·9 to 6·2 distributed on the column. With carbon tetrachloride most of the colouring matter was eluted together with the weakly basic material present in certain samples; hyoscine was then quantitatively eluted with ether and finally hyoscyamine was eluted with chloroform. The mixture of solvents issuing from the column at the change over of solvents from carbon tetrachloride to ether usually contained no alkaloid. From these observations, it became clear that the eluate could be collected in three main fractions.

Proposed Method of Assay

Moisten the drug, in moderately fine powder, with water (Note 1) and set aside overnight in a well-closed container. Triturate with 1 g. of calcium hydroxide until a uniform mixture is produced; transfer the mixture to a 100-ml. cylindrical separator, plugged with cotton wool, and complete the transference, using in all about 50 ml. of solvent ether. Close the separator securely and shake continuously for 1 hour. Allow the solid to settle, drain off the supernatant liquid, compress the marc and percolate with solvent ether until complete extraction of the alkaloids is effected (Note 2). If the percolate is turbid, filter through a No. 42 Whatman filter-paper. Remove the ether and dissolve the residue in 2 ml. of carbon tetrachloride.

Mix intimately 3·2 ml. of phosphate buffer (Note 3) with 10 g. of kieselguhr (Note 4). Compress a plug of cotton wool into a glass tube, 1·7 cm. in internal diameter and about 35 cm. length, fitted with a tap. Pour about 30 ml. of carbon tetrachloride into the tube and introduce about 3 g. of the kieselguhr mixed with buffer. Agitate the suspension by rapid vertical strokes of a perforated plunger and then by slow strokes gradually compress the solid; continue the packing using successive quantities of 1 to 2 g. of the remainder of the kieselguhr mixed with buffer; pack 1 g. of kieselguhr and a plug of cotton wool on top of the column. Allow the supernatant carbon tetrachloride to drain from the column and transfer the carbon tetrachloride solution of the residue obtained by evaporation of the percolate to the top of the column; complete the transference using successive quantities each of 1 ml. of carbon tetrachloride, allowing each to flow into the column before adding the next. Develop the column with carbon tetrachloride at a rate of about 3 ml. per minute until the liquid leaving the column just becomes pale yellow (Note 5). Continue the development with solvent ether and reject the first portion of the eluate if it is free from basic material (Note 6);

collect the subsequently issuing eluate in a cylindrical vessel until no more base is eluted from the column (Note 7). Add 5 ml. of water and titrate (Note 8) with 0·005N sulphuric acid, using solution of bromocresol green as indicator. Each ml. of 0·005 N sulphuric acid is equivalent to 0·00152 g. of hyoscine. Continue the development with chloroform until no further base is eluted (Note 9). Evaporate the chloroform solution to about 5 ml. (Note 10), add 10 ml. of solvent ether and 5 ml. of water and titrate with 0·005 N sulphuric acid, using solution of bromocresol green as indicator. Each ml. of 0·005 N sulphuric acid is equivalent to 0·00145 g. of hyoscyamine.

Notes 1. The quantities of drug and water are as follows: belladonna herb and root, stramonium 5 g.; 3 ml.; hyoscyamus 10 g.; 6 ml.

2. About 250 ml. of percolate is required; 0·05 N iodine is employed in testing for complete extraction.

3. The phosphate buffer (*p*H 5·9 to 6·2) is made by mixing 25 ml. of M potassium dihydrogen phosphate, 3·0 to 4·1 ml. of carbonate-free N sodium hydroxide and diluting with freshly boiled and cooled water to 100 ml.

4. "Hyflo Super-cel" or "Celite No. 545" are suitable grades.

5. About 30 ml. of carbon tetrachloride is required. Prolonged development may cause loss of hyoscine. Basic material eluted in the first, dark green eluate is not hyoscine.

6. It is preferable to reject as much as possible of the mixture of carbon tetrachloride and ether issuing from the column at the change over of solvents, since the presence of carbon tetrachloride reduces the sharpness of the end-point in the titration of the ether solution of hyoscine. Test portions of the eluate are collected and shaken with diluted solution of bromocresol green which has been adjusted to its transition tint; the eluate is collected as soon as basic material is detected. Occasionally elution of the hyoscine begins before all the carbon tetrachloride has been displaced.

7. About 100 ml. of eluate is collected. Solution of bromocresol green is used in testing for completion of the elution.

8. The two layers are thoroughly agitated with a glass stirrer during the titration. This titration is most conveniently carried out in a cylindrical vessel.

9. About 100 ml. of chloroform is required.

10. Chloroform interferes with the observation of the end-point unless most of it is removed. A pale brown colour in the chloroform causes no difficulty, since the end-point is observed in the aqueous layer.

The results obtained with this process are summarised in Table VII. Indian belladonna root contains an alkaloid additional to hyoscine which is eluted from the column with ether. The process described above is therefore not applicable to this drug unless the eluate is collected in small fractions and those corresponding to hyoscine and hyoscyamine are titrated separately.

TABLE VII

ALKALOIDS IN SOLANACEOUS PLANTS

Sample	Total alkaloids calculated as hyoscyamine by Pharmacopœial assay per cent.	Hyoscine per cent.	Hyoscyamine per cent.	Equivalent of total alkaloids calculated as hyoscyamine per cent.	Hyoscine/hyoscyamine ratio
Atropa belladonna					
1. English herb	0·48	0	0·47	0·47	—
		0	0·51	0·51	
2. Sample 1 plus 0·056 per cent. of hyoscine	—	0·052	0·051	—	—
3. English herb	0·43	0	0·45	0·45	—
		0	0·43	0·43	
4. Sample 3 plus 0·084 per cent. of hyoscine	—	0·078	0·43	—	—
5. Indian leaf	0·21	0	0·22	0·22	—
		0	0·22	0·22	
6. European root ..	0·62	0·02	0·58	0·60	0·035
		0·02	0·59	0·61	
Datura stramonium					
7. Herb	0·26	0·07	0·20	0·27	0·30
		0·07	0·21	0·28	
8. Herb	0·25	0·05	0·20	0·25	0·25
		0·05	0·19	0·24	
9. Herb	0·37	0·11	0·28	0·37	0·38
		0·10	0·28	0·36	
Datura tatula					
10. Herb	0·43	0·27	0·17	0·43	1·55
		0·29	0·18	0·44	
11. Herb	0·27	0·09	0·19	0·28	0·45
		0·08	0·19	0·27	
12. Herb	0·15	0·05	0·15	0·20	0·32
		0·04	0·13	0·17	
Hyoscyamus niger					
13. Herb	0·04	0·03	0·02	0·05	1·5
		0·03	0·02	0·05	
14. Herb	0·05	0·01	0·05	0·06	0·2
15. Herb	0·05	0·02	0·04	0·06	0·5

DISCUSSION

The ideal operation of a partition chromatographic system as a true partition process requires perfect transfer of the solutes between the two phases, constant partition ratios and complete equilibration during the transfer. In practical systems these conditions do not normally exist and the operating conditions must be determined empirically. Hyoscine and hyoscyamine differ sufficiently in those physical properties which are exploited in their partition chromatographic separation to permit considerable latitude in the operating conditions.

The results recorded in Table I show that wide variation in the rate of flow of developing solvent is permissible, although the rate of equilibration of the solute depends mainly on the time and area of contact of the phases and the concentration gradient between the phases.[16,17] From the data recorded in Tables II and III, it is apparent that satisfactory separations are possible over a considerable range of buffer-kieselguhr ratios and alkaloid-buffer ratios at *p*H 7. The degree of separation increases as the alkaloid-buffer ratio decreases. The position of the maximum of the hyoscine peak on the elution curve remains unaltered since the partition coefficient of hyoscine under the conditions studied is overwhelmingly in favour of the mobile phase (see Fig. 4).

