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BRITISH PHARMACEUTICAL CONFERENCE BLACKPOOL, 1949

Chairman : NORMAN EVERS

CHAIRMAN'S ADDRESS

PHARMACEUTICAL RESEARCH

I PROPOSE, this afternoon, to talk on the theme of pharmaceutical research, its aims and scope and its relationship to research in medicine, chemistry and other sciences. This topic was discussed by Dr. T. E. Wallis in his address to the Conference in 1943, and I feel therefore, that some apology is necessary, but as I propose to approach the matter from a direction rather different from that of Dr. Wallis, who was mainly concerned with academic research, perhaps I may be forgiven for referring to the subject again. In fact, my address is in the nature of an addendum to Dr. Wallis's and will underline much of what he said.

In considering the subject of Pharmaceutical Research, I am immediately confronted by the difficulty of definition. What is Pharmacy and what is Research? The word " research " has become very much overworked in these days. It seems to be applied to almost any type of enquiry. We hear of " listener research," " market research," " packaging research " and even " time-table research." Most people, I think, would hesitate to give the term " research " so wide a definition, but where is one to draw the line? Research cannot be defined according to the importance of the results achieved. The distinction between what is or is not research is something more intangible. Rather, it depends on the attitude of mind with which an enquiry is approached. A problem which appears at first sight to be simple and solvable by known methods and therefore not to be dignified by the name of research may turn out to involve a fundamental investigation into certain phenomena, which raises it to the plane of true research.

When I ask myself " What is pharmaceutical research? " I find it equally difficult to provide an answer. Pharmacy is not a science in itself. It is an art which makes use of many sciences. I would remind you of the words of Daniel Hanbury which were quoted by Dr. Wallis in his address and which I think are worth repetition. He said, " Our art, gentlemen, is ever progressive. All science is interesting to us since almost every scientific discovery may sooner or later, directly or indirectly, yield some results profitable to pharmacy."

It would be going too far to argue from this that all scientific research is pharmaceutical research. Here, I think, we must take into account the objective of the research, and say that pharmaceutical research is research carried out with a pharmaceutical objective, which is rather like saying that an archdeacon is one who performs archidiaconal functions.

What, then, is a pharmaceutical objective? I think that the best way to answer that question is to consider the different types of investigations

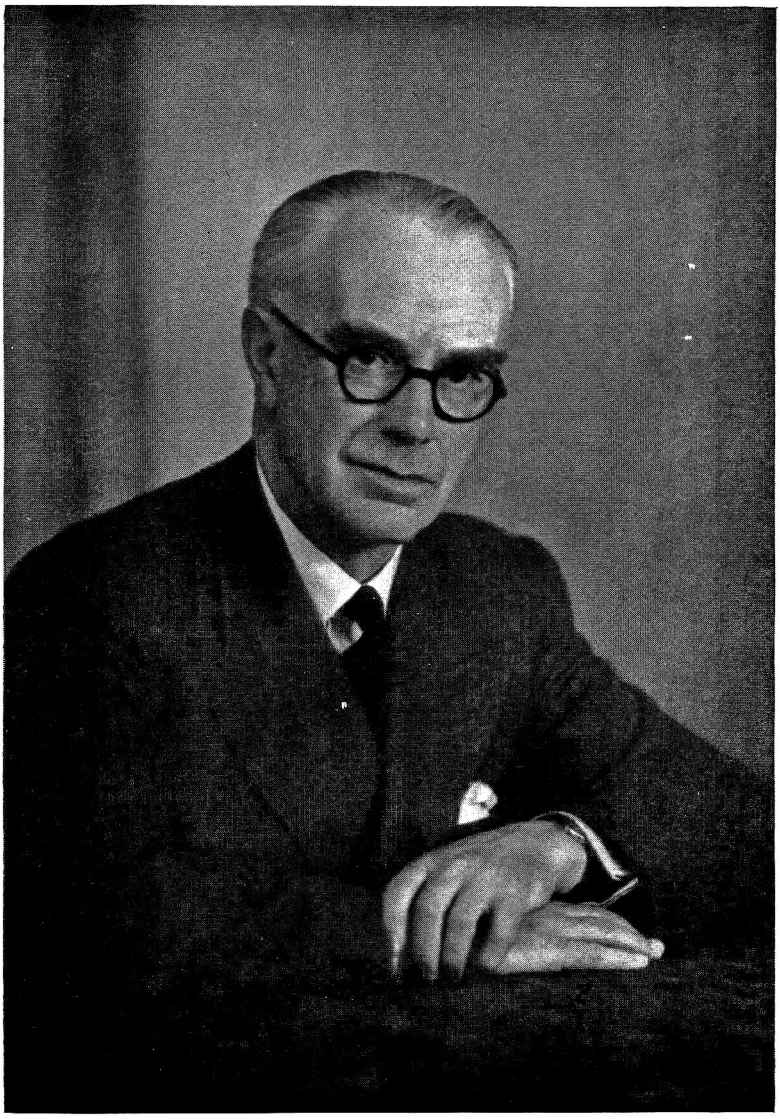
that can justifiably be claimed as pharmaceutical research. The following list is probably not complete, but, I hope, fairly comprehensive:

1. The synthesis of new organic compounds with the object of discovering new drugs, and the examination of known compounds for new pharmacological properties.
2. The isolation and purification of the active principles of naturally-occurring drugs, the elucidation of their structure and their synthesis.
3. Pharmacognostical research.
4. Research on methods of cultivation of vegetable drugs with the object of producing maximum potency.
5. Methods of preparation of drugs in a form suitable for administration, under which heading we may include the preservation and sterilisation of medicinal products.
6. The chemical and biological standardisation of drugs.

Let us consider these divisions in rather more detail.

The primary importance to medicine of organic chemical research needs no emphasis from me. The number of valuable synthetic drugs discovered in recent years provides sufficient evidence of this. The organic chemist must, of course, work hand-in-hand with the pharmacologist, who functions as a sort of compass and tells him whether he is moving in the right direction. Most research of this type is nowadays carried out by teams of workers, each approaching the subject with a special knowledge and skill. The organic chemist usually takes as his starting-point some compound, either natural or synthetic, of known pharmacological action, and by modification of its structure, produces new compounds which he hopes will have an improved or modified pharmacological action. Modification of structure in the direction of simplification has proved a fruitful source of new drugs. Such simplification may have the advantage of substantially reducing the cost of treatment. Examples of this are seen in stilbœstrol and related compounds which resemble in a much simplified form the structure of the natural œstrogens, and the new synthetic curarising compounds which are substitutes for the natural alkaloid, tubocurarine, are comparatively easily synthesised and are very much cheaper. Sometimes such researches lead the chemist far away from his starting point. The series of researches which started out to produce an improvement on the antimalarial drug, mepacrine, ended with paludrine, a compound of very different structure.

Research of this type sometimes involves the production of hundreds of compounds in the laboratory. The discovery of a successful new type of synthetic drug is followed by feverish activity in organic laboratories in the production of compounds of a similar structure in the hope of finding an improvement on the original. The number of sulphonamides which have been synthesised since the discovery of the value of sulphanilamide is legion, but the number of real value in medicine is probably less than a dozen. Sulphanilamide itself provides an example of a compound which has been known for many years before its valuable bacteriostatic properties were realised. This emphasises the importance of thorough pharmacological testing of all new compounds. Sometimes too, a varia-



NORMAN EVERS

Chairman, 1949

tion on a compound having a certain pharmacological action may prove to be valuable for a therapeutic effect of quite a different kind. On the other hand, valuable drugs are sometimes discovered which bear no relation in structure to those previously known. The analgesic, pethidine, is modelled on the structure of morphine, in amidone the relationship is scarcely recognisable. The new antihistamine drugs are of a type not hitherto used in medicine and have a novel pharmacological action. The most remarkable development of recent years has been the discovery of drugs which act selectively on species of living organisms or upon one particular type of cell or enzyme. The antibiotics, antimalarials, antrycide, etc., are examples of drugs acting selectively on certain organisms. Antihistamine, antithyroid, anticholinesterase and curarising agents are examples of selective poisoning of certain types of cells or enzymes. Increased knowledge of chemical constitution and its relation to pharmacological action, of the causes of disease and of the nutritional requirements of organisms makes the task of the organic chemist less subject to chance than it used to be, but we are still a long way from being able to design a new drug like a machine on a drawing-board. The element of luck has not yet been entirely eliminated, but as Pasteur wisely said, "In the fields of observation chance favours only the mind which is prepared." It may have been a lucky chance that led Sir Alexander Fleming to the discovery of penicillin, but unless his mind had been prepared by years of research and thought on such matters, who knows that he would have appreciated its significance?

It cannot be too strongly emphasised that the greatest possible care and the most thorough and exhaustive trials are necessary before placing a new drug on the market. Time and again a drug, which has been thought from preliminary tests to have an irreproachable character and has been launched with a flourish of trumpets, has proved to possess undesirable and even dangerous qualities.

The second type of research to which I have referred deals with the isolation and purification of natural drugs, the determination of their structure and synthesis. Recent discoveries have shown that Nature still has something up her sleeve. A few years ago it might have been thought that it was unlikely that any new natural drugs of value would be discovered and that the future lay with synthetic organic chemistry. Then came penicillin to show us an entirely new type of drug from an entirely new source—a compound with a new sort of structure, and one, moreover, that has not yet been synthesised except in minute quantity in spite of the efforts of some of the finest organic chemists in the world—a blow to the pride of those organic chemists who might have been inclined to think that anything can be synthesised once its structure is known—except, of course, cane sugar. Then, as if to heap coals of fire on the head of the organic chemist, Nature produces chloramphenicol or chloromycetin, a naturally occurring antibiotic with a comparatively simple structure, containing, above all things, a nitro-group and two

chlorine atoms, looking for all the world like a typical product of the organic laboratory, yet it is not among the hundreds of thousands of compounds which have been synthesised. Truly it behoves those who pry into the secrets of nature to preserve a spirit of humility.

Another natural product which promises to be one of the important drugs for the relief of human suffering is known as "Compound E," obtained from the suprarenal gland. The minute amount present in the gland precludes its extraction from natural sources in quantity, and all hopes are centred on a successful synthesis. The known synthesis is long and difficult, even if supplies of raw material from *Strophanthus sarmentosus* are forthcoming in sufficient quantity. The position is a challenge to the organic chemist, and those who solve it will, indeed, have deserved well of mankind.

I do not wish to spend more time on the contributions of organic chemistry to pharmaceutical research. Fundamental as these are, we must in these days regard organic chemistry as a highly specialised art and the organic chemist as a species apart. The pharmaceutical student who intends to become an organic chemist must realise that, henceforth, his whole mind will be steeped in organic chemistry and perhaps in one small section of this vast subject.

I cannot speak with any authority on the third section of pharmaceutical research on my list—pharmacognosy. Dr. Wallis has already done so, nor can I say anything on the fourth item—the cultivation of drugs, so I will pass on to the fifth, the section of pharmaceutical research which most properly merits the name, the preparation of drugs in a form suitable for administration. The introduction of a new drug into medicine entails four steps, (a) the chemical production by synthesis or other means, (b) the pharmacological investigation, (c) the pharmaceutical investigation and (d) the clinical trial. The chemical research should supply information on the physico-chemical properties of the drug, the solubility etc., and on its stability to heat, moisture and oxidation. The pharmacological research should give data on the best means of administration and the probable dose required, and some information about the method and rate of absorption and excretion, whether the effect is transient or prolonged. The clinician can advise from such data on the type of pharmaceutical preparation which is most suitable for administration, whether dosage should be frequent, or whether the action should be prolonged by some such means as the use of an oily medium for the injection. Nevertheless the pharmacist is by no means the least important link in the chain. Failure to provide a satisfactory pharmaceutical preparation may bring a new drug into discredit. The pharmacist out of his experience can and should give valuable assistance to the clinician as to the best method of administration of a new drug.

If an injection is required, a number of questions must be considered, such as—

(a) Is the drug sufficiently soluble and stable in water to make an aqueous injection possible? If not, can any other solvent be used or can

any addition be made to increase the solubility? Would any other form of the drug, such as another salt, be more suitable for injection? If these questions cannot be answered satisfactorily, would a dry ampoule be a suitable method? (b) Is the addition of sodium chloride or other material necessary to make the injection isotonic? If so, how does it affect the solubility or stability of the drug? (c) Is the natural *p*H of the drug suitable for injection, and does it ensure the maximum stability or must the *p*H be stabilised by the addition of a buffer? (d) What is the most suitable preservative, if one is required, and is it likely to react with the drug or affect its stability? (e) What is the most suitable method of sterilisation? Does sterilisation by heat cause any decomposition of the drug or production of toxicity? If heat is unsuitable, does sterilisation by filtration cause any loss of potency? (f) Should the injection be protected from oxidation by filling the ampoule with nitrogen? (g) What is the stability of the injection as finally formulated under ordinary conditions, under tropical conditions or under abnormally cold conditions? (h) If the injection is to be in a rubber-capped container, will contact with rubber affect the injection in any way? (i) Should the injection be protected from light by the use of amber containers?

If the required form is an oily solution or suspension, other problems arise such as— (a) What is a suitable composition for the oily base? (b) If a suspension is required, what is the most suitable particle size of the drug? (c) Does the drug remain easily dispersible in the base? (d) Is the viscosity suitable for drawing into a syringe?

If the new drug is likely to be used in combination with some other drug a study of any possible interaction between the two must be made.

The investigation of all these problems may involve a considerable amount of work. A large number of different formulae may have to be tried and each one checked either by analysis or by pharmacological tests or both. If the drug is a new one, analytical methods may have to be devised in order to detect decomposition. If the drug is administered orally, the problems are not usually so complex. The pharmacist must first consider whether a tablet is a suitable medium of administration or if not, whether a capsule would be a better form. If a tablet is chosen, the compatibility of the drug with the usual diluents or lubricants must be considered, and the amount and the type of diluent necessary to give effective disintegration of the tablet, the possible effect of the granulation process on the drug, and the stability of the drug in tablet form.

New drugs may sometimes call for new methods of administration. I should like, if I may, to take an instance from my own experience of what I regard as an example of pharmaceutical research, which called for all the resources of the pharmacist as distinct from the chemist, and in my opinion, was a type of problem which the pharmacist can tackle better than anyone else. I refer to the production of a chewing-gum containing penicillin. Here was a problem which was quite new. It was not a question of adding penicillin to the ordinary chewing-gum base, because the ordinary chewing-gum base contains water, and penicillin

would not last in it for more than a few days. A completely new type of water-free base had to be produced, and the penicillin had to be incorporated in such a way that it was liberated over several hours of chewing. The chief asset of the pharmacist, as I said in my address last year, is his knowledge of the properties of materials. It is said that the organic chemist with the aid of some coloured balls and bits of wire can design a new detergent and confidently prophesy its properties before the compound is made. A chewing-gum cannot be designed in this way, since so little is known of the effects of constituents on the rheological properties of solids. Many experiments had to be made before the right consistency was reached and the desired slow release of penicillin was attained, involving many lengthy chewings by rather unwilling human guinea-pigs and the taking of many samples of their saliva for penicillin assay. Not only must the desired consistency be attained, it must be retained under a variety of storage conditions, for the margin between chewing-gum which is too soft and sticks to the teeth, and chewing-gum which breaks up when chewed is a very narrow one. There is also the problem of flavouring to be considered; this, too, must be liberated slowly and must not react with the penicillin. Problems such as this can only be solved when the accumulated knowledge and experience of the trained pharmacist is brought to bear upon them.

Drugs which are used by external application provide some of the most interesting problems for the pharmacist. Many of the physical problems which arise in the formulation of such products were mentioned in my address last year, and I do not propose to repeat them now.

Much of the research in industrial laboratories is concerned with methods for large-scale production, not merely of new drugs, but of pharmaceutical preparations. Advice must be given to the production department on the most suitable type of plant and for this purpose batches of a size comparable with a production batch must be carried through. Transfer from the laboratory bench scale to the manufacturing scale is rarely achieved without difficulties, even though an intermediate or "pilot" stage is interposed. The closest co-operation between the research and production staff is essential to ensure success.

But research is not confined to new drugs or to new methods of presentation of drugs. There is a continual stream of problems arising from existing preparations. New ingredients are introduced and reformulation is required to produce a new and improved product. Improved types of plant may require a modification of a production process. Contact with the production department may lead the research worker to suggest an improvement in the production process which will give greater efficiency or reduce the cost. Existing formulae may be found to be unsatisfactory when subjected to certain conditions, or some slight alteration in the composition of one of the constituents may have caused trouble in the finished product. The question of stability provides more problems and more headaches for the pharmaceutical research laboratory than any other. The hospital and retail pharmacist is not so much concerned with this problem, but to the manufacturer it is all important, especially if

goods are to be exported. All sorts of conditions of temperature and humidity must be provided for, and in many cases it may be necessary to use a different formula for tropical countries. Suggestions from clinicians for improvements in the administration of drugs or for new combinations of drugs are a fruitful source of investigation for the pharmaceutical research worker. It is a curious fact, that, no sooner is a new drug introduced, than suggestions flow in for combining it with other drugs. The belief of some clinicians in synergism seems to be unbounded.

The proper organisation of research means a ready access to the published literature on the subject. Much of the research worker's time can be saved by an efficient library service and indexing system. The subject of patents is the bugbear of the industrial research worker. A knowledge of existing patents is essential before undertaking a piece of research. This is not the place to discuss the merits or demerits of patents for medicinal products, but certainly the number of patents granted in this field is increasing and rendering the task of the research pharmacist more difficult. One result of this is the multiplication of new drugs to the confusion of the medical man and the pharmacist. The success of a new drug induces rival manufacturers to produce a similar compound with a modification in structure which avoids the original patent, but may or may not possess any therapeutic advantage. The number of antihistamine drugs which have been put on the market in the United States has forced the Council on Pharmacy and Chemistry of the American Medical Association to refuse to accept new products unless they show a very marked superiority over the old.

Familiarity with recent advances in the pharmaceutical field is a necessary equipment for the research worker, but advances in other industries may provide the key to the solution of many a pharmaceutical problem. The greater part of pharmaceutical research is carried out in the laboratories of industrial firms, though much is done in the Universities and Schools and by hospital pharmacists. The days when the retail pharmacist can spare much time for research work have unfortunately passed; the time when men like Farr and Wright could spend laborious hours on the determination of alkaloids in drugs is no more. Most modern research requires expensive equipment which is not found in the pharmacy. Nevertheless, the retail pharmacist of an inquiring mind must meet many problems which he could solve for himself with some expenditure of time and ingenuity. Doubtless much work of this type is done, but with a few exceptions such efforts remain " unhonoured and unsung." It is to be hoped that the National Health Service will not still further discourage such efforts. Pharmacy must not become mass produced. The spirit of inquiry which is, or should be, engendered in pharmacists by their scientific training should not be stifled when they engage in practice.

Much of the work done in industrial laboratories necessarily remains unpublished. Consequently there is undoubtedly much duplication. A great deal of unnecessary effort could be avoided by a proper organisation

of pharmaceutical research by a committee such as was envisaged by the Executive Committee of this Conference in their report to the Council of the Pharmaceutical Society. Such a body, composed of men of distinction in pharmacy and the allied sciences, could do much to influence the direction of research towards the most pressing problems and to ensure co-operation between those interested in the same problem from different points of view. There seems to be a need for increasing the amount of research work which is carried out on behalf of the British Pharmacopœia and the British Pharmaceutical Codex. Pharmaceutical research must always be the hand-maiden of medical research, but this does not mean that it must be entirely dependent upon it. In fact, the introduction of new or improved pharmaceutical products can and does influence methods of administration. A method of prolonging the action of a drug by altering the form of presentation may so reduce the number of doses required that treatment is much less irksome to the patient.

I have not left much time to deal with the sixth item on my list—research on the chemical and biological standardisation of drugs. The discovery of a new drug is dependent on some method of testing its efficacy. The isolation of an active principle from a vegetable or animal source is dependent on some method of assaying its potency. New drugs require new methods of assay, whether chemical or biological. New methods of manufacture may introduce new impurities. Methods of testing are continually being improved. In parallel, therefore, with research proper must go research on methods of control. Such work can be just as fascinating as other types of research, though the prizes may not be so valuable.

The ideal type of research worker is rare and it is unlikely that all members of a research team will have the true research mentality. The laboratory might not be a very pleasant place to work in if they had. The plodder is needed as well as his more brilliant colleague. There is a general and laudable desire among students of science nowadays to become research workers. The first-class research worker must be a person of ideas, but ideas alone are not enough. He must have the capacity of sorting out the ideas which are practicable and those which are not. He must have the capacity for perseverance and concentration which are necessary to carry an idea into effect. He must be an enthusiast and be able to inspire enthusiasm in others, but his enthusiasm must not carry him so far that he ignores unpleasant facts. He must be prepared to meet continued failure and rise with head "bloody but unbowed." Above all, he must know when to stop. He must be prepared to accept much of his reward in satisfaction with work well done. He should be able to say, as was said by an old chemist a few hundred years ago, "The chymists are a strange class of mortals impelled by an almost insane impulse to seek their pleasure among smoke and vapour, soot and flame, poisons and poverty; yet among all these evils, I seem to live so sweetly. that, may I die, I would not change places with the Persian King."

RESEARCH PAPERS

THE ISOLATION AND IDENTIFICATION OF BEETLE FRAGMENTS FROM POWDERED VEGETABLE DRUGS

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Received May 31, 1949

INTRODUCTION

VEGETABLE drugs are required by the British Pharmacopœia 1948 to be free from insects and other animal matter and it follows from this statement that such matter is not to be tolerated under the term "foreign organic matter." Most vegetable drugs whether whole or powdered are very prone to attack by insect pests especially when carelessly stored. The presence of whole insects is readily detectable by examining the material directly or by sieving. If infested material has been subsequently powdered, any eggs present are usually killed and unless reinfestation occurs no whole insects will be found. Detection must then be based on whatever insect fragments are present and since most drug pests are very small creatures, the weight of the more common beetles being of the order of 1 mg. and that of the identifiable fragments much less, even high infestations may be overlooked by direct microscopical examination. Some method of concentrating the insect fragments must therefore be adopted. The identification of fragments as being of insect origin is not however sufficient evidence that the material was infested. Certain drugs, particularly herbs such as hyoscyamus, not infrequently contain small insects which were associated with the growing plant and were not removed during the preparation for the market. Large insects such as the cockroach may also occasionally find their way into drugs and unless their identity was realised, the powdered material might be reported as highly infested. Thus a study of the diagnostic microscopical characters of drug pests appears desirable. In the present paper, methods for the isolation of insect fragments are considered, and the diagnostic microscopical characters of some of the more common beetle pests of drugs described.

THE ISOLATION OF INSECT FRAGMENTS

For purposes of identification, the soft parts of insects may be ignored and only the exoskeleton need be considered. This consists of chitin impregnated with sclerotin and other substances, and is resistant to many chemical reagents, including boiling dilute mineral acids. Boiling caustic alkalies tend to remove the colouring and hardening materials without producing other visible changes. The exoskeleton is also water repellant but is readily wetted by petroleum and similar non-polar liquids.

Thus two methods of isolating insect fragments from powdered drugs are available; floating off with a non-polar liquid from an aqueous suspension, or solution of the vegetable material. Flotation methods are used extensively for the examination of foodstuffs and are particularly

suitable for starchy materials containing little cellulosic matter. In the author's experience, they have not afforded the complete separation of insect fragments when applied to powdered drugs. A method involving solution of the cellulosic material of infected drugs was devised by Greenish[1], but does not appear to have been generally adopted. It consists of boiling the defatted powder in 5 per cent. hydrochloric acid, macerating the washed residue for 18 to 48 hours in a mixture containing about 12·5 per cent. of sulphuric acid and 20 per cent. of chromic acid and separating the insect fragments by centrifugation. This method is not entirely satisfactory because solution of the vegetable material is not always complete and because of the time involved. The use of acetolytic methods appeared to the author to be more suitable and it was decided to investigate their practicability. Preliminary experiments with the usual laboratory methods of acetylation were made on absorbent cotton and on tow (lignocellulose). These materials dissolved most readily and completely in acetic anhydride containing 10 per cent. of concentrated sulphuric acid. With vegetable drugs however, solution was not always complete and an amorphous sludge sometimes remained. This was thought to be due to the non-cellulosic constituents since the crude fibre from these drugs was found to dissolve completely. After various trials the following method was evolved.

Method.—Boil about 5 g. of the powdered drug in a flask with 100 ml. of 10 per cent. w/w nitric acid in water for about 1 minute with frequent shaking. Filter through a No. 3 sintered glass filter, using suction, and wash the residue with hot water. Return the residue to the flask and boil for about 1 minute with 100 ml. of 2·5 per cent. sodium hydroxide solution, filter through the original filter and wash the residue with hot water. Remove the excess of water by suction and the last traces by passing a few ml. of glacial acetic acid through the filter. Transfer the residue as completely as possible to a flask of about 50 ml. capacity, and wash the remaining fragments into it with 10 ml. of acetic anhydride. Add a mixture of 10 ml. of acetic anhydride with 2 ml. of concentrated sulphuric acid, mix well and heat on a boiling water-bath until the crude fibre has dissolved. Solution is usually complete after about 10 to 15 minutes during which the liquid becomes dark reddish brown. Separate the residue by centrifugation, pour off the supernatant liquid, replace with glacial acetic acid and recentrifuge. After pouring off the glacial acetic acid, the residue of insect fragments may be mounted in any desired mountant for microscopical examination. Alternatively, the acetic anhydride can be hydrolysed before centrifugation by gradually adding the contents of the flask to about 10 ml. of water. Where infestation is heavy and the finest fragments are not required, the contents of the flask may be passed through the sintered glass filter and the residue washed with glacial acetic acid followed by water. It can then be removed by adding a few drops of water or mounting fluid to the filter and gently brushing with a small stiff brush. Passage of air the reverse way through the filter by connecting the side-arm of the receiving flask to a water tap facilitates the removal. If the drug contains much oil or fat it is pre-

ferable to remove most of it by maceration for a short time with one or two changes of light petroleum or similar solvent before preparing the crude fibre.

Identification of the Fragments

The beetles most frequently infesting stored vegetable drugs[2] are *Stegobium paniceum* L. (the drug-room beetle) and *Ptinus tectus** Boie. (the brown spider beetle). Less common but of frequent occurrence are *Niptus hololeucus* Fald. (the golden spider-beetle) and *Calandra granaria* L. (*the grain weevil*). *Lyctus brunneus* Steph., one of the powder post beetles, although primarily a pest of timber has been reported on vegetable drugs e.g. liquorice[3]. In these laboratories it has been found on Butea seeds and was recently introduced on Shensi rhubarb on which it appears to thrive. The following account is confined to these five species with the addition of a reference to the common cockroach *Blatta orientalis* L.

Materials.—A sample of Jamaica ginger was examined for freedom from infestation and then coarsely chopped. For each species 10 beetles were mixed with about 30 g. of the material and powdered in a steel laboratory end-runner mill until fine enough to pass through a No. 60 sieve. About 5 g. quantities were treated by the method described above and the residue mounted in cedar-wood oil for microscopical examination. Further mixtures with other drugs were subsequently prepared and the residue after removal of the vegetable material also examined. It consisted of fragments from the added beetles together with the acid-insoluble ash if any. With some drugs, especially leaves or herbs, the residue often contained pollen, some foreign to the particular drug, also various fungal spores and hyphæ.

The following descriptions were made from these fragments after reference to the whole insects cleared by boiling in 2·5 per cent. sodium hydroxide solution. With a few exceptions indicated by the absence of a surrounding line, the sketches were also made from the fragments. Drawing was done with the aid of a camera lucida at an original magnification of × 500, or for mandibles and antennal joints × 200.

Microscopical Characters. General.—The fragments consist of the cuticle of the beetle, the soft parts having been dissolved. Fragments from the body are irregular in outline, yellowish-brown and translucent. The larger joints of the appendages are also broken but the smaller ones such as those of the antennæ and tarsi are often intact. In life, the beetles are clothed with hairs all but a few of which become detached during the powdering process, but leave scars to mark their position and frequency. The detached hairs are usually too finely broken to be recognisable although occasional ones are found intact. The hairs of the species described are of two general types, bristle-like articulated setæ, and smaller and relatively more numerous clothing hairs. On the fragments, the positions of the former are marked by the setal scars which consist of a circular puncture enclosed within a concentric ring whereas the clothing

* Wallis mentions *P. brunneus* Dft. but *P. tectus* appears the more common of the two species.

hairs leave minute usually simple scars referred to subsequently as micro-punctures. The fragments proving of most value for identifying the beetles were derived from the elytra, the prothorax and the head, those from other segments being less characteristic. Elytral fragments are recognised by the presence of coarse punctures arranged in rows—the strial punctures, which usually have a densely coloured rim. They are surrounded by an oval or irregular area slightly darker than the general colour and apparently due to a local thickening or ingrowth of the cuticle. These areas may be absent from some fragments if the cuticle has laminated. In the majority of cases, the rows of strial-punctures are parallel except on fragments from the ends of the elytra. In certain species, coarse punctures resembling the strial punctures occur elsewhere than on the elytra. These can be recognised either by their irregular arrangement or if they are in rows, by their closeness to the rim of the segment which is usually also present.

The outer surface of the fragments may be smooth or raised into tubercles or ridges. The latter often enclose polygonal areas which probably correspond with the original cells of the epidermis. Mandibles are rarely found unbroken but the thick biting edge is often intact in which case the number and arrangement of the teeth is a useful character. The shape and size of the terminal joints of the antennæ is also of value, while their number together with that of the mandibles gives an indication of the number of beetles originally present.

Stegobium peniceum L.

Elytra: strial punctures 10 to 50μ apart in parallel rows, slit-like about 20 to 30μ long, with on each side one or rarely two rounded tubercles, each tubercle associated with a micro-puncture; the whole surrounded by an oval area about 30 to 50μ long: intervals between the rows 100 to 150μ broad, with setal scars about 10 to 15μ diameter, mainly in a single median row and separated by about 20 to 70μ: over the entire surface, numerous scattered micro-punctures separated by up to about 20μ. (Fig.1.C.)

Prothorax: tuberculate; tubercles prominent, rounded conical, furrowed and irregularly dentate at the base, about 10 to 20μ diameter, contiguous or separated by up to about 50μ, each closely associated with a setal scar; intervals micropunctate like the elytra. (Fig. 1.E.)

Head: dorsal surface similar to the prothorax but with less prominent tubercles; ventral surface ridged, ridges curved to semicircular, distinct or confluent forming crenate rows, each ridge overhanging a shallow depression containing a hair or its scar. (Fig. 1.A.)

Antenna: terminal joint ellipsoidal about 250μ by 70μ, constricted at the base, hairy. (Fig. 1.F.)

Mandible: biting edge about 150μ long, tridentate, the teeth diminishing in size from apex to base (Fig. 1.B); in the larva, the subterminal teeth prominent and connected by a cutting edge. (Fig. 1.G.)

Hairs: setæ; cylindrical, about 100 to 150μ long, acuminate, moderately thick walled, straight or slightly curved; clothing hairs similar but smaller, about 30 to 50μ long. (Fig. 1.D.)

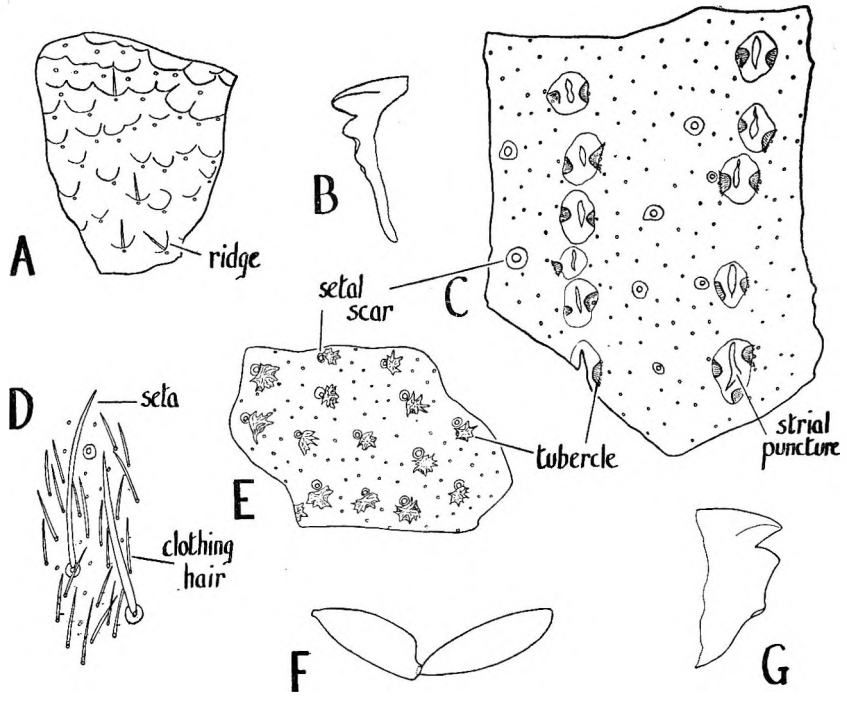

Fig. 1. *Stegobium paniceum* L.