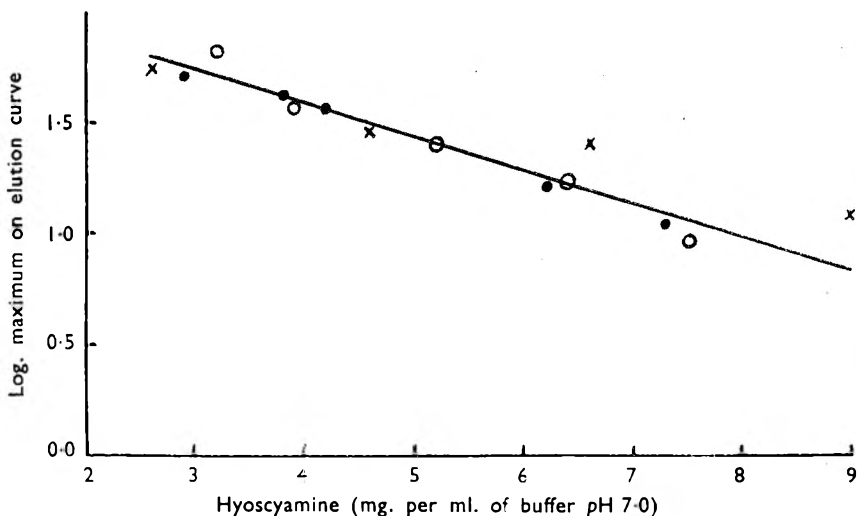


FIG. 1. Elution of hyoscyamine from columns with varying loads of alkaloid, sizes of the column and buffer-kieselguhr ratios.

×—variable load of alkaloid
○—variable size of column
●—variable buffer-kieselguhr ratio

Variation in the buffer-alkaloid ratio is one of the chief factors determining the position of the maximum of the hyoscyamine peak; this is demonstrated in Figure 1, from which it is clear that the buffer-alkaloid ratio is approximately linearly related to the logarithm of the position of the maximum of the hyoscyamine peak on the elution curve for differing buffer-kieselguhr ratios, column sizes and absolute amounts of alkaloid.

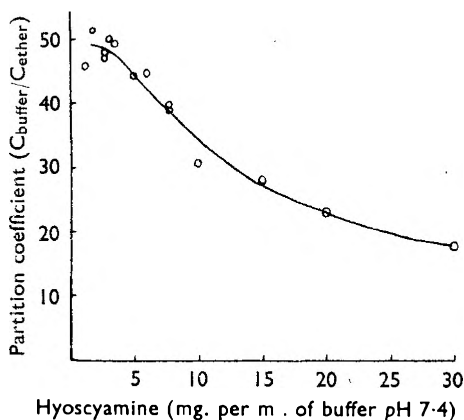


FIG. 2. Variation of partition coefficient of hyoscyamine with concentration.

The decrease in the total volume of ether required to elute the hyoscyamine for increasing alkaloid-buffer ratios recorded in Table III indicates that the partition coefficient of hyoscyamine between the two phases is not constant. Direct determination of the partition coefficient over a range of concentrations confirmed this (Fig. 2). Experimental difficulties prevented the accurate determination of the partition coefficient at very low concentrations of the alkaloid but some indication was obtained that the value approaches a constant with decreasing concentration. This change in partition

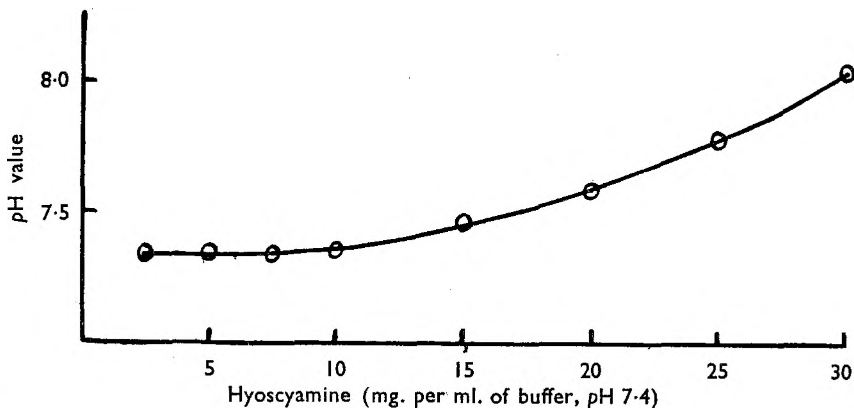


FIG. 3. Variation of *p*H value of buffer with concentration of hyoscyamine.

coefficient is in part accounted for by the relatively low buffering capacity of even 0·25 M buffer. From the curve illustrated in Figure 3, it is apparent that with concentrations of alkaloid in excess of 10 mg./ml. of buffer, the *p*H value of the buffer is changed. Evidence on the relationship of the partition coefficient to the *p*H value of the buffer is summarised in Figure 4. Buffer capacity is approximately proportional to the molarity of the buffer; the results recorded in Table IV therefore afford evidence of the effect of buffer capacity on the fractionation of the alkaloids. The shape of the elution curve for hyoscyamine is in agreement with these observations; as elution proceeds, it becomes progressively slower as the partition coefficient of the alkaloid increases in favour of the aqueous phase.

That the *p*H of the buffer has an important effect on the separation of hyoscine and hyoscyamine is shown by the results given in Table V; at *p*H 6·8 to 7·0 the alkaloids can be separated by fractional elution with ether, at *p*H 5·5 to 6·3 hyoscine can be eluted with ether and hyoscyamine with chloroform and at *p*H 5·0 the separation can be effected by

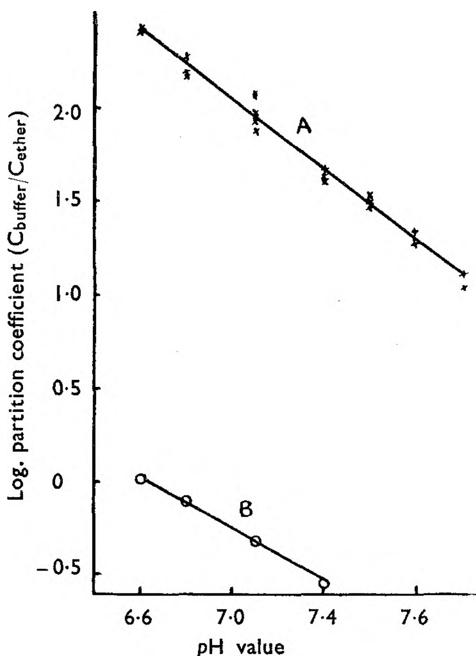


FIG. 4. Variation of partition coefficient of hyoscine and hyoscyamine with *p*H value of buffer.

A—Hyoscyamine
B—Hyoscine

fractional elution with chloroform. An explanation of the dependence of the process of fractionation on the pH of the buffer is afforded by a consideration of the relationship of this pH value and the partition coefficients of the alkaloids illustrated in Figure 4. Between pH 6·6 and 8·0, the partition coefficient of hyoscine changes but little with change in pH and favours the ether phase, whereas for hyoscyamine the value is very sensitive to changes in the pH of the aqueous phase.

Within the range of column sizes examined (Table VI), no significant effect on the ease of fractionation of the alkaloid mixture was observed. With long narrow columns, development is more even but greater pressure is required to obtain an adequate rate of flow of solvent.