A. Head, fragment from ventral surface showing ridges and an occasional hair, × 200. B. Biting edge of a mandible. G. The same from a larva, ×80. C. Elytron, fragment from the middle region showing two rows of strial punctures, × 200. D. Hairs from elytron of a whole insect, × 200. E. Prothorax, fragment showing tubercules with associated setal scars, × 200. F. Antenna, terminal and subterminal joints, hairs not shown, × 80.

Ptinus tectus Boie.

Elytra: strial punctures up to about 30μ apart in parallel rows, oblong to elliptical about 25 to 50μ long with a thickened rim and surrounded by an oval or rounded area about 60 by 55μ; intervals between the rows 80 to 100μ broad, with a single median row of setal scars about 5 to 7μ diameter and separated by 40 to 60μ; over the entire surface, numerous scattered micropunctures separated by up to about 20μ. (Fig. 2.A.)

Prothorax: central region of the pronotum coarsely punctate, punctures irregularly arranged, deep, circular to irregularly oblong about 40 to 80μ long, resembling the strial punctures of the elytra but sometimes lacking the surrounding area; intervals with scattered setal scars and micropunctate like the elytra, Fig. (2.D): remaining regions locally thickened, thickened areas isolated, more or less circular and surrounding a setal scar, or confluent, irregular and enclosing up to about 5 setal scars; surface over the areas micro-punctate, intervals impunctate. (Fig. 2.C.)

Head: coarsely punctate, punctures numerous scattered and separated by up to about 50μ, very shallow, circular to oval about 30 to 80μ diameter each associated with a setal scar; intervals micropunctate like

the elytra. (Fig. 2.B.) Similar fragments from the thoracic and abdominal segments also occur.

Antenna: terminal joint ellipsoidal, about 270 by 100μ and 30 to 40μ in diameter at the base, hairy. (Fig. 2.F.)

Mandible: biting edge about 120μ long with two teeth, apical tooth acute, basal tooth subacute. (Fig. 2.G.)

Hairs: setae; resembling those of *S. paniceum* but rather longer and

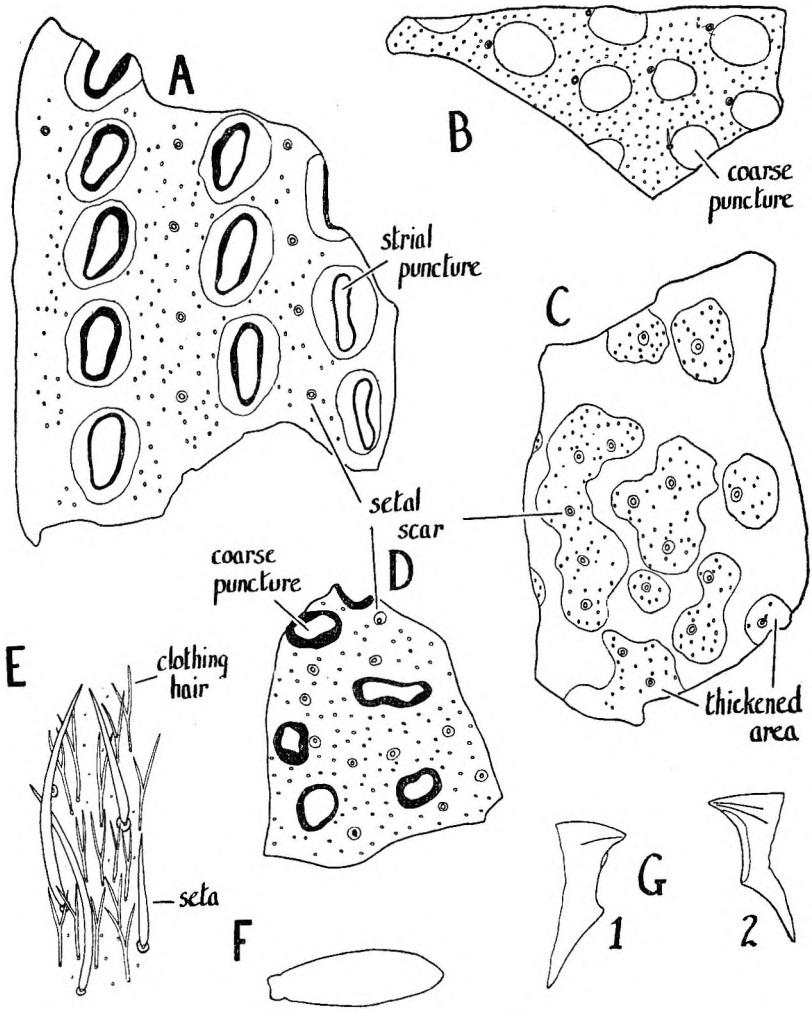

Fig. 2. *Ptinus tectus* Boie.

A. Elytron, fragment from middle region. B. Head, fragment showing coarse shallow punctures with associated setal scars. C. and D. Prothorax; C, fragment from near the head showing isolated locally thickened areas; D, from central region of the pronotum showing coarse deep punctures. E. Hairs from elytron of a whole insect. A to E, × 200. F. Terminal joint of antenna, hairs not shown, × 80. G. Biting edge of mandibles: 1. larval; 2. adult, × 80.

wider; clothing hairs Y-shaped, cylindrical with acuminate arms, about 40 to 60μ long. (Fig. 2.E.)

NIPTUS HOLOLEUCUS FALD.

Elytra: strial punctures about 100μ apart in parallel rows, circular about 5 to 10μ diameter with a deeply pigmented rim about 20μ diameter, each surrounded by a rounded to oblong concentrically striated area about 120 by 80μ having an indented margin and sometimes exhibiting a number of fissures radiating from the puncture; co-linear with the strial punctures, a row of setal scars, one associated with each puncture and separated from it by 20 to 40μ*; intervals between the rows about 200μ broad, with setal scars about 5 to 10μ diameter mainly in a single median row and separated by about 50 to 100μ; over the entire surface numerous scattered micropunctures separated by up to about 20μ. (Fig. 3.A.)

Prothorax: over the greater part, scattered setal scars and micro-punctures similar to those of the elytra; near the margin one or sometimes two rows of coarse punctures separated by about 30 to 70μ, punctures deep, circular to oblong about 20 to 40μ long with a thickened rim and sometimes an enclosing circular to oval area about 40 to 80μ long. (Fig. 3.C.)

Head: over the greater part, scattered setal scars and micro-punctures; near the margin tuberculate, tubercles contiguous or separated by up to about 20μ, conical, each subtending a short hair; extreme margin reticulately ridged, the ridges enclosing elongated polygonal areas about 15 to 60μ long and 15μ wide. (Fig. 3.F.)

Other regions; meso- and metosternites, some joints of the appendages: sparsely micropunctate and reticulately ridged, the enclosed areas more or less regular penta- or hexagonal, about 10 to 15μ long and 5 to 10μ wide. (Fig. 3.G.)

Antennæ: terminal joint ellipsoidal to subcylindrical, about 400 by 100μ and 50μ in diameter at the base, hairy. (Fig. 3.B.)

Mandibles: biting edge about 200μ long with two teeth and resembling that of *P. tectus*. (Fig. 3.E.)

Hairs: setæ, cylindrical to narrow fusiform, about 100 to 150μ long, acute or bifurcate at the tip, relatively thick walled; clothing hairs, flattened scale-like about 40 to 60μ long by 10 to 20μ wide, cleft into three or occasionally four long fine points. (Fig. 3.D.)

CALANDRA GRANARIA L.

Elytra: surface corrugate with intervals of 40 to 100μ: in the furrows, one or rarely two rows of circular punctures about 5 to 10μ diameter each situated at the bottom of a circular or oval saucer-shaped depression about 15 to 30μ long and separated by about 40 to 70μ, each depression also containing a circular puncture about 3 to 5μ diameter or an occasional hair: intervals, reticulately ridged the ridges enclosing regular or somewhat elongated hexagonal areas about 10 to 30μ long and 10μ wide, and also exhibiting an occasional punctured depression like those of the furrows. (Fig. 4.B.)

* Hinton[4] states that the setæ arise from the strial punctures.

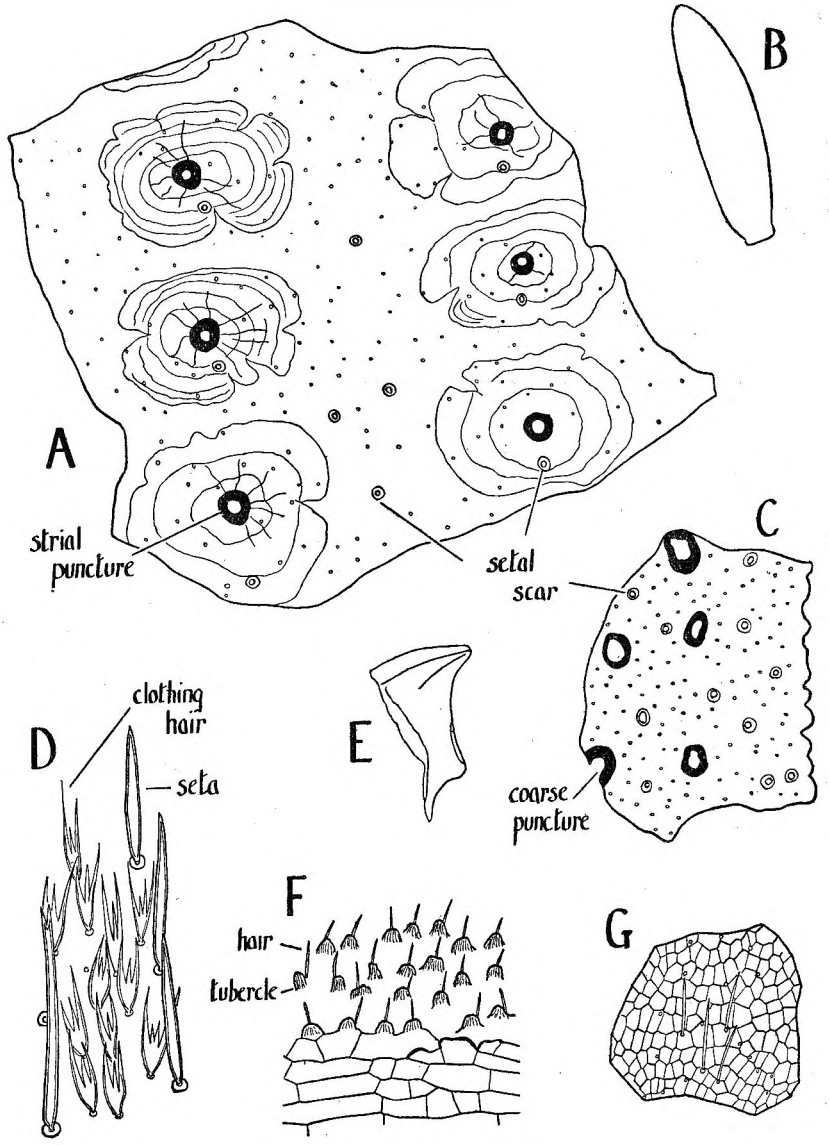

Fig. 3. *Niptus hololeucus* Fald.

A. Elytron, fragment from middle region showing strial punctures with surrounding areas and associated setal scars, x 200. B. Terminal joint of antenna, hairs not shown, × 80. C. Prothorax, fragment including the margin, showing coarse punctures, × 200. D. Hairs from elytron of a whole insect, × 200. E. Biting edge of a mandible, × 80. F. Head, fragment from near the margin showing tubercles with associated hairs and reticulate ridging, × 200. G. Fragment from mesosternite showing reticulate ridging and occasional hairs, × 200.

Prothorax, head and other regions: sculpture like that of the elytra, punctured depressions often larger, up to 60μ diameter, scattered irregularly or arranged in more or less regular rows, and separated by about

15 to 160μ, reticulations more regularly hexagonal than on the elytra, about 5 to 10μ long. (Fig. 4.A.)

Antennæ: terminal joint obovate, up to about 300 by 150μ, diameter at the base about 50μ, apical fourth with numerous very small hairs, remainder almost glabrous. (Fig. 4.D.)

Mandibles: biting edge curved, about 100μ long with 3 or 4 closely arranged serrate teeth. (Fig. 4.C.)

Hairs: arising from the punctured depressions, about 25 to 50μ long, somewhat flattened, multifid, with 5 to 10 linear truncate segments often unequal in length; the base sometimes elevated on a bun-shaped tubercle.

LYCTUS BRUNNEUS STEPH.

Elytra: strial punctures 10 to 25μ apart in parallel rows, narrow elliptical to slit-like about 5 to 15μ long, with a thickened rim and enclosed in an oval or irregularly oblong area about 15 to 30μ long: in-

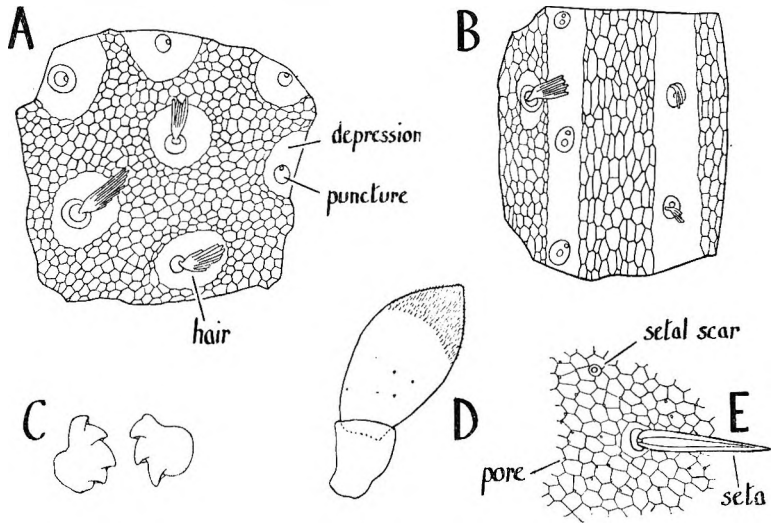

Fig. 4. *Calandra granaria* L.

A. Prothorax, fragment showing hairs arising from the saucer-shaped depressions, with reticulately ridged intervals, × 200. B. Elytron, fragment from middle region showing two furrows, × 200. C. Mandibles, × 80. D. Antenna, terminal and subterminal joints, × 80. E. *Blatta orientalis* L. fragment showing reticulate ridging, × 200.

tervals between the rows about 40 to 60μ broad with a single median row of setal scars about 5μ diameter and separated by about 20 to 40μ: surface smooth and impunctate. (Fig. 5.B.)

Prothorax, head and other regions: coarsely punctate, punctures numerous, contiguous or separated by up to about 20μ, very shallow, circular oval or irregular about 20 to 30μ diameter, each enclosing an eccentric setal scar: intervals smooth and impunctate. (Fig. 5.C.)

Antennæ: terminal joint rounded-conical about 160 by 100μ and 50μ diameter at the base, surface sparsely finely hairy, subterminal joint sometimes attached, obconical of similar dimensions. (Fig. 5.D.)

Mandibles: biting edge about 50μ long with two prominent closely set teeth. (Fig. 5.A.)

Hairs: setæ, about 80 to 100μ long, slender and thin walled but otherwise similar to those of *S. paniceum;* clothing hairs lacking from the body. (Fig. 5.E.)

BLATTA ORIENTALIS L.

Elytra lacking. All sclerites reticulately ridged, the ridges enclosing more or less regular hexagonal areas about 10 to 15μ long, some having a minute pore in one angle; setal scars, scattered at intervals of about 70 to 100μ, each about 5 to 15μ diameter and sometimes surrounded by a circular unridged area.

Setæ, about 80 to 100μ long, conical acuminate and thick-walled. Clothing hairs lacking. (Fig. 4.E.)

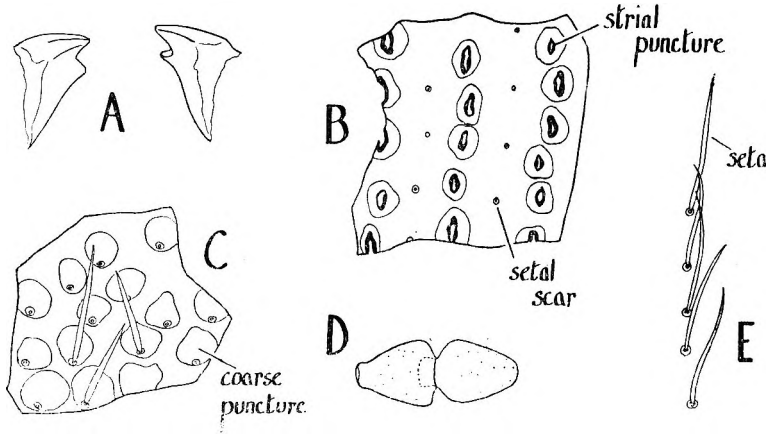

Fig. 5. *Lyctus brunneus* Steph.
A. Biting edge of mandibles, × 80. B. Elytron, fragment from middle region, × 200. C. Prothorax, fragment showing coarse shallow punctures with enclosed setæ or setal scars, × 200. D. Antenna, terminal and subterminal joints, × 80. E. Setæ from elytron of whole insect, × 200.

SUMMARY

1. A rapid and effective method of isolating insect fragments from powdered infested drugs is described.

2. The method depends on solution of the crude fibre of the drug in acetic anhydride containing 10 per cent. v/v of concentrated sulphuric acid.

3. The diagnostic microscopical characters of five of the more common beetle pests of drugs are described and illustrated.

In conclusion I wish to express my thanks to Mr. W. D. Hincks, M.P.S., F.R.E.S., of the Manchester Museum, for his assistance in confirming the identity of the species described.

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DISCUSSION

THE CHAIRMAN (Dr. Norman Evers) said that many people who had to deal with foods had been disturbed by the stringent regulations introduced in the U.S.A. for the absence of insect fragments, rodents' hairs, and so on, and he was afraid that few drugs in this country would pass. Nevertheless, he felt that it might be necessary to have before long, on the basis of a method such as the author had described, some such requirements for drugs.

DR. T. E. WALLIS (London) said that many of these small beetles were quite difficult to distinguish when entire, and the difficulty was much greater in the powdered form, when examination of fine details of the form and attachment of the setæ and hairs and fine markings on the exoskeleton was required. Dr. Melville's work made it possible to distinguish, and frequently to name, the insects with which a powder was infested. He asked the author how far it was possible to see these insect particles in the crude fibre.

DR. D. C. GARRATT (Nottingham) said that the quality of infestation was as important as the quantity. The American regulations applied to foodstuffs and spices, and it could not be long before they were extended to drugs. Kent-Jones and his co-workers in this country, in their work on cereals, had dealt with rodent hairs, but could not give any authoritative help on insect fragments. Dr. Melville's paper would be of considerable value in that connection. He asked the author whether, in clearing for the detection of insect fragments, the rodent hairs would be lost; if so two separate methods of separation would be required.

MR. H. DEANE (Long Melford) said that, in his own experience, moths were more troublesome than beetles in stored drugs owing to their greater rapidity of reproduction. Moths were softer than beetles, and fewer fragments would be left with this treatment. Had Dr. Melville done any work on that subject?

DR. J. M. ROWSON (London) asked whether the possibility had been considered of extending the work from the qualitative to the quantitative field, also whether Dr. Melville considered that the B.P. requirement of complete absence of insect fragments, and also of animal matter and animal excreta, was too stringent.

MR. T. C. DENSTON (London) asked what drugs had proved to be unsatisfactory for examination by the flotation method. In view of the considerable use of one or other modification of this method in the U.S.A. he was a little surprised to find that it had been discarded.

MR. R. MAXWELL SAVAGE (London) drew attention to recent work on the separation of lice from animal skins.

MR. V. REED (London) asked whether there was any easy way of preventing the beetles from getting into the drugs.

DR. G. E. FOSTER (Dartford) asked which fragments could be considered most characteristic of infestation. How much of the beetle should be found in order to be sure that beetle infestation had actually occurred?

DR. COLIN MELVILLE, in reply, said that the detection of the particles in the crude fibre depended on the extent of the infestation and on the bulk of the crude fibre. In ginger he had been able to see the particles in the crude fibre, which was relatively small in amount, but with the more lignified roots or some of the herbs he had not been able to see the particles in the crude fibre, but had found them after extraction. Rodent hairs would be lost by his method of clearing, because they would be soluble in caustic soda, but it might be possible to apply the method directly to the drug without the preliminary clearing. He thought that then the rodent hairs would stand up to the acetolytic treatment, but a rather higher residue might be found, due to the non-cellulosic matter of the drug, which might not be dissolved under acetolysis. He thought, however, that it should be possible to find the rodent hairs. He hoped to undertake that problem when he had more time. He had not yet done any work on moths, other than looking at the larvæ. He imagined that the majority of moths would leave the drug after they had laid their eggs, so that to detect infestation one would have to look for the larvæ or larval skins which were cast when the larvæ changed to moths. He had found larval skins, but he had confined the paper to beetles, because he hoped to do further work on the larvæ of beetles and moths. He thought that a quantitative method could be worked out on the area of some segment such as the elytron. As most of these insects did not differ very greatly in weight, one could take the area of the elytron as being more or less a standard figure. He had always been doubtful about the B.P. requirements that drugs should be entirely free from insects because one could always find insects in powdered drugs. Hyoscyamus frequently contained insects which were not pests, but which were associated with the growing plant, and they would, of course, pass into the powder. Flotation methods were definitely unsatisfactory with herbs. At the interface of the two liquids one got a high percentage of the powdered herb, and it was very difficult to get complete separation. In any flotation method some insect fragments remained in the sediment of the drug and would not float to the surface at the interface. For the prevention of infestation, cleanliness in storage, the removal of all spillage and storage in suitable containers were important. On a small scale, to remove infestation the best treatment was with a mixture of carbon tetrachloride and ethylene dichloride, or carbon disulphide alone. About 1 fl. ounce of those liquids was sufficient to fumigate about 10 cu. ft. of storage space. It could be placed on a wad of cotton wool at the top of the container, and the vapour, being heavier than air, sank through the drugs and killed any pests which might be present. On a large scale, fumigation by hydrocyanic acid or methyl bromide or an ethylene oxide-carbon dioxide mixture was used. The most characteristic fragment for identification was the elytron. As to how much would be detectable, 10 g. of a sample of nutmeg yielded by the process one beetle leg which was almost intact.

INDIAN HENBANE

PART I

BY J. L. FORSDIKE AND B. JOHNSON

From The Analytical Laboratory, Boots Pure Drug Co., Ltd.

Received July 1, 1949

DURING the war, and in the post-war years, a considerable quantity of henbane has been imported into this country from India. Amongst this material was some which differed in appearance and especially in microscopical characters from typical *Hyoscyamus niger*. This suggested the possibility that this henbane was derived from some other species of hyoscyamus and it was thought worth while to carry out some investigation of hyoscyamus from India ; with a view to ascertaining its botanical origin and characters. As a result, it has become apparent that there are several types of henbane growing in India and Pakistan in addition to *H. niger*. These other types have, in the past, commonly been referred to the species *H. reticulatus*, but they include plants whose habits are quite different from the typical form of this species and which, moreover, vary amongst themselves. It would appear, therefore, that there are a number of species of hyoscyamus growing wild in India, which might be collected as henbane. The question of the true botanical identity of these various species is still under consideration and it is hoped that more definite information will be available later.

The present paper is devoted to a description of one such species of hyoscyamus which grows wild in the North-West Frontier Province. This plant differs in habit from *H. niger* chiefly in the shape of its leaves, which are broadly ovate and much less indented (Fig. 1 and 2, A). It is hoped, in subsequent communications, to describe some further species of hyoscyamus which grow wild in India.

MATERIAL

The following description is based on six dried specimens, numbered H1 to H6, collected in Pakistan, in the area of the North-West Frontier Province, by Professor Qazilbash, of Islamia College, Peshawar, and on plants (H7) grown under glass at Lenton Experimental Station, Nottingham, from seeds sent by Professor Qazilbash.

For comparison, seven specimens of *Hyoscyamus niger* were used. These were :—

N1. Collected at Chelsea Physic Garden, 1937.
N2. Cultivated at Long Melford, 1948.
N3. Cultivated at Hitchin, 1947.
N4. Grown at Lenton, 1948.
N5. Cultivated in the North-West Frontier Province, 1948.
N6. From the Department of Botany, Government of Nepal, Katmandu, 1947.
N7. Cultivated in India, supplied by the Divisional Forest Officer, 1947.

Microscopical Characters

The plant is an erect, branching annual, 1 to 2 feet high. When dry, it has an odour resembling that of *Hyoscyamus niger* and a slightly bitter taste.

Stem. Cylindrical and smooth in the fresh state, but when dry flattened, longitudinally striated and usually showing one or more longitudinal furrows ; light green, 2 to 5 mm. in diameter, slightly swollen at the nodes and clothed with white viscid hairs, up to 7 mm. long ; leaf scars alternate. The transversely cut surface shows the remains of the pith with a central hollow, this is surrounded by a whitish ring of radiate xylem and a narrow, green bark.

Leaves. Ovate to oblong, 3 to 10 cm. long and 1·5 to 5 cm. broad, the lower two or three with petioles about a quarter the length of the leaf, the higher ones sessile, the uppermost clasping the stem ; outline varying from entire to coarsely toothed, there being from 1 to 5 teeth on each side of the leaf, usually more on one side than on the other ; apex acute, base acute in the lower leaves, obtuse to cordate in the upper ; venation pinnate, with a broad midrib and 5 or 6 secondary veins on each side, which make wide angles with the midrib and terminate in the teeth where these are present or, if teeth are lacking, anastomose near the margin (Fig. 2, A).

The surface is covered with long white trichomes and has a viscid feel, especially when fresh. The dried leaves are thin and brittle, usually crumpled and broken ; they are yellowish to greyish green in colour, the uppermost sometimes with a purple tinge.

Flowers. Typically solanaceous, the lower solitary in the axils of the leaves, the upper in a one-sided spike rolled back before flowering ; about 20 mm. long with a pedicel 2 to 3 mm. long. Calyx urceolate, 10 to 15 mm. long and 4 to 8 mm. wide, having 5 lobes, each with an apical spine and 10 main veins ; green, very hairy and persistent. Corolla funnel-shaped, 15 to 20 mm. long and 15 to 20 mm. in diameter at the mouth, the 5 lobes rounded and slightly unequal, very thin, pale buff with very prominent purple veins. Androecium of 5 free stamens, 2 long, about 12 mm. and 3 short, 8 to 10 mm., attached to the base of the corolla. Filaments very hairy in the lower half. Anthers 2 mm. long, black and dehiscing longitudinally. Ovary superior, and bilocular, consisting of 2 united carpels, smooth, cream-coloured, globular, about 2 mm. in diameter ; it contains numerous ovules, showing axile placentation. Style filiform ; stigma bilobed. Floral formula K(5).C(5).A5.G(**2**).

Fruit. An ovoid-oblong pyxis, 12 to 15 mm. long, green, surrounded by the calyx (Fig. 2, C), which becomes larger in size, thicker and more coriaceous in texture after fertilisation, with 5 broad, shortly pointed lobes protruding above the fruit, which contains numerous seeds.

Seeds. Reniform and flattened, 1 to 1·5 mm. in diameter and 0·5 mm. thick ; immature seeds green, mature seeds light brown. Testa finely reticulate, having 12 to 16 reticulations across the flat surface of the seed ; the walls of the reticulations corrugated and the surfaces of the

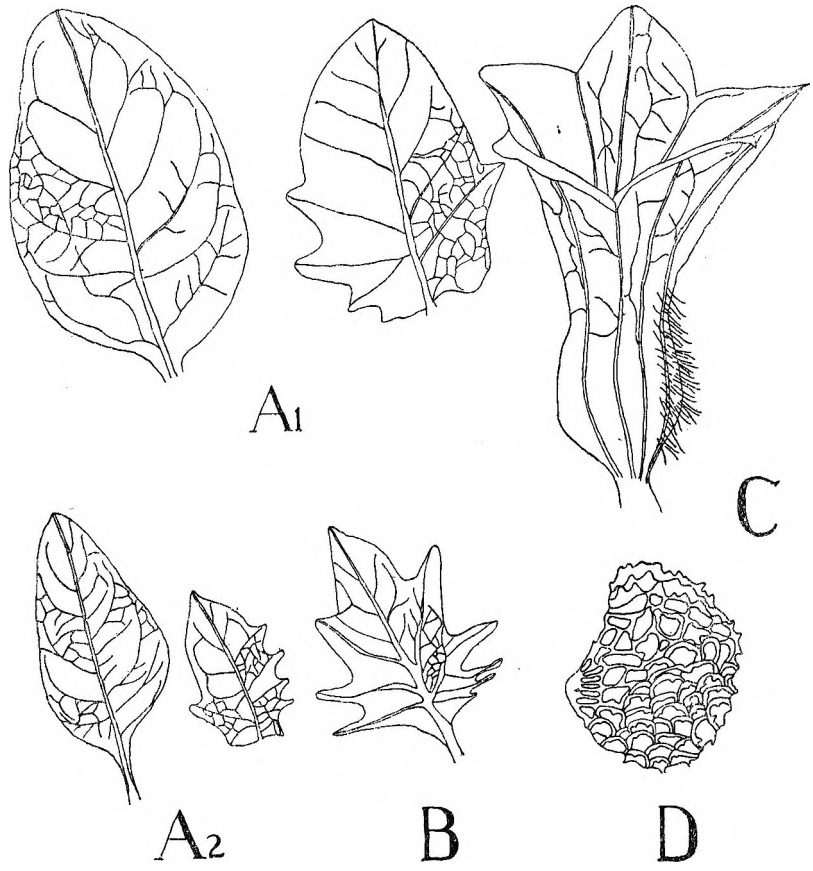


FIG. 2. A.1. Whole leaves, upper surface, natural size. A.2. Whole leaves, lower surface, natural size. B. *Hyoscyamus niger* leaf, natural size. C. Fruit × 4. D. Seed × 20.

depressions rugose (Fig. 2, D). The seed has a single scar of attachment to the placenta on one side. The weight of 100 seeds is 0·06 to 0·07 grammes.

MICROSCOPICAL CHARACTERS

STEM. *Epidermis.* Longitudinal rows of tetra- to hexagonal, tabular cells, 75 to 400μ long, 70 to 155μ wide and 25 to 55μ high. Outer walls 5 to 10μ thick, stratified and showing faint cuticular striations, parallel to the long axis of the stem ; inner walls also thickened ; anticlinal walls straight, thin, with occasional simple pits. Stomata rare, similar to those of the leaf, but lying at the same level as the epidermal cells. Trichomes similar to those of the leaf. Crystals absent.

Cortex. Consist of one outermost layer of ordinary parenchyma cells, containing chloroplasts ; about 5 layers of collenchyma, averaging 45μ radially, 60μ tangentially, and 200 to 500μ longitudinally, occasional cells very elongated, resembling fibres ; about 4 layers of colourless parenchyma, showing intercellular spaces.

The endodermis consist of 2 layers of cylindrical cells with the axis directed longitudinally, containing starch grains 1 to 5 microns in diameter, mostly simple but occasionally 2 to 4 compound. All the cells of the cortex have cellulose walls.

Pericycle. Consists of 1 or 2 layers of common parenchyma with, on the outside of the primary phloem, small groups of fibres, measuring 10 to 20μ radially, 25 to 50μ tangentially, and up to 5000μ longitudinally, with only slightly lignified walls. Fibres are lacking in the young stem.