Adsorption of the solute on the carrier of the stationary phase usually hinders fractionation.[18] Since it is not possible to predict adsorptive affinities, the suitability of any particular carrier must be found experimentally.

The application of the foregoing results to an assay of solanaceous drugs is based on earlier observations that in belladonna, stramonium and hyoscyamus, alkaloids other than hyoscine and hyoscyamine occur in only minor proportions[13,14,19]; atropine is determined as hyoscyamine. By the use of three solvents, carbon tetrachloride to separate colouring matter, ether to recover hyoscine and chloroform to recover hyoscyamine, the procedure is considerably simplified. The weak, non-volatile bases found in some samples of belladonna and hyoscyamus are eliminated in the course of purification of the alkaloids in the Pharmacopœial assay; in the present process, they are eluted from the column with the colouring matter and do not interfere with the determination of the alkaloids. The evidence presented in Table VII shows that by partition chromatographic determination of hyoscine and hyoscyamine results are obtained which are in satisfactory agreement with the results of parallel Pharmacopœial assays for total alkaloids.

The very significant variations found in the proportions of the two alkaloids even within a single species supplement other observations[10,11,12] and support the view that standardisation of solanaceous drugs for the individual alkaloids is desirable.

We are greatly indebted to Mr. J. L. Forsdike for the supply of a number of standardised samples of solanaceous drugs.

Summary

1. Factors influencing the separation of hyoscine and hyoscyamine by elution development of a partition chromatographic column have been studied.

2. A simple assay for hyoscine and hyoscyamine of belladonna, stramonium and hyoscyamus is described.

This communication is abstracted mainly from a thesis submitted by one of us (W.C.E.) in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of London.

REFERENCES

1. Hegnauer and Flück, *Pharm. Acta Helvet.*, 1948, **23**, 246.
2. Rubin and Harris, *J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 477.
3. Vallery, *C.R. Acad. Sci. Paris*, 1942, **214**, 171.
4. James and Roberts, *Quart. J. Pharm. Pharmacol.*, 1945, **18**, 29; 1947, **20**, 1.
5. Reimers, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 470.
6. Kuhn and Schäfer, *Apothekerztg, Berl.*, 1938, **53**, 405, 424.
7. Trautner, Neufield and Rodwell, *Austr. chem. Inst. J. Proc.*, 1948, **15**, 55.
8. Trautner and Roberts, *Analyst*, 1948, **73**, 140.
9. Schill and Ågren, *Svensk. farm. Tidskr.*, 1951, **55**, 781; 1952, **56**, 55.
10. Barnard and Finnemore, *J. Counc. Sci. Indust. Res. Austr.*, 1945, **18**, 277.
11. Hills, Trautner and Rodwell, *J. Counc. Sci., Indust. Res. Austr.*, 1945, **18**, 234; 1946, **19**, 295.
12. Hegnauer, *Pharm. Weekbl.*, 1951, **86**, 321.
13. Evans and Partridge, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 126.
14. Evans and Partridge, *J. Pharm. Pharmacol.*, 1949, **1**, 593.
15. Martin, *Biochemical Society Symposia No.* 3, 1949, 11.
16. Hunter and Nash, *J. Soc. chem. Ind. Lond.*, 1932, **51**, 285.
17. Barry, Santo and Craig, *J. biol. Chem.*, 1948, **174**, 209.
18. Chilton and Partridge, *J. Pharm. Pharmacol.*, 1950, **2**, 784.
19. Kussner, *Merck's Jber.*, 1938, **52**, 39.

DISCUSSION

The paper was presented by DR. M. W. PARTRIDGE.

DR. J. M. ROWSON (London) said that he fully supported the authors' final plea that solanaceous drugs should be standardised on the individual alkaloids. He was convinced that they should be standardised on their hyoscine content as well as on total alkaloids. He asked the authors for further comment on sample 10 of *D. tatula* which had a very large hyoscyamine content as compared with the others.

DR. M. MITCHELL (London) said he liked the use of lime in eliminating alkaloids, and he could see the point in using carbon tetrachloride because hyoscine and atropine are nearly insoluble in that solvent. He was interested to notice that, in the sample of European belladonna root which was examined, the amount of hyoscine was 0·02 per cent. and of total alkaloids 0·60 per cent., which was a ratio of 0·035. In 1948 the authors found 0·09 per cent. in a sample of drug showing 0·48 per cent. of total alkaloids, which was a ratio of 0·2, or some six times as much. It confirmed that there was a big variation in the species. It might be desirable to standardise the hyoscine content, but what should the standard be?

MR. J. E. CARLESS (Manchester) observed that the authors' results confirmed his own work using buffered filter paper strips. It indicated that the buffered filter paper technique might be a useful guide towards working on a larger scale. Had the authors any comments to make on the use of powdered cellulose, which could be otained in pure form, as opposed to the use of kieselguhr?

DR. M. W. PARTRIDGE, in reply, said that the values recorded for *D. tatula* were those found and the specimen was regarded as authentic. The result confirmed the suggestion that standardisation of the individual alkaloids was desirable. There was an extensive variation in the alkaloidal content of most of the solanaceous drugs, and there were quite important

variations throughout the life cycle. The chemist or pharmacist was not in a position to say what was the best type of standardisation to be adopted; that should be settled by those who used the drugs. He agreed that the *p*H was important. There was little doubt that powdered cellulose would work reasonably well. In earlier work with the alkaloids of pomegranate they had found that powdered cellulose and many of the usual inert supporters were buffers having a strong adsorptive capacity for alkaloids and vitiated partition chromatography.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ALKALOIDS

Curare Alkaloids. Purification of *d*-Tubocurarine Chloride and Isolation of *d*-Chondocurarine. J. D. Dutcher. (*J. Amer. chem. Soc.*, 1952, **74**, 2221.) The small but appreciable variations in the physiological potency of commercial *d*-tubocurarine chloride preparations have been found to be due primarily to the presence of additional quaternary alkaloids which accompany *d*-tubocurarine through the isolation procedure. A large sample of commercial *d*-tubocurarine was repeatedly recrystallised from 0·1N hydrochloric acid. After the fifth crystallisation a product of constant solubility properties was obtained, and although the physical properties, such as melting point, specific rotation, ultra-violet absorption, etc., of this product were not detectably different from the original, the physiological potency had now become constant as a value approximately 10 per cent. lower than the original. That the higher potency of the original material was due to the presence of more potent alkaloids was apparent from the increased activity of the molten liquor solids. These molten liquors were shown to contain a highly active related quaternary alkaloid which has been named *d*-chondocurarine. A. H. B.

Ergot Alkaloids, Paper Chromatography of. A. M. Berg. (*Pharm. Weekbl.*, 1952, **87**, 282.) The 3 ergotoxine alkaloids may be separated by paper chromatography. The author used the circular method, with buffered filter paper (Schleicher and Schull 2043B, McIlvaine buffer *p*H 3), the elution medium being benzene containing 10 per cent. of ethanol, saturated with water. The 3 bands obtained were, in order from the centre, ergocornine, ergokryptine and ergocristine. They were identified by comparison with chromatograms of the pure alkaloids. G. M.

ANALYTICAL

Acidimetric Titrations in Non-aqueous Media. C. G. van Arkel and J. Kroonenberg. (*Pharm. Weekbl.*, 1952, **87**, 137.) Many bases which are too weak to be titrated in water may be titrated in non-aqueous solutions, in particular in anhydrous acetic acid. This is prepared by adding to glacial acetic acid an amount of acetic anhydride corresponding to the amount of water present, as determined by titration, and allowing to stand for 24 hours. A standard 0·1N solution of perchloric acid is prepared similarly from 70 per cent. perchloric acid, and a 0·1N solution of sodium acetate is prepared by dissolving 5·300 g. of sodium carbonate (dried at 270° to 300° C.) in anhydrous acetic acid to 1 l. Excess of acetic anhydride is to be avoided, as it interferes in the titration of acetylisable amines. The titration is carried out by dissolving about 0·5 milli-equivalents of the substance in 15 ml. of anhydrous acetic acid, adding a drop of indicator solution (crystal violet in acetic acid) and titrating with the perchloric acid till the colour changes to blue-green. This colour change corresponds to the maximum potential jump in potentiometric titration. The perchloric acid is standardised against the sodium acetate solution. For potentiometric titration a glass electrode, type 6B2 and a Ag–AgCl electrode are used, both immersed

directly in the liquid. No salt bridge is required. The potential changes are noted on the *p*H scale. In some cases a sharper colour change is obtained by indirect titration, excess of perchloric acid being added and titrated back with sodium acetate. Applications are as follows: *direct titration*: atropine, codeine, strychnine, narceine, phenazone salicylate, alypine nitrate, strychnine nitrate, codeine phosphate, quinine sulphate or bisulphate, sodium salicylate, sodium acetate, sodium benzoate, sulphanilamide (potentiometric only): *direct or indirect titration*: morphine, quinine, phenazone, dihydro-codeinone bitartrate, atropine sulphate: *indirect titration*: papaverine, colchicine, quinidine, quinine ethylcarbonate, quinine carbonate, pyramidone, theobromine, amphetamine sulphate, calcium gluconate. Quinine and quinidine titrate as di-acid bases. Organic acids present as salts do not titrate, while sulphuric acid behaves as a monobasic acid so that when more than one basic group is present in a sulphate it can be titrated. Hydrochlorides do not appear to give satisfactory results. The limiting factor in the application of this method is the difficulty of dissolving certain compounds in the solvent. Temperature is also important, and a correction may be applied by using the formula:

$$\text{Normality at temperature } T_1 = \frac{N_0}{1 + 0{\cdot}001(T_1 - T_0)}$$

where T_0 is the temperature at which the solution is standardised, T_1 is the temperature during titration and N_0 is the normality at T_0. G. M.

Barbituric Acids, Titration of, in Pyridine. R. Heiz. (*Dansk Tidsskr. Farm.*, 1952, **26**, 69.) All the barbituric acids may be titrated in pyridine solution with sodium methylate, and in practice it is sufficient to use mixed pyridine bases purified from the technical material by standing over solid potassium hydroxide and distilling, the fraction 114° to 155° C. being collected. The titration solution is prepared by dissolving 6 g. of sodium in 100 ml. of methanol and diluting with 150 ml. of methanol and 1500 ml. of benzene. It must be protected from carbon dioxide. Electrometric titrations are carried out with a direct-indicating *p*H meter using an antimony-glass electrode combination. The applicability of the potentiometric and indicator method is shown in the table below.

Substance	Potentio-metric titration	Visual titration			Remarks
		Thymol blue	Phenol-phthalein	Thymol-phthalein	
Diethylbarbituric acid	+	+	+	+	
Ethylallylbarbituric acid	+	+	+	+	Thymol blue to green.
Diallylbarbituric acid	+	+	+	+	Thymol blue to distinct blue.
Ethyl-*N*-butylbarbituric acid	+	+	+		To distinct change.
Allyl-*iso*propylbarbituric acid	+	+	+	+	To distinct change.
Dipropylbarbituric acid	+	+	+		To weak red of phenolphthalein.
Methylphenylbarbituric acid	+				
Ethylphenylbarbituric acid	+		+		
Ethyl*cyclo*hexenylbarbituric acid	+				Not very sharp.
Allylphenylbarbituric acid	+		+		To first colour change.
N-methylethylphenylbarbituric acid.	+	+	+	+	
N-methylethyl*cyclo*hexenylbarbituric acid.	+	+	+	+	
Dimethylhydantoin	+				
Diphenylhydantoin	+	+	+	+	Thymol blue to distinct blue. Often precipitates.

G. M.

Bismuth, Titration of, with Versenate. O. Landgren. (*Svensk farm. Tidskr.*, 1952, **56**, 241.) For metallic bismuth, bismuth nitrate or carbonate, a quantity corresponding to about 0·2 g. of bismuth is dissolved in 10 ml. of 5M nitric acid and diluted to 100 ml.: 10·00 ml. is treated with 25·0 ml. of versenate solution, 4·0 ml. of 2M ammonia, 5·0 ml. of borax buffer and 3 drops of eriochrome black solution, after which the excess of reagent is titrated back with 0·01N magnesium sulphate to the first change from the original violet colour. In the case of bismuth salts of salicylic acid, tribromophenol or β-naphthol the acid component must first be removed by shaking into ether. The solutions required are as follows: eriochrome black—0·5 g. with 4·5 g. of hydroxylamine hydrochloride in methanol to 100 ml.; borax buffer—sodium borate 40 g., sodium hydroxide (50 per cent.) 20 g., water to 1000 ml.; versenate solution—18·60 g. of sodium versenate (sodium ethylenediamine tetra-acetate) in water to 1 l., standardised against 0·1N magnesium sulphate in presence of borax buffer. G. M.

Digitalis, Chemical Assay of. F. Neuwald and A. Diekmann. (*Arch. Pharm. Berl.*, 1952, **285**, 19.) The method of Neuwald (*Arch. Pharm. Berl.* 1950, **283**, 93) is based on the separation and colorimetric determination of the genins of the digitalis glycosides. In a modified form of the method the hydrolysis has been omitted and the glycosides extracted directly with chloroform from the solution after purification with lead acetate. Results from these two methods have been compared with those of the Soos method, which is based on the digitoxose fraction. The results show that the two forms of the genin method give the same average results, but that the scattering is greater with the modified method, while the digitoxose method gives results which are 10 per cent. lower. This is apparently due to differences in the extraction. Moreover, the Soos process has the disadvantage that it takes longer, that up to 5 hours are required for the determination of the maximum extinction, and that the reagent is variable. Thus in practice the original genin method is to be preferred.

G. M.

Gitoxigenin, Fluorimetric Determination of. K. B. Jensen. (*Acta Pharmacol. Toxicol.*, 1952, **8**, 101.) Paper chromatographic separation of the glycosides and aglycones in *Digitalis purpurea* and *Digitalis lanata* yielded the B series (purpureaglycoside B, gitoxin and gitoxigenin) displaying an intense blue fluorescence, and the A series (purpurea glycoside A, digitoxin and digitoxigenin) with a weaker and reddish yellow fluorescence; a fluorimetric method is described for the quantitative determination of gitoxigenin, and thus of gitoxin and purpurea-glucoside B, following paper chromatographic separation of the substances of the B series. A gitoxigenin solution containing 1 to 10 μg. of substance was evaporated on the water bath, cooled, and 10 ml. of a mixture of equal parts of hydrochloric acid and glycerol were added; after shaking, the solution was left for not less than 20 minutes and the fluorescence was then measured. The fluorescence curve was rectilinear for the given range of concentrations. Gitoxin and purpureaglycoside B were determined from the fluorescence of the aglycone, on the basis of equal intensity of fluorescence of equimolecular amounts of the B substances. The fluorescence develops under the dehydrating action of acids at suitable concentrations; the substances of the A series, digitoxigenin, digitoxin, and purpureaglycoside A, are not fluorescent under these conditions. The fluorescence is very stable in daylight, although the intensity decreases rapidly after exposure to ultra-violet rays and with increasing temperature. A reproducibility of ± 5 per cent. over the concentration range used is claimed. R. E. S.