Phloem. Primary phloem endarch, in bundles made up of groups of sieve tubes and companion cells, embedded in phloem parenchyma. The secondary phloem forms a continuous ring. Sieve tubes 12 to 16μ in diameter, transverse walls oblique, sieve plates and callus evident. Companion cells 3 to 4μ in diameter. All cells with cellulose walls.

Cambium. Consists of 1 or 2 layers of very thin-walled cells, tetragonal in transverse section. Average dimensions, radial 4μ, tangential 12μ, longitudinal 60μ.

Xylem. Primary xylem endarch, in groups opposite the primary phloem ; the secondary xylem forming a continuous ring. Consists of annular, spiral, reticulate and pitted vessels, 15 to 75μ in diameter ; wood parenchyma cells, usually 5-sided wtih pitted walls, averaging 15 μ radially and 20μ tangentially and wood fibres, with oblique slit-shaped pits, about 20μ in diameter. Medullary rays, uni- to triseriate, consisting of tangentially elongated pitted parenchyma cells. All elements of the xylem have lignified walls ; intercellular spaces absent.

Pith. Consists of large, thin-walled parenchyma cells with cellulose walls and showing numerous intercellular spaces, which usually appear triangular in transverse section. At the periphery, a ring of phloem bundles, similar in structure to the normal phloem and having small groups of fibres, resembling the pericylic fibres, on the inner side.

LEAF. *Upper epidermis.* Epidermal cells of the interneural region 100 to 200μ long by 50 to 100μ wide by 20 to 40μ high, with sinuate anticlinal walls and a thin cuticle. Epidermal cells of the main veins straight-walled and elongated in the direction of the vein, 150 to 400μ long by 25 to 50μ wide by 20 to 40μ high, (Fig. 3, B, ep. 1) the cells bearing the hairs are higher and wider than the normal epidermal cells and have rounded edges. Stomata 35 to 65/sq. mm., averaging 40 by 22μ, lying slightly above the level of the epidermal cells and surrounded by 3 to 5 subsidiary cells, one of which is markedly smaller than the others. Stomata absent over the midrib and the primary veins. Covering trichomes numerous, conical, uniseriate, of 2 to 4 cells, 75 to 290μ long (Fig. 3, D, c). Glandular trichomes fairly numerous, especially on the veins, 450 to 750μ long, with a uniseriate stalk of 2 to 6 cells, showing cuticular reticulations and a multicellular, glandular head (Fig. 3, D, gl). On the veins, in addition to the above types, are some very large trichomes, up to 7000μ long and containing up to 12 cells ; the basal cells being 500 to 1400μ long by 40 to 160μ wide ; the apical cell sometimes forming a rudimentary gland (Fig. 3, E).

Lower epidermis. (Fig. 3, A and B, ep. 2). Very similar to the upper,

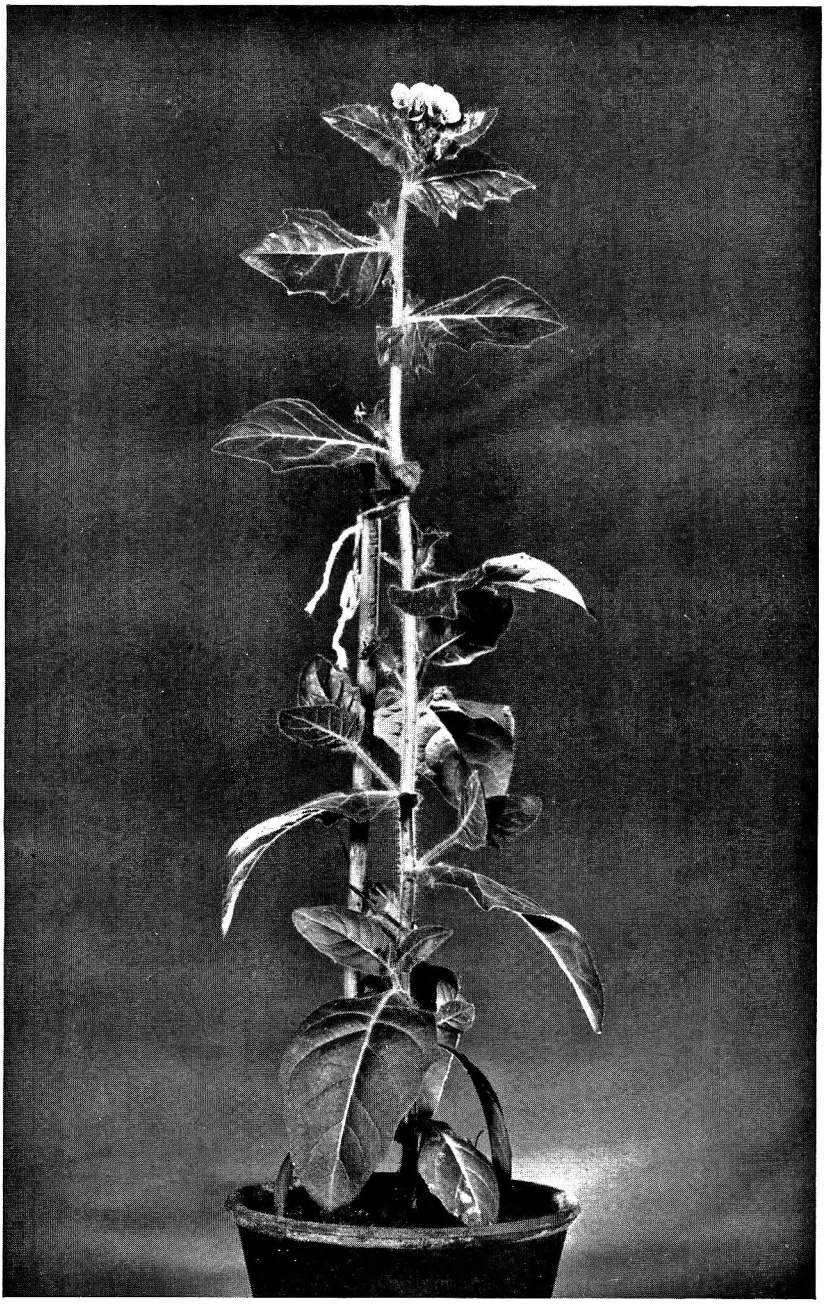


FIG. 1. Entire plant, grown at Nottingham. One-third natural size.

but the stomata are more numerous, 40 to 112/sq. mm. and slightly larger, 45 by 30μ. The trichomes are also more numerous.

Mesophyll. On the upper side of the leaf, one row of palisade cells, containing chloroplasts ; cells averaging 85μ high by 20μ wide (Fig. 3, B, p) ; chloroplasts 2 to 4μ in diameter. There are large intercellular spaces, especially under the stomata.

The spongy mesophyll consists of 3 or 4 layers of more or less stellate cells, 24 to 30μ in diameter, with large intercellular spaces (Fig. 3, B, sp). Idioblasts in the upper layer contain calcium oxalate crystals of three kinds :—

(*a*) Tetragonal prisms (Fig. 3, F, pr.).

(*b*) Cluster crystals. These are commonly composed of a small number of prisms. A type consisting of single large prism with a number of smaller ones attached is of frequent occurrence (Fig. 3, F, cl.).

(*c*) Microsphenoidal crystals.

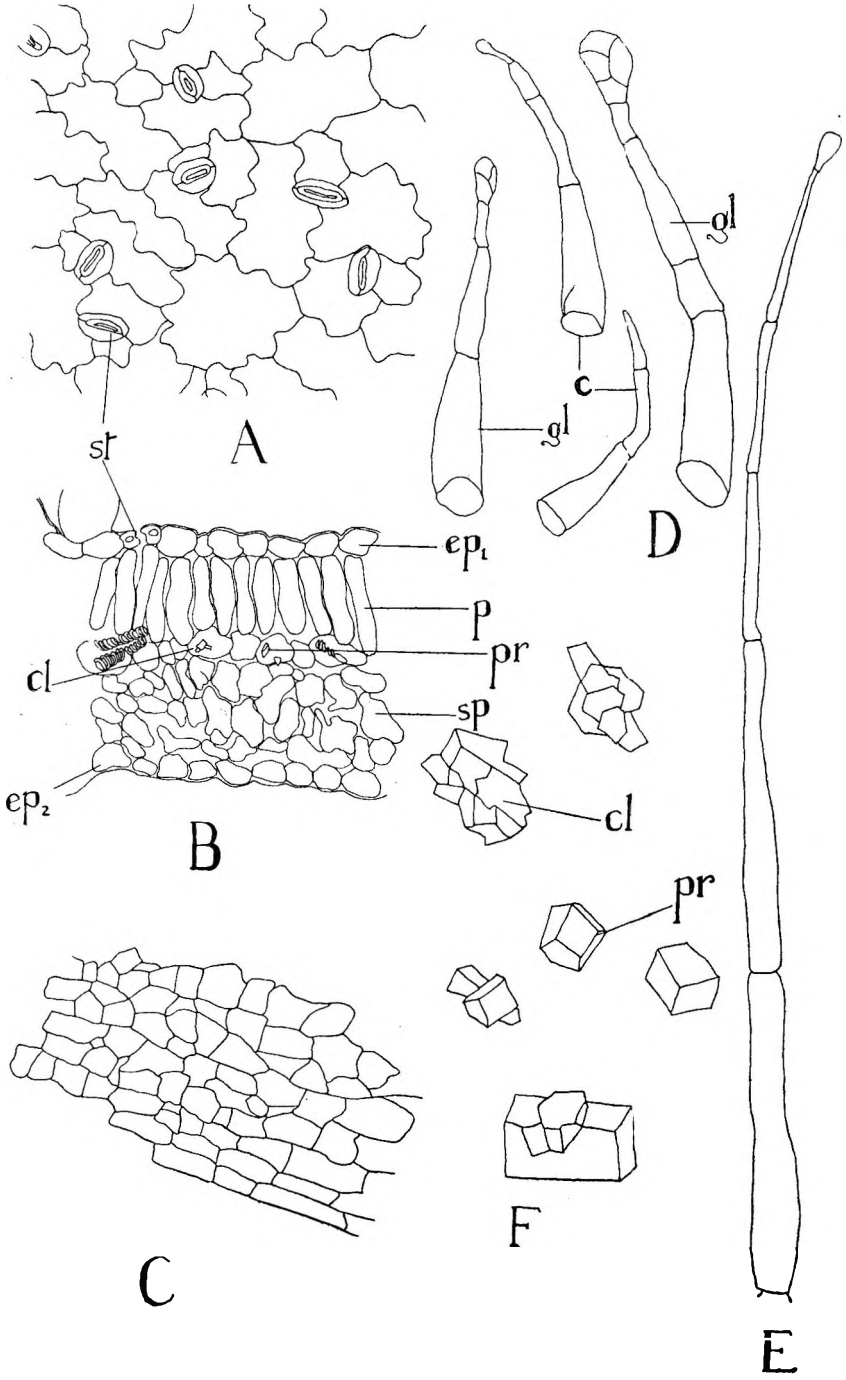
The prisms and clusters are 4 to 48μ long by 3 to 26μ wide.

Midrib. There is 1 layer of collenchyma on the upper surface and 1 to 3 layers on the lower ; cells about 30μ in diameter and up to 500μ long. Within the collenchyma is colourless parenchyma, about 10 layers on the upper and 4 on the under side, with large intercellular spaces ; the cells sometimes containing crystals of calcium oxalate. Cells average 60μ in diameter by 200 to 400μ long. Endodermis of 1 or 2 layers of cells with slightly thickened cellulose walls, containing starch grains and chloroplasts.

Meristele. In the form of an arc, made up of 8 to 10 simple, collateral vascular bundles separated by uni- or biseriate medullary rays. Xylem of 7 to 8 layers consisting of annular, spiral and reticulate vessels, 15 to 24μ in diameter, embedded in xylem parenchyma ; all elements lignified. Phloem consisting of sieve tubes and companion cells, embedded in phloem parenchyma. Perimedullary phloem, in groups corresponding roughly in number and position with the vascular bundles, and in form similar to the bundle phloem, is also present. The pericycle consists of 3 or 4 rows of somewhat thickened, fibre-like cells, 10 to 25μ in diameter and 200 to 300μ long, with cellulose walls.

FLOWER. *Calyx. Upper epidermis.* The basal cells are rectangular, with straight anticlinal walls, 15 to 25μ long, 15 to 25μ wide and 15 to 25μ high. Upwards the cells become larger, 30 to 65μ long, 15 to 30μ wide and 15 to 25μ high, the walls become more sinuate and the shape irregular, except over the veins, where the rectangular shape is preserved. Towards the top the cells are again smaller. The epidermis has a cuticle about 4μ thick which, over the main veins, sometimes shows cuticular striations, running parallel with the direction of the vein. The stomata increase in number towards the top of the calyx, where the number per sq. mm. is 80. Each stoma is surrounded by 3 subsidiary cells. The trichomes are very numerous and similar to those of the leaf. *Lower epidermis.* Similar to the upper (Fig. 3, C).

Mesophyll. Consists of 4 to 6 layers of thin-walled parenchyma cells,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containing chloroplasts ; cells more or less stellate in form, averaging 25μ in diameter, with very large intercellular spaces. Idioblasts filled with microsphenoidal crystals of calcium oxalate fairly numerous towards the base of the calyx only ; prisms and cluster crystals absent.

Meristeles. These are similar to the meristeles of the leaf.

Corolla. Upper epidermis. At the base, the epidermal cells are elongated and rectangular in shape, 35 to 90 μ long, by 15 to 30μ wide by about 20μ high ; the anticlinal walls are slightly wavy and the outer walls have a fairly thin, striated cuticle. Towards the apex, the cells become smaller and the anticlinal walls sinuate. Stomata very rare ; trichomes absent. *Lower epidermis.* Cells similar in shape to those of the upper epidermis, but slightly larger. Trichomes present, of two kinds :—(*a*) Short, conical, covering trichomes, 1 to 4 cells long. (*b*) Glandular trichomes, almost confined to the veins in the lower part of the corolla ; they have a 1- or 2-celled stalk and a well-developed multicellular glandular head.

Mesophyll. Consists of 4 or 5 layers of spongy parenchyma. Cells stellate, with large intercellular spaces.

Stamen. Filament. Epidermal cells elongated-rectangular with straight walls ; no stomata. Trichomes of 3 kinds are present :—(*a*) With a uniseriate stalk and a multicellular glandular head. (*b*) Covering trichomes, similar to those of the leaf. (*c*) Capitate trichomes, having a long apical cell. The mesophyll consists of about 5 layers of thin-walled parenchyma cells, with a central vascular strand containing lignified spiral vessels.

Anther. Epidermis of irregular cells with a few stomata. Mesophyll of ordinary parenchyma cells with fairly large intercellular spaces. The meristeles consist entirely of xylem, which contains lignified spiral vessels. There is a fibrous layer of 2 to 3 layers of cells, near the connective.

Pollen. The pollen grains have 3 pores and an irregularly pitted exine, diameter 45 to 55μ ; diameter of pores 10 to 15μ.

Pistil. Ovary. The epidermal cells of both surfaces are tetragonal, stomata rare. The mesophyll consists of about 10 layers of closely packed parenchyma cells, without intercellular spaces. The meristeles consist wholly of xylem, made up of spiral vessels, the walls of which are incompletely lignified.

Septum and Placentæ. Epidermis similar to that of the ovary wall ; mesophyll of spongy parenchyma with large intercellular spaces.

FRUIT. *Pericarp. Outer epidermis.* More or less tetragonal, tabular cells 55 to 95μ long, 15 to 30μ wide and 15 to 22μ high ; anticlinal walls thin and slightly wavy, outer walls with a very thin cuticle. Trichomes absent.

FIG. 3. A. Lower epidermis of leaf, surface view. B. Transverse section of interneural lamina of leaf. C. Lower epidermis of calyx, near base, surface view. D. Glandular and covering trichomes from the leaf. E. Very long trichome from midrib of leaf. F. Calcium oxalate crystals. c, covering trichomes; cl, cluster crystals; ep_1, upper epidermis; ep_2, lower epidermis; gl, glandular trichomes; p, palisade layer; pr, prismatic crystals; sp. spongy mesophyll; st, stomata. F × 300, remainder × 150.

Inner epidermis. Tabular cells, 75 to 130μ long, 20 to 40μ wide and 20 to 26μ high, anticlinal walls thin and wavy.

Mesophyll. Thin-walled, stellate cells, 13 to 26μ in diameter and 40 to 75μ long, with large intercellular spaces. Contains numerous vascular strands, each consisting of 1 to 3 spiral vessels, 4 to 12μ in diameter, with lignified walls. The parenchyma cells are filled with starch grains, polyhedral to subspherical in shape, 2 to 10μ in diameter, mostly simple, but including occasional compound grains of 2 to 4 components.

Septum and Placentæ. Epidermis similar to the inner epidermis of the pericarp ; mesophyll of thin-walled, stellate parenchyma, 30 to 60μ in diameter and 75 to 200μ long, containing starch similar to that in the pericarp.

Quantitative Data

Palisade ratio. The palisade ratio of the material under examination is from 4·25 to 5·5. This range is exactly the same as that found for *H. niger.*

Stomatal index. The range of stomatal index of the upper surface was from 19·2 to 24·0 and of the lower surface from 18·7 to 26·0. These figures compare with values of 21·0 to 22·5 for the upper surface of *H. niger* and 22·1 to 24·8 for the lower surface given by Rowson.[1]

Vein-islet number. The vein-islet numbers found for the species investigated ranged from 4·5 to 15·5 ; the range for *H. niger* was 6 to 16.

Calcium oxalate crystals. The prisms and cluster crystals in our unnamed species were, in general, larger than those of *H. niger* and this feature provides the best means of distinguishing the two species when broken or in the form of powder. In Table I are shown the ranges of sizes of the crystals in all the specimens examined and also the percentages of crystals in each sample which exceeded 25μ in length.

TABLE I

Dimensions of calcium oxalate crystals

Specimen	Size		Number less than 25μ long	Number greater than 25μ long	Percentage greater than 25μ long
	Minimum μ	Maximum μ			
Unnamed Species :—					
H.1...	6	42	390	120	23·6
H.2...	6	40	308	104	25·4
H.3...	4	40	352	146	24·4
H.4...	5	40	498	196	28·3
H.5...	6	45	317	142	31·0
H.6...	4	45	384	138	26·0
H.7...	4	48	360	220	38·0
All specimens ...	4	48	2609	1066	29·0
H. niger :—					
N.1...	5	35	364	37	9·2
N.2...	4	32	591	49	7·5
N.3...	4	28	549	37	6·3
N.4...	4	26	460	5	1·1
N.5...	6	32	480	20	4·0
N.6...	5	26	410	18	4·2
N.7...	5	30	374	15	3·8
All specimens ...	4	35	3228	181	5·3

These figures were obtained by the examination of powders, prepared from representative samples of the specimens. It was observed that, in both species, the size of the crystals varied markedly from leaf to leaf, one leaf containing large crystals and another smaller ones while, from some leaves, crystals were entirely absent. It is thus clear that no reliance, from this point of view, can be placed on the examination of single leaves, but, by examining representative samples of powders it was found that, while the crystals of *H. niger* never exceeded 35μ in length, and except in one specimen, did not exceed 32μ, all specimens of the Indian material contained crystals up to 40μ long and some up to 48μ. Moreover, the percentage of crystals exceeding 25μ in *H. niger* was never greater than 10 and averaged only about 5, while in the Indian material it ranged from 23·6 to 38·0 per cent.

Alkaloid contents. The content of total alkaloids, calculated as hyoscyamine, of the 6 samples, H1 to H6, grown in India, are shown in Table II. These were determined by the method of the British Pharmacopœia for Hyoscyamus.

TABLE II

ALKALOID CONTENTS

Specimen	Total alkaloids, as Hyoscyamine	Specimen	Total alkaloids, as Hyoscyamine
	per cent.		per cent.
H.1	0·038	H.4	0·034
H.2	0·038	H.5	0·046
H.3	0·031	H.6	0·055

Ash values. In Table III are shown the results of determinations of total and acid-insoluble ash, by the methods of the B.P., on the material under investigation and the amounts of ash soluble in dilute hydrochloric acid, obtained by difference.

The new material thus has a soluble ash ranging from 15·7 to 18·3 per cent. This quantity is distinctly higher than is normally found in

TABLE III

ASH VALUES

Specimen	Total Ash	Acid-insoluble Ash	Acid-soluble Ash
	per cent.	per cent.	per cent.
Unnamed Species :—			
H.1	21·5	4·9	16·6
H.2	21·5	4·8	16·7
H.3	22·3	4·6	17·7
H.4	22·7	4·4	18·3
H.5	23·5	6·1	17·4
H.6	21·7	6·0	15·7
H.7	20·2	3·2	17·0
H. niger :—			
N.1	10·0	3·0	7·0
N.2	13·7	1·7	12·0
N.3	15·3	1·3	14·0
N.4	16·0	4·6	11·4
N.5	19·8	3·6	16·2
N.6	21·7	3·3	18·4
N.7	25·1	6·0	19·1

H. niger grown in England. The 3 samples of *H. niger* obtained from India, however, had soluble ash up to or above this range.

Germination

Hyoscyamus seed is known to be difficult and erratic in germination. For this reason a germination test was carried out on the Indian seed. Three methods of preliminary treatment were tried :—(A) The seed was placed in the refrigerator, at approximately 5°C., overnight ; (B) The seed was soaked in a solution of hydrogen peroxide, approximately 2 per cent. w/v, for 18 to 24 hours. This method of treatment was suggested for belladonna seed by Sievers[2] ; (C) The seed was soaked in concentrated sulphuric acid for 2½ minutes and then washed with water. This method is recommended by Newcomb and Haynes[3].

The seeds so treated, together with untreated seed (D) were sown in pans on 30th July, 1948. Fifty seeds were put in each pan and 2 pans were sown with each type of seed. John Innes Seed Compost was used and the pans placed in the greenhouse. Counts were made after 10 and 17 days.

The results are recorded in Table IV.

TABLE IV

Germination of indian henbane seed

Pan	1st Count	2nd Count	Average of Two Pans 2nd Count	Germination
				per cent.
A. 1	2	2	2·5	5
A. 2	1	3		
B. 1	35	40	26·0	52
B. 2	9	12		
C. 1	1	2	1·5	3
C. 2	1	1		
D. 1	3	5	4·0	8
D. 2	3	3		

The results show that the treatment with hydrogen peroxide had a marked effect in stimulating germination. The other two treatments, that with sulphuric acid and refrigeration actually produced inferior germination to that obtained with the untreated seed. As regards the sulphuric acid treatment, this confirms the experience of Sievers[2] with belladonna seed. He states that treatment with sulphuric acid was of little use. It is probable that the period of refrigeration used in this experiment was too short. Melville and Metcalfe[4] found refrigeration successful in stimulating the germination of belladonna seed, but they used periods of 7 or 14 days. It is hoped to try their method with hyoscyamus seed this year.

The plants reached the flowering stage in 8 weeks after sowing and were then gathered and either dried or preserved in alcohol for examination.

SUMMARY

1. A type of hyoscyamus growing wild in India is described. The species to which this plant should be referred has not yet been determined.

2. This plant may be distinguished from *H. niger*, when unground, by the shape of the leaves, which are ovate to oblong and much less indented. The powder is distinguishable because the calcium oxalate crystals present are larger than those in *H. niger* ; the presence of any calcium oxalate crystals exceeding 35μ long, or of more than 10 per cent. exceeding 25 μ may be taken as indicating something other than true *Hyoscyamus niger*.

3. The total alkaloid contents of the specimens examined were, except in one case, less than the minimum required by the British Pharmacopœia for Hyoscyamus.

4. The acid-soluble ash was greater than that usually found in *H. niger* grown in England, but about equal to that found in *H. niger* from India.

5. A report of a germination test on the seeds is given. It was found that soaking the seeds for 18 to 24 hours in 2 per cent. w/v solution of hydrogen peroxide before sowing gave the best results.

Our thanks are due to Professor Qazilbash, of Islamia College, Peshawar and to Mr. K. C. Chaterjee, of Boots Pure Drug Company (India), Ltd., for the provision of material ; to Mr. A. W. Billitt, of Lenton Experimental Station, Nottingham, for carrying out the germination test and for growing the plants ; to Dr. R. Melville, of the Royal Botanic Gardens, Kew, for information ; and to Mr. H. O. Meek, for helpful criticism. We are indebted to the Directors of Boots Pure Drug Company, for permission to publish this paper.

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2. Sievers, *Amer. J. Pharm.*, 1914, **86**, 483.
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DISCUSSION

MISS B. JOHNSON read a summary of the paper.

DR. T. E. WALLIS (London) said that the authors seemed to indicate that this plant was different from *Hyoscyamus niger* or the other known species of *Hyoscyamus*, but he hoped they would be very careful in future work on the same material in deciding that it really was something different. Experience with plants in general showed to what an enormous extent details of structure varied in the same plant. The most extraordinary differences in the leaves of plants known to be of the same species might be found. He thought the shape of the calcium oxalate crystals was more important than their size. It was known that the size of crystals in henbane and similar plants varied enormously. Unless a careful review was made of their size, one might arrive at an inaccurate result. This plant closely resembled annual henbane, and it might better be compared with henbane generally than with *Hyoscyamus niger*.

DR. J. M. ROWSON (London) said that he had been interested in the

remarks on the action of hydrogen peroxide on the germination of seeds. He had himself had some experience of the erratic nature of the germination of *Hyoscyamus* seeds. He also was sceptical as to whether this was a different species of *Hyoscyamus*. He had grown annual henbane for the last ten years, with the same strain of seed and it was amazing to note the variation which occurred. He could produce leaves identical with those figured in the paper, broadly ovate, or with just one marginal tooth. He did not think there was any difference in that respect, nor was there much difference in the crystal sizes and total ash. The low alkaloidal content was not of great significance. The alkaloidal content of Hyoscyamus varied greatly with the environment in which it was cultivated, and it was possible that here they had poor cultivation. If the authors had details of the alkaloidal content of the Nottingham-grown material, it would be interesting to see how that compared with the Indian samples.

DR. W. MITCHELL (London) asked if there had been any attempt to characterise the alkaloids. It would be interesting to know whether they differed from those of normal Hyoscyamus.

MR. A. R. G. CHAMINGS (Horsham) said that, in the cultivation of belladonna in Leicestershire, he had found that successive refrigeration of the seed was much better than a single refrigeration.

DR. C. MELVILLE (Manchester) said that the use of calcium oxalate crystal determinations for identification purposes was interesting, and this could be extended. The illustration of the trichomes from the leaf gave the impression that the head of the gland was relatively simple, composed of not more than eight or ten cells. In *Hyoscyamus niger* the gland often contained upwards of twenty cells, with frequently two cells at the apex, one of which extended or projected beyond its companion; so that if the glands on this Hyoscyamus were relatively simple, it might prove a characteristic for differentiating it from *Hyoscyamus niger*.

DR. G. E. FOSTER (Dartford) asked whether the authors had investigated the volatile alkadoidal content of the drug.

MISS JOHNSON said that they would give further consideration to the suggestion that the plant was possibly a variation of annual henbane. The amount of material had been so small that it had been impossible to do anything more than the ordinary alkaloidal determination and the volatile alkaloids had not been determined. Regarding germination, they had stored their seeds in the refrigerator overnight at 5°C. and as stated, that had given very poor results. Since the paper had been written, they had tried longer periods of refrigeration. They would now try successive periods. Unfortunately, they had not yet had any reports on the effect of longer refrigeration.

MR. FORSDIKE said they had consulted authorities in this country and in India, and were satisfied that their material represented a species different from *Hyoscyamus niger;* it was hoped before next year to give it a definite botanical name. They had found in commercial material, during the war in particular, a henbane which differed in a number of ways from the typical *Hyoscyamus niger*, and they were sure that more than one species of *Hyoscyamus* was represented.

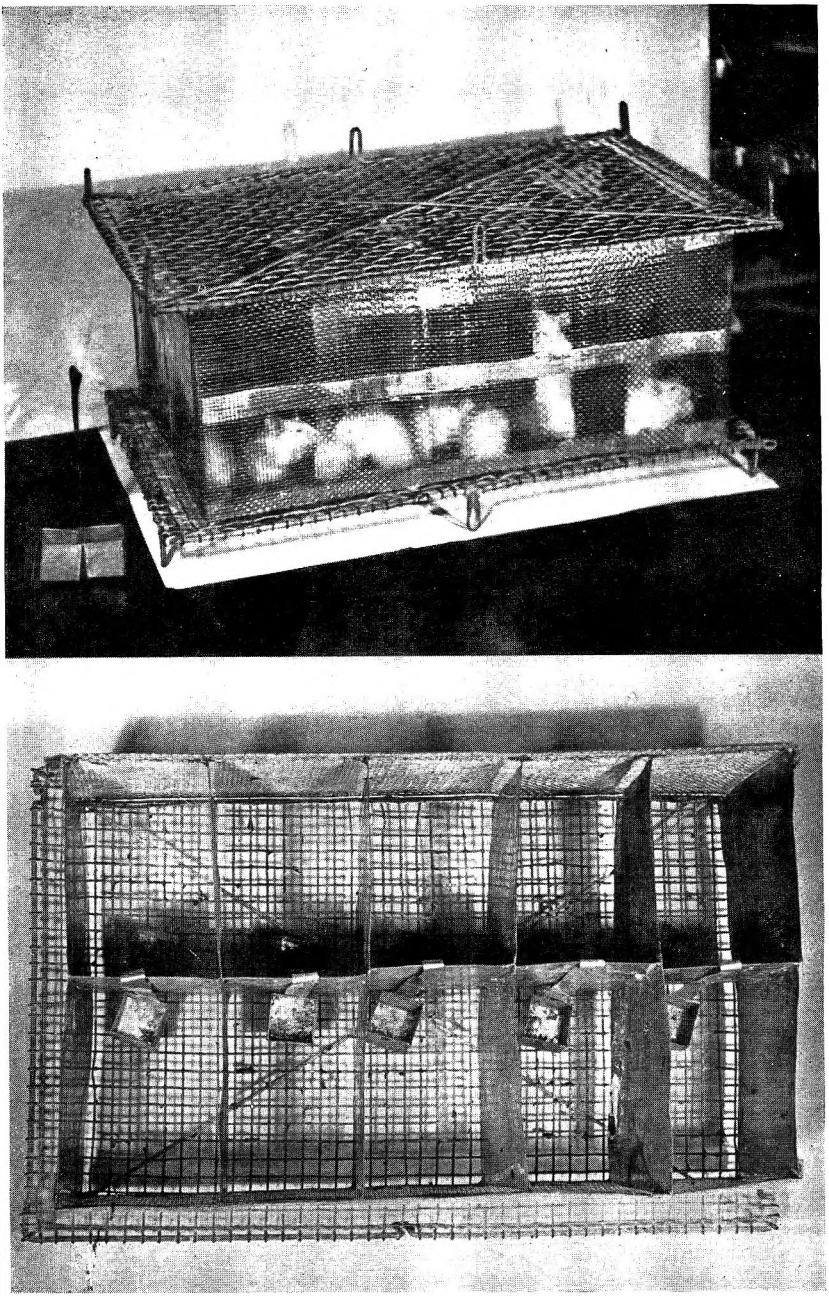

FIG. 1. Photographs of the cage. Upper: Side-view of the cage with a pair of food containers on the left. Lower: Top-view of the cage with the top grid removed.

THE BIOLOGICAL ASSAY OF VEGETABLE PURGATIVES

Part I.—Senna Leaf and Fruit and Their Preparations

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Received July 4, 1949

Introduction

As part of a general investigation on the vegetable purgatives containing anthracene derivatives, I was asked in 1947 to carry out biological assays of several of these drugs and their preparations. Several biological methods of assay have been described and reviewed by Munch[1] and Viehoever[2]. More recently, Loewe[3] used rhesus monkeys and claimed that his method is applicable to many types of laxatives. Straub and Gebhardt[4] used white mice and determined the minimum effective dose (ME) of senna infusions. The potency was expressed as the number of such doses per ml. of the infusion. Geiger[5] adopted a similar method but instead of using animal units the use of a standard (a 5 per cent. infusion of senna leaf) was introduced. The potency of a test preparation was compared with the standard by comparing the percentage of mice which produce positive response. Later, Hazleton *et al.*[6,7,8] introduced the term T.C.D. (Threshold Cathartic Dose—i.e. the dose which produces catharsis in approximately 50 per cent. of the mice), and Grote and Woods[9] introduced the use of powdered senna leaf as their standard of reference. Collier *et al.*[10] further modified the method by using the ratio of the number of unformed fæces (UFF) to the number of total fæces (TF) as the criterion of purgative activity.