Gitoxigenin, Gitoxin, and Purpurea glycoside B, Paper Chromatography of. K. B. Jensen. (*Acta Pharmacol. Toxicol.*, 1952, **8**, 110.) Gitoxigenin, gitoxin, and purpureaglycoside B were separated by one-dimensional filter paper chromatography and determined fluorimetrically, according to the method of Jensen (*Acta Pharmacol. Toxicol.*, 1952, **8**, 101). One-dimensional chromatography was used with a descending mobile phase and solutions of the substances in methanol-chloroform were applied to filter paper sheets, chromatography being performed partly at 22° C. and partly at 17° C; at 22° C. gitoxin and gitoxigenin were found to pass together, but separately from the primary glycoside, while at 17° C. all three substances separated. The chromatogram was developed by spraying with a trichloracetic acid solution, and heating at 100° C. for 2 minutes, the substances giving a blue fluorescence being marked under an ultra-violet lamp. Corresponding paper strips from the non-developed part of the chromatogram were cut out and transferred direct to the fluorescence-producing test solution (equal parts of hydrochloric acid and glycerol), the fluorescence being measured after 30 minutes. A correction for the adsorbent action of the filter paper was made by obtaining a blank reading from the use of a standard substance of the B series. R. E. S.

Iodine, Determination of Organically Bound. B. Zak and A. J. Boyle. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 260.) Chloric acid is preferable to sodium chlorate as a digesting reagent, as it avoids a high concentration of sodium perchlorate in the final solution. The process is rapid and loss of iodine is negligible. Phosphoric acid may be added to form complexes with any iron present which would otherwise tend to cause high results. The following procedure is recommended. Place the sample with 10 to 25 ml. of chloric acid reagent in a 150-ml. beaker and evaporate at a low heat until fumes of perchloric acid are evolved. Cool, dilute to 50 ml., neutralise to phenolphthalein, add phosphoric acid and hydrochloric acid and titrate with sodium thiosulphate using cadmium iodide-linear starch reagent as indicator. Alternatively, dilute the solution after digestion, with 0·2N sodium hydroxide containing 0·15 per cent. of sodium sulphite to remove dissolved oxygen and complete the determination polarographically. The digestion solution may also be assayed spectrophotometrically by adding potassium iodide and measuring the colour of the liberated iodine at 288 or 353 mμ. G. B.

Salicylates, Colorimetric Determination of. R. E. Pankratz and F. J. Bandelin. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 267.) Salicylates may be assayed by measurement of the violet colour obtained by reaction with ferric salts. For the best results the reaction of the solution should be between *p*H 4·0 and 6·0, but the great dilution required generally brings the hydrogen ion concentration within this range without special adjustment. A standard curve may be prepared by treating quantities of a standard solution corresponding to 0·125 to 1·125 mg. of salicylic acid with a 1 per cent. solution of ferric nitrate containing 1 per cent. of HNO_3, diluting to 50 ml. with water and determining the absorption at 525 mμ, with a spectrophotometer. Beer's law applies within this range of concentration. Sodium salicylate solutions of equivalent concentration may be treated in a similar manner and the result calculated from the salicylic acid data. Salicyl-, succinyl- and acetyl-salicylic acids and phenyl and methyl salicylates require hydrolysis with ethanol and potassium hydroxide before carrying out the absorption measurements. The method gives a reproducibility of ±1·0 per cent. and may be applied to the assay of elixir of sodium salicylate, theobromine sodium salicylate tablets and tablets of aspirin, phenacetin and caffeine. G. B.

Strychnine and Brucine, Spectrophotometric Study of. P. Demoen and P. Janssen. (*J. Pharm., Belg.*, 1952, 7, 80.) Absorption curves were determined for strychnine, strychnine nitrate and brucine tetrahydrate. For strychnine, ethanol (95 per cent.) was used as solvent; for strychnine nitrate and brucine, ethanol (95 per cent.) and water gave identical results. The following coefficients were obtained at the various absorption minima and maxima.

Wavelength mμ	$E_{1\,cm.}^{1\,per\,cent.}$ calculated with reference to the anhydrous alkaloid		
	Strychnine	Strychnine nitrate	Brucine
231	173	166±1	—
240	—	—	130
255	374±1	380±1	—
265	—	—	337±1
285	—	—	150
301	—	—	232±1

Absorption spectra for strychnine and brucine are given, from which it follows that mixtures containing strychnine and brucine may be assayed by absorption measurements at 305 mμ, at which wavelength strychnine exhibits negligible absorption and the quantity of brucine is proportional to the optical density. The total amount of strychnine and brucine is proportional to the optical density at 261 ± 1 mμ, and hence the quantity of strychnine can be calculated. G. B.

Tropine Alkaloids in Tablets, Determination of. K. Jentzsch. (*Scientia Pharm.*, 1952, **20**, 6.) 1 tablet is weighed and powdered, the weight of powder obtained being determined to check any loss. The powder is placed in a small percolator (6 to 7 mm. diameter) and percolated with 1 per cent. aqueous solution of tartaric acid until extraction is complete, testing with Mayer's reagent. The percolate is allowed to drip directly into a small separating funnel, and extracted with 3 quantities, each of 5 ml., of ether; the ethereal extracts are rejected. After the addition of ammonia, the alkaloids are shaken into 5 ml. of chloroform, the chloroformic extract being filtered through a little anhydrous sodium sulphate and then evaporated by repeated brief dipping in a water bath. The extraction is repeated 4 times or until all the alkaloid is extracted. The residue is dried at 103 to 105° C. and dissolved in chloroform so that the solution contains 60 to 100 mg. of base per ml. 1 ml. of this solution is evaporated to dryness, the residue is dried at 105° C. and treated with 7 drops of a reagent, prepared by dissolving 1 g. of pure *p*-dimethylaminobenzaldehyde, added in small portions, in 9·0 g. of 88 per cent. (w/w) sulphuric acid, cooled in ice. After standing for 2 minutes, the mixture is heated for 180 seconds in the water bath and then cooled for 15 seconds in ice and water. 5·0 ml. of acetic anhydride is added from a pipette in a rapid stream, and after 15 seconds the tube is taken out of the ice and water and allowed to stand for 1 hour. The extinction is then determined at 515 mμ against a blank treated in a similar manner. It is important that the strength of the sulphuric acid should be exactly 88 per cent. by weight, as a variation of only 1 per cent. alters the extinction by 10 per cent., while the purity of the reagent is also of great importance. The method works equally well with atropine, methylatropine, hyoscyamine and tropic acid, but with scopolamine the absorption maximum is displaced towards the short wave region, i.e., is at about 502 mμ. In this latter case the standardisation should be done with scopolamine at 502 mμ, but for other or mixed alkaloids standardisation is done with atropine at 515 mμ. G. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Terramycin and Aureomycin Hydrochloride, Isomorphism of. R. Pepinsky and T. Watnabe. (*Science*, 1952, **115**, 541.) Single crystal X-ray patterns for aureomycin and terramycin hydrochlorides show striking similarity, indicative of isomorphism. It appears from this and published analytical information that aureomycin differs from terramycin only in containing a chlorine atom in place of a hydroxyl group. Comparative crystallographic data are given. 3-dimensional X-ray scattering data have been collected, probable co-ordinates for the chlorine in aureomycin which replaces the hydroxyl in terramycin have been established and further elucidation of the structure is in progress. G. B.

Vitamin B_{12} in Liver Preparations, Stability of. B. Noer. (*Dansk Tidsskr. Farm.*, 1952, **26**, 47.) The stability of vitamin B_{12} in a purified liver extract was determined by heating at various temperatures. At the most favourable *p*H, 5·5 to 7, an initial content of 6·40 µg./ml. was reduced to about 4·8 by 30 minutes at 100° C., and to 2·0 after 20 minutes at 120° C. Outside this range the loss was considerably greater, especially on the alkaline side. The destruction is a mono-molecular process, and the constant is, at 100° C., $1{\cdot}0 \times 10^{-2}$; at 63° C., $1{\cdot}2 \times 10^{-4}$: and at 39° C., $0{\cdot}6 \times 10^{-6}$. Similar results were obtained for liver extracts to which were added vitamin B factors. In this case a *p*H of 5·0 was taken for the tests, since this is a suitable compromise between the optimum for aneurine (3·0), and that for lactoflavine and panthothenic acid (7·0). The addition of these vitamins produces an improvement in the stability of vitamin B_{12}. The redox potential is lower in liver extract than in extract to which B vitamins have been added, indicating that reduction is one of the factors involved in the destruction of vitamin B_{12}. G. M.

BIOCHEMICAL ANALYSIS

Adrenaline-like Substances in Blood, Estimation of. H. Weil-Malherbe and A. D. Bone. (*Biochem. J.*, 1952, **51**, 311.) A fluorimetric method for the estimation of adrenaline-like substances in blood is outlined. It consists of the following steps: (*a*) filtration of plasma-buffer mixture (*p*H 8·4) through acid-washed alumina and elution of the adsorbed amines by dilute acetic acid; (*b*) heating of the eluate at 50° C. with a mixture of ethylenediamine and ethylenediamine dihydrochloride; (*c*) extraction of a stable fluorescent condensation product with *iso*butanol; (*d*) measurement of fluorescence. The method has the advantage, compared with previous methods, that adrenochrome, a labile oxidation product of adrenaline formed as an intermediary, is trapped in the nascent state and quantitatively converted into a stable condensation product. Both adrenaline and noradrenaline were quantitatively recovered by the process at concentrations equal to or greater than 1 µg./l. The mean concentration observed in human venous blood under normal conditions was about 3 µg./l. Since the fluorescence formed from noradrenaline is 1/5 of that produced by adrenaline, figures for adrenaline concentrations have to be multiplied by 5 to convert them to noradrenaline concentrations. A study of the specificity of the method and of the action of amine oxidase led to the conclusion that the reactive material in blood consists entirely of amines derived from catechol. Whether this is adrenaline, noradrenaline or a mixture of both, remains to be further investigated. R. E. S.

Bromides in Blood and Spinal Fluid, Determination of. W. D. Paul, R. W. Knouse, and J. I. Routh. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 205.) A modification of the procedure for the colour reaction between gold chloride and bromides is described. The method necessitates only half as much plasma, serum, spinal fluid, and reagents as previous procedures, avoids centrifuging, uses all of the filtrate to obtain maximal colour intensity, and is generally less time-consuming. It retains the same degree of accuracy as previous methods; a spectrophotometer is used to measure the gold bromide colour. By using a 0·35 per cent. sodium chloride solution, and a wash solution containing 0·35 per cent. of sodium chloride and 5 per cent. of trichloroacetic acid, superimposable standard curves can be prepared for bromide levels in various body fluids.

R. E. S.

Fungistatic Agents, A Quantitative Method for the *in vitro* Assay of. F. Blank. (*Canad. J. med. Sci.*, 1952, **30**, 113.) Plates of a suitable medium containing peptone, maltose, yeast extract, bovine serum and agar, are prepared. Suspensions of thallospores and mycelium are made in distilled water to which has been added 1 or 2 drops of Tween 80/200 ml. For *Ctenomyces interdigitalis*, 5 ml. is added to a test-tube containing a 3-weeks culture on solid medium, the tube is rolled between the hands and the suspension decanted. For *Trichophyton* and *Sabouraudites* spp. and *Epidermophyton floccosum* the surface of the culture is scraped with a hooked needle. Cultures of *Candida* sp. in liquid media are used. Strips of filter paper are soaked in 0·1M and 0·02M solutions of the chemicals under test dissolved in methyl cellosolve, dioxan or ethanol, and dried. Control strips are prepared with the pure solvent. For ointments, a thin layer is spread on a strip of thin cardboard. Each strip is laid on the surface of the medium in a Petri dish and incubated at 37° C. for 16 hours. Streaks of the various inocula are drawn from the edge of the medium to the strip and the whole incubated at 30° C. For *Candida* sp. zones of inhibition are measured after 24 to 48 hours, and for *Ctenomyces* and *Trichophyton rubrum*, at the 3rd, 4th, and 5th days. *T. Schönleini*, *T. violaceum*, *T. glabrum* and *T. concentricum* are not suitable as test organisms. Fungistatic power is greatly affected by the presence of serum, which must therefore be included in the test medium to prevent misleading results. G. B.

Protamine Zinc Insulin, Estimation of Protamine and Insulin in. F. A. Robinson and K. L. A. Fehr. (*Biochem. J.*, 1952, **51**, 298.) It was found that protamine and insulin could be separated by paper chromatography using an upper phase obtained by equilibrating a mixture of *n*-butanol and glacial acetic acid (3:1 by volume) with an equal volume of water. The strips were developed in a descending manner producing 2 well-defined bands with R_F values of 0 and 0·43 respectively. The concentration of protamine and of various proteins in solution could be estimated on filter paper by retention analysis with a suitable dye, e.g., erythrosine. The area of the unstained wedge formed above the protein spot was proportional to the amount of protein present; the amount of protamine in protamine zinc insulin could be estimated after removal of insulin by paper chromatography. The insulin concentration of protamine zinc insulin could be estimated, after separating the insulin by paper chromatography, by staining with bromocresol green solution, eluting the insulin-dye complex and comparing the colour of the eluate with that given by a known amount of insulin treated in the same manner. Results are given for the composition of suspensions of protamine zinc insulin and on the distribution of the components between the precipitate and the supernatant liquor.

R. E. S.

CHEMOTHERAPY

Esters of Basic Bicyclic Alcohols as Antispasmodics. L. H. Sternbach and S. Kaiser. (*J. Amer. chem. Soc.*, 1952, 74, 2219.) The basic bicyclic alcohols 2-benzyl-3-quinuclidinol, 1-azabicyclo [3:2:1]-6-octanol, 1-azabicyclo [3:3:1]-4-nonanol, 1-azabicyclo [3:3:1]-2-methyl-4-nonanal and octahydro-1-byrrocolinol were esterified with diphenylacetic acid to produce compounds with possible spasmolytic activity. Several esters of 3-quinuclidinol were prepared including the diphenylacetate, benzilate and fluorene-9-carboxylate. The spasmolytic activities of the various esters were determined on the isolated rabbit intestine by measuring the relaxation produced by the drug against a spasm induced by acetylcholine bromide, and these results are tabulated. The esters of 3-quinuclidinol possess a much higher anti-acetylcholine activity than the analogous esters derived from diethylaminoethanol and other generally used basic alcohols, the most potent compounds being the benzolate, fluorene-9-carboxylate and diphenylacetate. It is of interest that the *lævo* isomer of 3-diphenylacetylquinuclidine had twice the activity of atropine while the *dextro* isomer was almost inactive, but the toxicity of the two isomers was equal. A. H. B.