The most promising method seemed to be that of Geiger as modified by others, since mice are convenient to handle and require only small amount of test material; this last point is particularly important when only small quantities of pure compounds are available for assay. Accordingly, work was commenced in 1947 on the basis of their methods; but experience soon showed that improvements were necessary for the following reasons: (a) the handling of large number of frog-jars or beakers used as mouse containers during test is inconvenient; (b) droplets of water occasionally appeared on the inside of the jars, indicating a high humidity due to bad ventilation; (c) it is impossible to use water-bulbs with the jars and previous workers have withheld drinking water during test, but this seems undesirable, since water plays a large part in purgation; (d) my experience showed that increase in dose of purgative produced a corresponding increase in the number of wet (or unformed) fæces (i.e. those which differ from the normal dry ones in being round and pasty and leaving a brown stain when placed on blotting paper). It appears therefore that a method based on a quantitative response, rather than the qualitative ("all or none") type used by Geiger and others, would give more accurate results. Collier[10] apparently had a similar idea as he determined the ratio of UFF/TF and used this value as a criterion

of purgative activity. This method, however, is tedious as it involves counting a very large number of fæces in each test.

To overcome these defects, (a) special cages were devised to avoid the use of large number of frog-jars or beakers, (b) a definite proportion of water was added to the feeds during test, and (c) counts of *wet* fæces *only* were used as criteria of activity. The resulting method described in this paper is not only free from the defects already mentioned, but is more convenient in use and gives results of a comparatively high degree of accuracy. Furthermore, this method has been found applicable to other purgatives apart from senna; Hazleton *et al.*[7] report that their method is unsuitable for cascara and aloes.

General Experimental Details

1. *The Standard.* It was decided to use the powdered crude drug as standard, wherever possible, rather than infusions, because infusions may not contain the entire activity. Moreover, owing to the unavoidable variation in preparation, successive infusions may vary in potency. According to Collier[10], an infusion made from 5 to 6 mg. of senna fruit (0·24 g./kg. body-weight) produced no response; I have found that the same dose of senna fruit given directly always produce marked response. While this may be due to my sample of senna fruit being highly active, it may also be due to the fact that the infusion (as made by Collier) does not contain all the activity. The standard, in powdered form, is kept in evacuated glass bottles in a refrigerator. To prepare a standard suspension for administration, a weighed amount of the powder is triturated in a mortar with a small quantity of boiling distilled water and made up to volume in a measuring cylinder with cold distilled water. For a mouse of 20 g. body-weight, a dose of 0·5 ml. is suitable.

2. *Design of the Cages.* The cage as shown in Figure 1 is 15 inches long, 9 inches wide, and 6 inches high. It is divided into 10 compartments with tinned plates. The outer walls were made of "window substitute" (wire gauze impregnated with transparent plastic). Each compartment has a food container made of tinned plate; these containers were connected in pairs by a ∩-shaped handle which hangs over the wall dividing two compartments. The floor and ceiling consist of loose (detachable) grids made of galvanised wire with a mesh of 1 cm. by 1 cm. Six feet are provided at the edges of each of these grids so that the bottom grid is raised about 1 inch from the table on which a sheet of white blotting paper is provided to receive the fæces. The advantages of this cage are (a) good ventilation is obtained, (b) the inconvenience of using a large number of jars is avoided and (c) the bottom grid can be easily removed (either for inspection of the adhering fæces or for washing) by inverting the cage; the top grid will now serve as a floor.

3. *The Test Animal and Diet.* White albino mice weighing not less than 18 g. are used in all tests. Owing to the variation in response of the sexes mentioned by Hazleton *et al.*[8], male mice only are used. They are housed in metal cages and fed with rat-cubes (Diet 41 supplied by the Associated London Flour Millers Ltd.). In addition, each mouse receives an unrestricted supply of fresh tap-water; green vegetables are given over

the week-ends only, thus avoiding the possible interference with the test during the week. The animals, if in good condition, can be used repeatedly after a resting period of not less than 1 week.

4. *The Test.* All food and water are withdrawn from the mice early in the morning and the animals are put singly or in pairs into each compartment of the cage. After 2 or 3 hours, the fæces are examined and any mouse having soft or wet fæces is discarded. The mice are then weighed to the nearest gramme and the weights recorded. They are evenly divided into 4 groups each of 10 mice. As will be shown, later, it is necessary to give two dose-levels each of the standard preparation and the test preparation for every assay. The dose is given into the œsophagus of the animal by means of a blunted needle attached to a 1 ml.-syringe. After dosing, the animals are kept under observation for at least 12 hours.

During this testing period, a special food made by mixing 10 parts of powdered rat-cubes and 7 parts of fresh tap-water is supplied in the food containers. This moist food has several advantages: it allows the animals a uniform intake of water in proportion to the diet; it ensures the normal working of the alimentary canal during the 12 hours of test; it, unlike the dry rat-cubes, when scattered during feeding, does not absorb water from the wet fæces. Moreover, the inconvenience of using a large number of water-bulbs is avoided.

Purgation is indicated by the excretion of wet fæces which are recognised by their somewhat rounded shape and the presence of a brown stain surrounding each on the blotting paper. They can be easily distinguished from the normal dry fæces which are elongated in shape and do not stain the paper. Counting of the wet fæces is usually started from the second hour after dosing and repeated every $1\frac{1}{2}$ hours until the fifth or sixth hour. The final counting is done early in the following morning.

Relation Between Dose and Response

One would expect that an increase in the dose of purgative would result in an increase in the number of wet fæces produced; in other words, the response evoked by purgatives is "quantitative" rather than the "all or none" type such as that evoked by digitalis where the animal

TABLE I

Numbers of wet fæces produced by groups each of 10 mice

Experiment No.	Body-weight of 10 Mice	Dose mg./kg.	Number of WF per Group	Number of WF per kg. of Mouse
	g.			
30	210	280	8	38·1
	245	350	15	61·2
	198	840	25	126·3
	233	1050	34	146·0
33	328	280	19	57·9
	305	350	23	75·4
	329	840	46	139·8
	320	1050	49	153·2

either lives or dies. The following experiments were designed to prove this assumption and to investigate the relation, if any, between dose and response.

4 graded doses of powdered senna leaf suspended in distilled water were given to 4 groups each of 10 mice and the number of wet fæces (WF) produced by each group was recorded as shown in Table I.

Table I clearly shows that an increase in dose produced an increase in the number of wet fæces. To determine the relation between the dose and response, graphs were constructed to illustrate the relation of response to (a) dose (Fig. 2) and (b) logarithm of the dose (Fig. 3). To eliminate

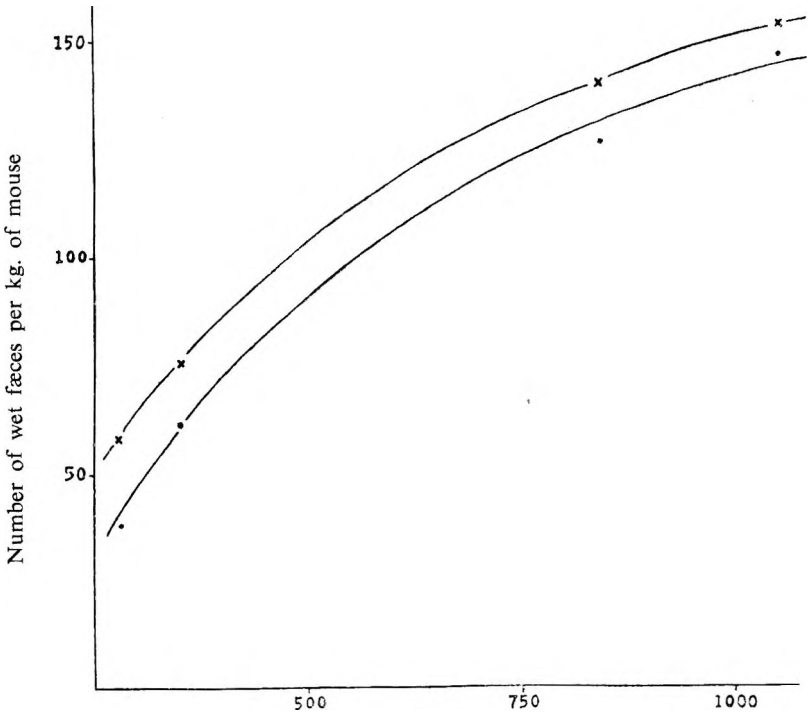

FIG. 2.—Relation of response to dose.

the variation in body-weight of different groups of mice, the response was expressed as number of wet fæces per kg. of mouse. These graphs show that the relation response/log. dose is linear, whereas response/dose is not so. This conclusion was confirmed by later experiments (using the same sample of senna leaf as above) involving 92 × 10 mice; the responses of groups of mice were averaged for each dose-level and the results are shown graphically in Fig. 4. It can be clearly seen that the response is proportional to the log. dose.

In Fig. 3, it will be noted that the log. dose/response lines representing

the same dose-levels given on different days are almost parallel. Though most subsequent experiments resulted in lines of similar slope this was not invariabley true. Hence, it is necessary always to give two dose-levels each of the test and of the standard for every assay, so that the slope of the log. dose/response line for that particular day can be determined. Furthermore, if the distance between the lines of response is unusually great or the slopes of the log dose/response lines of the standard and the test given on the same day differ to a great extent, one may suspect that either the choice of the dose-levels is unsuitable or the nature of the response is different due to different types of active constituents present in the standard and the test, or that the distribution of mice is uneven. It may be necessary, therefore, to repeat the test after due consideration.

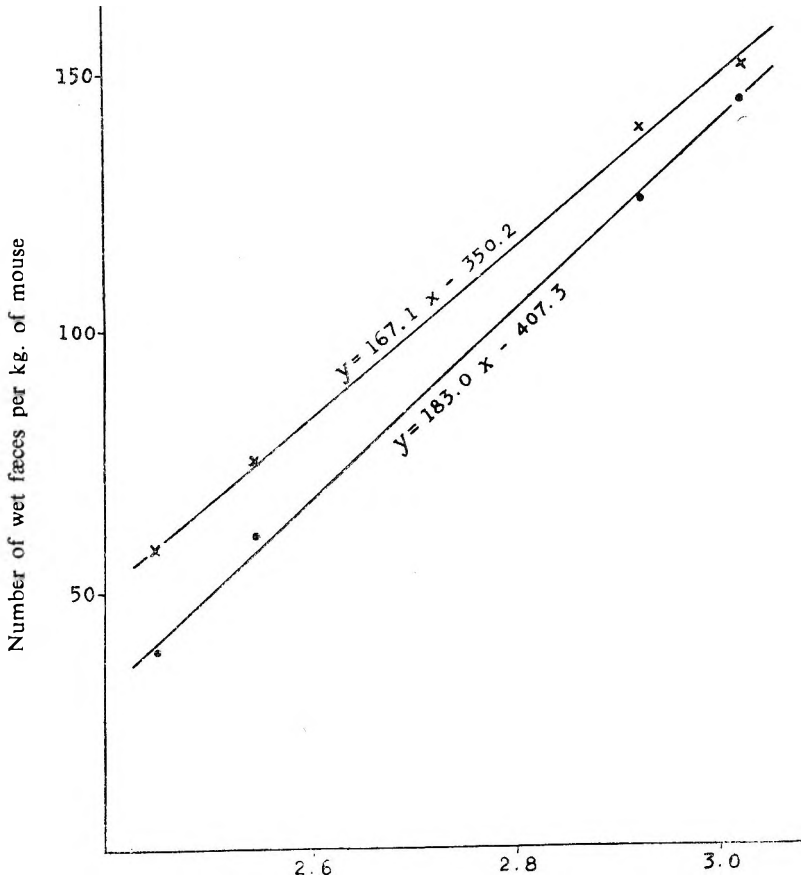

FIG. 3.—Relation of response to the logarithm of the dose.

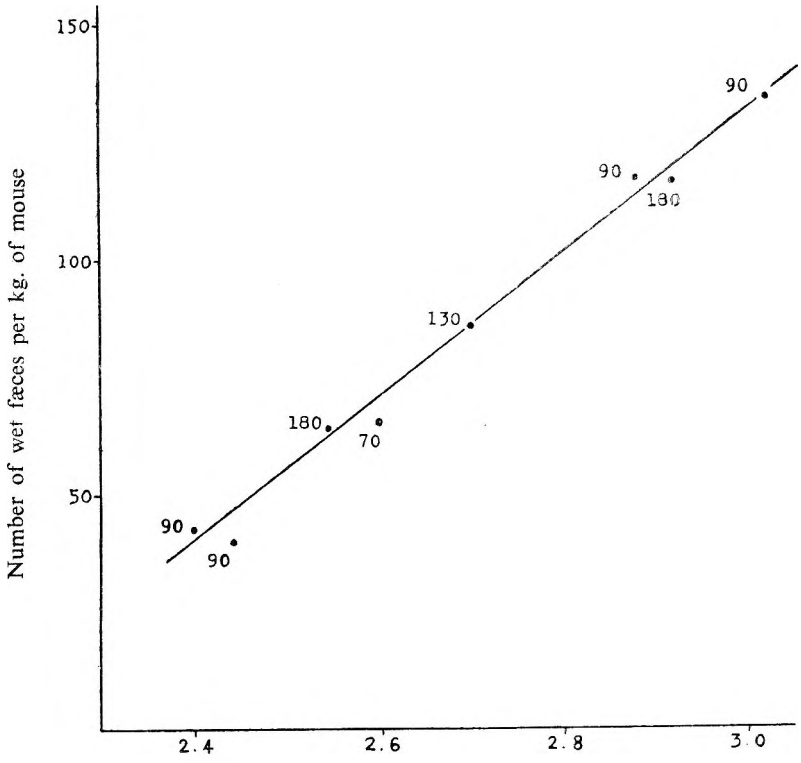

FIG. 4.—The average response/log. dose line. The figures indicate the number of mice used.

EXAMPLE OF THE METHOD: ASSAY OF DRY EXTRACT OF SENNA

A sample of dry extract of senna (E2) made in this laboratory was compared with the laboratory standard sample of senna fruit (Ps) from which the extract was made, by testing them on 4 groups each of 10 mice. In Table II are given the details of the test.

TABLE II

BIOLOGICAL ASSAY OF E2 (EXPERIMENT NO. 68)

	Body-weight of 10 Mice	Dose mg./kg.	Number of WF per Group of Mice	Number of WF per kg. of Mouse
	g.			
Ps	197	350	21	106·7
Ps	186	1050	47	252·7
E2	190	150	28	147·4
E2	185	450	51	275·7

Calculation: (At length to demonstrate the principle involved).

Tripling the dose of Ps caused an increase of 146·0 WF/kg.

Tripling the dose of E2 caused an increase of 128·3 „

Mean effect of tripling the dose ... ... = **137·15** „
Mean effect of the two doses of Ps ... ... = 179·7 „
Mean effect of the two doses of E2 ... ... = 211·55 „
Difference between the mean effects of Ps and E2 = **31·85** „

Since the number of *wet* fæces per kg. of mouse is proportional to the logarithm of the dose

$$\frac{137{\cdot}15}{\log.\ 3} = \frac{31{\cdot}85}{\log.\ r} \quad \text{or} \quad \frac{137{\cdot}15}{31{\cdot}85} = \frac{\log.\ 3}{\log.\ r}$$

where r is the ratio of the potency of the doses of Ps and E2.

Hence $\log.\ r = 0{\cdot}1109$
and $r = 1{\cdot}291$
i.e. $\frac{\text{potency of 150 mg. E2}}{\text{potency of 350 mg. Ps.}} = 1{\cdot}291$

Potency of 1 g. E2 = potency of 3 g. Ps.

i.e. the extract E2 possesses three times the purgative activity of the same weight of the standard senna fruit from which it was made.

Accuracy of the Method

In order to determine the accuracy obtainable by the method, doses of a laboratory standard of powdered senna leaf were given to 4 groups (1, 2, 3, 4) each of 10 mice on 9 different occasions. 2 of the 4 groups (1 and 3) each received 3 times the dose given to the other 2 groups (2 and 4). The responses of each of the 4 groups were noted. The responses of 1 high-level dose and 1 low-level dose (say groups 1 and 2) were taken to represent a potency of 100, the potency of the remaining two (groups 3 and 4) was calculated. This process was repeated by rearranging the groups so that the responses of groups 1 and 4 were taken to represent a potency of 100, and the potency of the groups 2 and 3 was calculated as before; hence, one single "assay" yielded two results. Altogether 18 such results were obtained and are recorded in Table III. The mean (M) of these 18 results was 99·44 with a standard deviation (σ) of 15·626 (or 15·714 per cent. of the mean). Limits of error (P = 0·99) for a single assay are therefore 100 ± 40·5 per cent.

Calculation :

Mean potency of B calculated $= \frac{1789{\cdot}94}{18} = 99{\cdot}44.$

Sum of squares of deviations from the mean $= \Sigma d^2 \quad 4150{\cdot}832.$

Standard deviation of a single determination

$$\sigma = \sqrt{\frac{4150{\cdot}832}{18\text{-}1}} = 15{\cdot}626 \text{ or } \frac{15{\cdot}626}{99{\cdot}44} \times 100 = 15{\cdot}714 \text{ per cent.}$$

Comparison of Accuracy with that of Other Methods

Previous workers used rats[11, 12], guinea pigs[13], daphnia[2], etc., for the evaluation of purgative activity but none of them gave any indication of the accuracy obtainable. Munch[1] who obtained his best results with cats claimed an accuracy of only 20 to 50 per cent. Loewe[3] using rhesus

TABLE III

ESTIMATION OF THE ACCURACY OF THE METHOD

Experiment No.	Group of Mice	Number of WF/kg. of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d^2
30*a*	A 1	126·3			
	A 2	33·5	108·93	9·49	90·0601
	B 3	136·6			
	B 4	38·1			
b	A 1	126·3			
	A 4	38·1	103·39	3·95	15·6025
	B 3	136·6			
	B 2	33·5			
31*a*	A 1	130·4			
	A 2	54·5	105·62	6·18	38·1924
	B 3	135·8			
	B 4	56·8			
b	A 1	130·4			
	A 4	56·8	102·29	2·85	8·1225
	B 3	135·8			
	B 2	54·5			
32*a*	A 1	173·0			
	A 2	82·0	89·15	10·29	105·8841
	B 3	187·3			
	B 4	43·1			
b	A 3	187·3			
	A 2	82·0	78·00	21·44	459·6736
	B 1	173·0			
	B 4	43·1			
33*a*	A 1	146·6			
	A 2	65·4	90·88	8·56	73·2736
	B 3	139·8			
	B 4	57·9			
b	A 1	146·6			
	A 4	57·9	100·54	1·10	1·2100
	B 3	139·8			
	B 2	65·4			
34*a*	A 1	99·2			
	A 2	41·7	72·91	26·53	703·8409
	B 3	82·7			
	B 4	25·0			
b	A 1	99·2			
	A 4	25·0	100·19	0·75	0·5625
	B 3	82·7			
	B 2	41·7			
35*a*	A 1	76·2			
	A 2	31·3	105·30	5·86	34·3396
	B 3	86·2			
	B 4	26·2			
b	A 1	76·2			
	A 4	26·2	117·52	18·08	326·8864
	B 3	86·2			
	B 2	31·3			
37*a*	A 1	119·5			
	A 2	42·9	100·50	1·06	1·1236
	B 3	109·4			
	B 4	53·6			
b	A 3	109·4			
	A 2	42·9	118·82	19·38	375·5844
	B 1	119·5			
	B 4	53·6			
38*a*	A 1	46·0			
	A 2	20·0	114·32	14·88	221·4144
	B 3	72·5			
	B 4	4·9			

Experiment No.	Group of Mice	Number of WF/kg. of Mouse	Potency of B calculated (A = 100)	Deviation from the Mean d	d^2
b	A {3	72·5	61·35	38·09	1450·8481
	A {2	20·0			
	B {1	46·0			
	B {4	4·9			
39*a*	A {1	123·3	112·97	13·53	183·0609
	A {2	22·1			
	B {3	142·3			
	B {4	27·2			
b	A {1	123·3	107·26	7·82	61·1524
	A {2	27·2			
	B {3	142·3			
	B {4	22·1			
TOTALS ...			1789·94		4150·8320

monkey as test animal claimed an accuracy of ± 15 per cent., however there is neither data nor statement as to how many monkeys are needed to achieve this accuracy.

Geiger's original method[5] involved the use of 72 mice per single assay; however, insufficient data is available to calculate the accuracy obtainable. Moreover, his method was improved by Grote and Woods who used 105 mice per single assay. Again, however, no figure for the degree of accuracy was given. This omission from the published methods of bioassay is a serious one, especially in view of the large number of mice used in each assay.

Collier[10] states that the standard deviation of his method is usually about 20 per cent. with a slope (b) of log. dose/response line of about 80 per cent. ($b^2/s^2 = 16$). The limits of error (P = 0·99) of each assay using 40 mice calculated from the formula quoted by him would be 63 and 160 per cent. However, when a ratio of $b^2/s^2 = 30$ is obtained, as he states occasionally occurred, the limits of error using 40 mice would be 71 and 141 per cent. As already stated the limits of error of the method described in this paper (P = 0·99) are 100 ± 40·5 per cent., which on the whole is a higher accuracy than that of Collier's method.

Applications of the Method

The method was found very satisfactory when used to assay senna leaf, senna fruit and extracts and commercial preparations made from these drugs, also the pure glycosides, sennosides A and B, and the pure anthracene compounds, aloe-emodin and aloe-emodin anthranol. The method was further applied to cascara, rhubarb and to preparations of these drugs also with satisfactory results, although in some instances slight modification is necessary. It is hoped to publish details of these investigations later.

The results of many of these assays are incorporated in the paper of Dr. J. W. Fairbairn[14].

Summary

1. A method for the biological assay of vegetable purgatives based on the number of wet fæces produced by groups of mice after dosing is described.

2. The relation of the number of wet fæces per kg. of mouse to the logarithm of the dose was found to be linear.

3. 40 mice divided equally into 4 groups were used in each assay. 2 groups received the standard preparation and the other 2 groups received the test preparation. The standard deviation of a single determination based on 9 such assays was estimated to be 15·7 per cent. The limits of error (P = 0·99) for a single assay are 100±40·5 per cent.

4. A special cage has been designed for this assay, and it has been found advantageous to incorporate a definite proportion of water in the diet, during test.

5. The method described is not only convenient in use but also gives a comparatively high degree of accuracy.

6. The method has been successfully applied to senna leaf, senna fruit and extracts and commercial preparations made from these drugs, pure glycosides (sennosides A and B), and pure anthracene compounds (aloe-emodin and aloe-emodin anthranol).

Acknowledgements

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THE ACTIVE CONSTITUENTS OF THE VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

Part I. Glycosides and Aglycones

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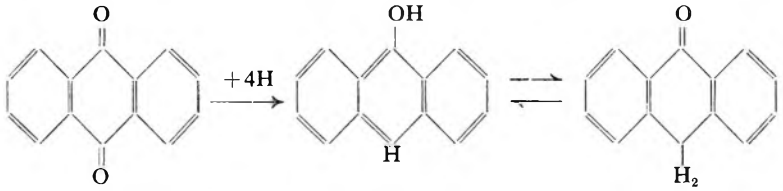
The following are the common vegetable drugs containing anthracene derivatives ; Senna, leaf and pod (*Cassia acutifolia* and *C. angustifolia*) ; Cassia pulp (*Cassia fistula*) ; Cascara sagrada (*Rhamnus purshiana*) ; Frangula (*R. frangula*) ; Rhubarb (*Rheum* spp.) and Aloes (*Aloe* spp.). These drugs act as irritant purgatives and all respond to the Bornträger test[1] or suitable modifications[2,3] by means of which the anthracene derivatives are converted into free anthraquinone compounds which give pink to red colours in alkaline solution.

Anthracene Derivatives

The anthracene derivatives occur either free or in the form of glycosides, usually with glucose, though glucofrangulin also contains the sugar rhamnose[4]. The following aglycones have been reported :—

(1) *Anthraquinone compounds*, e.g. emodin, aloe-emodin, rhein. These compounds occur in all the drugs mentioned above, sometimes in very small amounts as in aloes[4] and in senna leaf[5] and sometimes in quite large amounts as in cascara[6] and rhubarb.[7]

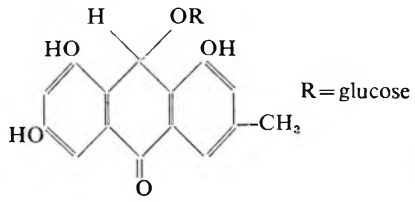
(2) *Anthranol compounds.* Anthranol (and its tautomeric isomer anthrone) is a reduced form of anthraquinone as shown



anthraquinone. anthranol. anthrone.

Hydroxy derivatives of anthranol and anthrone, corresponding to those of anthraquinone, occur frequently in these purgative drugs, e.g. aloe emodin anthranol in aloes.[8]

(3) *Oxanthrone compounds.* These are intermediate between anthraquinones and anthranols. Schindler[9] has shown that cascara bark contains a glycoside based on this structure :—



Glycoside from cascara.

On hydrolysis the aglycone is rapidly oxidised to emodin.

(4) *Dianthranol compounds.* Stoll, *et al.*[10] suggest that the aglycones in senna leaf may be dianthranol or dianthrone compounds which on oxidation in alkaline solution give a rhein-like compound.

For the purposes of this preliminary investigation these anthracene derivatives are classified as (*a*) glycosides, (*b*) free anthranol compounds, and (*c*) free anthraquinone compounds.

Active Constituents

Since all these anthracene purgative drugs can be made to give the Bornträger reaction under suitable conditions, it was natural that attempts should be made to determine the total content of anthracene derivatives as anthraquinones (calculated from the intensity of the red colour) and see if this amount corresponded to the biological activity of the pure anthraquinones prepared synthetically or otherwise. However, it soon became apparent that the " total anthraquinone content " could not account for the activity of these drugs. Thus Tutin and Clewer[11] found that 100 mg. of aloe emodin, or of emodin or of rhein were practically ineffective on human beings. This quantity of anthraquinones correspond to 4 g. of cascara bark, which is the maximum dose (B.P. 1932). Similarly, a recent attempt to correlate the colorimetric assay of frangula extract with its biological assay led the author to conclude that no parallelism exists between the two types of assay[12]. Similar conclusions were arrived at by Astruc and Giroux for cascara[31], and Ström and Kihlström for rhubarb[7].

An interesting series of experiments by Green, King, Beal *et al.* on cascara extract [14,15] seemed to offer an explanation of the superior activity of the crude drug and its preparation over the pure anthraquinones. They showed a definite synergistic action when the anthraquinones, aloe emodin, emodin and chrysophanol were given together to guinea-pigs. The response was much greater than with similar doses of these compounds separately, and as these compounds were stated to exist together in the drug extract it seemed reasonable to suppose that synergism of the anthraquinones accounted for the purgative action of cascara.

On the other hand Casparis and Maeder[4], working on the similar drug frangula bark, concluded that the total activity of the bark was due to the glycoside gluco-frangulin. In fact they found (by experiments on man) that this glycoside was much more active than the corresponding amount of bark ; the loss of activity, when in the crude drug, they attributed to the tannins present in the bark.

Straub and Gebhardt[16], working on senna leaf, discovered two active glycosides whose activity appeared to account for a large proportion of the activity of the leaf. Their work was continued by Stoll, *et al.*[10] who solated the glycosides in crystalline form and called them sennoside A and sennoside B.

Thus these recent series of experiments suggest that the activity of the anthracene purgatives may be accounted for by either (*a*) synergism of the free anthraquinone compounds as in cascara, or (*b*) highly active glycosides as in senna leaf and frangula bark. It is interesting to note that in both senna leaf[5] and frangula bark[17], more than nine-tenths of the anthracene

derivatives present occur as glycosides. Hence a determination of "total anthraquinones" would be virtually a determination of the glycosidal content, which (as stated above) is said to account for the total activity of these drugs. However, I have already quoted papers to show that such a correlation has not been found for every member of the group.

I decided therefore to investigate the whole series of anthracene drugs to see if any generalisation could be made as to what are the active constituents. The remainder of this paper describes the preliminary work towards this end, viz., the determination of the relative activity of (*a*) glycosides, (*b*) free anthranols and (*c*) free anthraquinones. The results of the experiments recorded show that for senna leaf, senna pod, sennosides A and B, rhubarb and cascara, the anthracene derivatives are highly active in the glycosidal form ; less active as free anthranols and much less active as free anthraquinones. A discussion of these findings is given at the end of the paper.

Experimental

Chemical and Biological Assays. Necessary requirements for this type of investigation, are reliable methods of chemical and biological assay. The *chemical assays* were based on the colorimetric methods of Kussmaul and Becker for senna[5], and Björling and Ehrlén for frangula[12]. Various modifications were necessary and it is hoped to publish details of chemical assays for each drug later. The glycosidal content of senna and preparations were estimated as sennosides A + B; that of cascara and of rhubarb as the oxidised aglycone, emodin. In order to make the figures comparable the glycoside content of senna is also given in terms of aglycone A + B. The *biological assays* were carried out by Mr. T. C. Lou[18].

1. *Preliminary experiments on Senna leaf.* Portions of powdered Tinnevelly leaf were subjected to increasing degrees of hydrolysis and the purgative activity of these fractions and that of the original leaf were compared by a bio-assay method based on that of Geiger[19]. The results, recorded in Table I, indicate that mild hydrolysis has little effect on the purgative activity but that more vigorous hydrolysis, and

TABLE I

Purgative activity of senna leaf fractions after varying degrees of hydrolysis

Treatment	Temperature and Time	Bio-assay
1. Untreated leaf	—	100
2. Warmed with 0·16N hydrochloric acid in atmosphere of nitrogen	20° C. for 18 hours 70° C. for ½ hour	88
3. Warmed with 0·1N hydrochloric acid in atmosphere of carbon dioxide	90° C. for ⅛ hour 20° C. for 22 hours	77
4. Boiled in water under reflux	100° C. for 2 hours	36
5. Boiled in 1·5N hydrochloric acid under reflux...	approx. 120° C. for 2 hours	0

possibly oxidation (produced either by boiling in water or warming in strong acid solution in air) led to a marked loss in activity.

2. *Quantitative experiments using senna leaf glycosides.* The preliminary experiments suggest that hydrolysis of the leaf constituents, and possibly oxidation, leads to loss of activity. I obtained the leaf glycosides sennosides A and B in pure form and decided to repeat the previous experiments on a more quantitative basis.

A solution of sennoside A of suitable concentration was divided into three portions. One portion was hydrolysed and the liberated aglycones extracted and purified (as in the chemical assay process[5]); these were administered to mice in suitable doses. The second portion of the sennoside A solution was hydrolysed and oxidised (as in the chemical assay process[5]) and similar doses of the purified products were given to mice. The third portion of the original solution was used as a control.

The results, recorded in Table II, show that the aglycone possesses about 1/3 of the activity of the parent glycoside, whereas the oxidised aglycone possesses no activity at all (when given in similar doses).

TABLE II

BIOLOGICAL AND CHEMICAL ASSAYS OF SENNOSIDE A AND FRACTIONS

Material	Bio-assay	Chemical assay
1. Sennoside A	100	100
2. Hydrolysed Sennoside A (3 N sulphuric acid at 95° C. for ½ hour)	32·5	96·4
3. Hydrolysed and oxidised sennoside A (above, oxidised in N sodium hydroxide and hydrogen peroxide)	0	85·3

Note : In theory, the results for the chemical assays should all be 100. The discrepancy in Expt. 2 is within experimental error ; that in Expt. 3 can be accounted for by having to heat with hydrogen peroxide longer than usual, owing to the high concentration of the aglycones.

3. *Quantitative experiments using senna pod.* Similar experiments to those done on senna leaf glycosides were repeated on senna pod which is also said to contain sennosides A and B.[20] A large sample of Alexandrian senna pod was thoroughly mixed and stored in a cool place; a few hundred g. reduced to No. 60 powder was used as standard (= P_8). A potent extract of some of this powder was made by evaporating an infusion* under reduced pressure to a solid extract (E_1). This extract was assayed biologically and chemically against the standard powder. Some of it was then hydrolysed by heating in 5 per cent. hydrochloric acid at 90°C. for ½ hour, cooled and neutralised with sodium hydroxide. This hydrolysed product was re-assayed chemically and biologically against the Standard powder to which a calculated quantity of sodium chloride was added to balance that formed during the neutralising of the hydrolysate. The results of these experiments are shown in Table III.