Polymyxins, Chemotherapy and Pharmacology of. G. Brownlee, S. R. M. Bushby and E. I. Short. (*Brit. J. Pharmacol.*, 1952, **17**, 170.) Polymixins A, B, C, D and E have been shown to have similar antibacterial spectra, their efficiency depending upon the size and phase of the inoculum. Comparisons of the chemotherapy and pharmacology of polymixins A, B and C were made. A was found to be slightly more active than B and E in mice against *S. typhosa* and *H. pertussis*. A sharp fall in the counts of viable fæcal aerobes was effected by oral administration. They were not absorbed from the alimentary canal except in the newborn animal. By parenteral injections, variable blood levels persisting from 3 to 6 hours were obtained in animals and man. Higher blood levels could be obtained with repeated dosage. After single doses only a fraction of the polymixin was detectable in the urine. Polymixin E was detectable in the cerebrospinal fluid of rabbits up to 24 hours after intracisternal injections. The LD50's of A, B and E in mice were found to be about the same by the intravenous as by the intraperitoneal route. An antidiuretic effect was observed with large doses of all three in rats. Polymixins B and E had less nephrotoxic action than A, little evidence of injury was observed with E except in prolonged experiments with large doses in dogs. E was also found to cause less local reaction at the site of injection than B. Results of the chronic toxicity of E in rabbits and dogs are recorded. J. R. F.

PHARMACY

DISPENSING

Calcium Lævulinate, Injection of. F. Ernerfeldt and E. Sandell. (*Pharm. Acta Helvet.*, 1952, 27, 48.) After autoclaving a 10 per cent. solution of calcium lævulinate there is a slight turbidity which consists of calcium carbonate. Attempts were made to prevent this by excluding atmospheric carbon dioxide, by the addition of a small amount of hydrochloric acid, or by the use of ethylenediamine tetra-acetic acid, but were unsuccessful. Apparently the formation of calcium carbonate is associated with a slight decomposition of the lævulinic acid resulting from oxidation during the heating. By the addition of 0·1 g. of

ascorbic acid per l. it is possible to prepare ampoules in which there is no deposition. The same result may be attained by the use of 0·1 g. of hydroxylamine, although this addition is toxic. G. M.

NOTES AND FORMULÆ

Thiamylal Sodium (Surital Sodium). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1952, **149**, 369.) Thiamylal sodium is sodium 5-allyl-5-(1-methylbutyl)-2-thiobarbiturate and is supplied mixed with sodium carbonate. The mixture occurs as odourless, pale yellow, hygroscopic, agglutinated masses of crystals, freely soluble in water; *p*H of a 2·5 per cent. solution, about 10·8. The acidification of an aqueous solution precipitates thiamylal, which after drying at 60° for 1 hour, melts at 130° to 134° and complies with the following tests: when heated with sodium carbonate, the fumes evolved turned moistened red litmus paper blue (presence of nitrogen); when treated with sodium hydroxide and treated with sodium nitroprusside a red colour develops (test for substituted thiobarbituric acids and distinction from thiobarbituric and barbituric acids); when shaken with carbon tetrachloride and filtered, the filtrate decolorises a solution of bromine in carbon tetrachloride. Thiamylal sodium loses not more than 1 per cent. of its weight when dried in vacuo at 56° for 18 hours; a 2 per cent. solution is bright yellow and free of haze, floaters, and other foreign particles. It contains 94·0 to 96·0 per cent. of thiamylal sodium, and is assayed by extracting an acidified solution with chloroform, removing the chloroform, drying the residue at 60° for an hour, and weighing. The specification also includes requirements for the thiamylal used in the preparation of thiamylal sodium. Thiamylal sodium is an ultra-short acting barbiturate.

G. R. K.

PHARMACOGNOSY

***Chenopodium ambrosioides* L., Ascaridole in.** E. Wegner. (*Pharm. Zentralh.*, 1952, **91**, 43.) The presence of ascaridole in *Chenopodium ambrosioides* L. has been disputed, and it has been supposed that it is only present in *C. ambrosioides* var. *anthelminthicum* Gray. Two new approximately quantitative methods for its detection are based on the loss of weight on shaking with ferrous sulphate solution (formation of ascaridole glycol) and the production of propane on heating with titanous chloride. The ascaridole was also identified by the formation of ascaridole glycol monobenzoate. The presence of considerable quantities of ascaridole in the fruit of *C. ambrosioides* was confirmed, although the quantity was less than that in the variety *anthelminthicum*. G. M.

Digitalis Extraction Studies. R. E. Hopponen and O. Gisvold. (*J. Amer. pharm. Ass. Sci. Ed.*, 1952, **41**, 146.) Fresh leaves of *Digitalis lanata* were frozen by packing in solid carbon dioxide and ground to a powder of leaves and solid carbon dioxide which could be stored until required. After removal of the carbon dioxide the powder was extracted with warm water to form a 2 per cent. extract, calculated on the dry weight of the leaves. This solution was extracted with methyl *iso*butyl ketone and the extract concentrated at 60° C. and evaporated at room temperature to give a crystalline material which after further purification melted at 198° to 200° C. The crystalline structure, the absence of sugars other than digitoxose, and the presence of acetic acid and digoxin on de-esterification suggested that this substance was α-acetyldigoxin, although the melting point and specific rotation were not in agreement with

those previously reported. A lower-melting substance was obtained from the methyl *iso*butyl ketone mother liquors and on purification appeared to be β-acetyldigoxin, although the melting point did not agree with that in the literature. A small amount of gitoxigenin-containing substance was detected by the red colour in the Keller test. G. B.

Indian Strophanthus, *Strophanthus wightianus* Wall. K. L. Handa and I. C. Chopra. (*Indian J. med. Res.*, 1951, **39**, 403.) The seeds of this Indian species of strophanthus, which grows wild in Malabar, have been investigated. Cardiac glycosides in 1·9 per cent. yield were extracted and provisionally named strophanthin-w. This glycosidal mixture, as well as the seeds and their tincture, can be distinguished from the seeds and corresponding preparations of *S. kombé* by qualitative chemical tests. Pharmacological study of strophanthin-w shows that it possesses cardiotonic properties and is more potent than strophanthin-k. The tincture is also more potent than that prepared from *S. kombé* seeds. J. W. F.

PHARMACOLOGY AND THERAPEUTICS

1-Amino-and 2-amino-octane, Respiratory Stimulant Action of. D. E. Hutcheon and L. McCullough. (*Brit. J. Pharmacol.*, 1952, **7**, 42.) In experiments on rabbits and cats in which the breathing had been depressed by pentobarbitone sodium, the respiratory rate was increased by intravenous injections of the amines (2·0 to 8·0 mg.) and amphetamine (1·5 to 6·0 mg.). The respiratory stimulant effect of the 1-amino-octane was present after denervation of the carotid body. Both amines increased the respiration rate of rabbits in which breathing was depressed by morphine sulphate. Their action is considered to be direct stimulation of the respiratory centre under the influence of medullary depressant drugs. Both had less stimulant activity than amphetamine on the isolated rabbit heart but the coronary outflow was increased with 1-amino-octane. Although the amines are less toxic than amphetamine the therapeutic ratio is still in favour of amphetamine as a respiratory stimulant. J. R. F.