These experiments on senna pod confirm the conclusions derived from the experiments on senna leaf and glycosides, in that hydrolysis leads to a marked loss in activity. They also show that a determination of the anthracene derivatives as " total anthraquinones " would be no guide

* Chemical assays showed that all the anthracene derivatives passed into an infusion made under suitable conditions.

to the biological activity. Thus in experiments 2 and 3:—biological activity is in ratio 6·8 : 1; glycosidal content is in ratio 8·3 : 1 ; " Total anthraquinones " is in ratio 1·1 : 1.

TABLE III

BIOLOGICAL AND CHEMICAL ASSAYS OF SENNA POD AND FRACTIONS

Material	Bio-assay	Chemical Assay (Results expressed as mg./g.)			
		Glycosides		Free	Total
		Sennosides A+B	As Aglycones	As Aglycones A+B	As Aglycones A+B
1. Standard senna pod powder P_s	100	32·0	20·0	2·0	22·0
2. Potent extract of Pod E_1 ...	(i)301 / (ii)376 / 339	132·4	82·8	8·2	91·1
3. Hydrolysed extract	Approx. 50	16·0	10·0	71·5	81·5

4. *Quantitative experiments using Rhubarb.* The experiments already described were made on senna and its preparations, where the aglycones were " artificially " produced by hydrolysis. In rhubarb and cascara, however, there is present " naturally " a large proportion of free aglycones, so that experiments carried out on these drugs will not only decide whether what is true of senna is applicable to other anthracene purgatives; but the proof of relative activity of the glycosides and aglycones can be obtained without resort to the drastic process of acid hydrolysis. Crude drugs and their preparations are complex mixtures and it is not always possible to forecast what effect hydrolysis of other constituents will have on the biological activity.

A weighed quantity of a Standard sample of powdered Chinese rhubarb (R_s) was exhausted with ether and acetone to remove the free emodins and the exhausted material was dried and re-weighed. There was a loss in weight of 15 per cent. The activity of this exhausted material (R_s) and of the Standard powder were compared by the biological and chemical methods already mentioned and the results are shown in Table IV. In calculating the potencies of R_{ex} allowance was made for the 15 per cent. loss in weight on exhaustion.

TABLE IV

BIOLOGICAL AND CHEMICAL ASSAYS OF RHUBARB AND FRACTIONS

Material	Bio-assay	Chemical Assay Total Anthracenes as Emodin : mg./g.
1. Standard rhubarb powder R_s	100	30·5
2. R_s exhausted with ether, etc. = R_{ex}	(i) 106 / (ii) 84 / (iii) 107 } = 99*	7·2*

* Allowance made for loss in weight of 15 per cent. on exhaustion.

These results fall into line with those already obtained. The removal of a large amount of free aglycones did not result in any loss of activity, thus indicating that the main activity lies in the (ether-insoluble) glycosides.

The free aglycones consisted almost entirely of anthraquinone compounds, which would account for the entire absence of activity in this fraction.

5. *Quantitative Experiments using Cascara Sagrada bark.* Cascara bark, like rhubarb, contains a large proportion of free aglycones as well as glycosides. Biological experiments indicated there was insufficient activity per g. of crude drug for bio-assay work. Accordingly, a potent extract (EC) was made in a similar manner to that used with senna pod. It was found that this dried extract could not conveniently be exhausted with ether, acetone or methylal; accordingly, a suitable solution in water was prepared and half of this was shaken with ether till the bulk of the free compounds was removed. The exhausted solution was warmed to remove ether and adjusted to volume. These two solutions were assayed biologically and chemically and the results are shown in Table V.

TABLE V

BIOLOGICAL AND CHEMICAL ASSAYS ON CASCARA AND FRACTIONS

Material	Bio-assay	Chemical assay mg./g.		
		Glycosides (as emodin)	Free Aglycones (as emodin)	Total Anthraquinones (as emodin)
1. Cascara extract (EC) ...	(i) 100	20·2	19·0	39·2
2. Exhausted cascara extract (EC*ex*)	(i) 110·5 / (ii) 89·3 } 100	20·2	4·0	24·2

The results are exactly similar to those for rhubarb; chemical tests also showed that the free compounds were in the anthraquinone form, which once more accounts for the lack of activity in this fraction.

6. *Experiments with pure anthracene derivatives.* In order to confirm previous findings and also to determine the relative activities of anthranols and anthraquinones, pure anthracene derivatives were prepared, as below, and tested by the bio-assay method.

1. *Aloe Emodin.* Prepared from aloin by the method of Cahn and Simonsen[21]. Obtained orange needles. M.pt. 223°C. (uncorrected); 231°C. (corr.) (Cahn and Simonsen[24] give 218°C.; Liddell, *et al.*[14] give 222 to 223°C.).

2. *Aloe emodin Anthranol.* Prepared from aloin by the method of Hauser[8]. Obtained yellow needles. M.pt. 200°C. (uncorr.) (Hauser gives 194° to 195°C.).

3. *Emodin* (*frangula emodin*). Prepared from chrysarobin by the method of Gardner[22]. Obtained silky orange needles. M.pt. 256·5°C. (corr.) (Gardner gives 254° to 256°C.).

4. *Chrysophanol.* Prepared from chrysarobin by the method of Gardner[23]. Obtained golden yellow crystals, M.pt. 197° to 198°C. (corr.). (Gardner gives 193° to 194°C. (corr.). Naylor and Gardner[24] give 195·6° to 196·2°C.).

5. *Rhein.* Prepared from rhubarb and purified by sublimation *in vacuo*. Obtained orange yellow needles. M.pt. 319° to 321°C. (uncorr.). (Oesterle and Tisza[25] give 321° to 321·5°C.)

(*a*) *Qualitative experiment*

5·0 mg. per mouse of each of these compounds was given to groups of 5 mice with the following results :—

1. Aloe emodin ... ... 9 wet fæces produced
2. Aloe emodin anthranol ... ... 23 wet fæces ,,
3. Emodin ... ... 0 ,, ,, i.e. no response
4. Chrysophanol ... ... 0 ,, ,, ,, ,,
5. Rhein ... ... 0 ,, ,, ,, ,,

These results are purely qualitative and indicate that aloe emodin and its anthranol are more active than the other compounds. It was decided to compare the activity of the two former compounds on a quantitative basis.

(*b*) *Comparison of Aloe emodin and aloe emodin anthranol with senna pod* (P_s)

The two compounds were assayed biologically against the Standard Senna Pod (P_s) with the following results :—

Aloe emodin

(i) 1 g. has same activity as 0·561 g. of P_s.
(ii) 1 g. ,, ,, ,, 0·494 ,, ,,
Mean = 0·527 ,, ,, (=10·5 mg. of aglycone A + B)

Aloe emodin anthranol

(i) 1 g. has same activity as 4·49 g. of P_s.
(ii) 1 g. ,, ,, ,, 4·68 ,, ,,
Mean = 4·58 ,, ,, (=91·6 mg. of aglycone A + B)

NOTE.—1 g. of senna pod (P_s) contains 20 mg. of aglycone A+B.

These results show that—

(*a*) Aloe emodin anthranol possesses about 9 times the activity of the corresponding anthraquinone, aloe emodin.

(*b*) The glycosides in senna pod (calculated as aglycones) possess about 11 times the activity of aloe emodin anthranol and about 100 times the activity of the anthraquinone.

(*c*) The (reduced) aglycone of sennoside A is about 1/3 as active as the glycoside (see Table II). Hence this aglycone is much more active than the simple anthranol of aloe emodin.

DISCUSSION

The experiments recorded in this paper show that the main purgative activity of the anthracene derivatives is shown when they are in the glycoside form; and that of the free aglycones the reduced (anthranol) form, though less active than the corresponding glycoside, is much more active than the oxidised (anthraquinone) form. A question raised by these results is what part the aglycones play in purgation. According to the experiments of Okada[26] and Straub and Triendl[27], on senna leaf, the free emodins (anthraquinones) are the active compounds provided they reach the large intestine. Apparently the bulk of the free emodins disappear during metabolism[28], and Straub and Triendl[27] suggest that the sugar moiety of the glycoside acts as a " transporter " for the active

aglycone. The glycoside is not hydrolysed in the stomach and so reaches the large intestine where it is hydrolysed and the liberated (and presumably oxidised) aglycone then exerts its action. The work described in this paper has not only confirmed this theory for senna leaf and shown that the same is true of senna pod, rhubarb and cascara, but also indicates that the sugar moiety plays the further role of "protector," preventing the orally active anthranol from oxidation during storage to orally inactive anthraquinone. In all the drugs studied it was found that nearly all the free aglycones are present in the anthraquinone form (though the glycosides contained "anthranol" aglycones), indicating that after hydrolysis the liberated anthranols are fairly rapidly oxidised during storage. Furthermore, if the pure senna glycosides are heated in N sodium hydroxide with hydrogen peroxide, no oxidation takes place after hydrolysis of the glycosides (in acid), however, the aglycones are rapidly oxidised under similar conditions. This "protector" theory is in line with what is known of the constitution of these glycosides (e.g. in cascara[9], aloin[29]). The sugar is attached to the meso group and so would protect the anthranol structure.

These theories appear to conflict with that of Liddell, King and Beal[14], who claim synergism of the anthraquinones as the explanation of the activity of cascara. While their experiments do show that synergism occurs, they fail to show that this synergistic effect accounts for the total activity of the crude drug. They used 1·5 ml. of a commercial sample of fluid extract of cascara (U.S.P.) as standard in the bio-assay, but unfortunately did not determine the amount of anthraquinones in this standard. Gibson and Schwarting[6] have shown that this amount varies considerably; their highest figure is 2·9 mg. per ml. Thus, at the most 1·5 ml. of standard may contain about 4·5 mg. of anthraquinones, whereas Liddell *et al* found it required 12·5 to 25 mg. of the synergistic mixture to produce a similar effect. However, if sufficiently large doses of free anthraquinones are given by the mouth, purgation results; indicating that a proportion has reached the large intestine. In such circumstances, synergism may be an important factor. To be effective, comparatively large quantities must be given, e.g. 100 to 300 mg. for man[11,27]. This quantity corresponds with the dose of the synthetic anthraquinone, 1 : 8 dihydroxyanthraquinone* (Istin) of which 150 to 450 mg. is necessary as a purgative.

The results also show that a chemical assay which merely determines the total content of anthracene derivatives, irrespective of their form of occurrence, will not correspond to the purgative activity. An interesting example of this occurs in a paper recently published from Finland on the chemical and biological assays of Chinese and rhapontic rhubarbs[7]. The former contains less "total anthraquinones" yet is twice as active as the rhapontic and the authors conclude that the chemical assay does not give a true picture of the laxative effect. However, their figures for "combined anthraquinones" (glycosides) are in the ratio of 9·25 to 5·08 respectively, which corresponds very closely to the biological assays.

Further Work

Having established that the glycosidal fraction of these crude drugs possesses the main activity, I propose to study this fraction in greater detail, as it is obvious that the aglycones of the various glycosides vary considerably in their activity, e.g. the aglycones of sennosides A and B are much more active than the simple anthranol of aloe emodin. It is further hoped to publish details of chemical assays and to examine galenicals made from these drugs to see whether all the glycosides are extracted and whether they are preserved during storage. Hazleton and Talbert [30] state that the U.S.P. fluid extract contains only 35 per cent. of the activity of senna leaf. Preliminary experiments which we have performed on a commercial sample of dry extract of cascara showed it was slightly less active than the crude drug; whereas it should be about four times as active. Similar experiments on a dried extract of senna pod made by the B.P. method showed that it had only 1/6 of the activity of the pod, instead of 3 to 4 times its activity.

Summary

1. A review of the published theories which seek to account for the activity of purgative drugs containing anthracene derivatives is given.

2. The relative activity of anthracene derivatives occurring as (*a*) glycosides, (*b*) anthranols and (*c*) anthraquinones, in certain crude drugs has been determined by biological means.

3. A similar investigation of isolated glycosides, anthranols and anthraquinones has been carried out.

4. The results show that the anthracene derivatives are highly active as anthranol glycosides; less active as free anthranols and much less active as free anthraquinones.

5. The more complex aglycone (dianthranol) of sennoside A is more active than the simple anthranol of aloe emodin.

6. The theory is advanced that the sugar moiety of the glycoside not only acts as a " transporter " of the active aglycone, enabling it to reach the large intestine, but is also a " protector " which prevents oxidation of the aglycone to the relatively (orally) inactive anthraquinone.

7. The experiments also show that a determination of " total anthraquinones " irrespective of their form of occurrence will be no guide to biological activity.

8. Further work on the glycoside fraction of these crude drugs is being carried out.

I would like especially to thank Mr. T. C. Lou for performing all the bio-assays included in this paper, for preparing the pure anthracene compounds and for much valuable assistance and many useful suggestions during the course of the work. I am also grateful to the Pharmacology Department of this School for facilities for carrying out the bio-assays; and to the Department of Physical Chemistry for the loan of certain spectrophotometric equipment necessary for the colorimetric assays. Acknowledgments are made to Sandoz Products Ltd. for the gift of sennosides A and B, and to Mr. A. H. Fenton for the preliminary biological work.

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DISCUSSION

The Papers on Vegetable Purgatives by Mr. Lou and Dr. Fairbairn were discussed together

THE CHAIRMAN said that until recently there had not been a really satisfactory assay of these drugs. Mr. Lou's method was a development of that described last year by Collier and Harris.

DR. I. MICHAELS (London) said that senna pods were said to be more certain than the leaves in their laxative action, and to cause less griping. It appeared that sennosides A and B were present in both, and it would be logical to conclude that the pods contained a higher proportion of active principles than the leaves. It would be interesting to know the comparative figures for the active principles of Alexandrian and Tinnevelly pods so as to be able to assess the reason for the market price of the Alexandrian pods being three times that of the Tinnevelly pods. Griping was said to be associated with the anthracene compounds. The seeds were said to be the cause of griping, and yet it was recorded that the seeds did not contain anthracene derivatives. Modern theory stated that the senna glycosides passed unchanged through the stomach to the intestines, where they were absorbed into the blood stream: here they were hydrolysed. There was a latent period of 10 to 12 hours before the active emodin reached the large intestine, where it stimulated the

peristaltic movements of purgation. The emodins in a badly prepared extract were responsible for the griping pains by stimulating peristalsis in the small intestine. Mr. Lou's method complied with the accepted principles of biological assay. There could be little disagreement with the choice of powdered senna as a standard for the assay of senna and its preparations, although there was room for controversy as to the method of administration. It should be regarded, however, as an intermediate stage in the establishment of a standard based on a compound of known composition.

DR. E. F. HERSANT (Dagenham) said if the glycoside were not hydrolysed in the stomach, was it not possible that after hydrolysis in the large intestine the unoxidised aglycone would partly account for the greater activity of the glycosides.

DR. F. SAID (Egypt) said that glycosides were usually hydrolysed by acids, and if they were not hydrolysed in the stomach they were unlikely to be hydrolysed by the alkali in the duodenum. If the glycosides were absorbed in the stomach and were excreted in the intestine, would it be possible to inject a solution of an anthraquinone and see if it was so excreted.

MR. V. REED (London) asked if the author had any explanation of why the public taste for senna preparations had altered so much in the last 30 or 40 years. At first there had been a big demand for senna leaves, then senna pods had come in. Now there were senna leaves, Tinnevelly senna pods and Alexandrian senna pods, all taken for the same purpose. Was there any real difference in the actual active principle of the three things?

DR. T. E. WALLIS (London) said he was glad to see a Department of Pharmacognosy taking an interest in the biological side, as was the case in similar departments in other countries. He asked for Mr. Lou's opinion on the use of *Daphnia* in this type of work.

DR. D. C. GARRATT (Nottingham) said that it was necessary to get an accurate chemical assay before they could fully appreciate the really good work that had obviously been done.

DR. WALKER (London) said that in the last twelve months he had had to make comparisons clinically of liquid extracts of senna and frangula. The experience had been that many of the senna extracts examined had shown very poor activity compared with senna pods themselves, whereas the difference in the activity in frangula extracts had not been so marked. It would appear that the conclusions reached had been on single samples of powdered senna and cascara. Had the author gone far enough yet to offer any information as to variations between different samples of the powdered drugs.

DR. F. HARTLEY (London) asked whether, in arriving at the conditions for the hydrolysis of glycosides in Table 1 the times and conditions had been arbitrarily chosen, or were they known to be the most favourable for hydrolysis? If Dr. Fairbairn could give supplementary data on the rate of hydrolysis of the glycosides, it would throw light on the

conditions. It was said that by boiling at 110° under a reflux with water for two hours, 60 per cent. of the senna glycosides had been hydrolysed. Could Dr. Fairbairn say whether substantially all that decomposition had occurred during a shorter period such as 15 or 20 minutes.

MR. H. DEANE (Long Melford) said that the method appeared to be accurate for testing purgative drugs. Human volunteers had so far seemed the only way of testing, but the staff of laboratories considered that it was not part of their duties. Senna was more sensitive to heat than rhubarb or cascara; a freshly-made infusion of senna pods was much more active than a liquid extract made from the same pods.

MR. H. B. MACKIE (Brighton) said that it had long been known that with cascara partial hydrolysis decreased therapeutic activity, but increased palatability. With a solid preparation which could be protected by coating palatability was unimportant. In liquid preparations, the therapeutic action had to be balanced with flavour and other factors, if the product was bitter and nauseating it could not be swallowed. In the preparation of decoction of aloes, the aloes was boiled with potassium carbonate and the dose of decoction was equivalent to twice the dose of aloes to get the same therapeutic result, but it was then pleasant to take; 50 per cent. of the activity of the aloes had been lost in boiling.

DR. FAIRBAIRN, replying, said that the glycosides were the active principles, but the particular glycosides present in different drugs might vary, thus giving different activities. Regarding anthranols, presumably as soon as the glycosides were hydrolysed the free anthranol enhanced the activity. It was true that the stomach contained acid, but Table I showed that it needed fairly strong acids at high temperature to produce hydrolysis. Dr. Collier had reported last year that he had injected liquid extract of senna into the veins of mice, but there had been no purgative action.

MR. LOU said it was, generally speaking, much better if a definite compound was used as a standard, but unfortunately these purgative drugs contained various glycosides which might be different in structure. If sennoside A or B was adopted as a standard, and the product assayed on either compound, the result might be in conflict with a chemical test. It was obvious that workers in this field ought to be careful about the element of biological variation. Statistical considerations had been applied to all the tests they had made and the errors were within 15 per cent. They had no experience with the *Daphnia* method. There was a wide zoological gap between *Daphnia* and human beings, and they had thought it preferable to use animals where the gap was much smaller. He had tested a few preparations on himself. Those preparations which were inactive in himself, or had very low potency, had no activity in mice. In Munch's book on bio-assays, there were data to show that the relation of dose between a mouse and a human being differed with different purgatives. For senna the ratio was about 1 to 300. For instance, if the minimum effective dose for mice was 6 mg. the human dose would be 1·8 g. which was within the dose range of the B.P. for senna. He had carried out laboratory experiments on extracting both pods and leaves, but the latter gave difficulty because of the large quantity of mucilage they contained.

NOTES ON MICROBIOLOGICAL ASSAYS USING *LACTOBACILLUS LACTIS* DORNER

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The announcement by Shorb[1,2,3] that *Lactobacillus lactis* Dorner responded to a factor present in liver extracts to an extent roughly proportional to their clinical value, opened up the possibility of a microbiological assay of anti-pernicious anæmia preparations. The isolation of the growth factor by Rickes *et al*[4], who named it vitamin B_{12}, and the demonstration by West[5] of the anti-pernicious anæmia potency of Vitamin B_{12}, strengthens this possibility.

Most types of bacteria moulds, etc., are able to synthesise vitamin B_{12} but several organisms have been found which lack this property and these have been tried for assay purposes. The present communication deals with a method of assay in liquid culture using *L. lactis* Dorner.

Shorb[2,3,6] and Shive[7] have drawn attention to a marked tendency for this organism to behave in an inconstant manner under certain conditions. This uncertainty has been confirmed by the unpublished experiences of many workers in this field and, for many months, investigations in these laboratories were hampered by erratic assay results. The technique finally evolved and described below seems to be free from the disabilities reported by other workers.

The principles utilised in the final method are identical with those influencing all other microbiological assays (Shaw[8]). The test inoculum must be minimal. The organisms in the test inoculum must be young, in a highly active phase of growth, and to have been maintained on a complete medium. The medium must be free from other growth factors. The difference between *L. lactis* Dorner and other organisms is that the former is much more prone to variation formation. It was found that when optimum conditions had been established, the assays were more consistent than with any other microbiological assay performed in these laboratories.

The basal medium employed consists of sulphuric acid-hydrolysed casein with added tryptophane, the usual members of the vitamin B complex group and tween 80. T.J. factor, another essential supplement, is supplied as a concentrate of potato extract consisting of the ether precipitate from a phenol extract of the fraction insoluble in alcohol (80 per cent.) but soluble in alcohol (50 per cent.). One advantage of this source of T.J. factor is that unlike with enzyme digested casein used by Shive,[7] or with tomato juice, used by Shorb,[1,2,3,6] it is possible to use a large excess of T.J. factor without at the same time adding *L. lactis* Dorner factor. Another advantage is that since the current batch of concentrate is used at a final dilution of 1/50,000, it is possible to use the same batch of potato extract over a considerable period. All batches of tween 80 are not equally suitable. One sample which did not give a clear 10 per cent. solution in water was useless.

The reaction of the final medium is adjusted to *p*H 5·5 and the glucose, potato extract and preparation to be tested are added aseptically to the previously autoclaved basal medium. If the glucose is added to the medium prior to autoclaving, the resultant broth may support the growth of *L. lactis* Dorner in the absence of liver extract or other vitamin B_{12} containing material. Under these circumstances the " blank " is high and may even approach maximum turbidity. Autoclave treatment at *p*H 7 to 8 is more likely to upset the assay than when the *p*H is approaching 5·5 at which *p*H the phenomenon is rarely observed. One does not always obtain this growth potentiation on autoclaving and one possible explanation of failure to do so may be that some samples of glucose solution may become acid on autoclaving. If the *p*H is lowered before the reaction between glucose and media constituents can occur, it would be equivalent to autoclaving at a low safe *p*H.

The strain used was isolated as a single colony from soya bean medium and has been maintained for upwards of 150 daily sub-cultures on peptone agar, fortified with proteolysed whole liver, tween 80 and potato extract. Its behaviour has remained constant throughout this series of cultivation.

For assay purposes an overnight agar slope culture is washed into 10 ml. of basal medium fortified with proteolysed whole liver, and this allowed to incubate for 5 to 6 hours at a temperature of 37°C. The broth culture is centrifuged, the sedimented organism washed 2 or 3 times with basal medium deficient in vitamin B_{12} and finally suspended to an opacity of approximately 1/5th, in either vitamin free basal medium or saline, immediately before use. Three drops of this suspension are used to inoculate 10 ml. of test medium.

Growth is usually satisfactory after 16 to 18 hours incubation, but, if the inoculum is too small or the organism is not in good condition, incubation up to 48 hours may be necessary. Exposure of the washed organism to saline solution for more than about $1\frac{1}{2}$ hours may convert the organism from a plump bacillus, staining solidly Gram-positive, into a thin one with the body of the organism Gram-negative showing Gram-positive beads simulating the appearance of a chain of streptococci. Further exposure to saline solution might even result in a completely Gram-negative filamentous organism from which it is difficult to get a satisfactory culture. In contrast to the report of Shorb[3] we have not found that the length of incubation affects the assay values. The general levels of growth are slightly higher but the test solution is affected equally with the control. Probably as a consequence of the aseptic addition of glucose, the test medium is water white, and during the period when glucose was autoclaved with the medium it was found that, in general, a low blank was obtained when the colour of the medium was minimal, the colour being presumably an index of glucose decomposition.

To ensure regular results, it is essential to use constant-bore test tubes in order to have a constant surface-area/volume ratio. Incubation is best performed in a large room-incubator and the test tubes must not be shaken during the growth period. Sterility of the glucose is ensured by

the candle filtration of a 40 per cent. solution. Liver extracts in ampoules are assumed to be sterile, otherwise dilutions are made from preparations which have been allowed to stand in the presence of 1 per cent. phenol. The final dilution of the liver extract in the final medium is usually so great as to eliminate the effect of any phenol carried over. Where necessary, the preparation to be assayed is autoclaved and this does not appear to have any deleterious action. It is however best to avoid autoclaving the test sample.

It has been found convenient to make the basal medium up to 9/10ths final strength, to distribute this into aluminium capped test tubes in 9 ml. quantities and then to autoclave these tubes in lots of about 500 tubes. The tubes are prepared for use by adding the requisite amounts of glucose, potato extract and vitamin B_{12} preparation all in a volume of 1 ml. The autoclaved tubes may be used at any time up to 14 days after autoclaving without affecting the assay in any way; thus enabling a large number of assays to be conducted with medium is known to be identical in every respect.

The optimum conditions for autoclaving are obtained by the use of a large autoclave in which the tubes are placed in special racks so arranged that there is 1 in. free space around each tube. The autoclave is fitted with a recording thermometer and so it is possible to heat up during 10 minutes, to maintain at a temperature of 115°C. for 10 minutes and to take the same period to cool down. There is also available a permanent record of the autoclave history of that batch of tubes.

According to Shorb[6], *L. lactis* Dorner maintained on yeast-extract-agar with or without tomato juice, gives irregular results, and growth may be inhibited by some liver extracts. The strain used in these laboratories with the described technique gives very reproducible results and the dose-response curve for all liver extracts assayed, for pure vitamin B_{12} and thymidine, have been found to be identical whether assayed individually or in mixture. Thymidine has only 1/10,000th of the activity of vitamin B_{12}. Values obtained from the assay of mixtures are invariably the sum of two components. Inhibitors might be expected to affect the shape of the growth response curve and the values obtained on blending.

Growth-response curves are established for each assay by using 5 levels of the standard preparation. It is desirable to put up an equal number of levels of unknown, endeavouring to arrange the dilutions so that the reading of each level of the test coincide with the corresponding levels in the control series. For accurate work it is desirable to use 6 tubes at each level and to repeat the assay at least 3 times. Ordinarily 3 tubes at each of 3 or 4 levels provide a fairly accurate estimation. A typical growth response curve is shown in Figure 1.

Lester Smith and Cuthbertson[9] reported the presence of four substances with growth activity for *L. lactis* Dorner which may be present in liver extracts. These substances have different migration velocities on paper strip chromatograms when developed with aqueous butyl alcohol. Two of the substances, both with anti-pernicious anæmia activity, migrate less rapidly than riboflavine, and two, with no anti-pernicious anæmia

potency, migrate more rapidly than riboflavine, one being thymidine, which is found very near to the advance front. The possibility of other *L. lactis* Dorner-active materials being present in a liver extract in addition to vitamin B_{12} must therefore be taken into consideration. This possibility has been dealt with by subjecting various extracts to paper strip chromatograph (Shaw[10]). A spot of extract or other preparation (1/200th ml. of an extract assaying at 10 μg/ml. is satisfactory) is placed on a 1 cm. strip of Whatman No. 1 filter paper and, after drying, this strip is suspended in normal butyl alcohol, saturated with water, so that the butyl alcohol level is about 2 cm. below the applied spot. The butyl

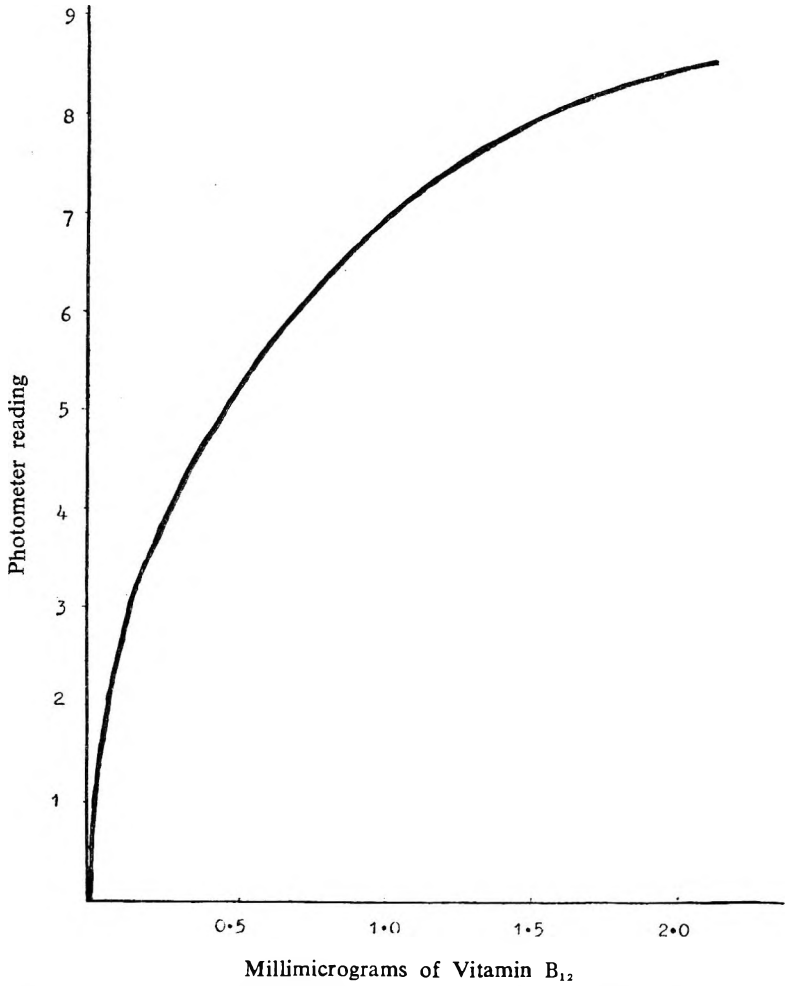

FIG. 1.—Growth-response curve vitamin B_{12}, *Lactobacillus lactis* Dorner. Average of 25 tests. 10 ml. tubes—1 cm. cell.

alcohol is contained in the bottom of a glass cylinder and the sides of the cylinder extend to about 13 cm. above the spot. The whole is protected

from light and the butyl alcohol travels up the strip, evaporates at the top of the cylinder and deposits all of the rapidly migrating material at this point of the strip at the advanced front.

A small amount of riboflavine may be placed on the same spot as the liver extract and after about 48 hours development, it will be found that the advance front is 13 cm. from the spot and a riboflavine belt (identified by examination under ultra violet light) is found about 6 cm. from the spot. The strip is cut at this point, each portion extracted with water and the extract assayed, thus giving the amount of activity which migrates both less and more rapidly than riboflavine.

It was found that the speed of migration of the main *L. lactis* Dorner-active fraction was so much slower than that of the other fractions that latterly the strip has been allowed to develop for a longer period, i.e., 14 to 21 days, when it has been found that most of the activity has left the spot, accumulating in a peak about $2\frac{1}{2}$ cm. from the spot. There is then a considerable length of filter paper which is devoid of activity and the rapidly migrating growth materials accumulate at the advance front. The strip is examined by cutting it up into 0·5 cm. sections, extracting each with water and assaying separately. A typical liver extract gives results as shown in the histogram in Figure 2.