Aureomycin, Toxicity of, to Guinea-pigs. P. Roine and T. Ettala. (*Nature, Lond.*, 1952, **169**, 1014.) During an investigation of the synthesis of vitamins in the intestines of guinea-pigs and rats it was found that the same proportional dose of aureomycin which had a growth-promoting effect on rats appeared to be toxic to guinea-pigs. Experiments were conducted by feeding a group of 9 guinea-pigs with aureomycin hydrochloride at the level of 100 mg./kg. of food. All began to lose weight on the second day. 6 died in 10 days while of the remaining 3, 2 died in 5 to 6 weeks. In 10 control animals not receiving aureomycin, no trouble occurred. Further experiments in which the animals were given 1 mg. of aureomycin subcutaneously per day had similar results, death occurring in 10 days. Food and water consumption of all guinea-pigs receiving aureomycin fell markedly soon after treatment began. It has been shown that aureomycin appears to be toxic to mice, lambs, steers and dogs, but no reasons have been established. The assumption is that harmful effects on the intestinal flora play an important part, but this seems unlikely in the case of guinea-pigs in view of the rapidity of the effect. The suggestion that the antibiotic owes its toxicity to its effect in inhibiting aerobic phosphorylation is thought to merit further consideration. H. T. B.

Choline Esters and Ethers, Relationships between Structure and Nicotine-like Stimulant Activity in. P. Hey. (*Brit. J. Pharmacol.*, 1952, 7, 117.) A hypothesis is proposed relating certain aspects of chemical structure to nicotine-like stimulant actions in choline derivatives. This states that increased activity is associated with a reduction of electron density of the "ether" oxygen atom of choline ethers and esters. A brief review of the literature is made and much evidence is produced in support. 21 compounds are used in a pharmacological study to subscribe further to this evidence. Investigations of the effects of modifications to the cationic head, to the chain structure of the molecule and of nuclear substituents are made. 3 compounds, the *m*-chlorophenyl, *m*-bromophenyl and 3:5-dibromophenyl ethers of choline, have twice or three times the activity of choline phenyl ether, the most powerful nicotine-like stimulant drug hitherto described, which itself has about twice the molar activity of nicotine.

J. R. F.

Cough-suppressing Drugs, Assessment of. B. R. Hillis. (*Lancet*, 1952, **262**, 1230.) Although the so-called reflex expectorants are widely used there is no evidence that they increase the flow of secretion from the bronchial mucosa. In troublesome cough reliance is placed on opiates and synthetic analgesics which depress the cough centre in the medulla. There is no doubt about their value but all produce side-effects such as nausea and drowsiness and there is no objective evidence of their relative value. An investigation was therefore carried out to determine the relative merits of the cough suppressants in common use. A long nasopharyngeal sprayer with a small adjustable nozzle was bent so that it could be inserted over the root of the tongue into the lower pharynx, and an extensive series of tests lasting over a year was carried out on the same individual, aged 42, who could tolerate the sprayer in his unanæsthetised pharynx for several hours. The larynx was then sprayed with irritant solutions to provoke coughing. Peppermint water and ether were found most convenient for this purpose. The efficiency of cough suppressants in common use was then determined by ascertaining the number of insufflations required to produce coughing before and after a dose of the drug under test. Control experiments were made throughout by using the identical methods after administration of an inert substance, namely physiological saline solution. The results are shown graphically and are analysed statistically. Morphine, diamorphine and amidone were found to be potent cough-suppressants. No evidence was obtained that codeine had any effect other than the psychological factor. An unexpected finding was the great importance of the psychological factor although the investigator took care to give no indication as to the expected potency of any preparation being given. This lends support to the view that the placebo has considerable importance in therapeutics. H. T. B.

Gold Sodium Thiosulphate, Influence of Drugs on the Acute Toxicity of. W. R. Byrum and J. L. Lichtin. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 105.) Doses of gold sodium thiosulphate were administered intravenously to mice, and intraperitoneally to rats, in quantities sufficient to cause a high percentage mortality. Antidotes were administered 20 minutes before the gold compound, in quantities previously found to be well tolerated. The following, listed in order of decreasing effectiveness, lowered the acute toxicity:—for rats, vitamin B complex, adrenal cortex extract, inositol, caffeine and sodium benzoate and mercurophylline, and for mice, vitamin B complex, theobromine and sodium acetate, adrenal cortex extract, caffeine and sodium benzoate and mercurophylline. Ammonium chloride, biotin and choline chloride were ineffective.

G. B.

Urethanes with Anæsthetic Properties. R. Hazard, J. Cheymol, P. Chabrier, Y. Gay and P. Muller. (*Thèrapie*, 1951, 6, 375.) Four derivatives of urethane were compared with procaine. Compounds (1) and (2) were prepared by heating glycol carbonate with the appropriate amine, compound (3) was prepared by condensing (2) with maleic anhydride and forming the sodium derivative and (4) by reduction of (3) with hydrogen and Raney nickel. The solubility of (2) in water is only 0·5 per cent., and (3) and (4) are more soluble derivatives.

Compound	Toxicity LD50 in mice mg. per 100 g.	Local anæsthetic activity Rabbit cornea
Procaine	7·5	1
(1) β-hydroxyethyl benzylcarbamate (242 H.C.)	30	1
(2) β-hydroxyethyl phenyl*iso*propylcarbamate (244 H.C.)	10	2
(3) Sodium derivative of maleic ester of (2)	4	0·5
(4) Sodium derivative of succinic ester of (2)	3·5	1

Compounds (1) and (2) had a depressant action on the central nervous system of rats, whereas comparable doses of the other compounds did not. All the urethane derivatives had a slightly less hypotensive effect than procaine on the arterial pressure of the dog. It is suggested that compound (1) which is as active as procaine, more stable, less hypotensive and has one fourth the toxicity be tested clinically. G. B.

Veratrum Alkaloids, Relative Hypotensive Activity of. G. L. Maison, E. Gotz and J. W. Stutzman. (*J. Pharmacol.*, 1951, **103**, 74.) Determination of the relative depressor potency of 15 substances derived from veratrum plants was made by comparison of hypotensive activity against a standard alkaloidal extract, veriloid. The derivatives were assayed for their equi-hypotensive dose at 32 per cent. fall of mean arterial pressure in anæsthetised dogs, the drugs being given by intravenous infusion of 10 minutes duration. In terms of the standard reference powder (veriloid) taken as 1 the following potencies were found: germitrine 11, neogermitrine 8·7, germerine 5·3, protoveratrine 4·7, germidine 2·4, veratridine 0·5, veratrine 0·3, veradine 0·18, veratramine 0·03. Germine, rubijervine, jervine, isorubijervine were so weak that accurate determination of potency was not possible. Dose-response curves suggest identity of mechanism of hypotensive action of protoveratrine, of veratridine and of mixtures of ester alkaloids. S. L. W.

Veratrum Derivatives, Emetic Properties of. E. D. Swiss. (*J. Pharmacol.*, 1952, **104**, 76.) The emetic dose, ED50 was determined in dogs. Using veriloid, an alkaloidal mixture of constant potency, and a large number of purified alkaloids of *Veratrum viride*, it was shown that the ratio of emetic to hypotensive doses was about the same for all the alkaloids, and that an assay based upon emetic activity would give a satisfactory assessment of the hypotensive potency. There was no significant difference in ED50 by oral and intravenous routes. Denervation of the gastrointestinal tract did not alter the intravenous emetic dose, so that intestinal irritation can be neglected as a cause of emesis. The larger oral emetic dose after gastrointestinal denervation may be due to decreased activity with poorer absorption. It is suggested that the alkaloids act on some specialised structure such as the vomiting centre in the medulla. Some drugs, such as hyoscine, atropine, ephedrine, etamon, dramamine and banthine were tested but no drug was found which might be of clinical use in alleviating the emesis. G. B.