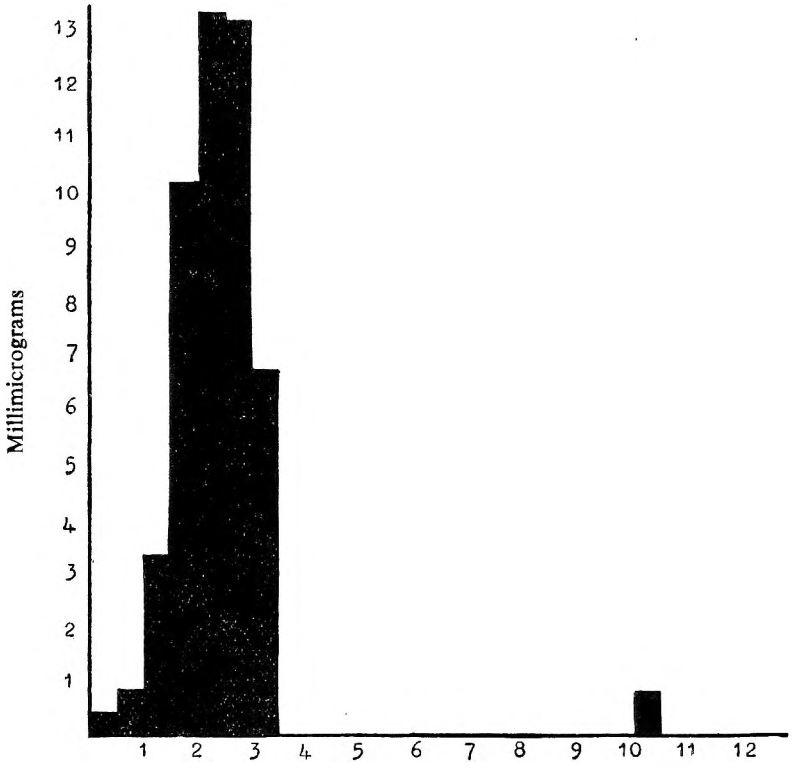

FIG. 2.—Paper chromatograph of vitamin B_{12} in liver extract Product "A," 0·005 ml. developed for 18 days.

Summary

A method has been described for conducting microbiological assay of material containing vitamin B_{12}. Over a long period regularly reproducible results have been obtained. Means have been described briefly for assessing the extent of interference with the assay of inhibiting materials and growth factors for *L. lactis* Dorner other than vitamin B_{12}.

Appendix

1. *HEPAMINO-PEPTONE-TWEEN AGAR.* (For strain maintenance.)

Evans Bacteriological Peptone	10 g.
Hepamino (Evans Medical Supplies)	1 g.
Tween 80	1 ml.
Glucose	50 g.
Potato Extract double the concentration used in final assay medium.	
Tap Water	to 1000 ml.

Adjust *p*H to 6·8, heat to 70°C. for 10 minutes, filter, add 10 g. of agar. Filter, tube in 10 ml. quantities. Autoclave for 10 minutes at 10 lb. steam pressure.

2. *ACID HYDROLYSED CASEIN.* (Modified from Mueller *et al*[11].)

Technical Casein	1000 g.
Concentrated Sulphuric Acid	400 ml.
Distilled Water	2600 ml.

Reflux for 50 hours. adjust *p*H with calcium hydroxide to *p*H 7·6, filter and wash precipitate with hot distilled water. Decolourise with activated charcoal. Dilute decolourised solution to the desired nitrogen content. t

3. *ASSAY MEDIUM* (Double strength).

Acid Hydrolysed Casein to provide in double strength medium	0·32 per cent. of nitrogen
Tryptophane Solution (0·1 per cent.)	200 ml.
Cystine Solution (0·4 per cent.)	50 ml.
Salt Solution "C"	40 ml.
Dipotassium hydrogen phosphate	5 g.
Potassium dihydrogen phosphate	5 g.
Distilled Water	to 900 ml.

Adjust *p*H to 6·9, heat to 70°C. for 10 minutes, filter, add 2 ml. of Tween 80, uracil, adenine, guanine and all vitamins as for *Lactobacillus Helveticus* (Shaw[8]). Dilute with distilled water to 1000 ml.

Note 1.—Potato extract is added to a concentration of double that which will give maximum growth with 2 μmg. of Vitamin B_{12}/10 ml. in 16 to 18 hours.

Note 2.—Most batches of glucose B.P. will substitute for glucose of A.R. quality.

Note 3.—A solid liver preparation standardised by Dr. Rickes of Messrs. Merck and Co., at 0·4 μg/mg. has been used as reference standard.

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THE GROWTH ACTIVITY FOR *LACTOBACILLUS LACTIS* DORNER OF COMMERCIAL LIVER EXTRACTS

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A Satisfactory method for the standardisation of commercial liver extracts in terms of their anti-anæmic potency would fulfil a long-felt want. Although it has not yet been definitely established that *Lactobacillus lactis* Dorner activity is an exact reflex of anti-anæmic potency, there is a definite correlation between clinical and Dorner effectiveness. The present communication deals with the growth-promoting efficiency for *L. lactis* Dorner of a series of commercial liver extracts of British, American and Continental origin.

Methods of assay used in this investigation are those detailed in the preceding paper[1]. The reference standard used was a solid liver preparation kindly supplied by Dr. Rickes and standardised by him at 0·4 μg of vitamin B_{12} per mg. The results are shown in Table I expressed in μg vitamin B_{12} per g. or ml. It should be emphasised that the values are referred to vitamin B_{12} as isolated by Rickes, and so should be comparable with the values for American liver extracts given by Rickes *et al.*[2]

TABLE I

Number	Manufacturer	Origin	Type	Vitamin B_{12} μg./ml or g.	Remarks
1	A	British	Whole Liver	18·0	Proteolysed
2	A	,,	Oral Liquid	18·0	,,
3	B	,,	Ext. Hepatis Liq. B.P.	14·0	
4	C	,,	,,	13·0	
5	D	,,	,,	9·0	
6	E	,,	Oral Liquid	7·6	Branded plus yeast
7	F	,,	Ext. Hepatis Liq. B.P.	0·5	Labelled Proteolysed
8	G	New Zealand	,,	7·5	
9	A	British	High Potency	10·0	Proteolysed
10	B	,,	,,	13·0	Batch 1
11	B	,,	,,	7·7	Batch 2
12	B	,,	,,	2·5	Batch 3
13	E	,,	,,	0·3	Batch 1
14	E	,,	,,	1·4	Batch 2
15	E	,,	,,	4·5	Batch 3
16	E	,,	,,	5·6	Batch 4
17	E	,,	,,	11·0	Batch 5
18	E	,,	,,	13·0	Batch 6
19	E	,,	,,	16·0	Batch 7
20	F	,,	,,	0·4	Batch 1 labelled Forte
21	F	,,	,,	0·4	Batch 2 labelled Forte
22	H	,,	,,	7·5	
23	I	,,	,,	9·5	Batch 1
24	I	,,	,,	8·0	Batch 2
25	J	U.S.A.	,,	30·0	20 U.S.P. units
26	K	,,	,,	12·0	15 U.S.P. units
27	L	,,	,,	10·0	15 U.S.P. units
28	M	,,	,,	9·0	15 U.S.P. units
29	J	,,	,,	7·4	15 U.S.P. units
30	N	,,	,,	10·0	10 U.S.P. units
31	O	S. America	,,	8·0	
32	P	Continental	,,	30·0	
33	Q	,,	,,	10·6	
34	R	,,	,,	7·4	
35	S	British	Crude Parenteral	3·0	
36	A	,,	,,	3·0	
37	C	,,	,,	2·3	
38	H	,,	,,	3·5	Batch 1
39	H	,,	,,	2·0	Batch 2

A direct comparison has not been made with the anti-pernicious anæmia factor of Lester Smith[3]. Dr. Lester Smith has stated that there might be a slight difference between his preparation and that of Dr. Rickes.

From Table I it will be seen that proteolysed whole liver assays at 18 μg of vitamin B_{12} per g., and that oral liquid liver extracts range from 18 μg. to 7·5 μg. of vitamin B_{12} per ml. It will also be noticed that one oral liver extract, which is stated to have been proteolysed, has little or no activity. Samples of parenteral products prepared by this manufacturer are also exceptionally low in *L. lactis* Dorner factor. From assays performed on comparatively small samples of liver, which had been subject to extensive laboratory treatment with papain, it would seem that proteolysed whole liver contains the entire potency of the liver used. Oral liver extracts in general are but lightly fractionated, and the final potency is probably a function of the extraction efficiency and the degree of accidental natural autolysis.

The vitamin B_{12} content of high potency refined parenteral extracts was found mainly to fall within the range of 7·5 to 16 μg. of vitamin B_{12} per ml. There are two exceptions, one of American and one of Continental manufacture, which assay at 30 μg. of vitamin B_{12} per ml. In a similar manner the crude parenteral liver extracts fall in a group containing 2 to 4·5 μg. of vitamin B_{12} per ml. A disturbing discrepancy was found when different batches of the same brand of high-potency parenteral liver extract were examined. Six batches from manufacturer "E" ranged from 0·3 to 16 μg./ml., three batches from manufacturer "B" varied from 2·5 to 13 μg./ml. These discrepancies were confirmed by repeated assays, taking care to assay the different batches of liver extract on the same day with the same batch of assay medium and against the same standard. In view of the large batch discrepancy shown, a search was made for the possible presence of inhibitory substances in the low-potency extracts. Chromatographic spectra for these extracts were normal with no unexpected increase in potency at any point. If there had been any inhibitory substance present one would not expect this "unknown" to migrate at the same speed as the *L. lactis* Dorner factor. Confirmatory evidence was obtained for the absence of inhibitors by blending low-potency extracts with a solution of crystalline vitamin B_{12}, and assaying the mixture. Values were obtained equal to the sum of the two components assayed separately. A difference would have been expected in the presence of an inhibitor unless the amount of inhibitor was just sufficient to inhibit the activity present in the liver extract alone.

Chromatographic analyses also provide a means of assessing the significance of other growth factor for *L. lactis* Dorner in liver extracts. By upward development there was in some preparations a small amount of activity present in the advance front after 14 days development. This is presumably due to thymidine, but in no case did it amount to more than 5 per cent. of the total Dorner activity. Other batches, although with similar spectra, showed no activity at the advance front. Proteolysed

whole liver, oral liver extracts, highly refined and crude parenteral liver extracts were similar. Downward development in the few samples examined gave entirely comparable spectra except that, since the advance front had proceeded beyond the limits of the strip, no advance front was present. In no case was there any evidence of a significant secondary peak. An illustrative histogram is shown in Figure 1. The absence of subsidiary peaks does not necessarily mean that the four *L. lactis* Dorner active substances described by Lester Smith and Cuthbertson[4] are absent, but provide evidence that they are not present in sufficient quantities to effect the assay.

As a consequence of batch variation considerable doubt is thrown on the utility of assaying one batch of a particular brand as a means of determining the general potency of that particular manufacturer's product. Rickes assayed a number of American liver extracts of an alleged potency of either 10 or 15 U.S.P. units per ml. and found a marked variation between the various brands and also a difference between the various batches of the same brand. The American preparations which have been tested in these laboratories have ranged between 7·5 and

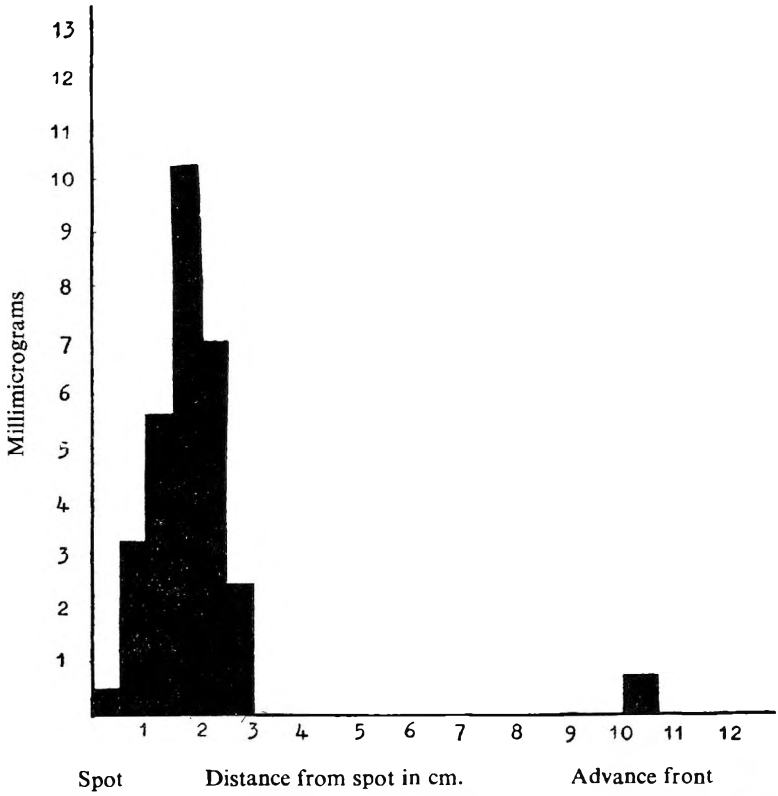

FIG. 1.—Paper chromatograph vitamin B_{12} in liver extract product "B," 0·005 ml. developed for 18 days.

13 μg. of vitamin B_{12} per ml. with the exception of the 30 μg. material already referred to. Manufacturer "F" issues 3 products, a liquid oral liver extract, a parenteral liver extract and a parenteral preparation labelled "Forte." All of these give exceptionally low values, and in this instance one is bound to suspect either the source of liver or else the primary extraction. In the case of manufacturer "E" and "B," both of which are highly reputable British firms, the explanation is not so obvious. Admittedly, one of the low-potency batches is known to be old, but others equally low were bought on the open market for current use. Moreover, we have been unable to find evidence that, in general, finished liver extracts deteriorate in potency on storage even when they have been stored under the adverse conditions which pertain in some of the export markets.

The only reasonable explanation is suggested by work in these laboratories concerned with investigating "process losses" by means of Dorner activity. It is now no secret that in a highly fractionated parenteral extract there may be very appreciable losses during processing, and that the magnitude of these losses may not be constant from sub-batch to sub-batch. Thus, for example, if a certain process is liable to give a 60 per cent. loss, it is not impossible that occasionally this loss is 90 per cent., so that unless a large number of sub-batches are combined in order to "iron out" the variations in process loss, the final product might be only one-third the potency of another.

Summary

1. A number of commercial liver extracts have been assayed for *L. lactis* Dorner activity in terms of vitamin B_{12}. A considerable variation from brand to brand has been found and also a serious variation between different batches of the same brand.

2. Although steps have been taken to eliminate artefacts due to method and the presence of inhibitors, it is not possible at the present time to correlate exactly *L. lactis* Dorner activity with clinical efficacy.

3. It is thus premature to advocate the establishment of a "Dorner" assay unitage applicable to all liver extracts, but it is not too much to expect that, in the future, branded preparations will maintain a reasonably constant "Dorner" activity.

4. The presumptive vitamin B_{12} content of "high potency" liver extracts is of the order of 10 μg./ml. whether of British, American or Continental manufacture.

It is a pleasure to acknowledge the technical assistance of Mr. G. B. D. Grafham.

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THE VITAMIN B_{12} CONCENTRATION IN LIVER EXTRACTS AND A NOTE ON THE RELATIONSHIP BETWEEN CLINICAL RESPONSE AND B_{12} DOSAGE

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Vitamin B_{12} Content of Liver Extracts

A number of different liver extracts have been assayed for vitamin B_{12} activity by the cup-plate method of Cuthbertson[1] employing *Lactobacillus lactis* ATCC 8,000. Some of these samples have also been assayed by the technique of Lees and Emery[2] employing the tube assay with *Lactobacillus leichmannii* 313. The results obtained are summarised in Table I.

TABLE I

B_{12} content of liver extracts for parenteral use

Distributor	Manufacturer	Extract	μg. B_{12}/ml.‡	Potency
	A	1	1·4	low
		2	5·0	high
	B	3	6·0	high
	C	4	2·9*	high
British		5	0·8	low
	D	6	1·0*	medium
		7	12*	high
	E	8	3*	low
		9	12*	high
	F	10	0·2	high
European	G	11	(*a*) 6*	low
			(*b*) 3·4	low
			0·23	low
	H	12	2·5*	high
	I	13	0·5	high
		14		
	J	15	0·1	medium
		16	0·1	high
			0·1	medium
	K	17	μg. B_{12} <0·05†	high
American	L	18	2·7	low
	M	19	3·5	medium
	N	20	22	high
	O	21	1·8	high
	P	22	11·8	high

* Average of 2 to 8 different batches. † No B_{12} detected even after chromatography.
‡ The essay results given in Tables I, III, IV and V represent the activity of one or more of the vitamin B_{12} group of factors expressed as the concentration of standard vitamin B_{12} (μg/ml) giving the same response as the sample.

Samples used for assay are all recent, having been obtained for the most part in June, 1949, except for samples from manufacturer G of

which (a) were obtained during 1936 to 1939 and (b) was captured during the war. Of the above samples N, O and P are known to have been manufactured in America. The other samples whose manufacturers are described as European or American were nevertheless presumably made in England, since we are informed that no liver extracts have been imported into this country for the last 6 years. The description used is thus the country of the manufacturers' headquarters, but not necessarily of the extract's origin.

The extracts have been classified into groups of low, medium and high potency on the basis of information supplied with the extracts by their manufacturers. This classification, shown in Table II, is necessarily very rough in the absence of any standard method of describing potency, but in assigning the extracts to low, medium or high potency groups, the suggested dosing schedules, U.S.P. units and liver equivalents have been taken into consideration.

TABLE II

Potency	Stated liver equivalent ml.	U.S.P. units
Low	<10 g.	2
Medium	10 to 20 g.	10
High...	>20 g.	15

No attempt has been made to distinguish between highly refined and highly concentrated extracts, although it is obvious that some manufacturers have attempted to produce potent refined materials, while others appear merely to have concentrated their liver extracts without any great degree of purification.

Table I demonstrates the wide differences between extracts from different manufacturers, the low potency extracts ranging from 0·23 to 6 μg./ml., while medium and high potency extracts vary over the ranges of 0·1 to 3·6 μg. and 0·1 to 22 μg. B_{12}/ml. respectively. These variations between samples of different origins would hardly be expected if adequate clinical trials had been carried out on all batches. This variation may be

TABLE III

BATCH TO BATCH VARIATION OF SAME BRAND OF EXTRACTS FROM DIFFERENT MANUFACTURERS

Manufacturer	Extract	Sample	μg. B_{12}/ml.	Manufacturer	Extract	Sample	μg. B_{12}/ml.
X	A	1	4·8	Z	C	12	15·0
		2	19·8			13	7·5
		3	11·3			14	10·0
Y	B	4	2·7			15	8·0
		5	3·6			16	5·0
		6	2·1			17	5·0
		7	3·0			18	15·0
		8	3·3			19	9·0
Z	C	9	24·0			20	17·5
		10	6·0			21	12·0
		11	9·0			22	12·0

ascribed to differences in manufacturing technique, quality of raw materials and clinical control (if any) of the products. Different batches of the same extract made by the same manufacturer may show a wide range of B_{12} concentrations, but it cannot be said whether these may not be due to alterations in procedure and materials available for manufacture. Table III shows the degree of variation encountered.

From the results it is clear that little value can be attached to estimates of potency unless better control is used than apparently at present. A number of these samples have been assayed by both of the different techniques employed in these laboratories, with the results shown in Table IV.

TABLE IV

MICROBIOLOGICAL ASSAY OF PARENTERAL LIVER EXTRACTS BY CUP-PLATE (*L. LACTIS*) AND TUBE (*L. LEICHMANNII*) METHODS

Extract	Tube assay μg. B_{12}/ml.	Plate assay μg. B_{12}/ml.
1	0·05*	<0·1*
2	0·04*	<0·05*
3	0·6*	<0·1*
4	ca 0·1*	<0·2*
5	0·14	0·22
6	0·4	0·8
7	0·7	1·0
8	1·5	1·4
9	2·8	2·7
10	3·0	3·5
11	3·3	3·3
12	9·7	10·5

* High concentrations of desoxyribosides present in these samples.

On the whole the agreement is reasonable for methods involving two different organisms and two different techniques (the cup-plate and tube method) having different sensitivities to interfering substances. The discrepancies encountered are being further investigated.

RELATIONSHIP BETWEEN CLINICAL RESPONSE AND MICROBIOLOGICAL ACTIVITY

All liver extracts prepared in these laboratories are clinically tested before they are released for sale. For this purpose typical cases of Addisonian pernicious anæmia in severe relapse are used, preferably those showing a red cell count between 1 and 2 million/cmm. The patients receive, by intramuscular injection, a single test dose of the extract. During the following 14 days the blood picture is determined on alternate days, but daily observations are made at the time when the peak of the reticulocyte response is expected. In interpreting the results particular attention is paid to the red cell response, which should increase at the rate expressed by the formula of $I = 0{\cdot}94 - 0{\cdot}214\ E_0$ (Della Vida and Dyke[3]), where I is the weekly increment in the red cells and E_0 is the initial cell count. In assessing the response of an individual patient, consideration is given to other factors that may bear on the test, e.g., hæmoglobin levels, shape of the response curves, reticu-

locyte response, infection and possible iron deficiency or abnormal red cell destruction. Foods that might have an anti-anæmic action are withheld before and during the period of test.

The results on 14 different liver extracts with 18 patients in a number of different hospitals are summarised in Table V, which gives only the red cell response as a percentage of that to be expected from the Della Vida and Dyke formula and the B_{12} content of the different extracts. The other observations made on these patients have been omitted for clarity.

If the responses of the individual patients are considered and if a response of 90 per cent. of that expected is taken as indicating activity of the liver extract, then it can be seen that samples containing less than

TABLE V

RELATION BETWEEN CLINICAL RESPONSE AND B_{12} ACTIVITY OF EXTRACT (1 ML. OF EXTRACT USED FOR THESE TESTS)

Sample	B_{12} activity μg./ml.	Increase in red blood cells expressed as percentage of expected response	Sample	B_{12} activity μg./ml.	Increase in red blood cells expressed as percentage of expected response
1	2·0	75	9	9·0	62
2	2·9	nil, 114 } 57	10	9·0	58
3	3·0	nil	11	12·0	46, 145 } 91
4	4·0	76	12	12·0	73, 138 } 106
5	4·1	nil			
6	4·5	54	13	15·0	93
7	6·0	50	14	17·5	96, 112 } 104
8	6·0	109			

10 μg./ml. are much less satisfactory than those containing more than 10 μg./ml. Of the 11 patients receiving less than 10 μg. of B_{12} 9 gave unsatisfactory responses, while of the 7 patients who received more than 10 μg. only 2 gave unsatisfactory responses. Several of these extracts were tested on more than one patient. The marked variation in response from patient to patient is very clearly seen in the results obtained with these. In particular the different patients receiving samples 2 and 11 show very wide differences in response. If the results obtained with the two patients on each sample are averaged, then it can be seen that only one extract out of 10 containing less than 10 μg./ml. would satisfy our criterion (and this result depends on the reaction of only one patient) while none of the four extracts containing more than 10 μg. B_{12}/ml. would have failed to do so.

SUMMARY

1. The vitamin B_{12} contents of a number of liver extracts have been reported.

2. Assay results using *Lactobaccillus lactis* and *L. leichmannii* have been compared.

3. The clinical responses to a number of extracts have been compared with their B_{12} contents, and the range of activity found by clinical test has been indicated.

We wish to express our thanks to the pathologists without whose enthusiastic co-operation the standardisation of liver extracts would not have been possible.

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DISCUSSION

THE three papers dealing with the micro-biological assay of liver extracts by Mr. Shaw and by Dr. Cuthbertson, Miss Lloyd, Dr. Emery and Mr. Lees were discussed together; the last paper was read by Mr. Lees.

MR. SHAW, in presenting his papers, stated that since submitting them he had found that on applying his assay to a commercial solution of crystalline vitamin B_{12} prepared for injection, the indicated vitamin B_{12} content appeared to be approximately three times as great as the labelled value, using as reference standard the solid liver preparation supplied by Dr. Rickes and standardised by him at 0·4μg. per mg. This observation indicated that the results quoted in the paper represented not necessarily vitamin B_{12} as such but were a measure of the growth activity for *Lactobacillus lactis* Dorner. This discrepancy along with the extremely slow speed of migration of the main constituent on paper chromatography, and the American view that microbiological assay of liver extracts for vitamin B_{12} is not reliable unless the vitamin B_{12} content of the liver solids in the preparation under test is of the order of 50 per cent. suggests the possibility that the clinical action of liver extracts may be due to a complex or conjugate of vitamin B_{12} more than to the presence of the free vitamin. It might well be that for the assay of liver extracts a standard liver preparation will be a more satisfactory reference standard than pure crystalline vitamin B_{12}.

The CHAIRMAN said that the three papers dealt with a subject which had been developed very considerably in the last year or so. If the figures given for assays of commercial extracts really represented their content of vitamin B_{12} then he thought that they gave a very disturbing picture of the state of affairs. The assay process required improvement before it was possible to place reliance on it, but the results given in the papers suggested that there might be some relationship between it and the clinical response. Mr. Shaw used a method of paper strip chromato-

graphy which was unusual, in that he did not protect the solvent from evaporation.

DR. C. H. HAMPSHIRE (London) commenting on the paper by Dr. Cuthbertson and his collaborators, remarked that he was a little sorry to see that they still found it necessary to make reference to the U.S.P. unit. The sooner that term disappeared the better. It was to be hoped that the work which was now being done by Dr. Cuthbertson and his colleagues and by other teams would enable a truly scientific method of assay of liver extracts to be arrived at by comparison with a standard.

DR. F. HARTLEY (London) said that the papers would be generally welcomed as helping to show something of the progress made on the microbiological side since the isolation of vitamin B_{12} last year, but a comparison of the two sets of papers as circulated revealed some startling discrepancies. However, Mr. Shaw had now clarified some of those discrepancies. The variations which became apparent immediately the papers were read would obviously be regarded by many people as being attributable to the essentially variant factors present in the microbiological method, namely the standard, the test organism, and the medium used. Mr. Shaw in his abstract, but not in the paper, indicated the very different interpretaion to be given to the results, when assaying preparations of pure vitamin B_{12} in contrast to those for the other materials examined. The standard which he used contained 0·4 μg./mg., or in other words 0·04 per cent. of vitamin B_{12}. It was well known, as Mr. Shaw himself had pointed out, that liver preparations contain other growth factors than vitamin B_{12}, and a relationship between the growth factors in the standard and in the test could be obtained. Mr. Shaw provided histograms for materials under test but not for the material used as his standard; however, he had pointed out that when he measured pure vitamin B_{12} against his standard he got a result about three times larger than he had expected. Therefore, making that allowance, the results of the two sets of results came more nearly into line. The marked influence of the medium on the growth-promoting and growth-inhibiting activities, and therefore on the results obtained, was fully discussed by Dr. Mary Shorb at the recent International Biochemical Congress. It became clear on looking at her growth response slopes that, according to factors such as would occur when comparing materials of totally different origin, some of which contained conjugates and others free vitamin B_{12} that one would obtain different interpretations according to which slope was used. To compare one kind of material with others of a different nature might be grossly misleading. It was now well known that different strains of organism responded differently to a particular standard and a particular unknown. Dr. Cuthbertson and his colleagues had suggested in Table IV that comparable results were obtained using *Lactobacillus lactis* ATCC 8,000 and *Lactobacillus leichmannii* 7,830 for tube assay, but other strains were available, and if the authors would look at that most recently made available, *L. lactis* Dorner ATCC 10,697, they would find a rather different picture. Both Jukes and Lester Smith had shown that there might be a group of vitamin B_{12} factors and Jukes had

already obtained in crystalline form a second vitamin B_{12} which he had called vitamin B_{12b}. Some or all of these different factors might well be clinically active. Mr. Lees had been understandably cautious in his interpretation of Table V, but it would be seen that 1 ml. of the extract containing 2·9 μg./ml. of vitamin B_{12} gave in one patient a response of a similar order to that given in another patient receiving 1 ml. of an extract containing 17·5 μg./ml. Such a contrast strongly suggested that there might be some factor additional to vitamin B_{12} exerting an erythropoietic effect. Standardisation in terms of vitamin B_{12} only, might not, therefore, wholly reflect the clinical potency of liver preparations. Mr. Shaw had suggested in his abstract, but not in his paper, that it might be desirable to assay the liver extract not against vitamin B_{12} but against a standard liver preparation. That was certainly a wise extension; but the difficulty lay in arriving at a standard preparation. Were they interested in the conjugated vitamin B_{12} or in free vitamin B_{12}? He thought it was important to obtain a great deal more information on the factors which could be separated from liver extracts chromatographically, and not necessarily limited to the coloured fractions. Under the conditions for chromatography which Mr. Shaw had described, most workers would have lost all their vitamin B_{12}. It would normally have moved far more than 2·5 cm. up the paper during elution for 18 days; overnight elution was sufficient for most people. Finally, it was important to recognise that if in fact the authors had measured growth-promoting factors for an organism and not vitamin B_{12} there should be revision of the headings in the tables in the papers.

DR. K. BULLOCK (Manchester) asked about the effects of autoclaving on the glucose. Mr. Shaw had said that the lactobacillus was very sensitive to the concentration of carbon dioxide and then later that autoclaving altered the redox potential. Were those two factors standardised in the medium which he recommended and stabilised during the method of preparation?

MR. T. D. WHITTET (London) commented that almost every physician had his favourite extract and pharmacists therefore had to stock a great variety of liver extracts. He inquired whether it had now been proved that both crude and refined liver extracts were necessary.

DR. G. E. FOSTER (Dartford) asked whether the culture could be kept in the dried state.

DR. LUMB (Nottingham) enquired whether there had been any attempt to remove the thymidine before carrying out the assays. If not, to what extent was the thymidine responsible for the growth of the lactobacillus?

MR. G. E. SHAW, in reply, said that the histogram of his standard was identical with the histogram of the extracts, there was no marked separation of the slow moving band. He had tried a normal downward development and had obtained exactly the same kind of histogram. It was owing to that slow moving band that he was beginning to wonder whether in an ordinary liver extract there was much free crystalline

vitamin B_{12}. In the course of work not yet published he had examined liver extracts from animals treated in certain ways. Two extracts, one from a treated and one from a normal animal, were adjusted to the same nominal vitamin B_{12} concentration. The normal one showed a normal crude liver extract histogram, but the other had multiple peaks. The normal one gave the expected clinical response, but the second one gave no response whatever. The mathematical formula used in the paper read by Mr. Lees was not an assay but a stipulation that a case which had been satisfactorily treated would on average give that response. There was no evidence whatever that the same dose of liver extract would give the same response in two patients, even if they had the same initial r.b.c. level. Apart from indicating that a weak liver extract was less likely to give the response than a strong one, he did not think that any reliance could be placed on the formula.

L. leichmannii, which was more subject to oxygen tension than *L. lactis,* Dorner was difficult to maintain under aerobic conditions. It must be grown on a medium in which the glucose had been autoclaved. He exposed the medium to air for 3 or more days after manufacture in order to enable it to reach equilibrium. He had also used a series of tubes which had been autoclaved six months before, or earlier, and had obtained a growth response curve identical with that which he had obtained six months before.

MR. K. A. LEES, also in reply, said that as the U.S.P. authorities had stated that they were not prepared to accept standardisation in terms of vitamin B_{12}, but only to accept the U.S.P. unit, it was necessary at this stage to incorporate the U.S.P. unit for purposes of comparison in any paper. He would like to thank Dr. Hartley for his suggestion with regard to *L. lactis* Dorner, ATCC 10, 697. With reference to redox potential, contrary to what Mr. Shaw stated, they had found that with *L. leichmannii* they could obtain satisfactory growth whether or not the medium was autoclaved containing glucose. They might get better growth when the glucose was autoclaved with the medium, but even if it was added as a sterile solution they still obtained the same result. The solution to the problem would be to determine the optimum E_H for initiation of growth, and to find a means of stabilising the medium at that particular E_H. Various methods had been suggested in the literature and they were examining them at the moment, particularly the effect of adding thioglycollic or ascorbic acid to the medium. Autoclaving thioglycollic acid and vitamin B_{12} together in the basal medium might result in a similar growth response to that obtained with aseptic addition of vitamin B_{12} to a sterile medium not containing thioglycollic acid, due to the growth response of thioglycollic acid compensating for any destruction of vitamin B_{12}. He did not think that it was possible to say at the present time whether there was any advantage in using a crude extract, except that Table I showed that in the more refined extracts the vitamin B_{12} activity tended to be higher than in the other extracts, though it could also be just as low.

Both Dorner and the *L. leichmannii* cultures could be dried, but when resuscitating the culture it was 7 to 14 days before a satisfactory growth response could be obtained.

They had not attempted to remove thymidine. They did a differential assay, determining vitamin B_{12} plus thymidine and then thymidine alone, after alkaline hydrolysis of the vitamin B_{12}. The Della Vida and Dyke formula was compiled from a large number of clinical tests, and so far had been the only scientific approach to clinical testing. As such, they had considered it to be the most suitable standard for their present work. He, personally, agreed with Dr. Hartley that 18 days was a long time for developing the chromatogram; they found that 24 hours was quite adequate. It was difficult to say whether the vitamin B_{12} content reflected the clinical activity. Medical opinion accepted vitamin B_{12} activity as the total anti-anæmia activity, but the scientific mind did not.

THE VARIATIONS IN COLOUR OF LIQUID EXTRACT OF LIQUORICE B.P.

By J. H. Oakley and R. E. Stuckey

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

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It is well known that variations in colour are found between batches of liquid extract of liquorice B.P. prepared from different samples of liquorice root.

Colour differences in liquid liquorice extracts are usually noticed in the dispensing of mixtures having the extracts as a constituent; the dilution obtained in such mixtures is often such as to bring it within what might be called the "critical" colour range. Variations in colour are not readily apparent until a dilution of 1 in 10 is reached, but differences can be detected in dispensing a number of common National Formulary mixtures. Thus Mistura Ammonii Chloridi contains a 15 minim dose and Mistura Ammoniae et Ipecacaunhae Composita a 10 minim dose of liquid extract of liquorice; these amounts represent dilutions of 1 in 16, and 1 in 24 respectively, and variations in the colour of the liquid extract of liquorice used will result in a different colour for the final dispensed mixtures, which may cause comment. In an endeavour to trace the reasons for these variations, details of the method of preparation have been examined, together with differences inherent in the drug and in the final product.

The British Pharmacopœia 1948 directs that unpeeled liquorice root, *Glycyrrhiza glabra* and other species of *Glycyrrhiza* shall be used in the preparation of liquid extract of liquorice. There are considerable differences in commercial samples of liquorice root and Wallis[1] gives figures of 15 to 27 per cent. for aqueous extractive, dried at 100°C.; the B.P. excludes samples of root with a water-soluble extractive below 20 per cent. Liquorice root of commerce is at present imported mainly from Anatolia, Syria or Iraq as ordinary or "natural" root; another type available is known as "cuttings" which are selected pieces of uniform

TABLE I

Analysis of commercial samples of liquorice root

Description of material	Moisture	Ash	Acid-insoluble ash	Water-soluble extractive (on material as received)
	per cent.	per cent.	per cent.	per cent.
Natural, unpeeled	8·3	4·4	0·4	20·5
Cuttings, unpeeled	8·6	5·1	0·6	31·6
Powdered decorticated*	7·0	3·9	0·1	41·2
Cuttings, unpeeled	9·1	6·9	0·6	35·3
Natural, unpeeled	10·7	5·4	0·6	25·1
Cuttings, unpeeled	8·5	5·2	0·2	33·1
Natural, unpeeled	8·4	7·6	0·9	25·9
Natural, unpeeled	7·0	5·1	0·3	21·0

* Described as "pulv. decort. elect."

size and diameter, the length of each piece being approximately 2 to 4 inches. A selection of samples of liquorice root obtained from different sources during recent years gave the analytical results shown in Table I and indicate the variations to be expected.

The figures are chiefly of interest in showing the wide variations in water-soluble extractive encountered in different samples of the crude drug; the remarkably high extractive in the single sample of commercial powdered decorticated root is also noteworthy. The method used for the determination of water-soluble extractive was similar to that described in the B.P. 1948.

Examination of the colouring matter

Representative samples of each of 6 lots of liquorice root, which complied with the requirements of the B.P. 1948, were reduced to a coarse powder and macerated with chloroform water (5 g. root to 100 ml.) for 24 hours, shaking frequently. The resulting product was filtered and aliquot portions of the filtrate were taken for determination of the water-soluble extractive and also for recording the colour in Lovibond units in a Lovibond Tintometer using a 1 cm. cell. The results obtained are given in Table II.

TABLE II

COLOUR VALUES OF SAMPLES OF LIQUORICE ROOT

Sample	Source and description of root	Water-soluble extractive	Lovibond Units using a 1 cm. cell			
			Yellow	Red	Blue	Neutral
		per cent.				
A.	Anatolian, natural	35·9	9·6	5·1	—	—
B.	Iraq, natural	26·9	10·1	6·2	—	0·1
C.	Syrian, natural	20·5	11·0	5·7	—	0·2
D.	Anatolian, cuttings	32·3	11·5	4·5	—	0·2
E.	Anatolian, cuttings	33·7	11·0	4·2	—	—
F.	Anatolian, cuttings	34·5	11·6	5·2	—	0·2

It can be seen from Table II that although the water-soluble extractive varied from 20·5 per cent. in sample C to 35·9 per cent. in sample A, the colour of the extracts did not vary appreciably; the composition and intensity of colour expressed as above were in fact relatively constant, despite the differences in origin and characteristics of the samples. Thus, under the conditions of experiment used, it can be concluded that the "depth" (composition and intensity) of colour extracted per unit weight of root appears to bear no relation to the percentage of water-soluble extractive obtainable from the material.

Usually most samples of extract of liquorice throw out a precipitate when added to acid solutions. For the preparation of liquorice extracts a dilute ammonia solution may be used in the process as, for example, in the preparation of liquorice root fluid extract, U.S.P. XIII, and Berg[2] used a menstruum containing ammonia for the extraction of liquorice by percolation. To study the effects on the resultant colour, further 5 g. quantities of the materials reported in Table II were macerated using a dilute (1 per cent. w/w) solution of ammonia in place of chloro-

form water. The proportion of extractive obtained was determined and the colour examined as before. The results obtained are given in Table III.

TABLE III

EXTRACTIVES AND COLOUR OF EXTRACTS OBTAINED WITH A DILUTE SOLUTION OF AMMONIA

Sample of Liquorice Root (see Table II)	Dilute Ammonia-soluble extractive	Lovibond Units using a 0·25 cm. cell			
		Yellow	Red	Blue	Neutral
	per cent.				
A.	34·5	12·3	3·8	—	0·1
B.	29·5	10·0	3·5	—	—
C.	21·8	13·4	5·5	—	—
D.	33·5	13·2	5·0	—	—
E.	36·4	19·0	3·3	—	0·2
F.	34·5	18·2	4·4	—	0·2

The use of dilute solution of ammonia did not increase significantly the total percentage of extractive, as can be seen by comparison of the extractive figures for the respective samples given in Table II and Table III.

It should be noted, however, having regard to the smaller cell width used, that the intensity of colour of the extract as expressed had been very appreciably increased. The question arises, therefore, whether the increased intensity of colour results from the extraction of additional coloured materials by the alkaline solution or is simply due to a darkening or " indicator " effect at alkaline *p*H values.

A sample of aqueous extract (5 g. of liquorice root, 100 ml. of water) was therefore adjusted to various *p*H values by the addition of small amounts of either sodium hydroxide solution or hydrochloric acid and the colour examined (see Table IV). The *p*H value of the extracts before addition of acid or alkali was approximately 6·5.

TABLE IV

EFFECT OF *p*H ON THE COLOUR OF AN AQUEOUS EXTRACT OF LIQUORICE ROOT

*p*H Value of extract after addition of acid or alkali	Lovibond Units using a 0·25 cm. cell			
	Yellow	Red	Blue	Neutral
2·0		Cloudy solutions		
4·0				
6·5	2·3	0·7	—	—
7·0	4·0	1·0	—	—
7·5	4·9	1·0	—	0·1
8·5	6·0	1·6	—	0·1
9·0	9·0	1·9	—	0·2
9·5	13·9	2·4	—	0·3
10·0	16·0	3·0	—	0·1

In acid solutions (low *p*H values) precipitation occurred which interfered with the examination of the colour of the liquid. It can be seen, however, that as the *p*H increased and especially on the alkaline side the extracts became considerably darker, both yellow and red components increasing in intensity. The colour obtained at each *p*H value was stable,

however, and showed no significant change over a period of two days. It was noted incidentally that on the addition of alkali a buffering effect occurred between *p*H 8·5 and 9·0 and the addition of a relatively large quantity of sodium hydroxide solution was required to increase the *p*H above 8·5.

Because of the difficulty of measuring directly the colour of the extracts at low *p*H values, the effect of acid and alkali on the colour isolated chromatographically was examined. A sample of aqueous extract of liquorice root was passed through a column of alumina when the colour was adsorbed. Washing down the column with quantities of water then produced a dilute aqueous solution of the coloured materials of the extract substantially free from other water-soluble matter. Aliquot quantities of this solution were then adjusted to various *p*H values and the colour examined. The results obtained are given in Table V.

It can be seen that there is a definite " indicator effect " obtainable with the colouring matter from extract of liquorice, the colour being much lighter in acid and darker in alkaline solution. This effect was found to be reversible. Addition of alkali to an acidified solution quantitatively restored the colour, and addition of acid to an alkaline solution diminished the colour, which could be restored on making alkaline once more.

TABLE V

EFFECT OF *p*H ON THE COLOUR OF AN AQUEOUS SOLUTION OF THE COLOURING MATTER ISOLATED FROM AN EXTRACT OF LIQUORICE ROOT

*p*H	Lovibond Units using a 0·25 cm. cell			
	Yellow	Red	Blue	Neutral
2·0	0·4	—	—	—
4·0	0·8	—	—	—
6·5	1·0	—	—	—
8·0	1·1	—	—	0·2
9·0	2·4	0·1	—	0·1
10·0	2·7	—	—	—
10·5	2·8	—	—	—

It will be realised from the results reported that, according to the conditions of preparation and use of liquid extract of liquorice B.P. so there can result variations in the colour of the products.

After evaporation according to the official instructions a *p*H slightly on the acid side is usually obtained, but the variation of acidity encountered is not such as to result in appreciable variations in colour due to the indicator effect referred to above. Since, however, the B.P. instructs " evaporate until the weight per ml. of the liquid at 20°C. is 1·198 " it follows that the standardisation of the liquid extract on total water-soluble extractive, though entirely logical for such a product, may cause relatively appreciable differences in colour between extracts prepared from samples of root showing a relatively high, and from those showing a relatively low, water-soluble extractive. Thus, for example, a sample of liquorice root giving a high water-soluble extractive will give a comparatively large volume of final product and since, as was shown in

Table II, the amount of coloured materials extracted is independent of the water-soluble extractive of the root, the resulting preparation will be relatively light in colour. Using water-soluble extractive as a criterion of quality of liquorice root it follows that a good quality root may, in fact, result in an official extract relatively light in colour. The yields and final colours of a number of prepared samples of liquid extract of liquorice B.P. were therefore noted and these are given in Table VI; the recorded yields were obtained using B.P. quantities and the Lovibond Tintometer readings are for 1 in 100 aqueous dilutions of the liquid extract. These results show that a high yield was associated in these examples with a relatively low colour intensity. Having regard, however, to the variations encountered between laboratory and industrial methods of evaporation and to lack of knowledge of the influence of heat on the constituents of the extract responsible for its colour, it would not be justifiable to expect such a relationship to be applicable generally.

TABLE VI

CORRELATION BETWEEN YIELD AND COLOUR FOR SAMPLES OF LIQUID EXTRACT OF LIQUORICE B.P.

Example	Yield	Lovibond Units using a 1 cm. cell			
		Yellow	Red	Blue	Neutral
	ml. per 1000 g. of root				
1	481	14·1	1·7	—	—
2	678	7·0	0·9	—	—
3	566	10·5	1·3	—	—
4	655	7·0	0·9	—	—
5	368	14·2	1·8	—	—
6	671	7·5	0·9	—	—
7	716	5·4	0·7	—	—
8	569	6·5	0·8	—	—

It is interesting to note that, while the British Pharmacopœia 1948 adopts the more logical procedure of standardisation on solid content, thus leading to the possibility of variations in colour according to quality of root used, the method of the United States Pharmacopœia XIII will lead to the production of extracts varying in solid content according to the water-soluble extractive of root used, but the extracts obtained are likely to be relatively constant in colour.

The colour measurements given in Table VI are, as mentioned, those obtained in aqueous dilutions only. The colour in dispensed mixtures will, of course, differ according to a number of factors as well as the *p*H of the solution; for example, the nature and concentration of the electrolytes present may also cause alterations in colour.

Certain Aspects arising during Manufacture. In the preparation of the liquid extract the B.P. directs that unpeeled liquorice root, in coarse powder, shall be exhausted by percolation with chloroform water, the percolate boiled and set aside for not less than 12 hours, the clear liquid decanted and the remainder filtered from the relatively light-coloured sludge present in the latter being rejected. This sludge is not sufficiently coloured, however, to affect the colour of the final preparation. It is not

advisable to set aside the preparation for any length of time at this stage, owing to the likelihood of fermentation. In practice on the manufacturing scale, evaporation may be done under reduced pressure so that changes in the colour of the product due to overheating are less likely to occur.

As it was thought possible that differences in the time for which the preparation is allowed to stand before filtration (the B.P. states " not less than forty-eight hours ") might cause slight differences in the colour of the final preparation, the effect of time of standing was examined. After standing for three weeks a small heavier layer collected, giving the following comparative results (1 in 100 dilution, Lovibond Units):

Extract immediately after preparation:	Yellow 8·0; Red 1·0
Extract (lower layer) after standing for three weeks	Yellow 10·6; Red 1·3

Although, as can be seen, the lower layer was relatively more intensely coloured, the actual volume of this layer was small in comparison with the bulk of the preparation and the amount, later rejected, did not appreciably affect the colour of the bulk of the extract.

SUMMARY

1. A study has been made of the colouring matter extracted from liquorice root and of some of the possible causes of variation in colour of liquid extract of liquorice B.P.

2. It has been shown that the colour variation occurring in samples of extract prepared according to the official instructions may result from the standardisation of the preparation on its content of total water-soluble extractive.

The effect of changes in *p*H value on the colour of liquid extract of liquorice has also been investigated.

The authors wish to thank Miss S. M. Stokes for assistance in carrying out the practical work, and the Directors of The British Drug Houses, Ltd., for permission to publish this paper.

REFERENCES

1. Wallis, *Textbook of Pharmacognosy*. 1946, Churchill, 334.
2. Berg, *J. Amer. pharm. Ass., Sci. Ed.*, 1924, **13**, 814.

DISCUSSION

The paper was read by MR. J. H. OAKLEY.

THE CHAIRMAN said that it was interesting to note the increasing use of chromatography, which was coming into a large number of Conference papers.

DR. J. M. ROWSON (London) remarked that nothing seemed to be known about the nature of the colouring matter present in liquorice root and the standard textbooks made virtually no reference to it. He wondered whether the authors had any idea of the chemical nature of

the colouring matter present. Did they consider that there was any possibility of caramelisation of the fairly large amount of sugar present? That in itself would tend to account for the darker colour of the extracts which had been prepared.

DR. W. MITCHELL (London) said that, although he had not made any colour measurements himself he assumed, by analogy with other cases that the red component was the dominant one in controlling the colour, and that the yellow was of relatively small importance. He wondered, therefore, whether the figures given in Table V were of any value, because, in exposing the material to chromatographic separation all the red component had been lost and one was merely measuring the yellow. Also, there was a buffer effect on the *p*H values corresponding to the very large change in the colour values at just about the same point. His experience did not confirm the wide variations in colour found by the authors. It was also necessary to consider the effect of contact with metals in large manufacture. Experiments with glass apparatus were not entirely realistic. A good deal might depend on the metal used in the plant, and even more on the heat treatment which the material had undergone. Caramelisation might play a very significant part in determining the final colour of the product, and it was most important to avoid the risk of overheating at any stage.

MR. J. H. OAKLEY, in reply, said that the authors had no information about the nature of the colouring matter present. In manufacturing practice caramelisation was not likely to be considerable, as it was customary to do the evaporation under reduced pressure. They had deliberately tried to produce a certain amount of caramelisation, and a small sample of extract was grossly overheated until actual charring occurred. They were surprised to find that very little increase in colour resulted.

They considered that the yellow was the more dominant factor and not the red. The buffer effect referred to was interesting. It was possible that some change took place at that stage, as it was coincident with a marked change in colour accurring there. Dr. Mitchell's somewhat different experience regarding the magnitude of the variation in colour might be because, to get a relatively constant colour, it was necessary to use the same type of root. If, however, roots of low and high water-soluble extractive were used a considerable variation in colour occurred. Though much of the work described had been done on a small scale in glass apparatus, it had also been compared with large-scale experiments done in large apparatus of various metals, and they had found no significant change in the colour.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Aldehydes, Determination of, with Hydrazine. L. Fuchs and O. Matzke. (*Scientia pharm.*, 1949, **17**, 1.) Hydrazine sulphate may be used for the acidimetric determination of aldehydes, details being similar to that of the hydroxylamine method, with methyl red as indicator. Examples are given of the application to benzaldehyde, piperonal, and vanillin.

G. M.

Atropine, Determination of Small Concentrations of. M. Tonnesen. (*Acta Pharmacol. Toxicol.*, 1948, **4**, 186.) The colorimetric reaction of Vitali as modified by Allport and Wilson is recommended for the chemical determination of small concentrations (50 μg. or more) of atropine, although the substance must be fairly pure. The conditions and precautions necessary to make the reaction quantitative are discussed and standard curves of extinction and time are given. Details of a biological assay depending on the dilatation of the mouse's eye are described, the technique being based on the method worked out by Pulewka (*Arch exp. Path. Pharmak.*, 1935, **178**, 439). The alkaloid is injected subcutaneously into each of 10 male white mice in an amount that in mydriatis effect corresponds to 0·75 μg. of *l*-hyoscyamine and the pupil diameter is measured microscopically after 1 hour. Dose response curves are given for atropine sulphate, hyoscyamine sulphate and scopolamine hydrobromide. A statistical analysis of results obtained using less than 10 mice has been made. After a large number of determinations it was found that a pupil diameter of 2 mm. corresponded to a minimum of 1·75 μg. and a maximum of 2·0 μg. of atropine sulphate. In forensic analysis an ether extract from an alkaline solution of the urine should be made evaporated to dryness, the residue dissolved in 0·001N hydrochloric acid and injected. An extraction of the contents of stomach and intestines with 0·001N hydrochloric acid should also be made, this procedure being more reliable than the " Stas-Otto " process. The untreated urine can also be injected. Amounts as small as 0·5 μg. can be determined by this method.

R. E.S.

Digitoxin and Digitoxigenin; Baljet Reaction. F. K. Bell and J. C. Krantz, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 107.) The Baljet test was applied using the original method, in which sodium hydroxide is the alkali, and the modified method, in which tetraethylammonium hydroxide is the alkali. The results indicate that under the conditions described there is no difference on a molar basis in the sensitivities of digitoxin and digitoxigenin towards the reagent, particularly if allowance is made for the digitoxose portion of digitoxin. If, therefore, digitoxin contaminated with digitoxigenin, which is physiologically less active, is assayed by the method described, erroneously high results will be obtained. Comparison of the results of the two methods supported the previous conclusions that the use of tetraethylammonium hydroxide as the alkali resulted in an increase of about 100 per cent. in the colour intensity. G. R. K.

Histamine, Microchemical Identification of Small Quantities of. G. Deniges. (*Bull. Soc. Pharm. Bordeaux,* 1949, 1, **87**, 3.) A solution of histamine dihydrochloride was used to study the microcrystalline reactions of histamine. A small drop of a solution (concentration, 0·1 mg./ml.) containing 0·0003 mg. of histamine was allowed to crystallise spontaneously in air, when a number of octahedral crystals and aggregates were obtained. These were examined microscopically and a diagram at a magnification of 130 diameters is given. To the crystals was added a small drop of a 1 per cent. aqueous solution of picric acid; at the edge of this drop bundles of fine yellow needles of histamine picrate formed, not to be confused with the picric acid crystals, larger and more squat, due to excess of picric acid which crystallised out on standing. The presence of chloride in the evaporated crystalline histamine dihydrochloride can be confirmed by the addition of a small drop of silver nitrate solution; the precipitated silver chloride was dissolved in ammonia solution and allowed to evaporate, when characteristic crystals of silver chloride were formed. R. E. S.

Pamaquin, Colorimetric Detection and Determination of. J. A. Sanchez. (*Ann. pharm. franc.,* 1949, **6,** 495.) Three reactions are described. *Quinone reaction.* On warming a few mg. with 2 drops of 33 per cent. sulphuric acid in a glycerin bath at 155° to 160°C. for 5 minutes, a carmine-red colour appears on the walls and bottom of the tube. This is soluble in alcohol (70 per cent.) and becomes violet with ammonia. Tablets should be extracted with alcohol before the reaction is applied. *Nitrous acid reaction*: A few mg. is dissolved in 2 ml. of alcohol (70 per cent.) and treated with 2 drops of acetic acid and 1 drop of 10 per cent. sodium nitrite solution. A red colour is produced. On diluting and extracting with chloroform the colour is extracted by the chloroform. *Diazo reaction.* A red colour is produced by coupling with diazotised *p*-nitraniline. This reaction may be used quantitatively as follows: Dissolve 0·01 g. with 10 drops of acetic acid in alcohol (95 per cent.) to 100 ml. To 1 ml. of this solution 0·5 ml. of diazotised *p*-nitraniline solution (0·5 g. of *p*-nitraniline and 0·5 ml. of sulphuric acid in 50 ml. of water) is added; to 7 ml. of this solution 1 drop of 10 per cent. sodium nitrite solution is added, and the mixture is shaken until decolorised. The volume is finally made up to 5 ml. with alcohol (95 per cent.). For tablets, the reaction should be applied to the alcoholic extract.

G. M.

ESSENTIAL OILS

Ascaridol in Oil of Chenopodium. A. Halpern. (*J. Amer pharm. Ass., Sci., Ed.,* 1948, **37,** 161.) Ascaridol is the only anthelmintic component of oil of chenopodium, but the U.S.P. X, gave no standard for ascaridol content, other than physical constants. A method of determination based on the oxidation of ascorbic acid in the presence of ascaridol and of oil of chenopodium, and subsequent back titration of the ascorbic acid with 2 : 6-dichlorophenolindophenol has been investigated. Uniform results were not obtained, indicating that some other substance in the oil also caused oxidation. L. H. P.

FIXED OILS, FATS AND WAXES

Shark Liver Oil, Deodorisation of. P. K. Mathew, P. V. Nair, T. A. Ramakrishnan and H. Sreemulanathan. (*Nature,* 1948, **162,** 494.) Methods of deodorisation of shark liver oil are examined. Steam treatment of the oil yields a product free from odour when freshly prepared but which reverts to its original character in a few days. Oils

deodorised by agitation with fermenting milk or toddy, were found to remain bland for several months. Tables giving data with regard to the chemical constants, potency, and stability of oils deodorised with fermenting milk and toddy, as compared with those of the original oil, showed that these characteristics were little changed in the process. Hydrogenation of the oil was carried out with 0·1, 0·25 and 0·4 per cent. of a nickel catalyst with promising results: 0·1 per cent. of catalyst was not sufficient to effect complete deodorisation within a temperature range of 100° to 180° C., but 0·25 per cent. of the catalyst effected fairly complete deodorisation of the oil at 120° C. within 30 to 45 minutes. A loss of vitamin A of about 7 per cent. was found although the keeping quality of the oil improved considerably. Increase of the concentration of the catalyst to 0·4 per cent. caused increased destruction of vitamin A. Hydrogenation results are given in a table which shows that with 0·25 per cent. of nickel catalyst at temperatures between 100° and 180°C. and reaction periods from 30 to 180 minutes, the loss of vitamin A increased progressively with rise in temperature and time of exposure.

R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Biocerin. C. W. Johnson, H. D. West, H. L. Jones and C. J. Long. (*J. Bact.*, 1949, **57**, 63.) The antibiotic material was obtained from a culture of *Bacillus cereus* isolated from soil and grown in a medium containing glucose, mineral salts, methionine and agar, by extracting the metabolism solution with ether and evaporating the ethereal liquid. The residue was insoluble in water but soluble in various organic solvents. The growth of all the following bacteria was inhibited by a concentration of 1 mg./ml. of the crude material, and all except the first three by 0·5 mg./ml.:—*Salmonella paratyphi A and B, Sarcina lutea, Salmonella typhosa, Corynebacterium diphtheriæ, Bacillus anthracis, B. subtilis, Staphylococcus albus, Escherichia coli, Brucella suis, Aerobacter aerogenes* and *Neisseria catarrhalis.* Rabbit serum caused a lowering of activity. A dose of 20 mg. in liquid paraffin suspension given intraperitoneally to mice did not produce any toxic effects. Further investigation is considered to be warranted.

H. T. B.

Circulin, A New Antibiotic. F. J. Murray, P. A. Tetrault, O. W. Kaufmann, H. Koffler, D. H. Peterson and D. R. Colingsworth. (*J. Bact.*, 1949, **57**, 305.) The antibiotic was isolated from an aerated broth culture of a soil organism resembling *Bacillus circulans*, but differing from almost all known strains of the latter by giving a positive Voges-Proskauer reaction. The unit of activity is defined as that amount per ml. that completely inhibits the growth of *Salmonella typhosa* for 18 hours at 37° C. in a broth containing 0·75 per cent. of peptone and 0·25 per cent. of yeast extract at *p*H 7·2. Material of the highest potency contained about 5000 units/mg. Circulin sulphate is exceedingly soluble in water, less soluble in the lower alcohols and insoluble in hydrocarbons or ether. It is not affected by autoclaving for 15 minutes. It is more active against Gram-negative than against Gram-positive organisms, thus differing from all other

antibiotics except aerosporin and polymyxin. In general, circulin is more active than streptomycin against Gram-negative bacteria. Doses of 27,000 units/kg. subcutaneously, protected mice against 1000 minimum lethal doses of *S. typhosa;* 43,200 uits per kg. intraperitoneally gave protection against 100 minimum lethal doses of *Klebsiella pneumoniæ.* It is less toxic than aerosporin but more so than polymyxin. The LD50 in mice for a preparation about 50 per cent. pure given subcutaneously is about 150 mg./kg.

H. T. B.

Penicillin, Antibacterial Activity of Synthetic Compounds of. G. Brownlee and M. Woodbine. (*Brit. J. Pharmacol.,* 1948, **3,** 305.) The compounds examined consisted of the following groups based on (1) penicillamines, (2) thiazolidine-4-carboxylic acid, (3) oxazolones, (4) derivatives of glycine, and a miscellaneous group of intermediate and associated products. All the compounds were found to possess poor antibacterial activity *in vitro* when compared with penicillin. Those penicillamine esters which were found to possess antibacterial activity were inactivated by the presence of 10 per cent. of blood or serum, and their mode of action is not related to that of penicillin. All the active compounds, when given intraperitoneally, were acutely toxic in small doses to mice, and none possessed chemotherapeutic value. S. L. W.

Penicillin, Cytochemical Action of. J. Dufrenoy and R. Pratt. (*J. Bact.,* 1948, **55,** 525.) Agar plates seeded with *Staphylococcus aureus, Bacillus subtilis* or *Proteus vulgaris* were incubated until the organisms reached the logarithmic phase of growth. Aqueous solutions of penicillin in the usual assay cylinders were then allowed to diffuse through the medium for a period too short to permit the development of clear zones. On flooding the plates with appropriate reagents inhibition zones were immediately visible. Application of Pappenheim's stain, which differentiates between ribo- and desoxyribo-nucleic acid derivatives, showed that the inhibition zones were free from ribo-nucleic acid. Application of redox indicators showed that the inhibition zones had low dehydrogenase activity. Cobra venom behaved similarly to penicillin, and since it is known to interfere with sulphydryl groups on which dehydrogenase activity depends it is possible that the same mechanism is involved in the action of penicillin.

H. T. B.

Penicillin Effectiveness, Enhancement by Traces of Cobalt. L. A. Strait, J. Dufrenoy and R. Pratt, with V. Lamb. (*J. Amer. pharm. Ass., Sci. Ed.,* 1948, **37,** 133.) The addition of traces of hydrated cobalt chloride to the agar medium used in the assay of penicillin by the cylinder-plate method caused a pronounced increase in the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on the plates infected with certain bacteria. Concentrations ranging from 0·1 to 10 p.p.m. were used and the effectiveness of the pencillin solution in producing inhibition zones was increased 4 to 8 times using *Staphylococcus aureus.* Similar increases in the bacteriostatic properties of penicillin on *Escherichia coli, Bacillus subtilis* and *Proteus vulgaris* were observed, *P. vulgaris* being especially sensitive to traces of cobalt. The presence of traces of cobalt also reduced the threshold concentration of penicillin detectable by the cylinder-plate method to one-third or less. L. H. P.

Vitamins, Antagonistic Action of, Towards Histamine. R. Lecoq, P. Chauchard, and H. Mazoué. (*C. R. Acad. Sci., Paris,* 1948, **227,** 1264.) Repeated administration of histamine produces a chronic state of nervous chronaxia which may be relieved by the subcutaneous administration of normal doses of vitamins A, B, C, nicotinamide or rutin. Of these, only vitamins C and D, and rutin, all of which have an antagonistic action towards acetylcholine, can suppress completely the chronaxic effects of anaphylactic shock. While vitamin A and nicotinamide neutralise the effects of histamine but not of acetylcholine, on the other hand vitamin H and inositol have a neutralising effect only towards acetylcholine. G. M.

BIOCHEMICAL ANALYSIS

Amino-acids, Ninhydrin Reagent in Determination of, by Paper Chromatography. A. J. Landua and J. Awapara. (*Science,* 1949, **109,** 385.) The modified ninhydrin reagent of Awapara (*J. biol. Chem.,* 1949, in the press) consisting of a 2 per cent. ninhydrin solution in methyl cellosolve water at *p*H 5 (citrate buffer) and containing stannous chloride, was used to develop amino-acid spots on a paper chromatogram. The spots were cut from the chromatogram and the colour intensity was measured using a Beckman spectrophotometer at 570 mμ. A table is given showing density readings obtained at various concentrations for solutions of glutamic acid, aspartic acid, glycine and alanine; in all cases the colour density was a straight line function of the concentration. The greatest source of error in this procedure was the filter paper which gave blank readings ranging from 0·070 to 0·100; this range of 0·030 units indicated an error of about 7 per cent. on a determination of 10 μg. of amino nitrogen. Analyses carried out on the same sheet of paper could, however, be reproduced with variations of about 1 to 2 per cent. In this connection the best results were obtained when an aliquot of the coloured solution was removed from the tube and made up to a convenient volume, thus avoiding the difficult removal of all the colour on the filter paper remaining in the tube. The amount of colour adsorbed on the filter paper was constant. R. E. S.

Insulin, Determination of Protein in. H. Cordebard and J. Schneider. (*Ann. Pharm. franc.,* 1949, **6,** 542.) To 10 ml. of a solution, corresponding to 100 I.U., 1 ml. of 5 per cent. solution of sodium tungstate is added, then 1 ml. of N sulphuric acid containing 20 per cent. of sodium sulphate. The mixture is centrifuged, and the residue, after washing, is heated for 15 minutes on the water bath with 2 ml. of N potassium dichromate and 5 ml. of concentrated sulphuric acid. After cooling and dilution to 150 ml., potassium iodide is added and the mixture is titrated with 0·1N sodium thiosulphate. In calculating the precentage of insulin protein, 1 ml. of 0·1N solution is taken as equivalent to 0·75 mg. of insulin. G. M.

Subtilin: Microbiological Assay. R. D. Housewright, R. J. Henry and S. Berkman. (*J. Bact.,* 1948, **55,** 545.) Details are given of a filter-paper disc method for the assay of subtilin using *Bacillus cereus* as the test organism. The medium used consisted of Difco peptone, beef extract, yeast extract and agar, and it was found necessary to add 2 per cent. of sodium chloride and to adjust the *p*H to 6·4 with hydrochloric acid. The method can also be used for the assay of subtilin in certain body fluids, consistent results being obtained in the presence of more than 10 per cent.

of blood and more than 12 per cent. of serum. With the procedure described the error of assay is approximately ± 5 to 10 per cent., depending on the number of dilutions of the unknown falling within the range of the standard curve. H. T. B.

CHEMOTHERAPY

Œstrogens related to Triphenylethylene. W. Tadros, K. Farahat and J. M. Robson. (*J. chem. Soc.*, 1949, 439.) A number of new triphenylethylene derivatives have been prepared and their œstrogenic activity has been studied by the method of Robson (*Quart. J. exp. Physiol.*, 1938, **28**, 195). The compounds were prepared by addition of different *pp'*-disubstituted benzophenones to an ethereal solution of *p*-chlorobenzylmagnesium chloride and decomposition of the product with aqueous ammonium chloride, when the corresponding carbinols were obtained. Dehydration of the carbinols gave the triphenylethylenes. The compounds were dissolved in olive oil and the œstrogenic activity was examined on injection subcutaneously and on administration orally by a stomach tube into groups of 5 ovariectomised mice.

TIME REQUIRED FOR ŒSTROGENIC ACTIVITY TO FALL TO HALF

No.	Substance	Dose (μg)	Time (days)
1	1 : 1-Diphenyl-2-*p*-bromophenylethylene	1000	2
2	2-Bromo-1 : 1-diphenyl-2-*p*-bromophenylethylene	1000	56
3	2-Bromo-1 : 1-diphenyl-2-*p*-chlorophenylethylene	100	11
4	Bromotri-*p*-chlorophenylethylene	5000	5
5	2-Bromo-2-*p*-chlorophenyl-1 : 1-di-*p*-bromophenylethylene ...	5000	10
6	2-Bromo-2-*p*-chlorophenyl-1 : 1-di-*p*-iodophenylethylene	5000	10
7	2-*p*-chlorophenyl-1 : 1-di-*p*-methoxyphenylethylene	1000	3
8	2-Bromo-2-*p*-chlorophenyl-1 : 1-di-*p*-methoxyphenylethylene ...	100	5
	,, ,, ,, ,,	10*	slight
	,, ,, ,, ,,	100*	12
9	2-*p*-Chlorophenyl-1 : 1-di-*p*-ethoxyphenylethylene	1000	slight
10	2-Bromo-2-*p*-chlorophenyl-1 : 1-di-*p*-ethoxyphenylethylene ...	100	3
	,, ,, ,, ,,	10*	3
	,, ,, ,, ,,	100*	16
11	2-Bromo-2-phenyl-1 : 1-di-*p*-ethoxyphenylethylene	10*	7
	,, ,, ,, ...	50*	21
	,, ,, ,, ...	500*	38

* Compounds in solution in olive oil administered orally by a stomach tube into groups of 5 ovariectomised mice. In all other cases compounds in solution in olive oil were injected subcutaneously into groups of 5 ovariectomised mice.

The time required for the œstrogenic activity of compounds 8 and 10 to fall to half was shorter than that of the corresponding compounds with a phenyl group only on the ethylene 2-carbon atom. At the dose level employed the compounds 4, 5, and 6 were active in contrast to the corresponding compounds with no halogen in the phenyl group, which were inactive. R. E. S.

Œstrogens, Synthetic, related to Triphenylethylene. W. Tadros. (*J. chem. Soc.*, 1949, 442.) Heating di-*p*-benzyloxyphenylbenzylcarbinol and 2-phenyl-1 : 1-di-*p*-*iso*propoxyphenylethylene *in vacuo* in the presence of a trace of sulphuric acid, or refluxing the solution in acetic acid in the presence of sulphuric or hydrobromic acids yielded 2-phenyl-1 : 1-di-*p*-hydroxyphenylethylene. 2-Bromo-2-phenyl-1 : 1-di-*p*-hydroxyphenylethylene and its diacetate (which can be obtained by bromination of 2-phenyl-1 : 1-di-*p*-acetoxyphenylethylene) were found to be highly active œstrogenic compounds when injected subcutaneously into ovariectomised mice. A number

of new triphenylchloroethylenes were prepared by chlorination of the corresponding ethylenes; di-*p-ethyl*thiophenylbenzylcarbinol and di-*p-n*-propylthiophenylbenzylmagnesium chloride were obtained by adding the appropriate ketone to the ethereal solution of benzylmagnesium chloride.

R. E. S.

PHARMACOGNOSY

Aloes, Preparation of Powdered. I. W. Spoon and W. M. Sesseler. (*Pharm. Weekbl.* 1949, **84**, 241.) The authors give a description of the method used in the Dutch Antilles (including Curaçao) for the preparation of aloes. The boiling of the juice is carried out in copper kettles, but it is not possible to control the process properly, so that some decomposition probably occurs. A sample of the sap, preserved with chloroform, and transported in cold storage, was obtained. The liquid showed a considerable amount of sediment, which redissolved on warming; the density was 1·0911, and the solid content 25·5 per cent. The liquid was spray dried at a temperature of 71° to 74°C. The powder obtained contained 2·4 per cent. of water, was lighter in colour, and more soluble than the ordinary block aloes, while it satisfied all official requirements. It was hygroscopic, but did not cake unless the humidity of the atmosphere exceeded 70 per cent. G. M.

PHARMACOLOGY AND THERAPEUTICS

Curare, Antagonism of by Congo Red and Related Compounds. C. J. Kensler. (*J. Pharmacol.*, 1949, **95**, 28.) The anticurare activity of congo red has been examined by using pure *d*-tubocurarine chloride and two pure erythrina alkaloids, β-erythroidine hydrochloride and dihydro-β-erythroidine hydrochloride. Congo red and the related dyes, Evans blue and chlorazol fast pink prevented paralysis in the frog when *d*-tubocurarine chloride in doses up to 10 mg./kg. is used as the curarising agent, but no protective action was observed when the erythrina alkaloids were used. Congo red also shortened the recovery time of frogs paralysed with *d*-tubocurarine chloride; it also prevented the inhibition by *d*-tubocurarine of the response of the isolated frog rectus abdominus muscle to acetylcholine and incubation with congo red potentiated the response of this muscle to acetylcholine. In contrast to the protection afforded by congo red when the erythrina alkaloids were used complete inhibition of the acetylcholine response was obtained, even though congo red was present throughout the exposure to the alkaloid. Congo red was found to be a moderately potent inhibitor of frog brain cholinesterase activity *in vitro* but *in vivo* it did not measurably inhibit activity. Related compounds varied in their anticholinesterase activity, some being moderately active inhibitors *in vitro*, but lack of any obvious correlation between this property and anti-curare activity, the lack of effect *in vivo*, and failure to exert any anti-curare activity with the erythrina alkaloids indicated that the anti-curare activity of the azo dyes is not due chiefly to their anti-cholinesterase activity. Evidence is presented which indicates that *d*-tubocurarine and congo red form a complex which is soluble or insoluble depending on the ratio of the two substances. The evidence which supports the hypothesis that congo red and related compounds are effective against *d*-tubocurarine because of a reaction with *d*-tubocurarine rather than an action on a functional component of the myoneural junction is discussed.

S. L. W.

Diparcol in Parkinsonism. R. S. Duff. (*Brit. med. J.*, 1949, **1**, 613.) Diparcol, a proprietary brand of diethylaminoethyl-N-thio-diphenylamine hydrochloride, was employed in the treatment of 8 cases of post-encephalitic chronic Parkinsonism of varying degrees of severity. Starting with small doses the amount was gradually increased up to 1 g. daily by mouth, and this dose continued for a period of 10 weeks until significant benefit was obtained, after which the dose was varied by small amounts to suit the requirements of the patient. It was found that treatment with two 0·25 g. tablets on awakening, followed by one tablet at noon, and one at about 5 p.m., brought the greatest reduction of rigidity. Diparcol seemed to offer some advantages over the tropine series of alkaloids (exemplified by stramonium), and all the patients derived a little benefit as compared with previous treatment. Improvement in the feeling of well-being was noteworthy in several of the patients. Oculogyric crises were not significantly reduced. Hypersalivation was not well controlled in 4 of the cases, in which the addition of small doses of belladonna became necessary. Undesirable effects in some patients included faintness, paræsthesiæ, transient blurring of vision, undue drowsiness. There was a tendency for the white cell count to fall to 4000 per c.mm. during the initial weeks with a subsequent rise to former levels. A method of recording and evaluating the results of treatment of patients with chronic disorders of locomotion is described. S. L. W.

Di-isopropyl Fluorophosphonate (D.F.P.) in Surgery. J. P. Quilliam and T. A. Quilliam. (*Lancet*, 1949, **256**, 603.) The use in post-operative paralytic ileus is illustrated in 12 successfully treated cases. It should be used as a supplement to other measures, 2 to 4 mg. being given by intramuscular injection, and the dose repeated after 12 to 24 hours if defæcation has not occurred. If the case is very urgent a maximum of 2 mg. may be given 4 hours after the first dose. It is more certain in its action on paralytic ileus than neostigmine and does not give rise to the unpleasant and sometimes dangerous side-effects which may attend the use of posterior pituitary extract, while the beneficial effects seem to last longer than those of either of these drugs. It was also used successfully in 5 cases of post-operative abdominal distension and 4 cases of intestinal obstruction and in post-operative retention of urine. As a simple biological test to assess potency a small measured volume is instilled into the right conjunctival sac of a rabbit, and a similar volume of a standard solution into the left sac. A comparison of the mean pupil diameters under constant conditions of illumination made at 10 minute intervals during the hour following administration will indicate the potency of the sample against the standard. There was little, if any, loss in potency of an arachis oil solution after storage for up to 2 years at room temperature. S. L. W.

Hexa-ethyltetraphosphate, Toxicology and Pharmacology of. S. Forssling. (*Acta Pharmacol. Toxicol.*, 1948; **4**, 143.) Comparative toxicity tests on hexa-ethyltetraphosphate with and without atropine prophylaxis were carried out on the mouse, rat, guinea-pig, rabbit, and cat. The administration was subcutaneous, also percutaneous for the rat and guinea-pig, and oral for the rat. The inhibitive action of hexa-ethyltetraphosphate on acetylcholinesterase was demonstrated by recording the blood pressure. The LD50 by subcutaneous administration was: for mouse 0·9, rat 0·7, guinea-pig 2·2, rabbit 2·0-2·5 and cat 2·5-3·0 mg./kg. In the rat the relation between LD50 by subcutaneous, oral and percutaneous administration

was 1:2·5:35. Certain animals, particularly mice are more protected than others with atropine prophylaxis. The mortality is hardly reduced in the rabbit although the survival time is considerably prolonged. The guinea-pig and rat, after atropine, tolerate a 3-fold increase in the dose of hexa-ethyltetraphosphate. With di-*iso*propylfluorophosphate and hexa-ethlytetraphosphate the animals investigated seemed to be better protected by magnesium sulphate and atropine than by tropine alone; physostigmine in large doses after a small dose of atropine gave the best protection. The inhibition of acetylcholinesterase is essential for the toxicity; in the cat this inhibition can be demonstrated *in vivo* by recording the momentary decrease in blood pressure after an intravenous injection of acetylcholineesterase before and after the administration of hexa-ethyltetraphosphate. The nicotine but not the muscarine activity in hexa-ethyltetraphosphate poisoning is eliminated in the cat by urethane narcosis, resulting in a considerable increase in tolerance.

R. E. S.

Paludrine, Some Pharmacological Actions of. J. R. Vane. (*Brit. J. Pharmacol,* 1949, **4,** 14.) The LD50 for acute intravenous toxicity in mice was 22 mg./kg., this figure being calculated after observing the mice for 72 hours after the injection; the simultaneous injection of neostigmine increased the immediate toxic effect. Paludrine antagonised the action of acetylcholine or of vagal stimulation; it inhibited the contractions of the isolated frog rectus muscle and guinea-pig ileum and abolished the action of acetylcholine on isolated rabbit auricles; it decreased the response of the cat's intestine to vagal stimulation, and caused depression of respiration in the rabbit. It lengthened the refractory period of auricular tissue and had a curariform action on the cat sciatic-gastrocnemius, the rat phrenic nerve-diaphragm, and the perfused superior cervical ganglion preparations. Paludrine caused vasodilatation of the perfused dog hind leg, and in the cat, this dilation was reduced by antihistamine agents, suggesting that paludrine might release histamine from the tissues. If this is so it presents a difficulty in assessing the relationship between paludrine and histamine: whereas paludrine inhibits gastric secretion evoked by histamine, and reduces the response of isolated guinea-pig ileum to histamine, it potentiates the action of histamine on guinea-pig lungs. On the other hand, antihistamine agents, which abolish the effect of histamine in most hormones but potentiate the gastric secretion caused by histamine, reduced the action of paludrine on the systemic vessels.

S. L. W.

Penicillin, Radioactive, Investigations with. P. D. Cooper and D. Rowley. (*Nature,* 1949, **163,** 480.) A penicillin uptake was detected by counting thin films of bacteria. Bacteria were suspended under various conditions in radioactive penicillin solutions, centrifuged, washed and the radioactivity on the bacteria measured directly by Geiger counter, the uptake being expressed as units of penicillin per g. of dry weight of the cells. The penicillin uptake was small, from a maximum of 40 units /g. of dry weight to the smallest detectable amount of 0·5 units /g. depending on the strain of the organism and on the conditions of experiment. It was found that in the range of 0·0 to 0·5 units/ml. much more penicillin was fixed by sensitive than by resistant bacteria, there being a direct correlation between the sensitivity of an organism and the amount of penicillin attached to it. The uptake increased when growth occurred in the presence of penicillin although if growth were halted by cooling, or with dead cells, there was still a rapid

but smaller uptake. The penicillin was strongly and irreversibly attached to the cells and could not be removed by washing or by incubation for 30 minutes with 0·1 per cent. of cetyltrimethylammonium bromide or "Aerosol OT," 5 per cent. of phenol, 1 per cent. of cysteine, 1 per cent. of sodium hydroxide, N hydrochloric acid, 1 per cent. or glucose or with ordinary penicillin (200 u./ml.), although it was removed by heating for 5 days at 60°C. in water. The uptake of penicillin was not prevented by pre-treatment with 3 per cent. of formalin, 0·1 per cent. of cetyltrimethylammonium bromide, 0·1 per cent. of euflavine, N hydrochloric acid, or by autoclaving, but heating for 5 days at 60°C. in water or pre-treatment with penicillin or acetic anhydride completely prevented any uptake. It is clear that rapid growth exposes more centres in the bacteria with which the penicillin can react. R. E. S.

Penicillin: Single Daily Dose in Treatment of Pneumonia. W. Weiss and I. Steinberg. (*Amer. J. med. Sci.*, 1949, **217,** 86.) Thirty consecutive pneumonia cases were treated by a single daily injection of 300,000 units of penicillin G in aqueous solution. Treatment was continued for from 4 to 13 days, usually from 5 to 7 days. In 18 cases, the temperature dropped to normal by crisis within 12 to 36 hours. In 9 patients the temperature fell by lysis, and longer treatment, up to 13 doses, was necessary. Of the remaining 3 patients, in one the condition was due to *Streptococcus viridans* and failed to respond; the other 2 terminated fatally, although in both cases the lobe involved was sterile. The results are considered to be at least as good as reported by others using smaller doses at more frequent intervals. H. T. B.

Stramonium Powders, Investigations into the Alkaloidal Content of Smoke from. *P. Terp.* (*Acta Pharmacol. Toxicol.*, 1949, **4,** 135.) The amount of tropa-alkaloid present in the smoke from burned stramonium powder has been investigated. The smoke was absorbed in a series of absorption bottles containing dilute hydrochloric acid and the final estimation of tropa-alkaloids was made on a biological basis, using Pulewka's method, (*Arch. exp. Path. Pharmak.*, 1932, **168,** 307) by measuring the pupil dilation of the mouse's eye. This gave an indication of how much biological activity present in the starting material had been recovered in the smoke, but not how much was due to atropine and hyoscyamine respectively; nor did it give information as to whether the burning caused racemisation. It was found that 8 to 11 per cent. of the amount of alkaloid in the starting material (as atropine sulphate) was present in the smoke. Less than 1 per cent. remained in the ash. Experiments on mice showed that the greater part of the alkaloid entering the respiratory organs with the smoke, was absorbed. R. E. S.

Streptomycin in Non-tuberculous Infections. P. H. Buxton, R. D. Simon and F. R. Selbie. (*Lançet*, 1949, **256,** 729.) This is a report on the treatment of 67 cases of infection by organisms insensitive to the sulphonamides and penicillin but sensitive to streptomycin. These included 44 cases of urinary infection, chiefly by *Pseudomonas pyocyanea* and *Bacterium coli,* 14 cases of wound infection, mainly by *Stapylococcus pyogenes,* and 9 miscellaneous infections. A dosage of 3 g. of streptomycin daily for 4 days in urinary infections due to a susceptible organism is likely to cure about one-third of the cases, provided there is no mechanical obstruction to drainage; all patients in whom the infecting organisms were eliminated had sterile urine within 72 hours, and usually within 24 hours,

of starting treatment, any organisms still present after 72 hours showing greatly reduced sensitivity to streptomycin. In the wound infections due to staphylococci the results were promising, of 14 patients treated 10 were cured and 2 improved. The usual dosage was 4 g. daily for 2 days, followed by 2 g. daily. In superficial infections a powder consisting of streptomycin 1 g., penicillin 45,000 units, and suphathiazole 9 g. was also given locally by insufflation every 3 or 4 hours to give a total daily dosage equivalent to 1 g. of streptomycin. If no improvement occurs in these cases within a week of starting streptomycin, further treatment by surgery to eliminate inaccessible foci of infection and provide adequate drainage should be considered. Side-reactions occurred in less than one-third of the cases, and in only 3, where there was severe vestibular disturbance, were they of serious significance. S. L. W.

***Strychnos lucida*, Alkaloids of.** F. H. Shaw and I. S. de la Lande. (*Austral. J. exp. Biol.*, 1948, **26**, 199.) *Strychnos lucida* is an Australian *Strychnos* species closely related to *Strychnos nux vomica*. In the present investigation the presence of strychnine (approximately 0·3 per cent.) and brucine (1·5 to 2·4 per cent.) in the seed has been confirmed by their isolation, and their probable presence in the leaf and bark indicated by assay data. In addition, non-strychnine-brucine alkaloid fractions have been isolated from the seed and leaf. These have been tentatively called lucidine-S and lucidine-L respectively. These alkaloid fractions are both amorphous and have similar solubilities to brucine. Both were isolated in small quantities from the plant by a method which is described, and both appear to be identical, though owing to the inability to isolate the bases or their salts in a crystalline condition it is not known whether they are homogeneous or not. The pharmacological investigation of lucidine-S was restricted to toxicity tests on mice and rabbits, which indicate that the intravenous LD50 is greater than 250 mg./kg. and 60 mg./kg. for these animals respectively. Toxic doses in mice produced strychnine-like convulsions. The tixicity of lucidine-L is of the same order as that of lucidine-S and toxic doses produced similar strychnine-like convulsions; it does not show any marked physiological activity. In anæsthetised cats and dogs (10 mg./kg.) it produces a fall in blood pressure similar to that produced by brucine. S. L. W.

BACTERIOLOGY AND CLINICAL TESTS

2:4-Dichlorophenyoxyacetic Acid, Differential Effects of, on Aerobic, Anaerobic and Facultative Anaerobic Micro-organisms. A. Worth, Jnr., and A. M. McCabe. (*Science*, 1948, **108**, 16.) This substance is biologically recognised as an auxin or growth-regulating substance. It is known to have bacteriostatic and bactericidal properties, and to inhibit growth of typically aerobic seeds, such as barley seeds, but not the growth of seeds like those of rice, which are able to germinate in the absence of oxygen. To demonstrate the effects of varying concentrations of 2 : 4-dichlorophenoxyacetic acid on the growth of micro-organisms which differ in their utilisation of oxygen, 10 organisms were chosen. The aerobes were *Rhizobium trifolii, R. phaseoli, R. japonicum and Azotobacter chroöcoccus;* the anaerobes were *Clostridium welchii, Cl. botulinum* and *Cl. tetani;* and the facultative anaerobes were

[*Continued on page* 733

BOOK REVIEWS

MATERIA MEDICA. PHARMACY, PHARMACOLOGY AND THERAPEUTICS, by W. Hale-White, 28th Edition, revised by A. H. Douthwaite. Pp. 507 with Appendix and Index. J. & A. Churchill, Ltd., London, 1949. 16s. 0d.

There are many books which attempt to present the subjects of materia medica, pharmacology and therapeutics in a form acceptable to the student and to the medical practitioner. The most frequent criticism of such publications is that they either provide too much or too little information. Probably the chief fault of a book such as Hale-White's "Materia Medica" is that the concise nature of the text does not afford a fuller presentation of the available evidence on the pharmacological action of drugs. This does not permit discussion of some aspects of the subject where it is perhaps desirable to distinguish between opinions arising from conjecture and statements based on fact. In conformity with the traditional nature of the book there is a comprehensive list of the drugs and preparations described in the British Pharmacopœia. For the student of pharmacy this may be a desirable feature, but it is doubtful whether the medical student can derive much benefit from such an array of names, except as a dictionary of reference. He might well be puzzled regarding the necessity for a strong tincture and a weak tincture of ginger; a strong and a weak solution of ammonium acetate; a liquid extract, two infusions and a tincture of senega, especially when in the latter instance the text indicates that they are only occasionally used as expectorants. A text-book which in the course of over half a century has provided on the average a revised edition every second year, requires no further comment on its popularity. The 28th edition of this book incorporates all the numerous changes in the drugs and preparations resulting from the appearance of the British Pharmacopœia of 1948. ANDREW WILSON.

GRUNDLAGEN DER PHARMAKOLOGIE, by K. W. Merz. Pp. 274 and Index. Wissenschaftliche Verlagsgesellschaft M.B.H. Stuttgart, 1948.

Professor Merz, who was formerly Director of the Institute of Pharmaceutical Chemistry in the University of Königsberg, has completed the fourth edition of his text-book of Pharmacology. It is primarily intended for pharmacists, chemists and biologists, and if chemical formulæ might be regarded as an inducement to the reader, there is much to attract his attention. The book is divided into 18 chapters which permits a systematic and quite comprehensive treatment of the subject. The first three chapters consist of a general discussion of definitions, types of pharmacological action and the mechanism of drug action. In the remaining sections the action and uses of drugs are discussed in relation to the systems of the body, in much the same fashion as in Clark's "Applied Pharmacology." There is a concise description of the anatomy, and physiology, and where appropriate, of the pathology relevant to each such system. A very useful chapter deals with the toxicology of the common organic solvents and gases. On the whole the account of the pharmacological actions is sound though in some instances the author appears content to concern himself only with what happens in the frog and not in the higher species, particularly in man. It is desirable also to indicate more clearly that the parasympathomimetic drugs do not stimulate nerve endings, though their action resembles this effect. The book is well illustrated with chemical formulæ, line drawings and photographs of original

tracings. It is unfortunate that the quality of the paper is poor and does not permit a reasonable reproduction of those photographs which are intended to display characteristic clinical features. In some instances the effect is spoiled by lack of definition in which important detail is not clearly visible. For those to whom the German language presents no difficulties the book is well worth reading; others may prefer to await a suitable English translation.

ANDREW WILSON.

BENTLEY'S TEXTBOOK OF PHARMACEUTICS, by Harold Davis. Pp. xiv + 1100 and Index. Fifth Edition. Bailliere, Tindall & Cox, London, 1949. 30s. net.

While welcoming the fifth edition of Bentley and recommending it to all students of pharmacy as an essential text-book, one wonders whether it is in fact possible to produce successfully in one volume, a satisfying account of the very numerous pharmaceutical phenomena, together with their explanations, descriptions of machinery and their uses, dispensing of medicines, surgical dressings, bacteriology, immunology, and pharmaceuticals even in 1100 pages. The numerous references to the original literature without which no modern text-book is of value, help in some measure to ease the path of the earnest student, but many of the less critical students will neglect the implied advice of the authors to read widely and the curriculum does not allow much time for work in the library. Having said this, in the hope that Dr. Davis and his collaborators will feel only partially satisfied with their efforts and from their wide experience and knowledge will be inspired in the near future to produce a series of volumes consisting of the sections of the present volume each expanded to a volume of its own, congratulations are extended to the authors for the accomplishment of a really formidable task and encouragement is offered to go even further. Such a work would help to meet the needs of critical students of whom there is an ever-increasing number.

The volume is well printed by modern British standards and is freely illustrated. Many of the illustrations are excellent but some of the photographs have not reproduced well and as sources of information the sketches on pages 489 and 490 would convey little to one not already familiar with the equipment concerned. The proof reading has been well done although the reference to chapter LXXVI on page 47 should read LXXVII. J. P. TODD.

ABSTRACTS (*Continued from Page* 731)

Escherichia coli, Staphylococcus albus and *Candida albicans.* Different media were used for different organisms, but the reaction was adjusted in all cases to *p*H 7·4. The concentrations tested were 2, 1, 0·2, 0·02, 0·002 and 0·0002 per cent. in 5 ml. of medium; this was poured into Petri dishes containing about 10 to 15 ml. of the corresponding medium. Each micro-organism was grown on these six concentrations, and on a control plate, the aerobes were cultivated at room-temperature, the anaerobes and facultative anaerobes were incubated at 37·5°C., the anaerobes in Brewster anaerobic jars. The amount of growth was compared with the control at intervals of 24, 48 and 72 hours. Growth of *R. phaseoli* and *R. japonicum* appeared to be inhibited after 48 hours, but some growth appeared in the lower concentrations after 72 hours; whereas *R. trifolii* and *A. chroöcoccus* were inhibited only temporarily by the higher concentrations, lower concentrations increased the amount of growth. No inhibitory effects were observed with the facultative anaerobic organisms or with the anaerobes. L. H. P.

LETTERS TO THE EDITOR

The Inactivation of Penicillin by Oil of Theobroma

SIR,—During the development of penicillin formulations for human and veterinary use we have had occasion to investigate the effect of incorporating various penicillin salts in bases containing oil of theobroma. The rapid fall of potency in these preparations led us to suspect that the latter was exerting a definite inactivating effect. A series of dispersions of penicillin in oil of theobroma was accordingly prepared, using separately calcium penicillin and crystalline potassium penicillin at concentrations of approximately 10,000 I.U./g. These were assayed at time intervals after storage at room temperature and a progressive potency fall was observed. For calcium penicillin a fall of 27 per cent. was recorded after 3 months and for crystalline potassium penicillin a fall of 90 per cent. after 12 months. Further work is in progress to investigate this change in more detail but meanwhile we think it advisable to draw attention to what may be a serious incompatibility.

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August 16th, 1949.

Vitamin B_{12} as a 5:6-Dimethylbenziminazole Derivative

SIR,—A paper chromatogram of an acid hydrolysate of vitamin B_{12} (*n*-butyl alcohol-acetic acid being employed as the irrigation solvent) was exposed to the light of a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter, when three blue fluorescent spots were revealed. Spectroscopic examination of eluates from these areas indicated their close chemical similarity. The compounds responsible for the fluorescent zones have accordingly been termed by us *components* α-, β-, and γ. Comparison of their ultra-violet absorption spectra with those of known heterocyclic ring systems led to their identification as derivatives of benziminazole. Spectroscopic comparison with 22 methylated benziminazoles synthesised for this purpose led to the identification of *component* γ with 5:6-*dimethylbenziminazole*, and of components α- and β- with 1-substituted 5:6-dimethylbenziminazoles. Moreover, both spectroscopic and chemical work has led us to the conclusion that vitamin B_{12} itself contains one preformed benziminazole residue in the molecule. It may therefore be inferred that *components* α-, β-, and γ represent different stages of degradation of a common precursor. It is interesting to note that vitamin B_{12} and riboflavine may thus both be regarded as derived chemically from 4:5-dimethyl-*o*-phenylenediamine, and speculation on the biogenesis of vitamin B_{12} thus becomes possible.

Both Dr. K. Folkers and one of us (V.P.) have to-day simultaneously reported at the 1st International Congress of Biochemistry held at Cambridge the identification of hydrolytic fragments of vitamin B_{12} with 5:6 dimethylbenziminazoles. It therefore seems desirable to place these observations on

record at this stage. Our detailed results will be submitted shortly for publication in your Journal.*

It is a pleasure to acknowledge the encouragement of the Directors of The British Drug Houses, Ltd., in this work.

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V. PETROW.

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August 22, 1949.

*Received August 30, 1949.—EDITOR.

The "Ninhydrin-Reacting" Hydrolytic Fragment of Vitamin B_{12}

SIR,—We have previously reported[1] that hydrolysis of vitamin B_{12} with 20 per cent. hydrochloric acid at 100°C. for 6 hours followed by examination of the hydrolysate by unidimensional paper-strip chromatography, reveals the presence of *one* "ninhydrin-reacting" substance which could not be identified with any of the known amino-acids. Our studies have hitherto been handicapped by incomplete separation on paper chromatograms of the "ninhydrin-reacting" fragment from other products of vitamin B_{12} hydrolysis. By using *n*-butyl alcohol-acetic acid as the irrigation solvent, however, complete separation has now been obtained. The "ninhydrin-reacting" area occupies a position well removed from zones which fluoresce under the light of a low-pressure mercury resonance lamp fitted with a Corning 9863 glass filter[2], and which form the subject of a separate communication (*vide infra*). Elution of the "ninhydrin-reacting" area with dilute hydrochloric acid gives a solution transparent to ultra-violet light. From this and other observations we concluded that the "ninhydrin-reacting" substance was probably an aliphatic base.

We now find that the "ninhydrin-reacting" substance and 2-aminopropanol show identical behaviour on paper chromatograms irrigated with four different solvent systems. The two substances thus have the same partition coefficients in each of these solvent systems, and it is therefore reasonable to conclude that they are identical. A final decision, however, must rest on a direct chemical comparison. Full details of this work have already been submitted for publication[3].

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

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London, N.1.

B. ELLIS,
V. PETROW,
G. F. SNOOK.

August 22, 1949.

REFERENCES

1. Ellis, Petrow and Snook, *J. Pharm., Pharmacol.*, 1949, **1**, 60.
2. Holiday and Johnson, *Nature*, 1949, **163**, 216.
3. Ellis, Petrow and Snook, *J. Pharm. Pharmacol.*, in the press.

NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Kina-Redoxon* tablets contain quinine and vitamin C and are indicated for the prophylaxis and treatment of influenzal conditions, the common cold, coryza, etc. Sugar-coated tablets are supplied in bottles of 50 and 100.

S. L. W.

Lipo-Adrenal Cortex. (*New and Nonofficial Remedies. J. Amer. med. Ass.*, 1949, **139**, 849.) Lipo-adrenal cortex is an oil-soluble extract of hog suprarenal glands containing crystalline, biologically active constituents of what are considered to be 17-hydroxycorticosterone, 11-dehydro-17-hydroxycorticosterone and corticosterone, and a non-crystallisable amount of 11-dehydrocorticosterone. It is almost free from adrenaline and is assayed biologically. It has about 10 times the suprarenal cortical activity of adrenal cortex extract N.N.R. and is used for the same purposes when a more prolonged action is required. It is supplied as a sterile solution in cotton-seed oil and is administered intramuscularly in doses up to 3 ml., depending on the degree of cortical insufficiency and clinical response. It should be supplemented by the administration of sodium salts. The method of preparation is described. G. R. K.

Parvestin* is a concentrated desiccated extract made from the small intestine of the pig, one heaped teaspoonful corresponding to about 4 ounces of the fresh intestine. It is indicated in the treatment of chronic ulcerative colitis. The dose is 1 or 2 teaspoonfuls daily, reduced to 1 teaspoonful daily as improvement occurs. If preferred, the powder may be stirred into warm beverages or mixed with food. Treatment usually requires to be continued for 4 to 8 weeks. Parvestin is supplied in 4-oz. bottles. S. L. W.

Tyroderm* is an antibiotic cream containing 0·5 mg. of tyrothricin per g. in a water-soluble base. It is indicated in the treatment of pyodermatoses, including acne vulgaris, dermatitis and other dermatoses due to Gram-positive organisms. It may also be of value in varicose, decubital and ischæmic ulcers infected with Gram-positive organisms, and in the treatment of accessible post-surgical wounds and potentially contaminated minor burns. The cream should be applied at least once daily and may be covered with a dressing if necessary. It is supplied in 2 oz. tubes. S. L. W.

Unden* is a brand of œstrone, available either in pellets, ointment or aqueous solution, 1 mg. of which is equivalent to 10,000 international œstrone units, or (as œstrone benzoate) in oily solution, 1 mg. of which is equivalent to approximately 10,000 œstradiol benzoate units. The ointment is rubbed thoroughly into the affected areas of skin several times a day. The packings are as follows:—pellets containing 0·01, 0·05 or 0·1 mg. in bottles of 15 or 100; ampoules (aqueous) in boxes of 5 × 1 ml., each ampoule containing 0·1 mg.; ampoules (oily) 1 ml., each containing 5 mg. of œstrone benzoate solution (oily); bottles of 10 ml., each ml. containing 1 mg. of œstrone benzoate; ointment in pots of 20 g., containing 0·5 mg./g.

S. L. W.

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